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BRIEF COMMUNICATION

NOVEL FLUORESCENCE METHOD TO VISUALIZE ANTIBODY-DEPENDENT HYDROGEN PEROXIDE-ASSOCIATED "KILLING" OF LIPOSOMES BY PHAGOCYTES

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ABSTRACT We have developed a new methodology to examine effector-cell–mediated immune attack using liposomes as targets. Hydrogen peroxide-associated killing of liposomes was observed with fluorescence intensification microscopy. Liposomes were composed of 98–99 mol % egg phosphatidylcholine and 1–2 mol % dinitrophenyl lipid hapten. Anti-dinitrophenyl IgG antibody was used to opsonize liposomes. Liposomes were loaded with dihydroxymandelic acid (DHMA) and peroxidase. Macrophage- or neutrophil-mediated recognition of liposomes triggers the release of H_2O_2 and other oxidative products. Upon interaction of H_2O_2 or OH radical with liposome contents, DHMA dimerizes forming a fluorescent derivative. Our studies indicate that individual living neutrophils and macrophages deposit oxidative products in a heterogenous fashion among bound targets.

INTRODUCTION

Model membranes containing antigens or lipid haptens can participate in both afferent and efferent immune reactions in vitro and in vivo (1–9). Considerable interest has been focused on the use of liposomes or lipid vesicles as targets of immune effector cell function (6–20). Studies employing T cells (6–9), neutrophils (10, 11), and macrophages (11–20) have been reported. In particular, recent studies on phagocyte-liposome recognition have explored the kinetics of internalization (12), respiratory burst (11), cell surface receptor activity (13), membrane protein composition (14), and surface morphology (16). Moreover, modifications of lipid (17), glycolipid (18), and carbohydrate (19) composition have been examined. These studies have provided insight into the chemical nature of recognition, triggering, and degradation mechanisms. However, the direct observation of living cells depositing chemical effector molecules, such as H_2O_2 and hydroxyl radicals, into a liposome or any other target has not yet been reported. Although the molecular details of the events responsible for killing are not completely understood, it is likely that reactive oxygen metabolites play an important role in one mechanism (21). Therefore we employ the word "killing" in a more restricted, operationally defined sense as the delivery of reactive oxygen metabolites to the internal volume of a target. Here we describe a novel fluorescence technique to follow the activated oxygen-mediated killing of liposomes.

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EXPERIMENTAL STRATEGY

The experimental approach adopted was to follow the oxidation of 3, 4-dihydroxymandelic acid (DHMA, Fig. 1, compound I). The overall experimental scheme is shown in Fig. 1. Because of its three hydroxyl groups and its carboxylic acid group, DHMA is membrane impermeable except at low pH. The presence of hydroxyl radical (generated by either exogenous or endogenous peroxidase) initiates a free-radical dimerization of DHMA (22–24). The dimer (compound II) is fluorescent. The spectral properties of II are very close to those of fluorescein, i.e., ex. 472 nm and em. 532 nm (23). Although experimentally convenient, the quantum yield of this fluorophore is low.

MATERIALS AND METHODS

Neutrophils and Macrophages

Peripheral blood leukocytes were prepared from clot preparations. Briefly, drops of fresh blood obtained from a finger prick were placed on glass coverslips. Samples were kept for 30 min in a 95:5% air/CO_2 humidified incubator. The coverslips were gently rinsed with buffered saline to remove the clot. Clot preparations contained 92–95% neutrophils, ≤3% eosinophils, and 4–8% monocytes. Since neutrophils have a distinct morphology, they can be easily distinguished from the contaminating cell types.

Human peritoneal macrophages were obtained from peritoneal dialysis fluid (gift of Dr. D. Sillix, Department of Nephrology, Harper-Grace Hospital, Detroit, MI). Cells from the first outflow were collected on ice. The peritoneal cells were concentrated by sedimentations and resus-

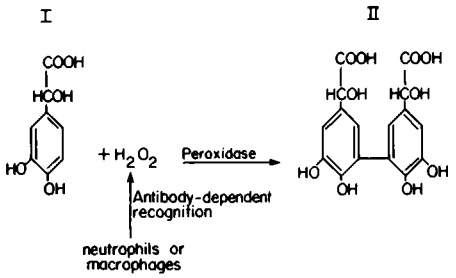

FIGURE 1 The experimental scheme employed in this work is shown.

pended in Hank's balanced salt solution (HBSS; Gibco Laboratories, Grand Island, NY) without phenol red. Mononuclear cells were purified by step-density gradient centrifugation on Ficoll-Hypaque separation medium. The mononuclear cell layer was washed by centrifugation and allowed to adhere to glass coverslips for 30 min at 37°C in HBSS. The monolayer was gently rinsed to remove nonadherent cells (primarily lymphocytes). Wright-Giesma staining demonstrated that 90–95% of the cells were monocytes/macrophages. This cell preparation was observed to phagocytose *Staphylococcus aureus*. Phagolysosome formation was found in >85% of cells using acridine orange staining and fluorescence microscopy.

Liposomes

Liposomes were prepared as previously described (16) with several modifications. Liposome composition was 98–99 mol % egg phosphatidylcholine (Avanti Polar Lipids, Inc., Birmingham, AL) and 1–2 mol % dinitrophenyle-aminocaproyl-phosphatidylethanolanine (DNP-cap-PE; Avanti Polar Lipids, Inc.). Solvents were removed with a Buchi roto-vap followed by 30 min under vacuum. Hydration buffer was degassed 0.14M Tris (Sigma Chemical Co., St. Louis, MO) at pH 8.5. Typical hydration media were composed of: 10 mg/ml DHMA, 10 mg/ml horseradish peroxidase, and buffer. Lipids were allowed to hydrate in the presence of media for 2 min, then were vigorously vortexed for 1 min. Liposomes were washed in degassed buffer five times by centrifugation to insure complete removal of substrate. Liposomes were prepared fresh each day. They were stored under argon until use. Liposomes were opsonized with rabbit anti-DNP antibodies (Miles Ames Div., Miles Laboratories, Inc., Elkhart, IN). An IgG fraction was obtained from antiserum using Protein-A affinity chromatography (Pharmacia Molecular Biology Div., Pitscataway, NJ). Heat-inactivated antiserum or an IgG fraction were employed to opsonize liposome targets.

Incubation

Opsonized liposomes were allowed to settle on effector cells bound to the glass surface. Incubation of liposomes with surfaced-adherent cells was carried out in HBSS for 5–30 min at 37°C. Samples were then transferred to a microscopic stage kept at a nominal temperature of 37°C for observation.

Fluorescence Microscopy

To observe liposome-cell interaction a Zeiss fluorescence microscope equipped with special excitation and detection instrumentation was required (Carl Zeiss, Inc., Thornwood, NY). The device is a combination of fluorescence recovery from photobleaching and video-intensified microscopy (25, 26). An argon-ion laser (164-09; Spectra-Physics Inc., Mountain View, CA) tuned to 488 nm was used for excitation of DHMA dimer. The intensity was adjusted by plasma tube current density, neutral density filters, and a beam-splitter assembly as previously described (25). For observation of dimerization, a laser power level of roughly 100 mW was employed. No significant changes in cell morphology or fluorescence level was observed during typical periods of observation (30–60 s). The beam was spatially filtered, expanded (Ealing Corp., South Natick, MA), and reflected into the epifluorescence port. The cells were observed unfixed. A Leitz 50× water-immersion objective was employed (NA = 1.0). For these experiments, the image was reflected onto an RCA silicon-intensifier tube held in a Dage-MTI camera (model 65; Dage-MTI Inc., Michigan City, IN). Video signals were recorded on a high-resolution video recorder (NV-8050, Panasonic Co., Secaucus, NJ) and displayed on a monitor (Audiotronics Corp., North Hollywood, CA). The photographs reported were taken from the screen using a Polaroid camera (Polaroid Corp., Cambridge, MA).

RESULTS AND DISCUSSION

In Fig. 2 we show a series of photomicrographs of phagocyte-liposome interactions. Bright-field images and fluorescence-intensified microscopic data are shown in alternating panels. The binding and/or ingestion of liposomes is apparent in all cells. Anti-DNP antibody or heat-inactivated antiserum was titered to yield optimum binding under our conditions using adherent cells while maintaining a sub-agglutinating dose. Human neutrophils and macrophages have the ability to generate products capable of oxidizing DHMA (see Fig. 1). We have found that both bound and internalized liposomes can become fluorescent. This is consonant with the observation that the activation of the cell surface NAD(P)H oxidase precedes internalization (27). Opsonized liposomes are not fluorescent. We have found that prolonged exposure of liposomes to HBSS results in unacceptably high background levels. As a consequence, samples are discarded when negative controls become affected. Positive controls are performed by the addition of oxidizing agent (e.g., H_2O_2) to the sample. Positive controls indicate that all liposomes are capable of becoming fluorescent, as expected.

An interesting aspect of this study was the consistent heterogeneity in the fluorescence intensity of different liposomes bound to the same cell. This heterogeneity is apparent in each micrograph of Fig. 2. Unbound liposomes are not fluorescent. Microscopic data were quantitated using a videotape library of experiments. Most cells demonstrating one or more fluorescent liposomes did so in a heterogenous fashion (69 ± 11% of all bound liposomes were fluorescent). For cells binding two or more liposomes, 55 ± 19% of bound liposomes per cell were fluorescent. As mentioned above, this cannot be due to the absence of substrate from some liposomes. Another possible origin of heterogeneity is the time sequence of binding. The liposomes bound first are attacked first. This is likely a partial explanation. It does not seem to be a complete understanding since the heterogeneity observed in panels C–F persisted for up to 1 h. Setting an upper level to persistence times is not possible due to increasing background levels. In any case, the persistence times are at least 10-fold longer than the initial incubation period. If the stepwise killing suggestion is true, then all cells expend all resources between the first and second binding events. This possibility is remote. A complete understanding may involve concepts such as compartmental cell triggering (cf. mast

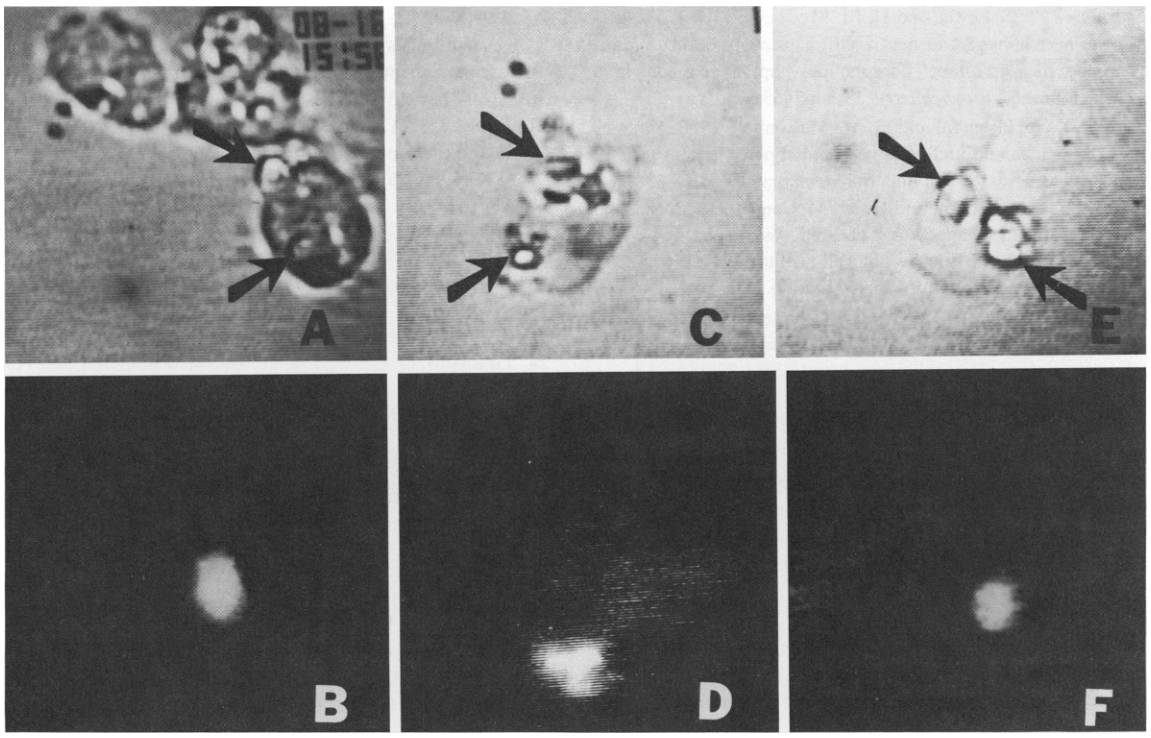

FIGURE 2 Representative fluorescence microscopy studies of phagocyte-liposome interactions are shown above. *A*, *C*, and *E* are bright-field photomicrographs, whereas those of *B*, *D*, and *F* show corresponding fluorescence intensification microscopy. *A–D* show neutrophils. *E* and *F* show macrophages. All micrographs focus upon the liposome, not the cell. Regions of liposome binding and/or phagocytosis are indicated in *A*, *C*, and *E* (*arrows*). Corresponding cell areas with fluorescent liposomes are indicated in *B*, *D*, and *F*. It is apparent that some liposomes become bright (i.e., have been exposed to significant levels of oxidizing agents) while nearby (1–4 μm) liposomes are unaffected. All photomicrographs are at a magnification of 1,200.

cell degranulation), down-regulatory mechanisms, or asymmetric localization of effector molecules in the plasma membrane. These possibilities can now be tested with this new tool.

Previous investigations have examined other cellular events associated with effector cell triggering and killing mechanisms (20, 28). Liposome lysis has been observed by self-quenching fluorescence studies (20). Initial events in metabolic stimulation have been observed by fluorescence flow cytometry (28). Here we describe the first in situ method to observe the release of reactive oxygen metabolites from living effector cells and their deposition within targets. The technique is useful in the analysis of target-effector interactions.

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