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SUPEROXIDE ENHANCES PHOTOBLEACHING DURING CELLULAR IMMUNE ATTACK AGAINST FLUORESCENT LIPID MONOLAYER MEMBRANES

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Lipid hapten-containing monolayer membranes with bound, anti-hapten antibody molecules serve as model immunological target membranes. Targets with bound-IgG trigger guinea pig macrophages to (a) adhere, (b) spread, (c) release lysosomal enzymes, and (d) increase cyanide-insensitive oxygen consumption. When the target membranes are derivatized with fluorescein, there is a 2–3-fold enhancement in the rate of fluorescein photobleaching in regions of cell-monolayer contact. This effect is due to release of O_2^- from macrophages, as shown by inhibition with superoxide dismutase and by the fact that enhanced photobleaching is not observed with cells of the RAW264 macrophage line, which undergo responses (a)–(d), but do not release O_2^- extracellularly. The O_2^--dependent photobleaching reaction appears to be relatively specific for fluorescein, as it did not occur with two other fluorophores, 4-nitrobenz-2-oxa-1,3-diazole and tetramethylrhodamine. Because stimulated neutrophils release large quantities of O_2^-, the photobleaching of fluorescein-labeled target membranes in response to neutrophils was examined. Monolayer membranes with specifically bound IgG caused neutrophils to adhere and become markedly motile during incubation at 37°C. Like macrophages, neutrophils induced O_2^--dependent photobleaching of fluorescein-labeled IgG in regions of cell-monolayer contact. In addition, neutrophils gave rise to a slower, nonphotochemical loss of fluorescence in the same contact regions. The latter effect is apparently due to cleavage of target-bound fluorescent IgG by proteolytic enzymes secreted by the neutrophils in response to the target surface.

Introduction

We have used planar lipid monolayer membranes on solid supports as models of cell surfaces and have used these models to study receptor-specific regulation of cell-mediated immune responses. Lipid haptens in the monolayer membrane mediate specific binding of anti-hapten antibodies (e.g., IgG or IgE). The antibodies, in turn, mediate binding of cells bearing appropriate Fc-receptors (e.g., macrophages or mast cells) and trigger antibody-dependent immune attack [1,2]. Similarly, monolayer membranes bearing the extracellular antigenic portion of the H-2K^k molecule are specific binding targets for cloned, allogeneic cytotoxic T-lymphocytes (anti-H-2^k) [3]. The phospholipid monolayer membranes serve both to present cell-surface ligands to interacting cells and to minimize nonspecific interactions between cells and the ligand-supporting surface. Glass, quartz, or tissue culture polystyrene surfaces induce a propagated clustering of C3b receptors

Abbreviations: NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; DNP-cap-PE, dinitrophenylaminocaproylphosphatidylethanolamine; DNP, 2,4-dinitrophenol; FITC, fluoresceinisothiocyanate; TRITC, tetramethylrhodamineisothiocyanate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

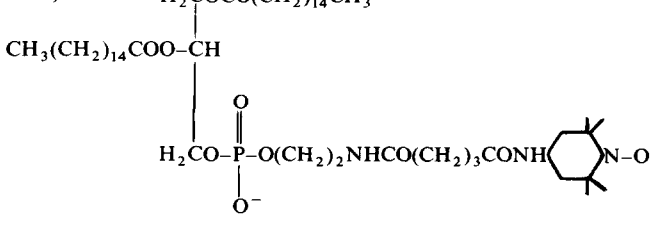
on the surface of neutrophils (Ref. 4, and Hafeman, D.G., Smith, L.M. and McConnell, H.M., unpublished data), whereas receptor redistribution does not take place when cells rest on pure lipid monolayers.

The specific antibody-dependent triggering of immune attack by monolayer membranes leads to serotonin release from mast cells, and release of lysosomal enzymes and stimulation of the respiratory burst in macrophages [1,2]. In addition, we have observed enhanced photobleaching of monolayer-membrane-bound, fluorescein-labeled IgG in the regions of macrophage-monolayer contact [1]. The enhanced photobleaching leads to the characteristic appearance of 'black holes' in the homogeneously fluorescent substrate and is indicative of immune attack by individual macrophages. In the present report, we quantitate and characterize further this effect, which is attributed to superoxide anion (O_2^-) release by stimulated macrophages. We also report here that certain cells, such as human neutrophils, mediate a dramatic loss of monolayer-bound fluorescent IgG through an additional (non-photochemical) mechanism.

Materials and Methods

Preparation of cells. Human neutrophils [4], guinea-pig peritoneal macrophages [1], and the RAW264 macrophage cell line [5] were obtained as described previously.

Monolayer membranes. Lipid monolayer membranes were deposited at room temperature from an air/water interface (pressure, 30 dyn/cm) onto alkylated glass coverslips as described previously [1,6]. All membranes were composed of dipalmitoylphosphatidylcholine. Unless otherwise noted, these membranes also contained 1 mol% nitroxide lipid hapten I. The dipalmitoyl hapten I was synthesized by D. Carter, according to a modification of Brûlet's original synthesis [7] (Scheme I).



Scheme I. Structure formula of dipalmitoyl hapten I.

The fluorescent phospholipid probe *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (NBD-PE), prepared from dimyristoylphosphatidylethanolamine, was purchased from Avanti Biochemicals, as was the dinitrophenylaminocaproylphosphatidylethanolamine (DNP-cap-PE). Dipalmitoylphosphatidylcholine, grade A, was purchased from Calbiochem and stored at 10 mM in methanol at −20°C.

Antibodies. Anti-nitroxide IgG was prepared by immunizing rabbits according to a procedure described by Humphries and McConnell [8] with the modification that an alum precipitate of 100 μg of spin-labeled keyhole limpet hemocyanin was used in the boosting intravenous injections. Rabbit anti-DNP bovine serum albumin antiserum was obtained from Miles-Yeda, Elkart, IN. Anti-nitroxide and anti-DNP IgG were fractionated from serum, using protein A coupled to cyanogen bromide-activated Sepharose as described previously [9]. The IgG was conjugated to fluoresceinisothiocyanate (FITC) as described [10].

Tetramethylrhodamineisothiocyanate (TRITC) was obtained from Research Organics, Inc., Cleveland, OH and conjugated to IgG according to the manufacturer's instructions. Anti-rabbit IgG (whole molecule) FITC conjugate was purchased from Sigma Chemical Co.

Inhibitors of photobleaching. Bovine liver superoxide dismutase (more than 3000 units/mg protein) was obtained from Diagnostic Data (Mountain View, CA) and used at a concentration of 500 μg/ml. Bovine liver catalase (more than 75000 units/mg protein) was purchased from Calbiochem (La Jolla, CA) and used at 50 μg/ml. Mannitol was used at a final concentration of 80 mM.

Incubations for microscopy and photobleaching measurements. The lipid monolayer on an 18 × 18 mm, No. 2, cover glass was mounted in a 60 × 15 mm glass petri dish with two double thickness strips of 0.25-inch double-coated tape (3M, St. Paul, MN). The mounting was carried out under water to avoid the disruptive effect of an air/water interface on the monolayer. For a schematic diagram of this system, see Fig. 1 of Ref. 1. The water held by surface tension between the coated cover glass and the petri dish was replaced with cell buffer (2.0 mM $CaCl_2$/1.5 mM $MgCl_2$/5.4 mM

KCl/1 mM NaH_2PO_4/5.6 mM glucose/120 mM NaCl/25 mM Hepes/0.2% bovine serum albumin (fatty-acid poor; Calbiochem-Behring, San Diego, CA); pH 7.4). Solution replacement, without introduction of air bubbles, between the monolayer and petri dish was facilitated by tilting the entire mounted preparation with respect to the horizontal. Next, 100 μl of a preparation of fluorescently labeled (either anti-nitroxide or anti-DNP) IgG was added. After 10 min, the monolayer was washed with cell buffer and was ready for either observation or addition of cells. Unless otherwise noted, cells were added at $2-5 \cdot 10^6$ per ml in 100 μl of cell buffer, the dish was inverted and the cells allowed to settle onto the monolayer. After 10 min at room temperature, the dish was righted for cell observation. Temperature was maintained at 37°C during observation by means of a water-jacketed microscope stage.

Photobleaching measurements were carried out with an epifluorescence microscope (Zeiss Photomicroscope III; Carl Zeiss, New York). An argon ion laser (Spectra Physics model 164-05; Spectra Physics, Mountain View, CA) was used to provide bleaching radiation at 488 nm. Laser radiation is not required for these measurements but was used because of convenience. The approx. 100-fold attenuated laser beam passed through a 400 μm diameter pinhole aperture placed in a real-image plane of the microscope. The narrow collimated beam was then reflected by an appropriate dichroic mirror into a 40×, N.A. 0.75, water immersion objective so that the final illuminated spot was 10 μm in diameter. The fluorescent light, passed by a 520 nm long pass filter, was measured with a cooled photomultiplier tube (RCA C31034-02). The anode current was amplified and the signal displayed on a storage oscilloscope and also sent to a computer for digitized storage and analysis. Recovery of fluorescence in the photobleached region due to translational motion of the specifically bound fluorescent IgG or the fluorescent lipid is negligible under these conditions due to a negligible rate of antibody dissociation-reassociation [1] and a negligible rate of diffusion of lipid haptens in the 'solid' dipalmitoylphosphatidylcholine monolayer membranes. Fluorescence recovery after periodic pattern photobleaching measurements [11] showed that the coefficient for lateral diffusion of fluorescent lipids in these membranes was about $5 \cdot 10^{-10}$ cm^2/s at 37°C.

Membranes bearing FITC- or TRITC-labeled anti-DNP IgG were illuminated with a 200 watt Mercury arc lamp through interference excitation filter sets for epifluorescence (FITC, 445–475 nm; TRITC, 515–560 nm bandpasses). Color photographs of fluorescence (not shown) were made with Kodak Ektachrome (400 ASA, daylight) film with 1–2 min exposure and developed to give a final ASA rating of 1600. Black and white photos were made with Kodak 2475 Recording Film (ASA 1000) also with 1–2 min exposure.

Results

Fluorescein-labeled rabbit anti-nitroxide IgG binds to monolayer membranes which contain 1 mol% lipid nitroxide hapten; the binding is observed as a uniform distribution of fluorescence over the entire monolayer membrane. Fluorescent IgG was not bound in control experiments, either where lipid hapten was omitted from the lipid monolayer or where nonimmune FITC-IgG was used instead of antinitroxide IgG. Guinea-pig peritoneal macrophages adhere when they are allowed to settle onto monolayer membranes with specifically bound IgG. Subsequent incubation at 37°C causes many of the cells to spread and thus increase their contact area with the monolayer membrane. Macrophage adherence and spreading are not observed in the absence of bound IgG (e.g., when lipid hapten is omitted or when nonimmune IgG is used in lieu of antinitroxide IgG). The monolayer-bound IgG remains uniform during incubation with macrophages at 37°C. The uniformity of fluorescence, however, is rapidly lost during excitation of FITC. The nonuniformity is manifested by the appearance of 'black holes' from 10 to 20 μm in diameter, each of which corresponds to a region occupied by an individual macrophage, as viewed by phase-contrast microscopy. Shown in Fig. 1, this phenomenon is caused by a 2–3-fold increase in the initial rate of photobleaching in areas of macrophage-monolayer contact. 'Black holes' are observed under only a fraction of adhering macrophages. The effect occurs with higher frequency under macrophages that are extensively spread on the monolayer compared to

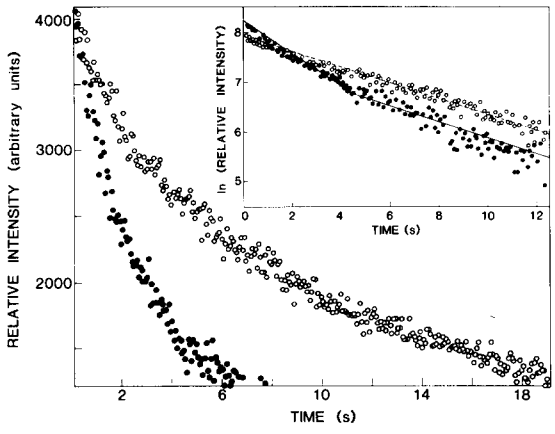


Fig. 1. Rates of fluorescence photobleaching of FITC-anti-nitroxide IgG bound to monolayer membranes. The open dots (○) show the photobleaching rate in a region devoid of cells. The closed dots (●) show the photobleaching rate in a region of monolayer membrane directly apposed to a bound macrophage. The inset shows a semilogarithmic plot constructed from the same sets of data after subtraction of nonbleachable fluorescence.

those that are adherent but not spread. The fraction of cells producing the effect (from 0.1 to 1.0) is relatively constant with a given macrophage preparation, but varies with different preparations obtained daily from single guinea pigs. Macrophage viability, as measured by Trypan blue exclusion, was always larger than 95%, and thus was not a factor in determining the cell response.

Photobleaching in cell-free regions of the monolayer is a pseudo-first-order process. The enhanced rate of photobleaching in areas of cell-monolayer contact, in contrast, does not follow a single exponential. As the reaction progresses, the enhanced rate of photobleaching slows to become nearly identical to that observed in areas devoid of cells (see the inset to Fig. 1). The biphasic kinetics suggest that reactant(s) other than fluorescein are depleted during the photobleaching.

We next examined the role of active oxygen intermediates in the enhanced photobleaching process. Compounds capable of depleting O_2^-, H_2O_2, or OH were added to macrophage suspensions and were present both during adherence of macrophages to the monolayers and during measurement of photobleaching rates. Superoxide dismutase eliminated the enhanced rate of photobleaching and the formation of 'black holes'. Boiling of superoxide dismutase destroyed the inhibitory effect. Addition of catalase (to remove H_2O_2) or mannitol (to remove hydroxyl radical), both at relatively high concentrations, had minimal effect (Fig. 2).

Enhanced photobleaching was not observed when cells of the RAW264 macrophage line were used instead of guinea-pig macrophages. The RAW264 cells bear Fc receptors, are phagocytic [12–14], and undergo a respiratory burst in response to anti-nitroxide IgG specifically bound to hapten-bearing phospholipid vesicles [15]. The RAW264 cells are, however, unusual in that they do not release O_2^- during their respiratory burst, but instead produce a delayed release of H_2O_2 (Ref. 15 and Hafeman, D.G., Orencole, S., and McGown, E.L., unpublished observations).

Enhanced photobleaching was also observed when guinea-pig macrophages were allowed to adhere to membranes containing a fluorescein-labeled lipid *. For these experiments the monolayer membrane contained 1 mol% fluorescein-labeled lipid, 1 mol% hapten I, and 98% mol% dipalmitoylphosphatidylcholine. Nonfluorescent, anti-nitroxide IgG was bound to these membranes instead of FITC-anti-nitroxide IgG (used in previous experiments) so that the fluorescence of the lipid could be observed. In analogy with results obtained with FITC-IgG, the bound IgG induced macrophage adherence and spreading on the hapten-containing monolayer membrane. Similarly, enhanced photobleaching of the fluorescein-labeled lipid occurred under many of the adherent, spreading macrophages and was inhibited by superoxide dismutase.

Of the three fluorophores tested, fluorescein was most sensitive to O_2^--dependent photobleaching. In experiments otherwise identical to the one described above, no enhanced photobleaching was observed when the fluorescent lipid, NBD-PE, was substituted for the fluorescein-conjugated lipid. Adhesion and spreading of the macrophages on monolayer membranes containing either one of these two fluorescent lipids was morphologically

* Fluorescein-labeled lipid was a conjugation product of dichlorotriazinylaminofluorescein and phosphatidylethanolamine-glycine-glycine-glycine, kindly provided by Dr. John C. Owicki of the Department of Biophysics and Medical Physics, University of California, Berkeley, CA.

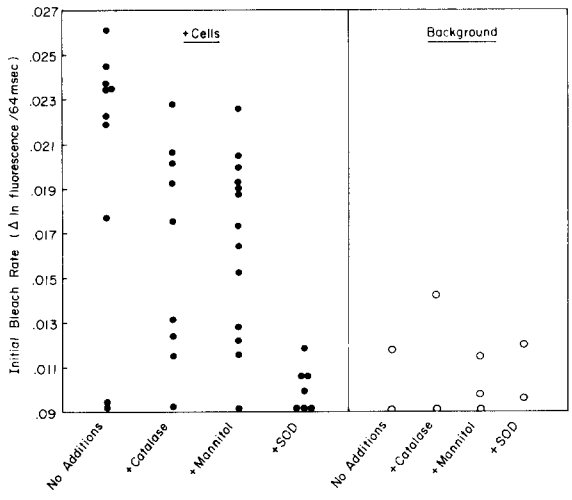
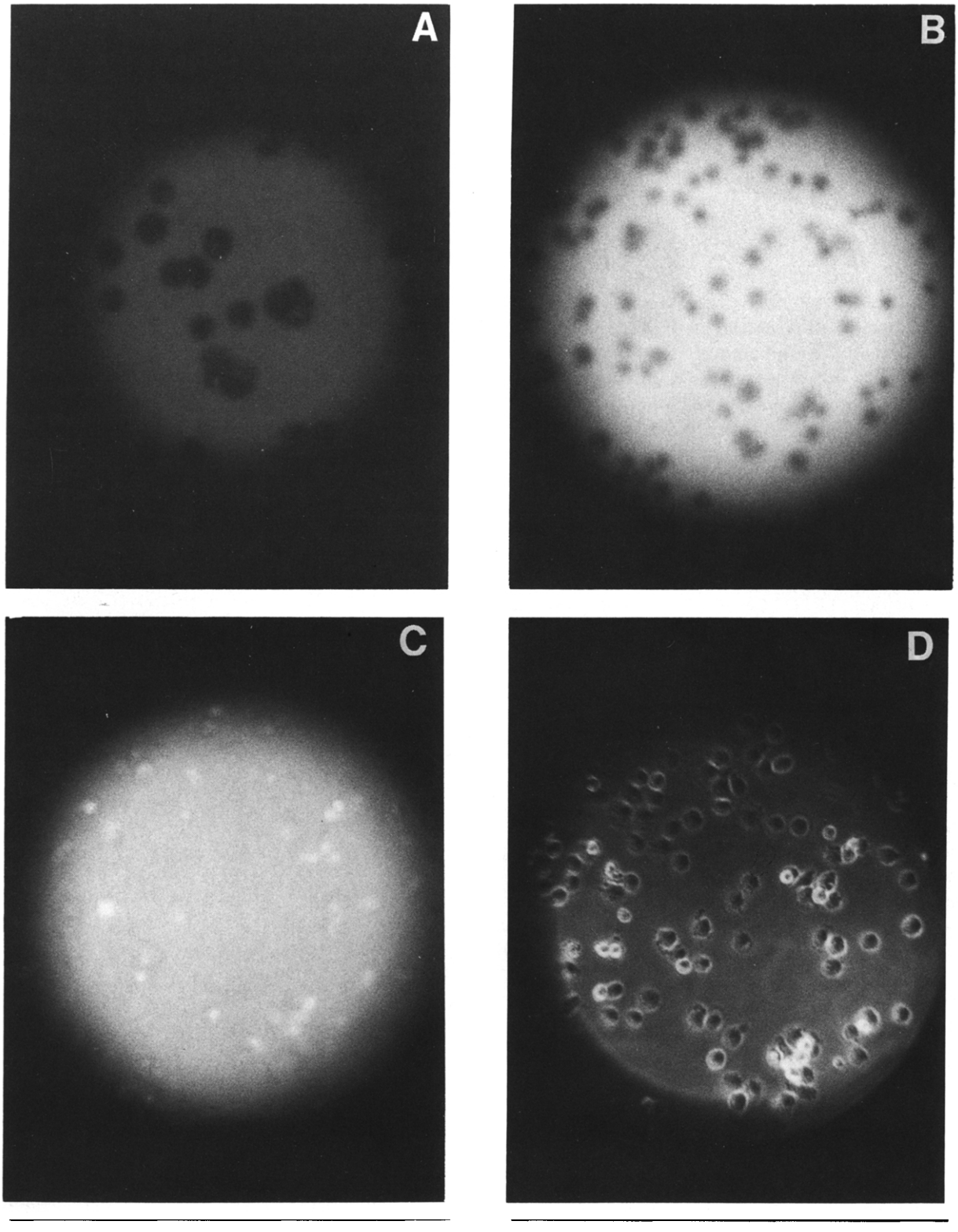


Fig. 2. Effect of catalase, mannitol and superoxide dismutase (SOD) on the rates of photobleaching of monolayer membrane-bound, FITC-anti-nitroxide IgG. Each dot represents the initial photobleaching rate of a separate region of the monolayer membrane, either devoid of cells (Background) or directly apposed to an individual bound macrophage (+Cells). The initial, first-order, decay in fluorescence during the first 2.0 seconds of each bleach was computer fit by least squares regression analysis to give the initial rate of photobleaching.

indistinguishable. Photobleaching of tetramethylrhodamineisothiocyanate (TRITC)-labeled IgG was tested on membranes containing 2 mol% of the lipid hapten, DNP-cap-PE, to which TRITC-anti-DNP IgG was bound. The TRITC-IgG under these conditions showed little, if any, enhanced photobleaching in the regions of immunologically-bound, guinea-pig macrophages. Similar membranes with bound FITC-anti-DNP IgG instead of TRITC-anti-DNP IgG showed the characteristic enhanced photobleaching with the same preparation of macrophages (Fig. 3A). In a similar series of experiments, the monolayer membranes were doubly labeled with both TRITC- and FITC-IgG. First, TRITC-anti-DNP was added, the non-bound fraction was washed away, and then a FITC-labeled, anti-antibody (goat, anti-rabbit IgG) was added. After washing to remove free anti-antibody, separate analysis of FITC and TRITC fluorescence showed that both types of IgG were bound. Incubation of macrophages with these doubly labeled membranes resulted in enhanced photobleaching of FITC-IgG, but not of TRITC-IgG in the same areas of macrophage-monolayer contact (Fig. 3B–D).

Because neutrophils secrete large quantities of O_2^- in response to phagocytic or various soluble stimuli (for reviews see Refs. 16–18), the response of neutrophils to specific antibody-coated monolayer membranes was tested. Fluorescein-labeled, anti-nitroxide IgG was specifically bound to monolayer membranes containing 1 mol% nitroxide hapten I in an identical fashion to the first series of experiments with macrophages. Incubation of neutrophils in contact with these membranes at 37°C resulted in a marked increase in cell elongation and motility. These cellular responses were not observed when neutrophils settled onto membranes devoid of IgG. Enhanced photobleaching, similar to that observed with macrophages occurred during initial incubation at 37°C. After 3–5 min of incubation in the dark, however, loss of fluorescence in areas of neutrophil-monolayer membrane contact was already evident when a new microscope field was first inspected under illumination. Neither superoxide dismutase, catalase, nor mannitol inhibited this non-photochemical loss of fluorescence. The non-photochemical loss of fluorescence, in combination with the dramatic increase in motility of neutrophils, resulted in the appearance of 'black tracks' in a uniformly fluorescent field of bound FITC-IgG after a 30 min, 37°C incubation in the dark. The 'black tracks' appear to be a record of cell migration; each track terminated at a cell. Periodic observations during the 37°C incubation directly confirmed this interpretation. To test whether neutrophils were destroying the lipid monolayer, membranes with 1 mol% of the fluo-re-

Fig. 3. Fluorescence 'black holes' produced by guinea-pig macrophages in monolayer-bound FITC-IgG during fluorescence illumination at 37°C. (A) fluorescence of FITC-anti-DNP IgG bound to monolayers with 2 mol% DNP-cap-PE; (B). FITC emission from similar monolayer membranes bearing both TRITC-anti-DNP IgG and FITC-anti-IgG IgG; (C). TRITC emission from the same preparation; (D). phase contrast, transmitted-light photomicrograph of the same preparation shown in B and C. The phase-dark cells are adherent and spread, whereas phase-light cells are adherent but not spread; in general, some, but not all, phase-dark cells show the O_2^--dependent photobleaching of fluorescein. It is possible to map the bleached areas in the fluorescence photomicrograph (B) to corresponding cells in the transmitted-light photomicrograph (D).


A
B
C
D

scent lipid, NBD-PE, 1 mol% nitroxide hapten I, and bound nonfluorescent antinitroxide IgG were used. These membranes triggered the same elongation and increased motility of neutrophils, but there was no loss of lipid fluorescence under the cells. Thus, the 'black tracks' are not due to modification of the lipid monolayer but instead are due to alteration of membrane-bound FITC-IgG.

Discussion

Model membranes with specifically bound IgG stimulate the respiratory burst and O_2^- release in macrophages and neutrophils [1,15,19]. We have shown that this stimulation results in enhanced photobleaching of fluorescein bound in the area of cell-membrane contact. The following observations indicate that this reponse is due to O_2^-: (1) enhanced photobleaching does not occur in the presence of superoxide dismutase; (2) RAW264 macrophages, which have a respiratory burst without the secretion of O_2^-, do not produce enhanced photobleaching.

Under the experimental conditions, fluorescein, but neither TRITC nor NBD fluorophores, exhibited O_2^--facilitated photobleaching. This property of fluorescein was demonstrated with both dichlorotriazinylaminofluorescein-labeled phospholipid incorporated in the monolayer membrane and with membrane-bound, FITC-labeled IgG. The mechanism of O_2^--facilitated photobleaching is not known. As an oxygen-peroxide redox intermediate, O_2^- readily acts as either a one-electron acceptor or donor [20–22]. Fluorescein similarly is susceptible to both one-electron photo-oxidation and one-electron photo-reduction [23–26]. Kinetic studies have shown that the relatively long-lived triplet excited state of fluorescein ($^3FH_2$) is the photo-reactive species in either case [23]. The following reactions can be predicted on the basis of O_2^- and fluorescein chemistry

$$^3FH_2 + O_2^- + H^+ \rightarrow FH + H_2O_2 \quad (1)$$

or

$$^3FH_2 + O_2^- + H^+ \rightarrow FH_3 + {}^1O_2 \quad (2)$$

In Reaction 1, O_2^- acts as a one-electron oxidant of triplet fluorescein. The oxidized fluorescein radical (FH) is assumed to be a phenoxy radical and has a life-time of less than 1 ms. This unstable species rearranges to form unknown bleached products [23]. In Reaction 2, O_2^- acts as a one-electron reductant of triplet fluorescein to generate the semi-reduced fluorescein free-radical (FH_3). Semi-reduced fluorescein, a triphenylmethyl type radical, is relatively stable in the absence of available electron acceptors [23,27,28]. Molecular oxygen, present in aerated solutions, acts as an electron acceptor that reoxidizes the semi-reduced radical to ground state fluorescein, as shown in Reaction 3

$$FH_3 + O_2 \rightarrow FH_2 + O_2^- + H^+ \quad (3)$$

Thus, no net bleaching occurs as the net result of Reactions 2 and 3. It is possible, however, that singlet (Δ) oxygen, is formed as a result of the reduction of triplet fluorescein by O_2^- in Reaction 2. The electrophilic $^1O_2$ species adds to olefins and aromatic compounds to form peroxides [29] and thus is expected to oxidize both excited and ground state fluorescein

$$\text{fluorescein} + {}^1O_2 \rightarrow \text{oxidized (bleached) products} \quad (4)$$

Therefore, Reaction 2 may lead indirectly to photobleaching via generation of $^1O_2$. In contrast to the slower rate of O_2^--independent photobleaching, the O_2^--enhanced reaction does not follow simple pseudo-first-order kinetics. Thus, a reactive species other than fluorescein (such as O_2^-) is apparently depleted during the photobleaching process. This would seem to rule out the photo-reductive mechanism (Reactions 2–4) where O_2^- is continually regenerated from semi-reduced fluorescein and oxygen. We cannot, however, exclude the possibility that oxygen, instead of O_2^-, becomes rate-limiting as the photo-reductive reaction proceeds.

Various antioxidant or reducing compounds such as *n*-propyl gallate, *p*-phenylenediamine, ascorbic acid, or dithionite have been used to decrease the rates of photobleaching of stains such as fluorescein that are used in fluorescence microscopy [30–33]. Analogous to O_2^- in Reaction 2, these compounds are electron-donors for triplet

dye species, and thus lower the concentration of reactive triplet dye during photo-excitation [23]. Although not tested because of possible deleterious effects on the O_2^--producing cells, the antioxidant compounds would be expected to inhibit O_2^--dependent photobleaching of fluorescein.

Lipid monolayer membranes are useful in studies of cell-cell recognition processes at the level of membrane-membrane interaction. The present study demonstrates the use of fluorescent monolayer membranes in detection of immune attack by O_2^--releasing cells. Various types of cells, including neutrophils and macrophages [15–17], eosinophils [34,35], and certain NK cells [36] release large quantities of O_2^- during immune attack. Smaller amounts of O_2^- may be released by other cells mediating immune attack but could be difficult to detect. The present photobleaching technique offers promise as a sensitive method for detection of the O_2^- released into the small aqueous volume between cytotoxic immune cells and target membranes.

Neutrophils in contact with target membranes bearing FITC-IgG caused both a photochemical and a nonphotochemical loss of fluorescence. Experiments with added superoxide dismutase showed that the nonphotochemical loss of IgG fluorescence was not dependent on O_2^-. Experiments with the fluorescent lipid, NBD-PE, and bound nonfluorescent IgG ruled out the possibility that neutrophils destroyed the lipid monolayer membrane. Although not directly demonstrated, we suspect that the nonphotochemical loss of fluorescence was due to cleavage of FITC-IgG from the monolayer surface by protease(s) released from neutrophils during lysosomal degranulation. Elastase and cathepsin G, two major serine proteases contained in azurophilic granules of human neutrophils [37], cleave IgG [38–41].

Acknowledgements

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