Phototoxicity of halogenofluorescein derivatives in *Dictyostelium discoideum* amoebae: comparison of 2',4',5',7'-tetrabromofluorescein- and 4',5'-diiodofluorescein dextran

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The halogenated fluorescein derivatives: 2',4',5',7'-tetrabromofluorescein isothiocyanate dextran (Br₄FD) and 4',5'-diiodofluorescein isothiocyanate dextran (I₂FD), were found to be efficient photosensitizers for the production of singlet oxygen. The singlet oxygen quantum yields were determined by reaction with acceptors in aqueous solution. Their comparison showed that the singlet oxygen quantum yield of Br₄FD was threefold higher than that of I₂FD. Br₄FD was more resistant to photobleaching than I₂FD. Both derivatives were internalized by fluid-phase pinocytosis in amoebae of the cellular slime mould *Dictyostelium discoideum*. Subsequently, illumination of cells led to a dose-dependent loss of viability consistent with a role for singlet oxygen generated inside the endosomal compartments in the mechanism of photoinjury. Br₄FD showed a three-fold higher efficiency than I₂FD for photoinduced cytotoxicity.

Introduction

Endocytosis is the process characteristic of eukaryotic cells that internalizes extracellular components in vesicles derived originally from their plasma membrane (for reviews, see Steinman *et al.*, 1983; Gruenberg & Howell, 1989; Van Deurs *et al.*, 1989; Courtoy, 1991). In the slime mould *Dictyostelium discoideum*, endocytosis constitutes the major pathway for nutrient uptake in amoebae (North, 1983). This haploid organism is well adapted for genetic studies and mutants defective in phagocytosis and fluid-phase pinocytosis have already been obtained (Vogel, 1980; Waddell *et al.*, 1987; Ebert *et al.*, 1989; Bof *et al.*, 1992; Labrousse & Satre, 1993). Clathrin-deficient amoebae, created by antisense RNA technique, are severely impaired in fluid-phase pinocytosis (O'Halloran & Anderson, 1992). One of our objectives is to characterize efficient photosensitizer molecules targeted selectively towards the endosomal compartments in order to select new mutants of endocytosis in *Dictyostelium* amoebae. The underlying

strategy relies on the fact that in a mixed population, wild-type amoebae having their endosomal compartments loaded with the sensitizer will be killed upon illumination, but endocytosis mutants will be spared as they are unable to internalize the sensitizer. Thus, photodynamic action will lead to an enrichment for endocytosis mutants. Among halogenofluorescein derivatives (Fig. 1), we characterized previously 4',5'-diiodofluorescein dextran (I₂FD) as an effective photosensitizer (Labrousse & Satre, 1993). In this work, we report that the related compound: 2',4',5',7'-tetra-

Fig. 1. Structure of FD. The backbone of glucose units in dextran is indicated by the Glc chain. The derivatives used in this study were either iodinated on positions 4' and 5' (I₂FD) or brominated on positions 2', 4', 5' and 7' (Br₄FD) of the fluorescein moiety.

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Abbreviations: Br₄FD, 2',4',5',7'-tetrabromofluorescein isothiocyanate dextran; FD, fluorescein isothiocyanate dextran; I₂FD, 4',5'-diiodofluorescein isothiocyanate dextran.

bromofluorescein dextran (Br₄FD) has improved characteristics for the above purpose.

Methods

Synthesis of fluorescein-, 4',5'-diiodofluorescein-, and 2',4',5',7'-tetrabromofluorescein dextran. Fluorescein dextran (FD) and Br₄FD were prepared by the dibutyltin dilaurate-catalysed reaction of dextran (70000 average *M_r*) with either fluorescein isothiocyanate or 2',4',5',7'-tetrabromofluorescein (eosin) isothiocyanate (De Belder & Granath, 1973; Cherry, 1978). Iodination of FD on positions 4' and 5' of the fluorescein moiety was performed with I₂ (Labrousse & Satre, 1993; Devanthan *et al.*, 1990). The degree of substitution of dextran was determined spectrophotometrically (Table 1) and was 5.9 for both FD and I₂FD, and 1.9 for Br₄FD. Data were normalized on the basis of chromophore per dextran.

Singlet oxygen measurement. Singlet oxygen was trapped with histidine to form an endoperoxide that, in turn, bleached *p*-nitrosodimethylaniline (Kraljic & El Moshni, 1978). I₂FD, Br₄FD or Rose Bengal (2',4',5',7'-tetraiodo-3,4,5,6-tetrachlorofluorescein) solutions in 50 mM-potassium phosphate buffer, pH 5.8, containing 33 µM-*p*-nitrosodimethylaniline and 10 mM-histidine were illuminated at 25 °C with a 75 W tungsten lamp giving a power density of 0.25–0.3 mW cm⁻² at the level of the samples. Variation of *A*₄₄₀ was measured against an identical sample kept in the dark.

Cell culture. *Dictyostelium discoideum* amoebae (strain AX2, ATCC 24397) were grown axenically on orbital shakers rotating at 170 r.p.m. at 21 °C in a peptone–yeast extract medium containing maltose (18 g l⁻¹) as carbon source (Watts & Ashworth, 1970).

Fluid-phase pinocytosis assay. Fluid-phase pinocytosis assay was conducted with amoebae (5 × 10⁶ ml⁻¹) suspended at 21 °C in axenic medium containing 1–10 mg ml⁻¹ Br₄FD as described previously for FD (Klein & Satre, 1986). Fluorescence excitation and emission wavelengths of Br₄FD are indicated in Table 1. The amount of intracellular Br₄FD was determined by comparison with a standard curve and converted to an equivalent volume of internalized fluid (endocytic index).

Table 1. Characteristics of I₂FD, Br₄FD and FD

	Br ₄ FD	I ₂ FD	FD
Absorption properties			
$\lambda_{\max}$ (nm)*	521	511	493
ϵ (M ⁻¹ cm ⁻¹)*†	8 × 10 ⁴	5.94 × 10 ⁴	8.04 × 10 ⁴
pKa	≈ 2.5	5.1 ± 0.2	6.3 ± 0.2
Fluorescence properties			
λ_{exc} (nm)*	523	513	497
λ_{em} (nm)*	543	537	518
Quantum yield (Φ_F)*‡	0.19	0.022	1
Photodynamic properties			
Singlet oxygen quantum yield (Φ_Δ)	0.49	0.17	ND
Bleaching constant, <i>k</i> (min ⁻¹)¶	0.0090	0.0189	0.0032

ND, Not determined.

* $\lambda_{\max}$, ϵ , Φ_F , λ_{exc} and λ_{em} were determined at pH 8.

† ϵ , Molar absorption coefficient at $\lambda_{\max}$.

‡ Φ_F values are relative to Φ_F (FD) taken as unity.

|| Φ_Δ values were measured at pH 5.8, scaled to Φ_Δ (Rose Bengal) = 0.75 (Gandin *et al.*, 1983).

¶ $A(t) = A(t_0) \cdot \exp(-k \cdot t)$, measured at pH 5.8.

Photocytotoxicity measurements. *Dictyostelium* amoebae were incubated for 3 h at 21 °C with either I₂FD or Br₄FD as described for the fluid-phase pinocytosis assay. Washed amoebae (1 × 10⁷) were suspended in 2 ml potassium phosphate buffer and transferred to a 5 cm diameter Petri dish maintained at 0–4 °C. They were illuminated for 10 min with a 150 W tungsten halide lamp (Schott KL1500) connected to a flexible 4 mm diameter light guide fitted with a 20° lens and focused to a 6 cm diameter spot. At the level of cells, the power density of this light setting was 8–10 mW cm⁻². In parallel, a control sample was kept in the dark. Samples were taken for plating in association with the bacterium *Klebsiella aerogenes* (Sussman, 1987). After 4 d at 21 °C, the number of plaques was counted and divided by the number of cells obtained initially from Coulter counter measurements to give the proportion of viable cells. Four identical plates were used for each dilution and the number of plated amoebae was adjusted to give between 30–60 plaques on a 10 cm diameter Petri dish.

Results and Discussion

Optical properties of FD, I₂FD and Br₄FD

Light absorption characteristics of Br₄FD were determined as a function of pH and compared to FD and I₂FD. Selected spectra are shown in Fig. 2 both at pH 8, where the dianionic forms of the fluorescein derivatives predominate, and at pH 5.8, which corresponds to the average endosomal pH in *Dictyostelium* AX2 amoebae (Bof *et al.*, 1992). Absorption characteristics are summarized in Table 1, together with apparent pKa values for the equilibrium: monoanion = dianion + H⁺. The spectra of Br₄FD (pKa ≈ 2.5) were identical at both pH 5.8 and pH 8. I₂FD (pKa = 5.0) had a slightly reduced absorbance at pH 5.8 as compared to pH 8. In contrast, FD (pKa = 6.3) showed a two-fold stronger absorption at alkaline than at acidic pH. The changes in $\lambda_{\max}$ of the chromophores on conjugation to dextran were minimal. Fluorescence properties were also examined as halogeno-substituted fluorescein derivatives have reduced fluorescent quantum yields (Φ_F) (Ullman & Khanna, 1981). Emission and excitation wavelengths are indicated in Table 1. Although Φ_F (Br₄FD) was five-fold lower than Φ_F (FD), the value was still about 10-fold higher than Φ_F (I₂FD) and allowed an easy fluorimetric detection of small quantities of Br₄FD. Fluorescence properties were in full agreement with data on fluorescein and 2',4',5',7'-tetrabromofluorescein (Adelman & Oster, 1956).

Photobleaching

When a photosensitizer solution is illuminated, some of its molecules are bleached. As bleached products remained inert as photodynamic agents, the effective concentration of sensitizer will decrease. We have determined the kinetics of bleaching of FD, I₂FD and Br₄FD in buffer adjusted to *Dictyostelium* endosomal pH (pH 5.8) and using the illumination conditions described for cytotoxicity experiments (Fig. 3). Time constants for

Fig. 2. Absorption spectra of FD, I_2FD and Br_4FD . FD (A; 0.15 mg ml^{-1}), I_2FD (B; 0.2 mg ml^{-1}) and Br_4FD (C; 0.5 mg ml^{-1}), were suspended in 50 mM -potassium phosphate buffer at pH 8 (a) or pH 5.8 (b).

Fig. 3. Kinetics of FD, I_2FD and Br_4FD photobleaching. FD (0.43 mg ml^{-1}), I_2FD (0.18 mg ml^{-1}) and Br_4FD (0.38 mg ml^{-1}) solutions in 50 mM -potassium phosphate buffer, pH 5.8, were illuminated as described for photocytotoxicity determinations (see Methods). At indicated times, absorbance was measured at 483, 511 and 521 nm for FD (■), I_2FD (▲) and Br_4FD (●), respectively.

bleaching were calculated on the basis of a simple exponential decrease in absorbance and are shown in Table 1. Bleaching was increased by substitution and was fastest for I_2FD , whereas Br_4FD was two-times less

Fig. 4. I_2FD -, Br_4FD -, and Rose Bengal-mediated singlet oxygen production. The rate of decrease in absorbance of the *p*-nitrosoaniline solution was measured as a function of concentration of Rose Bengal (■), and of the dextran derivatives I_2FD (▲) or Br_4FD (●). The inset plot shows the dependence of the rate of *p*-nitrosoaniline bleaching on the concentration of histidine, in the presence of 0.04 mg l^{-1} I_2FD (▲) or Br_4FD (●) ml^{-1} .

sensitive than I_2FD . Results were in agreement with earlier data on fluorescein derivatives showing that iodine atoms conferred more sensitivity to bleaching than bromine groups (Valenzano & Pooler, 1982).

Singlet oxygen generation by I_2FD and Br_4FD

Fig. 4 illustrates the abilities of I_2FD and Br_4FD to mediate bleaching of *p*-nitrosoaniline in the presence of histidine, in comparison with that of Rose Bengal, a classical singlet oxygen producer (Valenzano, 1987). The inset shows the characteristic bell-shaped dependence of rate of bleaching on histidine concentration (Kraljic & El Moshni, 1978); maximum bleaching was obtained at 10 mM -histidine. Singlet oxygen generation by I_2FD and Br_4FD was determined as a function of their concentrations and the efficiency of the two derivatives was compared to that of Rose Bengal. The initial slopes are proportional to the relative values of singlet oxygen quantum yield Φ_A (Gandin *et al.*, 1983; Blum & Grossweiner, 1985). Data were scaled to $\Phi_A(\text{Rose Bengal}) = 0.75$ (Gandin *et al.*, 1983) and normalized to identical substitution to give $\Phi_A(I_2FD) = 0.17$ and $\Phi_A(Br_4FD) = 0.49$. These values are lower than the corresponding values for free chromophores: $\Phi_A(4',5'$ -diiodofluorescein) = 0.48 and $\Phi_A(2',4',5',7'$ -tetrabromofluorescein) = 0.57 (Gandin *et al.*, 1983).

Br_4FD as fluid-phase marker in *Dictyostelium amoebae*

FD and I_2FD have been characterized as typical fluid-phase markers in *Dictyostelium* (Labrousse & Satre, 1993; Thilo & Vogel, 1980; Klein & Satre, 1986).

Fig. 5. Fluid-phase pinocytic uptake of Br₄FD. *Dictyostelium* amoebae (5×10^6 cells ml⁻¹) were incubated either at 21 °C (○) or 0 °C (●) in growth medium with 2 mg Br₄FD ml⁻¹ and the amount of internalized Br₄FD was determined at the indicated times. Uptake of Br₄FD was expressed as fluid entry volume (V , pl per cell) as a function of time (t , min) and fitted to a single compartment model. The line drawn through the data points represents the function $V = 0.63[1 - \exp(-0.016 \times t)]$. The inset graph shows the amount of internalized Br₄FD after 3 h incubation at 21 °C in growth medium as a function of extracellular Br₄FD concentration.

Fig. 6. Phototoxicity of I₂FD and Br₄FD. *Dictyostelium* amoebae were incubated in axenic growth medium containing 1–10 mg I₂FD (▲) or Br₄FD (●) ml⁻¹. After 3 h at 21 °C, internalized I₂FD or Br₄FD were measured. Then, washed cells were illuminated at 0–4 °C for 10 min before cell viability measurements.

Kinetics of entry of Br₄FD in *Dictyostelium* amoebae (Fig. 5) followed a course similar to that reported for FD or I₂FD (Ebert *et al.*, 1989; Bof *et al.*, 1992; Labrousse & Satre, 1993; Klein & Satre, 1986). Br₄FD was internalized with an influx rate of 0.007 pl per cell min⁻¹ up to a final plateau corresponding to an apparent volume of 0.63 pl per cell. The intracellular uptake of Br₄FD increased in proportion to its extracellular concentration and showed no evidence of saturation up

to 10 mg ml⁻¹. At 0 °C, the amoebae did not accumulate Br₄FD appreciably. These properties fully support the hypothesis that Br₄FD enters in *Dictyostelium* amoebae by fluid-phase pinocytosis.

Phototoxic effect of I₂FD and Br₄FD on *Dictyostelium* amoebae

The phototoxicity of Br₄FD towards *Dictyostelium* amoebae was determined by cell viability measurements and data were compared to I₂FD phototoxicity (Fig. 6). Amoebae were incubated for 3 h with I₂FD or Br₄FD to ensure the full loading of endosomal compartments, then washed cells were illuminated at low temperature (0–4 °C) to avoid any exocytic efflux of I₂FD or Br₄FD as well as photothermal effects, and the proportion of viable cells was measured. Amoebae incubated with I₂FD or Br₄FD and, kept in the dark, remained fully viable. In illuminated samples, phototoxicity increased as a function of the amount of internalized fluid-phase markers. The slopes of the viable cell–sensitizer concentration relationships were similar for I₂FD and Br₄FD and the 50 % viability values were used to determine the relative photosensitizer effectiveness. It was apparent that Br₄FD was about three-fold more efficient than I₂FD, in agreement with *in vitro* data for the production of singlet oxygen (see above and Fig. 4).

We believe that this compound will be useful for the isolation of a battery of endocytosis mutants. It should then be possible to identify genes involved directly in the endocytosis pathways as well as genes that may have secondary control functions.

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References

- ADELMAN, A. H. & OSTER, G. (1956). Long-lived states in photochemical reactions. II. Photoreduction of fluorescein and its halogenated derivatives. *Journal of the American Chemical Society* **78**, 3977–3980.
- BLUM, A. & GROSSWEINER, L. I. (1985). Singlet oxygen generation by hematoporphyrin IX, uroporphyrin I and hematoporphyrin derivative at 546 nm in phosphate buffer and in the presence of egg phosphatidylcholine liposomes. *Photochemistry and Photobiology* **41**, 27–32.
- BOF, M., BRÉNOT, F., GONZALEZ, C., KLEIN, G., MARTIN, J. B. & SATRE, M. (1992). *Dictyostelium discoideum* mutants resistant to the toxic action of methylene diphosphonate are defective in endocytosis. *Journal of Cell Science* **101**, 139–144.
- CHERRY, R. J. (1978). Measurement of protein rotational diffusion in membranes by flash photolysis. *Methods in Enzymology* **54**, 47–61.
- COURTOY, P. J. (1991). Dissection of endosomes. In *Intracellular Trafficking of Proteins*, pp. 103–156. Edited by C. Steer & J. Hanover. Cambridge: Cambridge University Press.
- DE BELDER, A. N. & GRANATH, K. (1973). Preparation and properties of fluorescein labeled dextrans. *Carbohydrate Research* **30**, 375–378.
- DEVANATHAN, S., DAHL, T. A., MIDDEN, W. R. & NECKERS, D. C. (1990). Readily available fluorescein isothiocyanate-conjugated anti-

- bodies can be easily converted into targeted phototoxic agents for antibacterial, antiviral, and anticancer properties. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 2980–2984.
- EBERT, D. L., FREEZE, H. H., RICHARDSON, J., DIMOND, R. L. & CARDELLI, J. A. (1989). A *Dictyostelium discoideum* mutant that missorts and oversecreted lysosomal enzyme precursors is defective in endocytosis. *Journal of Cell Biology* **109**, 1445–1456.
- GANDIN, E., LION, Y. & VAN DE VORST, A. (1983). Quantum yield of singlet oxygen production by xanthene derivatives. *Photochemistry and Photobiology* **37**, 271–278.
- GRUENBERG, J. & HOWELL, K. E. (1989). Membrane traffic in endocytosis: insights from cell-free assays. *Annual Review of Cell Biology* **5**, 453–481.
- KLEIN, G. & SATRE, M. (1986). Kinetics of fluid-phase pinocytosis in *Dictyostelium discoideum* amoebae. *Biochemical and Biophysical Research Communications* **138**, 1146–1152.
- KRALJIC, I. & EL MOSHNI, S. (1978). A new method for the detection of singlet oxygen in aqueous solutions. *Photochemistry and Photobiology* **28**, 577–581.
- LABROUSSE, A. & SATRE, M. (1993). Photodynamic killing of *Dictyostelium discoideum* amoebae mediated by 4',5'-diiodofluorescein isothiocyanate dextran. A strategy for the isolation of thermo-conditional endocytosis mutants. *Photochemistry and Photobiology* **57**, 531–537.
- NORTH, M. J. (1983). Solute uptake by *Dictyostelium discoideum* and its inhibition. *Journal of General Microbiology* **129**, 1381–1386.
- O'HALLORAN, T. J. & ANDERSON, R. G. W. (1992). Clathrin heavy chain is required for pinocytosis, the presence of large vacuoles, and development in *Dictyostelium*. *Journal of Cell Biology* **118**, 1371–1377.
- STEINMAN, R. M., MELLMAN, I. S., MULLER, W. A. & COHN, Z. A. (1983). Endocytosis and the recycling of plasma membrane. *Journal of Cell Biology* **96**, 1–27.
- SUSSMAN, M. (1987). Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions. *Methods in Cell Biology* **28**, 9–29.
- THILO, L. & VOGEL, G. (1980). Kinetics of membrane internalization and recycling during pinocytosis in *Dictyostelium discoideum*. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 1015–1019.
- ULLMAN, E. F. & KHANNA, P. L. (1981). Fluorescence excitation transfer immunoassay (FETI). *Methods in Enzymology* **74**, 28–60.
- VALENZENO, D. P. (1987). Photomodification of biological membranes with emphasis on singlet oxygen mechanisms. *Photochemistry and Photobiology* **46**, 147–160.
- VALENZENO, D. P. & POOLER, J. P. (1982). Cell membrane photomodification: relative effectiveness of halogenated fluoresceins for photohemolysis. *Photochemistry and Photobiology* **35**, 343–350.
- VAN DEURS, B., PETERSEN, O. W., OLNES, S. & SANDVIG, K. (1989). The ways of endocytosis. *International Review of Cytology* **117**, 131–177.
- VOGEL, G., THILO, L., SCHWARZ, H. & STEINHART, R. (1980). Mechanism of phagocytosis in *Dictyostelium discoideum*: phagocytosis is mediated by different recognition sites as disclosed by mutants with altered phagocytotic properties. *Journal of Cell Biology* **86**, 456–465.
- WADDELL, D. R., DUFFY, K. & VOGEL, G. (1987). Cytokinesis is defective in *Dictyostelium* mutants with altered phagocytic recognition adhesion and vegetative cell cohesion properties. *Journal of Cell Biology* **105**, 2293–2300.
- WATTS, D. J. & ASHWORTH, J. M. (1970). Growth of myxamoebae of the cellular slime mould *Dictyostelium discoideum* in axenic culture. *Biochemical Journal* **119**, 171–174.