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-

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

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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.
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 Mₓ) 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; Devathan 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, Br₂FD 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 (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; Devathan 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.

**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 (9 g l⁻¹) as carbon source (Watts & Ashworth, 1970).

**Fluid-phase pinocytosis assay.** Fluid-phase pinocytosis assay was conducted with amoebae (5 x 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 (endoctylic index).

**Table 1. Characteristics of I₂FD, Br₂FD and FD**

<table>
<thead>
<tr>
<th></th>
<th>Br₂FD</th>
<th>I₂FD</th>
<th>FD</th>
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<tbody>
<tr>
<td>Absorption</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>λmax (nm)*</td>
<td>521</td>
<td>511</td>
<td>493</td>
</tr>
<tr>
<td>ε (M⁻¹ cm⁻¹)†</td>
<td>8 x 10⁶</td>
<td>5.94 x 10⁴</td>
<td>8.04 x 10⁴</td>
</tr>
<tr>
<td>pKa</td>
<td>≈ 2.5</td>
<td>5.1 ± 0.2</td>
<td>6.3 ± 0.2</td>
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<tr>
<td>Fluorescence</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>λexc (nm)*</td>
<td>523</td>
<td>513</td>
<td>497</td>
</tr>
<tr>
<td>λem (nm)*</td>
<td>543</td>
<td>537</td>
<td>518</td>
</tr>
<tr>
<td>Quantum yield (Φₐ)*‡</td>
<td>0.19</td>
<td>0.022</td>
<td>0.1</td>
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<tr>
<td>Photobleaching</td>
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<tr>
<td>Singlet oxygen</td>
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<tr>
<td>quantum yield (Φₐ)</td>
<td>0.090</td>
<td>0.0189</td>
<td>ND</td>
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<tr>
<td>k (min⁻¹)¶</td>
<td>0.0032</td>
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<td></td>
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</table>

ND, Not determined.

*λmax, ε, Φₐ, AₚK and λem were determined at pH 8.
† ε, Molar absorption coefficient at λmax.
‡ Φₐ values are relative to Φₐ(FD) taken as unity.
¶ Φₐ values were measured at pH 5.8, scaled to Φₐ(Rose Bengal) = 0.75 (Gandin et al., 1983).
¶ A(t) = A(t₀)exp(−k·t), measured at pH 5.8.

**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...
Photodynamic killing of Dictyostelium amoebae

Fig. 2. Absorption spectra of FD, I₂FD and Br₄FD. FD (A; 0.15 mg ml⁻¹), I₂FD (B; 0.2 mg ml⁻¹) and Br₄FD (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, I₂FD and Br₄FD photobleaching. FD (0.43 mg ml⁻¹), I₂FD (0.18 mg ml⁻¹) and Br₄FD (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 (■), I₂FD (▲) and Br₄FD (●), 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 I₂FD, whereas Br₄FD was two-times less sensitive than I₂FD. Results were in agreement with earlier data on fluorescein derivatives showing that iodine atoms conferred more sensitivity to bleaching than bromine groups (Valenzeno & Pooler, 1982).

Singlet oxygen generation by I₂FD and Br₄FD

Fig. 4 illustrates the abilities of I₂FD and Br₄FD to mediate bleaching of p-nitrosoaniline in the presence of histidine, in comparison with that of Rose Bengal, a classical singlet oxygen producer (Valenzeno, 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 I₂FD and Br₄FD 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 Φₐ (Gandin et al., 1983; Blum & Grossweiner, 1985). Data were scaled to Φₐ(Rose Bengal) = 0.75 (Gandin et al., 1983) and normalized to identical substitution to give Φₐ(I₂FD) = 0.17 and Φₐ(Br₄FD) = 0.49. These values are lower than the corresponding values for free chromophores: Φₐ(4',5'-diiodofluorescein) = 0.48 and Φₐ(2',4',5',7'-tetrabromo-fluorescein) = 0.57 (Gandin et al., 1983).

Br₄FD as fluid-phase marker in Dictyostelium amoebae

FD and I₂FD have been characterized as typical fluid-phase markers in Dictyostelium (Labrousse & Satre, 1993; Thilo & Vogel, 1980; Klein & Satre, 1986).
Kinetics of entry of Br₂FD in *Dictyostelium* ameobae (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). Br₂FD was internalized with an influex 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 Br₂FD 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 Br₂FD appreciably. These properties fully support the hypothesis that Br₂FD enters in *Dictyostelium* ameobae by fluid-phase pinocytosis.

Phototoxic effect of I₂FD and Br₂FD on *Dictyostelium* ameobae

The phototoxicity of Br₂FD towards *Dictyostelium* ameobae was determined by cell viability measurements and data were compared to I₂FD phototoxicity (Fig. 6). Ameobae were incubated for 3 h with I₂FD or Br₂FD 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 Br₂FD as well as photothermal effects, and the proportion of viable cells was measured. Ameobae incubated with I₂FD or Br₂FD 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 Br₂FD and the 50% viability values were used to determine the relative photosensitizer effectiveness. It was apparent that Br₂FD 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**


