Effects of benzyl alcohol on transferrin and low density lipoprotein receptor mediated endocytosis in leukemic guinea pig B lymphocytes

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We demonstrated that benzyl alcohol, a neutral local anesthetic drug, inhibits the uptake and degradation of lowdensity lipoprotein and endocytosis of transferrin receptors of guinea pig leukemic B lymphocytes (L₂C). This inhibition is very rapid, concentration dependant and reversible by simple washing. Membrane fluidity of the living cells is also modified.

Receptor endocytosis; Inhibition; Benzyl alcohol; L₂C cell

1. INTRODUCTION

Local anesthetic drugs have been observed to affect the activity of numerous membrane proteins (enzymes [1–3], D-glucose transport [4], membrane receptors [5]) probably by modulating membrane fluidity [1–5]. Benzyl alcohol, an amphiphilic molecule, has often been used to investigate the relationship between enzyme activities and membrane fluidity; being neutral, it does not selectively interact with charged lipid species. This agent can readily partition into membranes from aqueous solution [6], and has well-defined effects on the fluidity of isolated plasma membranes [1] and living cells [2]. It was suggested from a study on asialoglycoprotein receptors that benzyl alcohol may be a selective inhibitor of the fusion step between lysosomes and endocytic vesicles [7]. In this study we demonstrate that benzyl alcohol strongly and reversibly inhibits endocytosis of two other receptors: low density lipoprotein (LDL) and transferrin (Tf) receptors of guinea pig leukemic B lymphocytes (L2C leukemia). These leukemic lymphocytes possess a large amounts of well-characterized LDL and Tf receptors [8,9]. Benzyl alcohol inhibition is very rapid, total and reversible.

2. MATERIALS AND METHODS

2.1. Biological materials

L₂C leukemia, affecting the B lymphocytes used in this study, arose spontaneously in a strain 2 guinea pig [10] and was passed to syngeneic animals. L₂C lymphocytes were harvested and purified at 20°C by lymphoprep (Pharmacia) gradient centrifugation, as described [11]. Cell viability was analysed by the Trypan blue method.

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2.2. Preparation and radiolabeling of LDL and Tf

Human LDL (d = 1.019–1.063) were isolated from healthy donors by the methods of Havel et al. [12] and radiolabeled (sp. act. about 200 cpm/ng prot.) as previously described [9].

Human Tf (Sigma) was radiolabeled either by ^{59}Fe or by ^{125}I (Amersham). Labeling by ^{59}Fe was performed as described in Dautry-Varsat et al. [13] (sp. act. varied from 20 to 40 cpm/ng of Tf). After saturation with iron, iodination of human Tf was performed with immobilized Enzymobead lactoperoxidase-glucose oxidase (Bio-Rad) as described in Vidal et al. [8]. Specific activity varied from 500 to 1000 cpm/ng protein. Protein content was determined by the Lowry method [14].

2.3. Binding and uptake assays

Binding, uptake and degradation of ^{25}I-LDL were performed in RPMI 1640 medium (Gibco) 150 μm CaCl₂ and MgCl₂ and MgCl₂ plus 10% delipoproteinized fetal calf serum plus 5 mg/ml bovine serum albumin (BSA), pH 7.4 (medium A), as previously described [9], in the presence or absence of an excess of unabeled LDL, and various concentrations of benzyl alcohol. Stock benzyl alcohol solutions in RPMI were prepared 10 times more concentrated than the final experimental concentrations. Benzyl alcohol was removed by 3 centrifugations (1000 × g) of cells incubated for 10 min at room temperature with 20 ml PBS-BSA (5 mg/ml) medium. Binding of ^{125}I-Tf was carried out as previously described [8] in the presence or absence of benzyl alcohol. For the ^{59}Fe-Tf uptake measurements, 5 × 10⁶ lymphocytes were incubated at 37°C in RPMI 1640 containing 50 μg/ml of ^{59}Fe-Tf (i.e. about 50-fold the K_d value calculated from the preliminary assay) bovine serum albumin (BSA) 5 mg/ml, pH 7.4 (medium B), in a total volume of 1 ml. At the end of incubation periods, samples were centrifuged (Beckman minifuge 11) and the radioactive supernatants removed. The cell pellets were washed in phosphate-buffered saline (PBS; Gibco), resuspended with 50 μl PBS and treated as previously described [9]. Final cell pellets were solubilized in NaOH (0.1 M), then protein contents were assayed by the method of Lowry [14] and radioactivity was determined using a Beckman LS 5000 TD model liquid scintillation counter. All data points in the figures represent average triplicate determinations (± SD) and were corrected for nonspecific measurements in the presence of an excess of unlabeled ligand.

2.4. Determination of the cell membrane lipid fluidity

5-Doxylpalmitic acid (10,3 F.A.) was dissolved in ethanol (10⁻³ M) and 10 μl aliquots were dried with a stream of N₂ in Eppendorf tubes. Samples (100 μl containing 2 × 10⁸ cells), preincubated with various

Table 1

Site numbers and dissociation constants for control and benzyl alcohol (30 mM) treated L2C cells

	Control		Benzyl alcohol	
	K_d	No.	K_d	No.
Tf	1.9×10^{-8} M	28 000	1.6×10^{-8} M	28 000
LDL	0.9×10^{-7} M	16 000	1.7×10^{-7} M	16 000

concentrations of benzyl alcohol (0–50 mM), were added to the probe and gently shaken for several minutes, then the mixture was aspirated in a disposable precalibrated pipette (50 μl). ESR spectra were immediately obtained at 37°C using a Brucker ER 200 D spectrometer. The order parameters (S and S(T∥)) were calculated as described in [1].

3. RESULTS

^{125}I-Tf and ^{125}I-LDL binding was observed at 4°C in the presence or absence of 30 mM benzyl alcohol. The number of receptors and their affinities for each ligand were determined by the method of Scatchard [15]. As shown in table 1, the presence of benzyl alcohol did not modify binding properties of the receptors. These values were in accordance with previous results [9].

Experiments have shown that benzyl alcohol acted very quickly: receptor internalization was inhibited after 2 min incubation at 37°C (data not shown).

Fig.1 shows the relationship between benzyl alcohol concentration and 1 h ^{59}Fe accumulation within the cells: 10 mM provoked more than 40–50% inhibition and 40 mM induced complete inhibition. The viability of cells measured by Trypan blue exclusion (insert fig.1) was not affected throughout the experiment (1 or 3 h), demonstrating that inhibition was not the result of a toxic effect of benzyl alcohol. The same inhibitory ef-
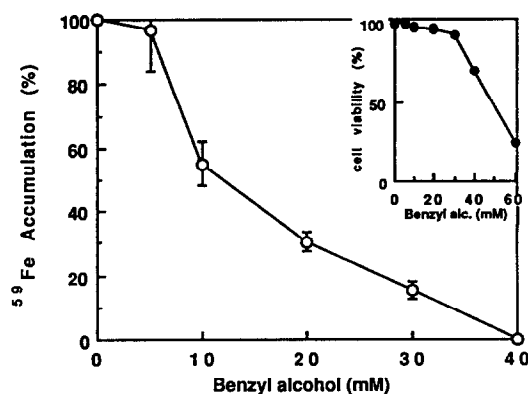
Fig.2. Relationship between ^{125}I-LDL degradation, uptake and benzyl alcohol with L2C lymphocytes. 5 × 10^6 cells were incubated at 37°C for 3 h, in medium A, 1 ml, with 20 μg ^{125}I-LDL, in the presence or absence of benzyl alcohol. The results (uptake (O), and degradation (●)) were expressed as % of control. (Insert) ^{125}I-LDL uptake in the presence of benzyl alcohol 30 mM (◆) and control (□).

fect was observed in the 3 h ^{125}I-LDL uptake (fig.2) which was reduced to 65% of control. There was a stronger degradation inhibition: 85% inhibition for 10 mM benzyl alcohol. Insert of Fig.2 shows the effect of 30 mM benzyl alcohol on the ^{125}I-LDL uptake time course.

Fig.3 presents the time course of ^{59}Fe accumulation into L2C cells at 37°C. In control cells, ligand uptake increased linearly with time. However, both concentrations of benzyl alcohol (20 mM for 1 h incubation and

Fig.1. Benzyl alcohol concentration/effect on ^{59}Fe accumulation within L2C lymphocytes. 5 × 10^6 cells were incubated at 37°C for 1 h, in medium B, 1 ml, with 50 μg ^{59}Fe-Tf in the presence or absence of various benzyl alcohol concentrations. The data (O) were expressed as % of control. Viability (insert (●)) was carried out by the trypan blue exclusion method after incubation in the same conditions as above.
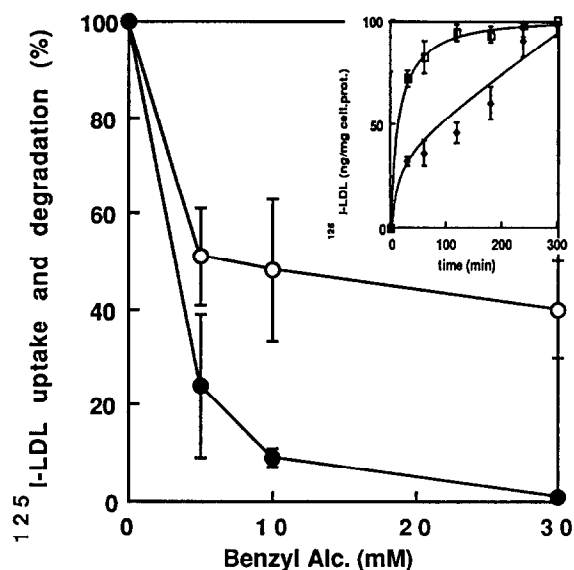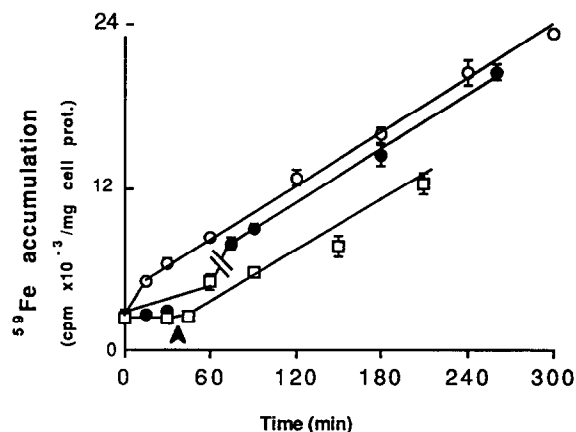
Fig.3. Time course of ^{59}Fe accumulation within L2C lymphocytes and reversibility of the benzyl alcohol effect. 5 × 10^6 cells were incubated at 37°C, in medium B, 1 ml, with 50 μg ^{59}Fe-T, with 0 (O), 20 mM (●), and 30 mM (□) benzyl alcohol. After 30 (▲) or 60 min (↘) incubation, cells were washed (as described in section 2), and the incubation was continued in the presence of ^{59}Fe-Tf, 50 μg.
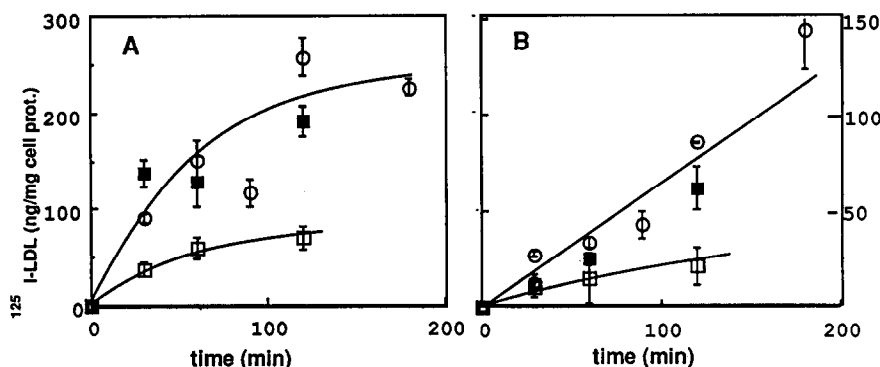
Fig.4. Reversibility of the benzyl alcohol effect on ^{125}I-LDL uptake (A) and degradation (B). Aliquots of 5 × 10^7 cells were incubated at 37°C in medium A with benzyl alcohol 10 mM, for 30 min and washed. From this aliquot (■), control cells (O), and cells in the presence of benzyl alcohol 10 mM (□), uptake and degradation of ^{125}I-LDL 10 μg were determined.

30 mM for 30 min at 37°C) reduced the uptake rate to less than 40% and 0%, respectively, of the control test. After 1 h incubation, the samples incubated with benzyl alcohol (20 mM) were washed (see section 2) and the incubation with ^{59}Fe-Tf was continued, samples exposed to 30 mM benzyl alcohol were similarly treated after 30 min incubation. After washing, in both samples, ^{59}Fe accumulation rate became linearly related to time, parallel to the control. Reversibility of the inhibition was also studied on ^{125}I-LDL uptake and degradation (fig.4); after 30 min incubation at 37°C in the presence of 10 mM benzyl alcohol, cells were washed as previously described in section 2. The experiment was carried out simultaneously on nontreated and 10 mM benzyl alcohol treated cells. The uptake and degradation inhibition (about 70% and 85%, respectively) was reversed by washing.

Fig.5 demonstrates the functional dependance of cell membrane order parameters on benzyl alcohol concentration. The presence of 20 mM benzyl alcohol increased membrane fluidity as indicated by decreases in S and S(T∥). According to Gordon et al. [1], further addition of benzyl alcohol progressively decreases the order parameter and hence increases the bilayer fluidity, up to the highest tested concentration (50 mM).
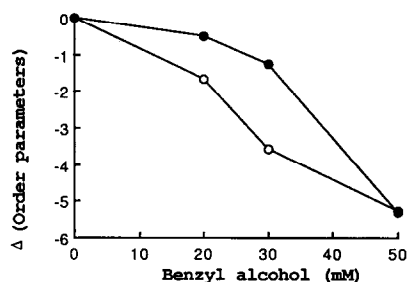

Fig.5. The effects of benzyl alcohol on the order parameters of (10,3 F.A.) labeled L₂C lymphocytes at 37°C. ΔS (O) ΔS (T∥) (●), the percentages changes in the order parameters from base values measured without benzyl alcohol are plotted as a function of benzyl alcohol concentrations.

4. DISCUSSION

In the present report, we show that benzyl alcohol inhibits the endocytosis of Tf and LDL on L₂C lymphocytes. This inhibition was not related to variations of the binding properties of receptors: no consistent changes in receptor numbers or K_d values were observed after benzyl alcohol activity (table 1). Inhibition was dose dependant (figs.1,2). The inhibition of ^{59}Fe accumulation was apparently stronger (90% for 30 mM) than ^{125}I-LDL uptake (65% at the same concentration). The ^{125}I-LDL degradation was also much more strongly affected (85%), than the uptake (60–70%). Kinetic experiments (insert fig.2), carried out in the presence of benzyl alcohol, showed that ^{125}I-LDL accumulated within the cells in spite of the internalisation inhibition. This means that ^{125}I-LDL degradation was more inhibited than the internalisation, as previously observed with asialoglycoprotein [7]. The ^{125}I-LDL accumulated within the cells represents the additin of the endocytosed ligand plus those which are not degraded, thus the internalisation inhibition could be more important than was apparent in our observations. As on purified plasma membranes [1], all effects were obtained rapidly (2 min) and were reversible (figs.3,4) by washing. The effect of 30 mM benzyl alcohol, which provoked a very strong inhibition of Fe accumulation, was also fully reversible (fig.3). Benzyl alcohol modified the plasma membrane fluidity: the effect on living cells was similar to that observed on isolated membranes [1]. LDL receptor endocytosis was also shown to be influenced by very subtle changes in the membrane fluidity. This was related to indirect and slow modifications of the cholesterol content in lymphoblasts [16], or to the fatty acid unsaturation in fibroblasts [17].

The rapid, amplified and reversible action of benzyl alcohol at nontoxic concentrations makes it a good tool for studying the endocytosis process.

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