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BRIEF COMMUNICATION

Role of macrophage cell surface sulphydryl groups in endocytosis, but not recognition of immune complexes

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Summary. Mononuclear phagocytes play a central role in the removal of immune complexes from the circulation. Cell surface receptors for the Fc domain of IgG mediate, at least in part, the recognition and internalization of particulate and soluble immune complexes. Recently, there have been significant advances in understanding the structural and functional aspects of Fc receptors (Leslie & Alexander, 1979; Zuckerman & Douglas, 1979). However, the post-receptor binding events which trigger endocytosis of immune complexes are not well understood. In the present communication, we employ the membrane-impermeable reagent 5,5′-dithiobis (nitrobenzoic acid), DTNB, to demonstrate that cell surface sulphydryl groups are required for the endocytosis, but not recognition of immune complexes.

The RAW264 macrophage cell line (Raschke *et al.*, 1978) was grown in cell culture as previously described (Petty *et al.*, 1980a). Cells were removed from tissue culture plates by established protocols using EDTA (Petty *et al.*, 1980a). Cell viability was routinely 95–98%, as judged by trypan blue exclusion. The reagent DTNB was obtained from Sigma Chem. Co. (St Louis, MO). Ferritin was labelled with fluorescein isothiocyanate using previously described methodology (Petty *et al.*, 1980b). An anti-ferritin antibody fraction was obtained by affinity chromatography on Sepharose-protein A (Pharmacia, Pitscataway, NJ). Immune complexes formed from these reagents have been characterized by precipitin analysis, electron microscopy, enzyme release, and inhibition of Fc receptor rosetting (H. R. Petty & W. Dereski, manuscript in preparation). Complexes were formed in four-fold antigen excess. Video intensification microscopy was performed as described (Willingham & Pastan, 1978) except that an argon-ion laser (488 nm line) was used as the excitation source (Petty *et al.*, 1980b). Fluorescence bound to the cell periphery, but not internalized, was quenched with crystal violet as described by Hed (1977). The percentage of cells possessing internal fluorescence is given as percent phagocytosis (% Ph). Three to six independent experiments were conducted for the conditions described. This measure may include both actively internalized complexes and those bound in the cytosol of dead cells.

Our experiments have been designed to test the hypothesis that macrophage surface sulphydryl groups are involved in triggering Fc-receptor dependent endocytosis of immune complexes. The reagent DTNB is particularly suitable as it is cell membrane impermeable. Other sulphydryl reagents such as N-ethyl maleimide, although inhibiting phagocytosis (Babior & Cohen, 1981; Ralph & Nokoniz, 1980), may affect cytoskeletal and metabolic systems. Immune complex dose was adjusted to give 95% inhibition of EA rosetting (Petty *et al.*, 1980a). In Fig. 1a, we show macrophages incubated with fluorescein-labelled immune complexes at 4° for 1 hr (range 5–7·5% Ph).

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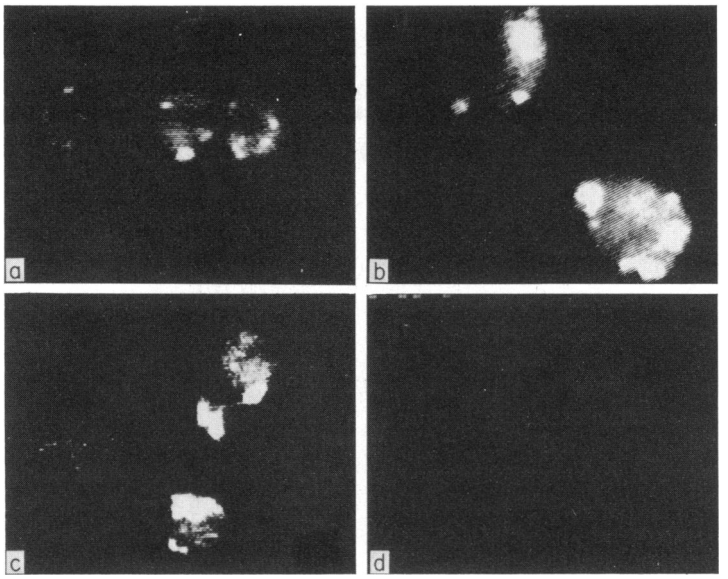

Figure 1. Representative video-intensification microscopic data on fluorescent immune complex binding and internalization are shown. (a) 4°; (b) 37° + crystal violet; (c) 37° + 2·5 mM DTNB; (d) 37° + 2·5 mM DTNB pretreated macrophages + crystal violet. (For additional controls, see text.) (Final mag.: a, b, c = 1200, d = 1500.) Magnification: (a), (b) and (c) × 960; (d) × 1200.

Immune complexes attached to the macrophage surface can be seen. The addition of crystal violet abolishes fluorescence (data not shown). This procedure provides a simple method of dissecting immune complex binding from internalization. Incubation of macrophages with immune complexes at 37° for 1 hr leads to complete internalization (100% Ph; Fig. 1b, with crystal violet). However, when cells are pre-incubated with 2·5 mM DTNB at 4° for 30 min, washed three times with buffer, followed by incubation at 37° with immune complexes, internalization is abolished. In Fig. 1c, we show a typical example of this DTNB experiment; Fig 1d shows an identical experiment with crystal violet as quenching reagent. No significant levels of internalized fluorescence can be discerned (range 0–4% Ph). Dose-response studies from 5 n*M* to 10 mM gave similar inhibition of endocytosis (range 4–9% Ph). Half-maximal inhibition of internalization occurs at 50 pM of DTNB; this corresponds to roughly 10,000 molecules of DTNB per macrophage. These studies demonstrate that endocytosis of immune complexes is exquisitely sensitive to the reagent DTNB.

Two lines of evidence indicate that the site of action of DTNB is the cell. Firstly, preincubation of macrophages with DTNB at 4° inhibits immune complex endocytosis to the same degree as co-incubation of DTNB, cells, and immune complexes at 37° (see above). A second line of evidence supporting the notion of a direct effect of DTNB on cell membranes was obtained. Fluorescein-labelled immune complexes were incubated with 10 mM DTNB on ice for 1 hr. Free reagent was rapidly removed by Sephadex G-25 (Pharmacia) column chromatography. The concentration of complexes was adjusted, followed by incubation with macrophages. The results obtained in this experiment were indistinguishable from blank incubations at 37° (100% Ph; data not shown).

Our experiments with the membrane-impermeable reagent DTNB indicate that a macrophage surface sulphydryl group is associated with endocytosis, but not binding of immune complexes. This sulphydryl is likely a component of the Fc receptor, or some other plasma membrane protein participating in the transduction of membrane signals. Our observations may be limited to Fc-receptors mediated endocytosis of immune complexes. Receptor-independent pathways such as latex bead phagocytosis may be unaffected. Unlike earlier methods for dissecting binding and internalization, such as cytochalasin B, the above discussion may provide a means to examine molecular events triggering endocytosis directly at the level of the plasma membrane.

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