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Inhibition by Glucocorticoids of Endocytosis in a Macrophage-Like Cell Line

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A macrophage-like cell line ($P388D_1$) has been used to demonstrate that glucocorticoids inhibit the fluid-phase endocytosis of fluorescein-labeled dextran (FITC-dextran). Initial experiments demonstrated that the interaction of FITC-dextran with cells had all the features of fluid-phase uptake, ie, the amount taken up was proportional to the concentration in the medium, the uptake proceeded continuously with time and was blocked at 4°C. Dexamethasone (10^{-7} M) had no effect on endocytosis until 11 hours after addition of the steroid, when it inhibited the uptake of FITC-dextran by 35%. The amount of inhibition increased with longer exposure times to the hormone up to 50% after 22 hours. Although this effect on endocytosis was observed prior to any effect on growth of the cells, endocytosis as well as cell proliferation were inhibited in a dose-dependent fashion. A preliminary survey of selected steroids has established that the inhibition of endocytosis was restricted to steroids of the glucocorticoid class. The key experiments were also performed using horseradish peroxidase instead of FITC-dextran with, essentially, identical results.

Key words: endocytosis, macrophage-like cell line

Most eukaryotic cells are capable of internalizing soluble material by both fluid-phase and absorptive or receptor-mediated endocytosis (for review see [1]). Absorptive endocytosis is a selective, saturable process while fluid-phase endocytosis is a bulk uptake process during which the amount of solute taken up is directly related to its concentration in the extracellular fluid.

The uptake of fluorescein-labeled dextran (FITC-dextran) by cells illustrates all the features of fluid-phase endocytosis [2,3]. The uptake is proportional to the concentration of FITC-dextran in the medium, it proceeds continuously with time and is blocked at 4°C. Its uptake is similar to other markers commonly used to measure fluid-phase uptake such as ^{3}H-sucrose [4], ^{3}H-inulin [5], ^{125}I-polyvinylpyrrolidone [6], and horseradish peroxidase (HRP) [7].

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Steinman et al [8], using HRP to measure fluid-phase endocytosis, found that mouse peritoneal macrophages internalized an amount of plasma membrane equivalent to 200% of their surface area per hour. This extensive internalization of plasma membrane does not result in a reduction of the cell's surface area implying that internalized membrane is replaced at a corresponding rate. Since the amount of membrane internalized is in vast excess of cellular biosynthetic capacity, it is generally assumed that internalized membrane is recycled to the plasma membrane. This idea of recycling of plasma membrane was initially proposed by Palade [9] as a result of electron microscopic observations.

Glucocorticoids are known to alter the lipid metabolism of a variety of cultured cells [10,11]. In HeLa cells the first measurable difference, occurring within 2 hours after steroid addition, was an inhibition of sterol synthesis [12,13]. By 6 hours dexamethasone (dex) caused the total cholesterol content to be lower in treated cultures than in the control cells. Such alterations in the biosynthesis of lipids by glucocorticoids may lead to specific impairment of specialized membrane functions such as endocytosis.

In this paper, we report on the initial phase of a study using a macrophage-like cell line ($P388D_1$) as a model system for studying the effects of glucocorticoids on endocytosis. We show that uptake of FITC-dextran by fluid-phase endocytosis is inhibited after dex treatment and that the effect on endocytosis precedes any effect on growth of the cells. Similar effects are seen when horseradish peroxidase is substituted for FITC-dextran.

MATERIALS AND METHODS

Cells

The $P388D_1$ line was originally isolated by Dawe and Potter [14] from a methylcholanthrene-induced lymphoma (P388) of a DBA/2 mouse. Studies on the physical and morphological properties of these cells by Koren et al [15] demonstrated that this culture line possesses most, if not all, of the characteristics of normal macrophages. These cells were provided by Dr. T. Suzuki, Department of Microbiology, University of Kansas Medical Center. The $P388D_1$ cell line is routinely carried as monolayer cultures in Eagle's minimal essential medium (MEM) supplemented with 5% fetal calf serum (FCS).

Fluorescein-Dextran Uptake

The $P388D_1$ cells were plated at 1×10^6 cells per 35-mm plastic dish and were pregrown for 24 hr in MEM supplemented with 5% FCS followed by an additional 24 hr incubation in the presence or absence of dexamethasone. Control cultures received an equivalent amount of ethanol (final concentration 0.1%). Fluid-phase endocytosis was measured by aspirating the medium and replacing it with complete medium containing FITC-dextran (Sigma FD-70, average molecular weight 62,000), typically at a concentration of 1 mg/ml. After various periods of FITC-dextran uptake the medium was aspirated and the cell monolayer was washed 6 times with 0.85% NaCl at 4°C. The cell monolayer was scraped into saline and collected in 1.5 ml microfuge tubes. The cells were pelleted using a microfuge (Brinkman 3200) and the supernatant fluid aspirated off. The cell pellet was solubilized by the addition of

0.1% Triton X-100 (v/v), and aliquots were taken for protein and fluorescence determinations. In some experiments we used horseradish peroxidase (Sigma Type II) in parallel cultures to compare against the results obtained with FITC-dextran. Handling and processing of the samples was identical for the two systems. The amount of HRP taken up by the cells was quantified essentially as described by Steinman and Cohn [7]. Protein was determined by the method of Lowry et al, [16], and fluorescence was measured with an Aminco-Bowman Corrected Spectra Spectrophotofluorometer (excitation wavelength 470 nm, emmission wavelength 520 nm). All experiments were carried out in triplicate and expressed as mean ± SD.

RESULTS

FITC-Dextran Uptake

Uptake of FITC-dextran at 37°C was linear as a function of concentration (Fig. 1). Cells exposed to increasing concentrations of FITC-dextran at 4°C gave fluorescence values equivalent to background cell fluorescence. The 37°C uptake of 1 mg/ml FITC-dextran as a function of time (Fig. 2) also demonstrated kinetics that are consistent with internalization by fluid but not adsorptive endocytosis.

Dexamethasone Effect on Growth

In this experiment P388D$_1$ macrophages were plated at a concentration of 5 × 10^5 cells per 60-mm dish. The cells were pregrown for 24 hr before the addition of the steroid and samples were taken every 24 hr up to 72 hr. The rate of proliferation of the P388D$_1$ cells, even in the presence of 5% FCS, was markedly inhibited by dex in a dose-dependent fashion (Fig. 3). Although a 48-hr incubation with 10^{-6} M dex inhibited growth by 52%, a 24-hr incubation with the steroid resulted in no significant difference in protein per culture between control and treated cells at any of the concentrations of dex examined.

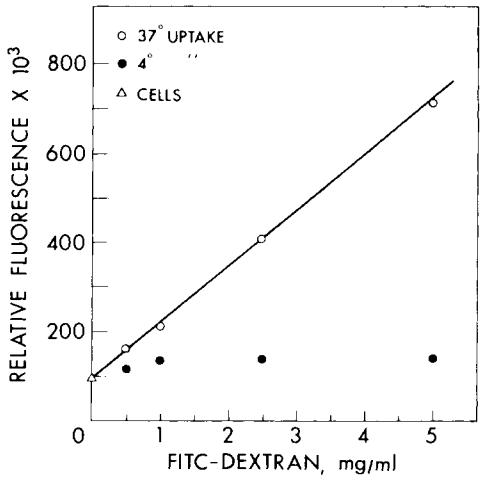

Fig. 1. Uptake of FITC-dextran as a function of concentration. Cells were incubated for 1 hr in the presence of 0.5, 1.0, 2.5, and 5.0 mg/ml FITC-dextran.

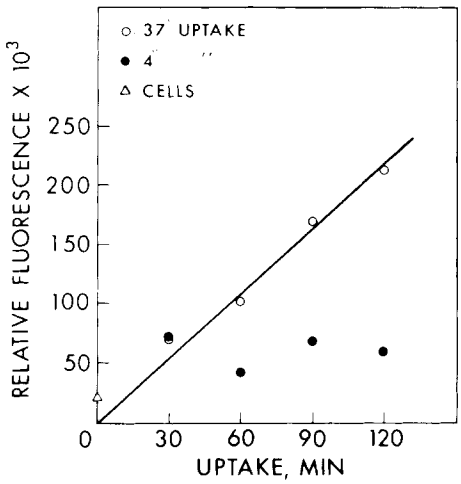

Fig. 2. Uptake of 1 mg/ml FITC-dextran as a function of time.

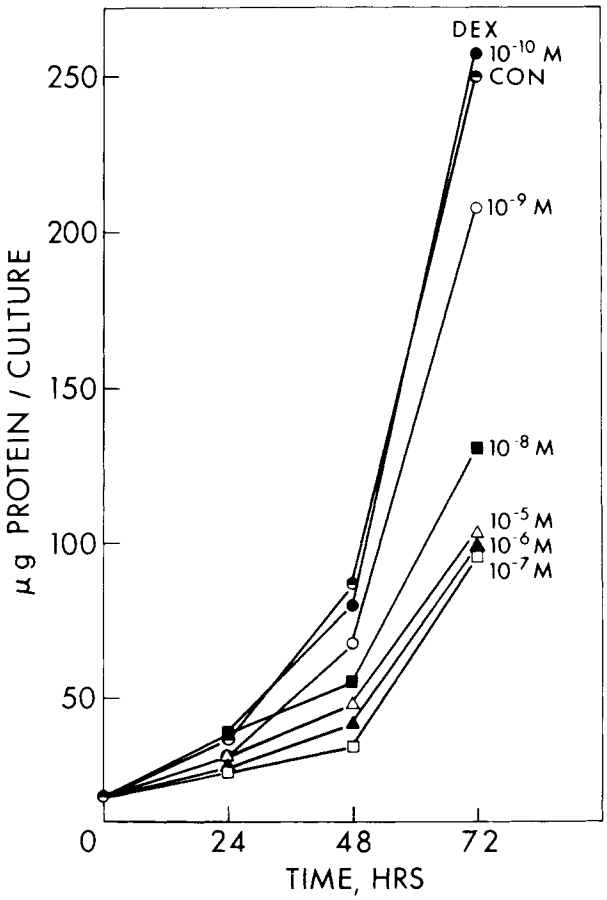

Fig. 3. Effect of dexamethasone on proliferation of P388D$_1$ macrophages. Cultures were plated at 5 × 10^5 cells per 60-mm dish and pregrown for 24 hr before the addition of the steroid at zero time.

Dexamethasone Effect on Endocytosis

To eliminate the possibility of artifactual effects, the established criteria for fluid-phase uptake [7] were evaluated in dex-treated cultures as well as in control cells. When cells were incubated with 10^{-7} M dexamethasone for a 24-hr period following which the uptake of 1 mg/ml FITC-dextran was determined as a function of time, the uptake in dex-treated cultures showed an initial increase over background cell fluorescence as measured at 60 min but did not increase further with time. Consequently in dex-treated cultures a 2-hr uptake period reflected an inhibition of FITC-dextran uptake by 63% (data not shown). Since 2 hr was the longest uptake period examined, it was conceivable that FITC-dextran was continually being taken up by the dex-treated cultures but at a low rate not detectable due to limitations in the sensitivity of the fluorescent assay. To examine this possibility, the cells were incubated with 10^{-7} M dexamethasone as before. After the usual 24-hr preincubation, the uptake of 1 mg/ml HRP and FITC-dextran was determined as a function of time in parallel experiments.

When HRP was used as a fluid-phase marker (Fig. 4A), glucocorticoid treatment resulted in a linear rate of uptake that was reduced by approximately one-half (slope = 5.07) when compared to control cultures (slope = 10.73). Uptake of FITC-dextran (Fig. 4B) demonstrated results consistent with the data obtained with HRP. One other point demonstrated by this experiment was the discrepancy in the ability to detect differences in uptake with time periods of less than 1 hr. It is clear that during short uptake periods the sensitivity of the fluorescent assay is limited.

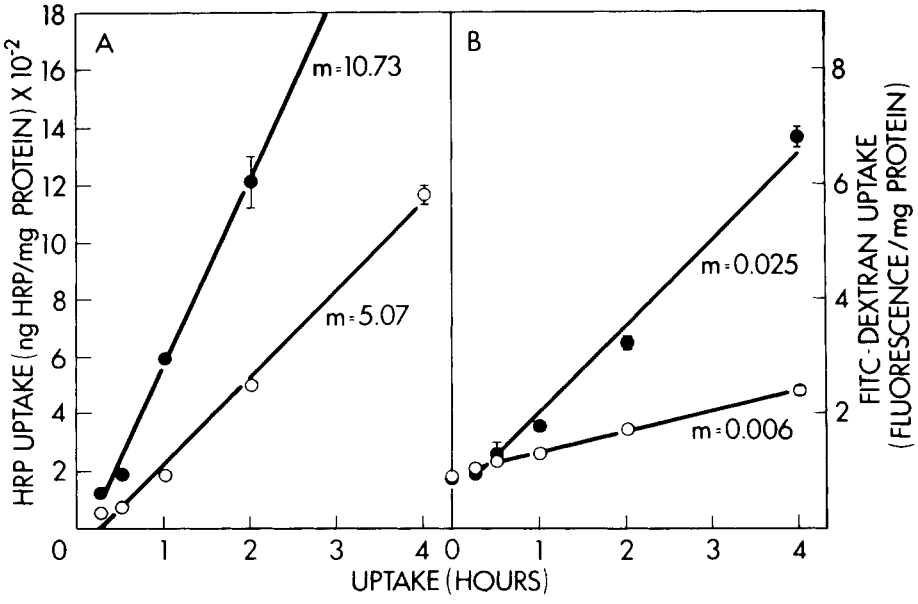

Fig. 4. Effect of dexamethasone on rate of fluid-phase uptake. Parallel cultures were incubated with 10^{-7} M dexamethasone for 24 hr and the uptake of 1 mg/ml HRP (A) and FITC-dextran (B) was determined as a function of time. Solid and open circles represent control and dex-treated cultures, respectively.

However, a 2-hr uptake period gave comparable inhibition of uptake between the two markers. For this reason all of the following experiments were done with the uptake period held constant at 2 hr.

Next the concentration dependency of dex for this effect on endocytosis was examined. The results from this experiment are shown in Figure 5. The ability of dex to inhibit endocytosis of FITC-dextran was strikingly concentration dependent. Thus, dex at a concentration of 10^{-9} M inhibited endocytosis in this experiment by ~ 20% while concentrations of 10^{-5} M to 10^{-7} M resulted in ~ 50% inhibition. Again, at concentrations used, there was no significant effect on cell protein content. The time course of the steroid effect was examined throughout a 10-hr period after the addition of 10^{-7} M dex followed by another sample at the 24-hr point. There was no difference in FITC-dextran uptake between control and treated cultures during the 10-hr period, but the 24-hr sample gave ~50% inhibition (results not shown). In a separate experiment the effect between 11 and 22 hr after addition of dex was examined. The amount of inhibition increased from 35% at 11 hours to 50% after 22 hr (Fig. 6). It is noteworthy that cell protein was unaffected by dex and that it increased with time in both control and treated cultures.

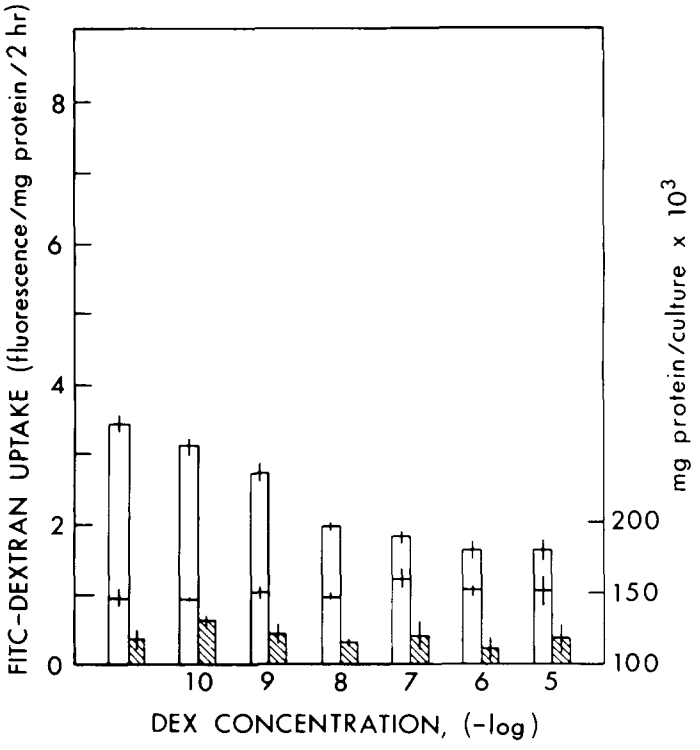

Fig. 5. Effect of dexamethasone concentration on fluid-phase uptake of 1 mg/ml FITC-dextran. Cells were incubated with the indicated molar concentrations of dexamethasone for a 24-hr period and the endocytosis was measured (open bars) after 2 hr of incubation in the presence of added FITC-dextran. Background values with cells only are represented by the bar within the open bar. Protein per culture represented by hatched bars.

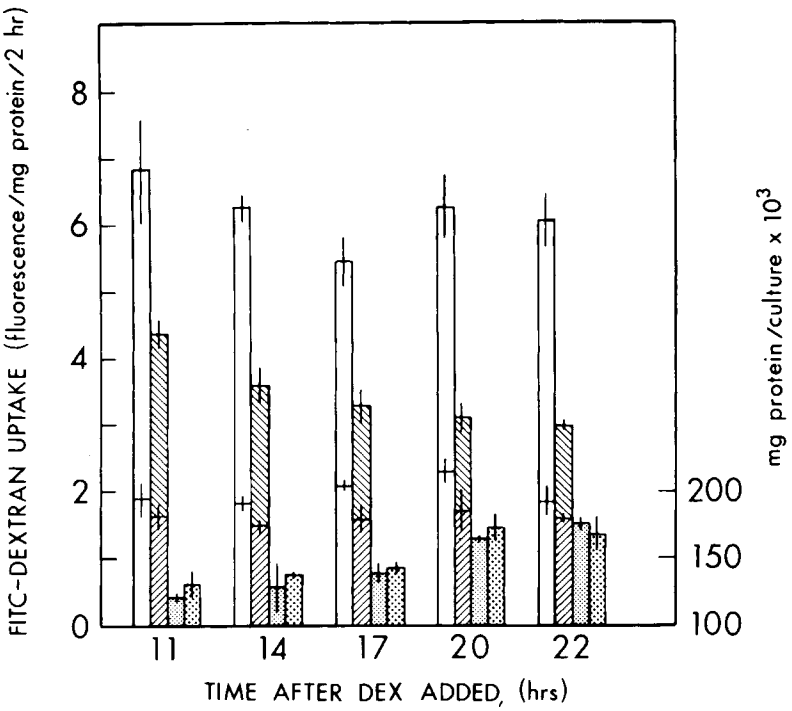

Fig. 6. Inhibition of endocytosis by 10^{-7} M dexamethasone as a function of time of exposure to the steroid. Open bar and hatched bar represent uptake by control and dex cultures, respectively. Bars within the bars represent background cellular fluorescence. Dotted bars represent control and dex-treated cultures, respectively.

A preliminary steroid survey with 10^{-6} M final concentration demonstrated (Fig. 7) that only steroids of the glucocorticoid series were capable of inhibiting endocytosis in this cell line. Cortexolone appeared to be slightly active relative to control but the deviations overlap with cortisone, progesterone, and deoxycorticosterone. Cortisone may be inactive because this cell line presumably lacks the enzymatic ability to convert it to cortisol.

DISCUSSION

We have shown that in the macrophage-like cell line P388D_1 FITC-dextran is a suitable marker for measuring fluid-phase endocytosis. In agreement with the work by others [2,3] uptake of FITC-dextran was proportional not only to time but also to the concentration in the extracellular medium and was blocked at 4°C. We have used this technique to demonstrate that treatment of P388D_1 cells with low concentrations of dexamethasone resulted in a reduced rate of fluid-phase endocytosis.

This reduced rate of endocytosis after glucocorticoid treatment was demonstrated in parallel experiments where the uptake of HRP was compared with that of

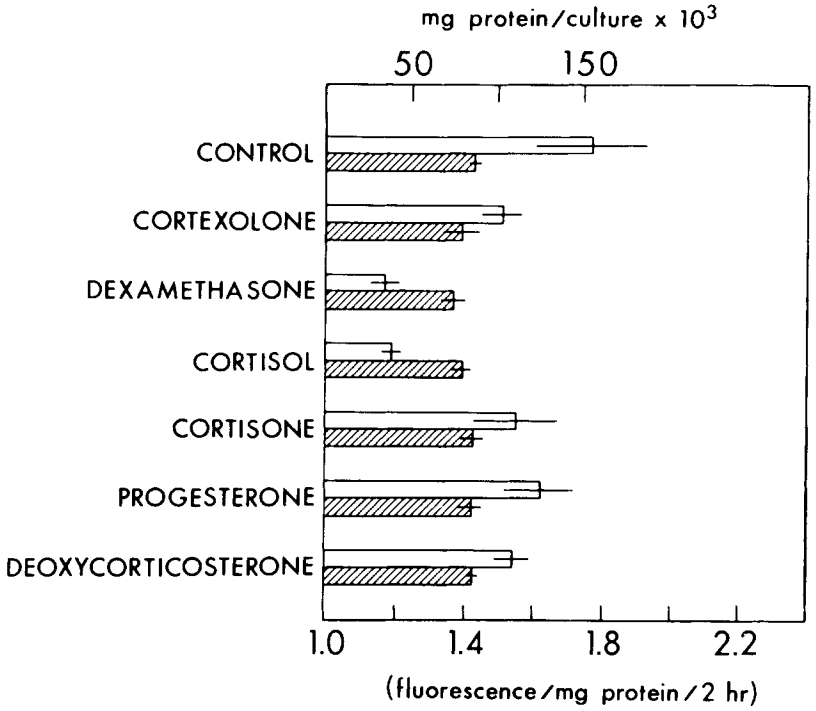

Fig. 7. Effect of various steroids (10^{-6} M) on fluid-phase uptake of 1 mg/ml FITC-dextran. Cells were incubated with the indicated steroids for a 24-hr period and the uptake of FITC-dextran during a 2-hr incubation was determined. Open bars represent endocytosis and hatched bars represent protein per culture.

FITC-dextran (Fig. 4A, B). Using a 2-hr uptake period these fluid-phase markers gave comparable inhibition of uptake. There are advantages and disadvantages to both techniques. For this phase of the study we have chosen to use FITC-dextran to follow fluid-phase endocytosis because its uptake can be seen readily with the fluorescent microscope even after short uptake periods [3]. Another advantage of FITC-dextran is its resistance to biological degradation. This feature becomes important if glucocorticoids as suggested by others [17] stabilize lysosomal membranes. Such an effect might also lead to a decrease in endocytic activity. This possibility is currently being explored using HRP by following its degradation after glucocorticoid treatment.

Other investigators using P388D_1 cells have established that this cell line contains receptors that bind glucocorticoids with a Kd of 4 nM [18]. They have also shown that secretion of neutral proteinases is inhibited after a 24-hr exposure to the hormone. In their system, in contrast to ours, cortexolone and cortisone both inhibited secretion in a concentration-dependent manner. Cortexolone is usually considered an antiglucocorticoid but has been shown to exhibit glucocorticoid activity in some systems [19]. The apparent activity of cortisone could be explained by its activation through metabolism to cortisol. The discrepancy between our inability to detect activity by cortexolone or cortisone may in part be due to our survey using only one steroid concentration. A more comprehensive survey testing the ability of

various steroids to inhibit endocytosis as a function of concentration may clarify this point.

The ability of glucocorticoids to inhibit endocytosis as well as the secretion of neutral proteinases may be related to alterations in lipid metabolism. In this regard, Mahoney et al [20,21] found that increasing the content of saturated fatty acids in cellular phospholipids results in a reduction in the rate of fluid-phase endocytosis as well as a reduction in membrane fluidity. A role for sterols is provided by the work of Heiniger et al [22], who demonstrated that cells depleted of sterols by 25-hydroxycholesterol treatment are unable to take up HRP by endocytosis. These results indicate that vesicle formation and internalization of the plasma membrane is dependent on the maintenance of a suitable level of rigidity in the lipid core of the membrane. It may be that the failure to produce cholesterol for membrane formation after glucocorticoid treatment [12,13] directly affects the cells' ability to carry out endocytosis. Experiments are in progress to examine this possibility.

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