# Cellular Handling of a Dexamethasone-Anti-E-Selectin Immunoconjugate by Activated Endothelial Cells: Comparison with Free Dexamethasone

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***Purpose.*** For selective inhibition of endothelial cell activation in chronic inflammation, we have developed a dexamethasone-anti-E-selectin immunoconjugate. The present study was performed to evaluate the cellular handling of this immunoconjugate by activated primary endothelial cells and to compare its drug delivery capacity with free dexamethasone.

***Methods.*** The binding, uptake, and degradation of $^{125}$I-radiolabeled dexamethasone-anti-E-selectin immunoconjugate by TNFα-activated endothelial cells were studied for different time periods and at different concentrations, as well as in the presence of inhibitors for E-selectin binding and lysosomal degradation. Its drug delivery capacity was compared with the uptake of unconjugated $^{3}$H-labeled dexamethasone.

***Results.*** The immunoconjugate was internalized by E-selectin expressing activated endothelial cells and degraded in the lysosomal compartment. The receptor-mediated binding and uptake was saturable, implying a maximal attainable intracellular concentration of the drug. In contrast, free dexamethasone entered both resting and activated endothelial cells by passive diffusion.

***Conclusions.*** The dexamethasone-anti-E-selectin immunoconjugate is capable of selective delivering the coupled drug into activated endothelial cells. This targeting concept enables disease-induced drug delivery in which intracellular concentrations can be reached comparable with those obtained after incubation with 3 μM dexamethasone.

**KEY WORDS:** drug targeting; immunoconjugate; activated endothelial cells; lysosomal degradation; chronic inflammation; glucocorticoids.

## INTRODUCTION

The endothelium plays an important role in the recruitment and infiltration of immune cells into chronically inflamed tissues. Activation of endothelial cells with pro-inflammatory cytokines, such as TNFα results in induced expression of adhesion molecules on the endothelial cell surface and secretion of a broad variety of cytokines and chemokines, which all contribute to the adhesion and transmigration of leukocytes into the underlying tissue (1,2). Activated endothelial cells are therefore considered an attractive target for pharmacologic intervention. Several established as well as more experimental therapeutics exert (part of) their anti-inflammatory effects by acting at the level of the activated endothelium. They either interfere directly with leukocyte binding to adhesion molecules, or inhibit immune cell recruitment indirectly, by inhibition of endothelial cell activation. Antibodies that bind selectively to endothelial adhesion molecules (3–5) and low molecular weight ligands, such as sialyl Lewis X mimetics (6) block leukocyte recruitment by acting outside the endothelial cell. However, such compounds often fail to enter clinical practice, mainly due to the occurrence of redundant pathways in leukocyte adherence. As an alternative to interfering in the leukocyte-endothelium interaction using blockers of the induced adhesion molecules, inhibitors of signal transduction pathways are investigated as anti-inflammatory drugs (7,8). If such inhibitors act early in the activation cascade, they can inhibit multiple events in the inflammatory process. In this respect, inhibitors of the nuclear factor κ-B (NF-κB) pathway and mitogen-activated protein kinase pathways have been studied extensively (7–10). Although many promising lead compounds have been developed, these compounds often suffer from toxicity, which hampers their therapeutic application.

We recently reported on the development of a dexamethasone (dexa)-anti-E-selectin immunoconjugate, which is intended for selective drug delivery to endothelial cells in chronically inflamed tissues (11). E-selectin, which is expressed by endothelial cells during cell activation, is rapidly internalized after its expression on the cell surface, thereby delivering the bound immunoconjugate into the target cells (12). Targeting strategies that deliver the coupled drug into the lysosomal compartment of the cells have shown promising results for tumor targeting (13), but have not yet been explored for the targeting of drugs into endothelial cells. Eventually, such a targeting strategy will improve the therapeutic profile of the coupled drug by preventing drug-induced side effects in non-target tissues. In addition, the immunoconjugate may furthermore display an improved anti-inflammatory profile by combining drug action and inhibitory effects of the anti-E-selectin antibody. As such, the combination can greatly increase the therapeutic potential of both parts of the immunoconjugate.

In the present study, we used a $^{125}$I-radiolabeled dexa-anti-E-selectin immunoconjugate to quantitate endothelial cell binding, uptake and degradation properties of the conjugate at different concentrations, for different periods of time and in the presence of inhibitors of binding and cellular processing. We compared the cellular handling of the immunoconjugate with $^{3}$H-labeled free unconjugated dexa. The results will gain insight in the expected onset of pharmacologic activity after uptake and processing of the conjugate and the amount of drug that can be delivered via the E-selectin internalization pathway.

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**ABBREVIATIONS:** $Ab_{Esel}$ mouse anti-human E-selectin antibody (H18/7); $Ab_{Ctrl}$ isotype control antibody (MOC31, mouse anti-human EGP-2); dexa dexamethasone; HUVEC human umbilical vein endothelial cells; TCA trichloroacetic acid; TNFα tumor necrosis factor-α.

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## MATERIALS AND METHODS

### Antibodies

Anti-E-selectin monoclonal antibody (mouse anti-human E-selectin antibody, hereafter referred to as $Ab_{Esel}$) was isolated by protein A affinity chromatography from culture medium of the H18/7 hybridoma (kindly provided by Dr. M. Gimbrone Jr; Boston, Mass, U.S.A.). An irrelevant control antibody (MOC31, mouse anti-human EGP-2, hereafter referred to as $Ab_{Ctrl}$) was kindly provided by IQProducts (Groningen, the Netherlands).

### Preparation and Characterization of Dexa-Ab Conjugates

Dexa was conjugated via a succinate linker to the $Ab_{Esel}$ and $Ab_{Ctrl}$ antibodies as described previously (11). Briefly, dexa (2.5 mmol; Genfarma; Maarssen, the Netherlands), excess succinic anhydride (42 mmol; Sigma; St. Louis, Mo, U.S.A.) and 4-dimethylaminopyridine (2.6 mmol; Fluka; Buchs, Switzerland) were dissolved in 80 mL of anhydrous acetone and reacted for 24 h at room temperature. After removal of the solvent by evaporation under reduced pressure, the crude product was recrystallized from ethanol:water (3:7). The identity of the synthesized dexa-21-hemisuccinate was confirmed by mass spectrometry (theoretical molecular mass: 492.5 Da; molecular mass found: 492.1 Da).

Dexa-Ab conjugates were prepared by coupling the carboxylic acid group of dexa-hemisuccinate to primary amino groups of the protein. Typically, dexa-hemisuccinate (3 μmol) dissolved in 300 μL 1,4-dioxane, tri-n-butylamine (16 μmol; Fluka) and isobutyl chlorocarbonate (15 μmol; Acros Organics; Geel, Belgium) were allowed to react for 30 min at room temperature. A 5.2 μl aliquot of this solution (corresponding to 52 nmol dexa-hemisuccinate) was slowly added to a stirred solution of the antibody (13 nmol, 0.5 mg/mL in PBS). The mixture reacted at room temperature for 4 h, after which the product was purified by dialysis against PBS at 4°C. The final product was filtered through an 0.2 μm filter and stored at –20°C. The dexa-Ab conjugates were analyzed for protein content (Lowry), dexa content (HPLC), molecular size (SDS PAGE and anti-dexa Western blotting) and reactivity with E-selectin (E-selectin BIAcore and immunohistochemical staining of activated endothelial cells) as described (11).

### Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as previously described (14) and provided by the Endothelial Cell Facility, Groningen University/Academic Hospital Groningen. Primary isolates were cultured on 1% gelatin-precoated plastic tissue culture plates or flasks (Costar Europe; Badhoevedorp, the Netherlands) at 37°C under 5% $CO_2$/95% air. The culture medium consisted of RPMI 1640 (BioWittaker; Verviers, Belgium) supplemented with 20% heat-inactivated fetal calf serum (Integro BV; Zaandam, the Netherlands), 2 mM L-glutamine (GIBCO-BRL; Paisley, Scotland), 5 U/mL heparin (Leo Pharmaceutical Products BV; Weesp, the Netherlands), 100 U/mL penicillin (Yamanouchi Pharma BV; Leiderdorp, the Netherlands), 100 μg/mL streptomycin (Radiumfarma-Fisiopharma; Milan, Italy), and 50 μg/mL endothelial cell growth factor supplement extracted from bovine brain. After attaining confluence, cells were detached from the surface by trypsin/EDTA (0.5/0.2 mg/mL in PBS) and split at a 1:3 ratio. HUVEC were used up to passage three.

### Radiolabeling of Dexa-$Ab_{Esel}$

Dexa-$Ab_{Esel}$ was radiolabeled with $^{125}I$ by a chloramine-T method to a specific activity of 0.4 MBq/μg (15). Prior to each experiment, the radiolabeled conjugate was purified by gel filtration on a PD10 column (Amersham Pharmacia; Uppsala, Sweden). After gel filtration, the $^{125}I$-dexa-$Ab_{Esel}$ conjugate contained less than 5% free $^{125}I$, as determined by precipitation with 10% trichloroacetic acid (TCA). $^{125}I$-radioactivity was counted in an LKB multichannel counter (LKB; Bromma, Sweden).

### Cell Binding, Uptake, and Degradation Experiments

Radioactive binding experiments were performed with confluent HUVEC monolayers in 12-well culture plates (Costar). Viability of the cells was monitored by microscopic evaluation and consistently found to be unchanged throughout the experiments.

Appropriate dilutions of TNFα (recombinant human tumor necrosis factor-α; Boehringer; Mannheim, Germany), radiolabeled compounds, and other compounds included in the experiments were prepared in endothelial cell culture medium. Unless otherwise stated, cells were incubated for 6 h at 37°C in a humidified atmosphere (5% $CO_2$), in the absence or presence of TNFα (100 ng/mL), in a total volume of 1 mL. At the end of the incubation period, medium was removed and wells were washed with ice-cold 1% BSA/PBS. Total cell-associated radioactivity (extracellularly bound plus internalized immunoconjugate) was determined by lysing the cells with 1 N NaOH (30 min at 37°C). In some experiments, surface-bound radioactivity was released by acid wash (10 min 0.1 N HCl) before lysis of the cells. Radioactivity retrieved by acid wash was expressed as extracellularly bound radioactivity, whereas radioactivity in the lysis fractions was expressed as internalized radioactivity.

#### *Determination of Binding Specificity of Dexa-$Ab_{Esel}$*

The specific binding of dexa-$Ab_{Esel}$ to E-selectin and subsequent internalization was investigated by examining the binding of $^{125}I$-dexa-$Ab_{Esel}$ (10 ng/mL at a specific activity of 400 Bq/ng) to resting or activated HUVEC as such, and in the presence of excess of unlabeled proteins $Ab_{Esel}$, dexa-$Ab_{Esel}$ or dexa-$Ab_{Ctrl}$ (all at 10 μg/mL).

#### *Time Course of Binding, Uptake and Degradation of Dexa-$Ab_{Esel}$*

We investigated the time course of binding and uptake of the conjugate by incubating HUVEC with TNFα and $^{125}I$-dexa-$Ab_{Esel}$ (10 ng/mL at a specific activity of 400 Bq/ng) for different time periods between 0–6 h.

In a separate experiment, the time course of degradation was investigated after loading of the cells, followed by a 16 h chase period. For this, HUVEC were incubated with TNFα and $^{125}I$-dexa-$Ab_{Esel}$ (10 ng/mL at a specific activity of 400 Bq/ng) for 6 h at 37°C. After washing, the cells were incubated with 1 mL of fresh medium for 16 h at 37°C in the

absence or presence of lysosomal pathway inhibitors chloroquine (100 μM, Sigma; St. Louis, Mo, U.S.A.) or ammonium chloride (20 mM, Merck; Darmstadt, Germany). The release of $^{125}$I in the medium was monitored in time using TCA precipitation of a 100 μL aliquot of the supernatant to determine the release of degraded immunoconjugate. At the end of the chase period, remaining cell-associated radioactivity was determined after lysis of the cells as described above.

*Saturation of Binding and Uptake of Dexa-Ab$_{Esel}$*

Saturation of binding and internalization of dexa-Ab$_{Esel}$ by activated endothelial cells was studied during a 6-h incubation period at 37°C. This allows determination of the capacity of the E-selectin internalization pathway to bind and transport the dexa-Ab$_{Esel}$ conjugate into the cells. HUVEC monolayers were incubated simultaneously with TNFα and $^{125}$I-dexa-Ab$_{Esel}$ (10 ng/mL-10 μg/mL, at different specific activities ranging from 400–0.8 Bq/ng). The total amount of cell-associated dexa-Ab$_{Esel}$, i.e., extracellularly bound plus internalized conjugate, was calculated from the radioactivity found after lysis of the cells. From these values, the amount of delivered dexa was calculated using the 2:1 dexa:Ab molar ratio of the immunoconjugate.

*Uptake of Dexa*

We investigated the binding and uptake of free (i.e., unconjugated) dexa by resting and activated HUVEC using a similar experimental setup as described above for the dexa-Ab$_{Esel}$ immunoconjugate. HUVEC were incubated for 6 h with or without TNFα and, simultaneously, with $^{3}$H-dexa (100 pM–10 μM, at different specific activities ranging from 2.4 MBq/nmol to 240 Bq/nmol; Amersham Pharmacia). After washing, cells were lysed and the lysis fraction was added to 3.5 mL scintillation liquid (Ultima gold XR, Packard Biosciences; Groningen, the Netherlands) and mixed vigorously for 1 h. $^{3}$H-radioactivity was counted in a calibrated scintillation counter (Packard Instruments; Meriden, Conn, U.S.A.).

*Time Course of Dexa Release*

To determine the fate of freely administered dexa after cellular uptake, HUVEC were incubated for 4 h with $^{3}$H-dexa (3.5 nM at a specific activity of 2.4 MBq/nmol), washed and incubated with 1 mL of fresh medium at 37°C. The release of $^{3}$H-dexa from the cells was monitored in time by harvesting supernatant and cell lysates at regular intervals, followed by scintillation counting of the samples as described above.

## STATISTICS

Unless mentioned otherwise, data are presented as the mean ±SD of triplicate experiments. Statistical significance of differences was tested using the two-sided Student's *t* test, assuming normal distribution of the data and similar variances. Differences were considered significant at $p < 0.05$.

## RESULTS AND DISCUSSION

We prepared dexa-Ab$_{Esel}$ and dexa-Ab$_{ctrl}$ immunoconjugates by reacting dexa-hemisuccinate via its succinate linker to primary amino groups of the antibodies. Covalent conjugation of dexa was determined by SDS-PAGE in combination with immunoblotting with an anti-dexa antiserum, and by HPLC after alkaline hydrolysis of the ester linkage in the conjugate. This latter analysis demonstrated an average dexa-content of 2 drug molecules per antibody (11). Higher drug loading of the protein was not pursued, since this can interfere with the E-selectin recognition (observed at a dexa:Ab ratio of 10:1; data not shown). Furthermore, modification of side chain (primary amino) groups with a relatively high number of drug molecules increases the hydrophobicity and negative charge of the protein, which may result in enhanced uptake of the conjugate in the liver or spleen via scavenger receptors (16,17).

After radiolabeling with $^{125}$I, we investigated binding and subsequent cellular handling of dexa-Ab$_{Esel}$ by resting and activated endothelial cells. As expected, activation with TNFα increased the binding/uptake of the conjugate (Fig. 1). Competition studies with unmodified Ab$_{Esel}$ demonstrated that $^{125}$I-dexa-Ab$_{Esel}$ bound to the same receptor as the native antibody. Dexa-Ab$_{ctrl}$, which consists of dexa coupled to a mouse IgG recognizing an irrelevant antigen, did not inhibit binding of $^{125}$I-dexa-Ab$_{Esel}$. Therefore, we conclude that $^{125}$I-dexa-Ab$_{Esel}$ specifically binds to E-selectin on activated endothelial cells.

Cytokine-induced expression of E-selectin by endothelial cells is regulated by *de novo* synthesis of the adhesion molecule, dictating a lag-time between activation and actual expression of the E-selectin on the cell surface (18). After its expression, E-selectin either becomes internalized and degraded via the lysosomal pathway, or is shed from the cells into the supernatant (19). Thus, both the kinetics of induction and down-regulation of E-selectin will affect the capacity of activated endothelial cells to bind and take up the dexa-Ab$_{Esel}$ conjugate. We, therefore, investigated the cellular

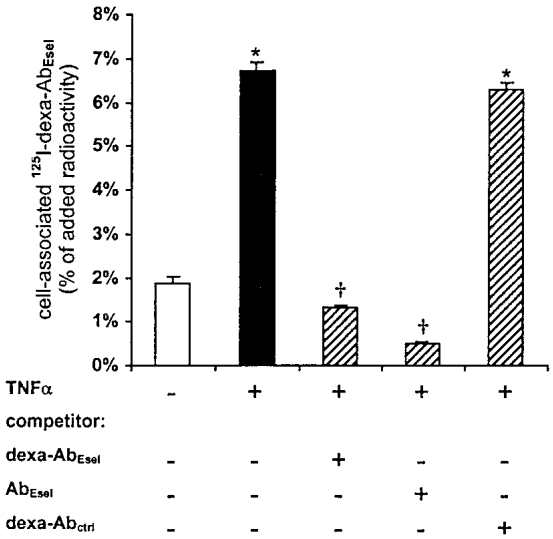


**Fig. 1.** Binding specificity of dexa-Ab$_{Esel}$ to endothelial cells. Resting or TNFα-activated HUVEC were incubated for 6 h with $^{125}$I-dexa-Ab$_{Esel}$ in the absence or presence of different competitors for binding to E-selectin. Cell-associated radioactivity was expressed as the percentage of the added radioactivity and was significantly increased after TNFα activation (*: $p < 0.05$ vs. resting HUVEC). Binding/uptake could be inhibited by excess Ab$_{Esel}$ or dexa-Ab$_{Esel}$, but not by control immunoconjugate dexa-Ab$_{Ctrl}$ (†: $p < 0.05$ vs. activated HUVEC).

handling, i.e., binding, internalization, and degradation, of $^{125}$I-dexa-Ab$_{Esel}$ under various experimental conditions. When the conjugate was added to the cells together with TNFα at the start of the activation period, binding and uptake could not be detected during the first h (Fig. 2A). At later time points, extracellularly bound and internalized radioactivity gradually increased to maximal values at 4–6 h. Degradation products of the radiolabeled conjugate in the supernatant were mainly detected at the end of this period. In addition, we studied the uptake of the conjugate by preactivated HUVEC, i.e., by cells that were pretreated with TNFα for 4 h before addition of the radiolabeled dexa-Ab$_{Esel}$ (Fig. 2B). Under these conditions, binding and subsequent internalization took place as early as 15 min after addition of $^{125}$I-dexa-Ab$_{Esel}$. This result is in good agreement with recent qualitative data of our group, using confocal laser scanning microscopy for the detection of internalized immunoconjugate (11), and previous data on the internalization kinetics of E-selectin itself (20).

To further determine the fate of the bound and/or internalized conjugate during the 6 h incubation period, we analyzed the release of radioactive products in the supernatant during a 16 h chase period. Ideally, such studies should be performed using a conjugate prepared with radiolabeled dexa, allowing investigation of the release of the drug from the carrier and its subsequent distribution. However, we were not able to prepare $^{3}$H-dexa-Ab$_{Esel}$ due to the high costs of tailor-made $^{3}$H-dexa-hemisuccinate and technical hurdles of the radioactive synthesis of the conjugate. As it is generally assumed, however, that the kinetics of carrier degradation reflects the kinetics of drug release, we followed the fate of the $^{125}$I-radiolabeled carrier protein and assumed similar drug kinetics of release and distribution. Fig. 3 shows the cumulative recovery of radioactivity in the medium and the cell-associated fractions. When the cells were incubated at 37°C, about 20% of the loaded radioactivity was released in the medium as intact protein during the first h of the chase period (Fig. 3A). In contrast, low-molecular weight degradation products were released during the total 16-h period, amounting to almost 80% of the radioactivity present in the cells at

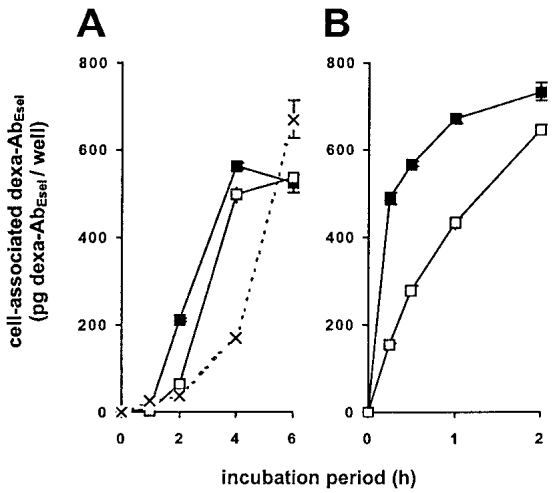


**Fig. 2.** Time course of binding, uptake, and degradation of dexa-Ab$_{Esel}$. Closed symbols: surface-bound dexa-Ab$_{Esel}$; open symbols: intracellular dexa-Ab$_{Esel}$; dotted line: dexa-Ab$_{Esel}$ degradation products in supernatant. Panel A: dexa-Ab$_{Esel}$ was added at the start of the experiment, simultaneously with TNFα. Binding and subsequent uptake and degradation is observed starting at 2 h after activation with TNFα. Panel B: dexa-Ab$_{Esel}$ was added at 4 h after the addition of TNFα. Immediate binding and uptake was observed due to the presence of E-selectin on the cell surface at the start of the incubation.

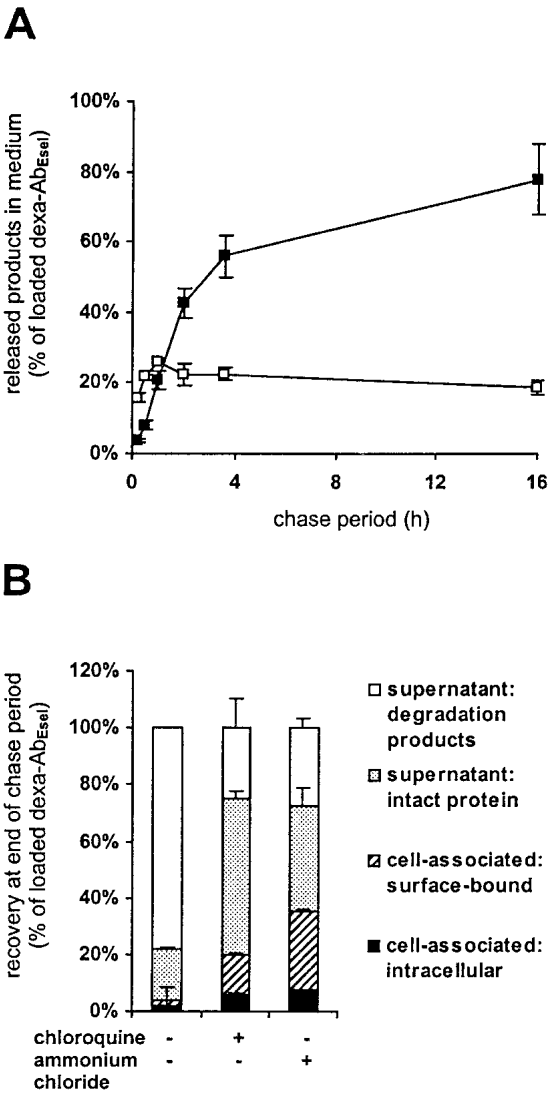


**Fig. 3.** Cumulative recovery of dexa-Ab$_{Esel}$ degradation products from loaded cells. HUVEC were simultaneously incubated for 6 h with TNFα and $^{125}$I-dexa-Ab$_{Esel}$, washed and incubated with EC medium for 16 h at 37°C, with or without lysosomal inhibitors. Released and cell-associated dexa-Ab$_{Esel}$ products were expressed as percentage of the cell-associated amount at the beginning of the chase period. Panel A: time course of release of products in medium. Closed symbols: low-molecular weight degradation products; open symbols: intact immunoconjugate. Panel B: Cumulative recovery at the end of 16 h chase period. Released radioactivity in the supernatant was precipitated with TCA to determine low- molecular weight degradation products (white bars) and intact immunoconjugate (light gray bars). Cellular radioactivity at the end of the experiment was subjected to an acid-wash procedure, followed by lysis of the cells to determine surface-bound immunoconjugate (hatched bars) and intracellular immunoconjugate products (black bars). The results found after incubation in the presence of chloroquine or ammonium chloride indicate lysosomal routing of the $^{125}$I-dexa-Ab$_{Esel}$ immunoconjugate.

the start of the chase period. At the end of the experiment, less than 5% of the radioactivity was still found in the cell-associated fraction (Fig. 3B). When inhibitors of lysosomal acidification chloroquine and ammonium chloride were added to the cells, the release of degradation products in the medium was reduced, while release of cell-associated radioactivity and intact protein increased. Apparently, these inhibitors of the lysosomal pathway prevented the degradation but also the internalization of $^{125}$I-dexa-Ab$_{Esel}$, as is indicated by the increase in surface-bound immunoconjugate.

As mentioned, dexa-Ab$_{Esel}$ conjugate is a preparation intended for the targeted delivery of dexa to activated endothelial cells. To estimate the delivering capacity of this type of targeting constructs, we investigated the concentration dependent binding/uptake of $^{125}$I-dexa-Ab$_{Esel}$. As expected for receptor-mediated endocytosis, saturation of the uptake was observed at increasing concentrations of the conjugate (Fig. 4). From these data, we calculated a maximal delivery capacity of approximately 3 pmol dexa/230,000 cells, which corresponds to $7.5 \times 10^6$ molecules/cell. Only cell-associated radioactivity was used to estimate the capacity of the delivery process, ignoring the release of degradation products from the cells into the medium during the incubation period. Thus, the value observed probably underestimates the actual delivery capacity of the immunoconjugate. To extrapolate this value to the *in vivo* situation, several other parameters must be known, such as the E-selectin expression level of activated endothelial cells in inflamed tissue, as well as the kinetics of E-selectin expression and internalization in disease situations. Such quantitative parameters are not available, neither from human experiments nor from animal models of chronic inflammation, since most studies only report qualitatively on E-selectin expression.

To our knowledge, this is the first paper in which the delivery capacity of a drug targeting preparation is compared directly with the amount of free drug that can enter the target

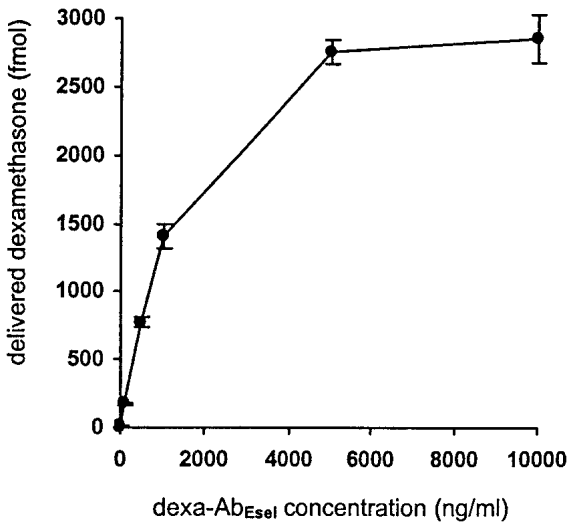


**Fig. 4.** Binding/uptake of dexa-Ab$_{Esel}$ by activated endothelial cells. HUVEC were simultaneously incubated for 6 h with TNFα and different concentrations of dexa-Ab$_{Esel}$. Cell-associated dexa-Ab$_{Esel}$ was determined as described in material and methods and used to calculate the amount of delivered dexamethasone. The observed saturation at higher concentrations indicates entry of the cells by receptor mediated endocytosis.

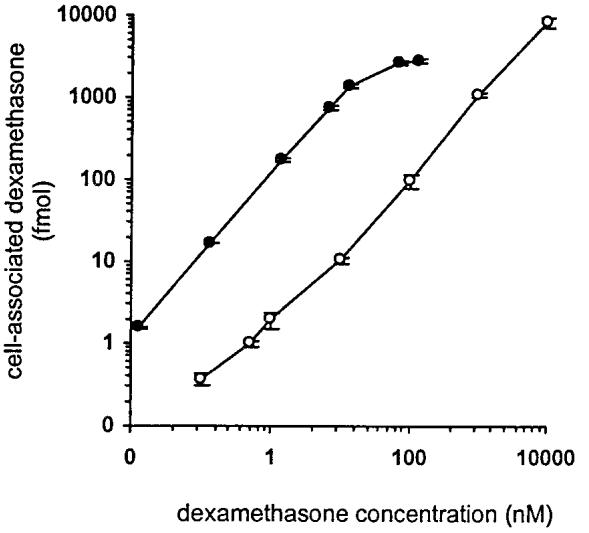


**Fig. 5.** Comparison of cellular delivery capacity of dexa-Ab$_{Esel}$ and freely administered dexamethasone. HUVEC were incubated for 6 h with TNFα and $^{125}$I-dexa-Ab$_{Esel}$ (closed symbols) or $^{3}$H-dexa (open symbols). Cell-associated dexa was determined as described in material and methods. The concentration of dexa-Ab$_{Esel}$ was expressed as dexa concentration using the loading ratio of 2 moles dexa per mole immunoconjugate.

cells. We determined the uptake of free dexa using $^{3}$H-labeled dexa (Fig. 5). Approximately, 0.1% of the added amount of dexa was bound/internalized by the cells. No saturation was observed throughout the studied concentration range, as expected for transport into the cells by passive diffusion. In comparison with the immunoconjugate, higher dexa uptake could be achieved at concentrations above 3 μM, whereas the uptake of dexa-Ab$_{Esel}$ was superior at low concentrations. An important difference between the immunoconjugate and free dexa is that the free drug will also enter other cell types than activated endothelial cells, as was demonstrated by the uptake into resting endothelial cells (data not shown). Furthermore, free dexa will enter non-endothelial cells as well, while dexa-Ab$_{Esel}$ will only accumulate in the designated target cells.

Finally, we studied the fate of dexa once intracellularly accumulated. Rapid loss of cell-associated $^{3}$H-dexa from the loaded cells was observed (Fig. 6), indicating that diffusion of this drug over the cell membrane is a bidirectional process. Likely, redistribution will also occur upon dexa delivery by dexa-Ab$_{Esel}$. Following uptake of the immunoconjugate, the intracellular concentration of free dexa will be determined by a slow-release process from the carrier through hydrolysis of the succinate ester linkage, binding to glucocorticoid receptors and other proteins, as well as the efflux of dexa from the cells. Although little is known about the exact concentrations of dexa necessary for glucocorticoid-mediated effects, results from our group demonstrate pharmacologic effects of dexa-Ab$_{Esel}$, e.g., on mRNA transcription of interleukin-8 and interleukin-6 (Asgeirsdottir *et al.*, *submitted*). These data indicate that the delivered dexa escapes from the lysosomes to the cytosol, binds to the glucocorticoid receptor, followed by translocation of the glucocorticoid receptor to the nucleus, which is a prerequisite for pharmacologic activity (21).

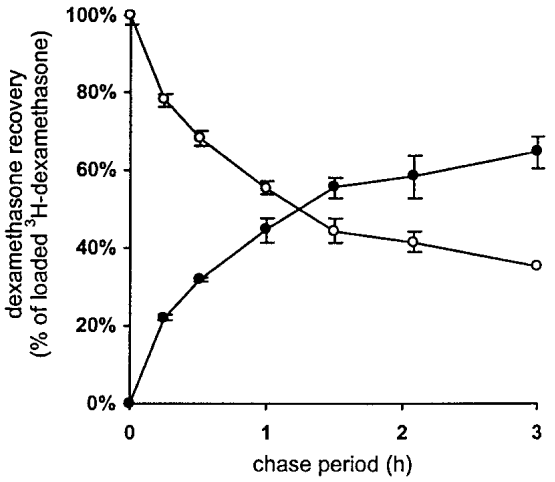


**Fig. 6.** Release of $^3$H-dexa from loaded cells. Resting HUVEC cells were incubated for 4 h with $^3$H-dexa, washed, and incubated with EC medium. Released (closed symbols) and cell-associated $^3$H-dexa (open symbols) were determined in the supernatant and cell lysates at indicated times and expressed as percentage of the cell-associated amount at the beginning of the chase period.

Currently, we aim to optimize endothelium-directed drug targeting strategies, among others by exploring other types of carrier systems, like E-selectin directed immunoliposomes. The use of such immunoliposomes may effectuate higher intracellular concentrations of dexa than the presently described immunoconjugate, that is if their internalization rate and drug-release characteristics are sufficient (22). Furthermore, we are investigating the preparation of $Ab_{Esel}$ immunoconjugates with other anti-inflammatory drug molecules.

To conclude, we have demonstrated that the dexa-$Ab_{Esel}$ immunoconjugate binds to and is internalized by activated endothelial cells. Subsequently, the conjugate is degraded in the lysosomal compartment. Potential saturation of the uptake process dictates the capacity of the E-selectin entry system, while the residence time of the released drug is governed by the rate of drug release, protein binding and cellular efflux of dexa. Further studies evaluating the capacity of the E-selectin carrier system *in vivo* including the use of alternative carrier modalities, such as immunoliposomes, will be undertaken to determine the therapeutic value of E-selectin drug targeting strategies for anti-inflammatory therapy.

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