# Cellular uptake of liposomes targeted to intercellular adhesion molecule-1 (ICAM-1) on bronchial epithelial cells

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## Abstract

Previously, it was demonstrated that immunoliposomes, bearing anti-intercellular adhesion molecule-1 (ICAM-1) antibodies (mAb F10.2), can specifically bind to different cell types expressing ICAM-1. In this study, we have quantified the amount of immunoliposomes binding to IFN-γ activated human bronchial epithelial cells (BEAS-2B) in vitro and studied the subsequent fate of cell-bound anti-ICAM-1 immunoliposomes. We demonstrate that binding of the immunoliposomes to the epithelial cells depends on the liposome concentration used. After binding to the cell surface, the anti-ICAM-1 immunoliposomes are rapidly internalised by the epithelial cells. Sixty percent of cell-bound immunoliposomes were internalised by the epithelial cells within 1 h of incubation at 37°C. The results indicate that ICAM-1 targeted immunoliposomes may be used as carriers for the intracellular delivery of anti-inflammatory drugs to sites of inflammation characterised by an increased expression of ICAM-1. © 1999 Elsevier Science B.V. All rights reserved.

*Keywords:* Immunoliposomes; Adhesion molecules; Drug delivery

## 1. Introduction

Adhesion molecules are cell surface glycoproteins that mediate physical and functional interactions between two cells or between cells and their extracellular matrix. They play an important role in many biological processes as diverse as the complex organisation of tissues and organs, the migration of cells during embryogenesis and the regulation of immune responses by mediating the communication between different immune cells and the extravasation of inflammatory cells into inflamed tissues [1–3]. The process of leukocyte extravasation is induced by the local release of pro-inflammatory mediators (vasoactive amines and cytokines) at the site of inflammation. This results in a locally induced or increased expression of a variety of adhesion molecules on endothelial cells, which allows multiple adhesive events with circulating leukocytes to occur.

Intercellular adhesion molecule-1 (ICAM-1) is an important adhesion molecule involved in the process

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of leukocyte adhesion and extravasation. ICAM-1 is an immunoglobulin-like transmembrane glycoprotein that is constitutively expressed at low levels on vascular endothelial cells, epithelial cells and on a subset of leukocytes and its expression is increased by the pro-inflammatory cytokines interferon-$\gamma$ (IFN-$\gamma$), tumour necrosis factor $\alpha$ (TNF-$\alpha$) or interleukin-1 (IL-1) [4–9]. Numerous studies have demonstrated a locally enhanced ICAM-1 expression in inflammation-related diseases, such as rheumatoid arthritis [10], asthma [11,12], nephritis [13,14], inflammatory dermatosis [15,16], and atherosclerosis [17,18]. The increased expression of ICAM-1 and other cell adhesion molecules at sites of inflammation is under investigation for therapeutic intervention. Up-regulated expression of adhesion molecules may be utilised as targets for drug carrier systems for selective delivery of anti-inflammatory compounds to sites of inflammation. In this light, we have previously constructed liposomes bearing antibodies to ICAM-1 [19]. These antibody-containing liposomes (immunoliposomes) were shown to strongly bind to different cell types expressing ICAM-1 on their surface and their binding was positively correlated with the expression level of ICAM-1 on the target cells. After this first report on immunoliposome targeting to cells expressing adhesion molecules, two other reports have appeared confirming this possibility [20,21].

Specific binding of immunoliposomes to their target cells is an important prerequisite to fulfil but on its own not enough for effective drug delivery. As many drugs act at intracellular sites, it is required that immunoliposome-encapsulated drugs are delivered intracellularly. In principle, three routes that may lead to intracellular delivery of encapsulated compounds can be discerned. Firstly, immunoliposomes may, after specific cell binding, release their contents in close proximity of the target cells, with subsequent cellular uptake of released molecules. Secondly, immunoliposomes may fuse with the cell membrane, thereby releasing their contents into the cytosol. Thirdly, cell-bound immunoliposomes may be internalised by the target cells via receptor-mediated endocytosis, followed by the intracellular release of encapsulated agents. Whether immunoliposomes will be internalised or not is dependent on a variety of factors, such as liposome size [22–25], type of cell and type of target receptor [26–28]. Targeting of im-

munoliposomes to receptors with known internalising capacities (e.g. transferrin receptor, low density lipoprotein-receptor, epithelial growth factor receptor) will likely result in internalisation of bound immunoliposomes, provided that they are relatively small in size ($< 0.2$ $\mu$m) [29–31]. On the other hand, it has been shown that immunoliposomes targeted to non-internalising cell surface molecules on tumour cells remain located at the cell surface [32].

Here, we report on the interaction of ICAM-1-directed immunoliposomes with ICAM-1 expressing human bronchial epithelial cells in vitro. We have quantified the degree of cell binding of ICAM-1-directed liposomes and we demonstrate that specific binding of these immunoliposomes to ICAM-1 on the bronchial epithelial cell line BEAS-2B triggers rapid internalisation of cell-bound immunoliposomes. The results are discussed in the context of targeted drug delivery to sites of inflammation, characterised by an increased expression of ICAM-1.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Liposome-related materials

Cholesterol (CHOL), *N*-succinimidyl-*S*-acetylthioacetate (SATA), *N*-ethylmaleimide, and calcein were obtained from Sigma (Rockford, IL, USA). Chloroform and methanol (pro analysi) were obtained from Merck (Darmstadt, Germany), and *N*,*N*-dimethylformamide (DMF) and hydroxylamine hydrochloride from Janssen Chimica (Geel, Belgium). Partially hydrogenated egg phosphatidylcholine with an iodine value of 40 (PHEPC; Asahi, Ibarakiken, Japan) was prepared as described previously [33]. Egg phosphatidylglycerol (EPG) was kindly donated by Nattermann (Cologne, Germany), *N*-[4-(*p*-maleimidophenyl) butyryl]phosphatidylethanolamine (MPB-PE) was synthesised as described before [32,34].

#### 2.1.2. Cell-related materials

IFN-$\gamma$ was purchased from Boehringer-Mannheim (Mannheim, Germany), keratinocyte medium (keratinocyte-SFM) and supplements for keratinocyte-SFM, containing recombinant human epithelial

growth factor (rhEGF), and bovine pituitary extract came from Gibco, Grand Island, NY, USA. Bovine serum albumin (fraction V), fluorescein isothiocyanate (FITC)-labelled goat anti-mouse F10.2 IgG (Fab′-specific) and *R*-phycoerythrin (PE)-labelled goat anti-mouse IgG were obtained from Sigma. Anti-ICAM-1 mAb (IgG$_1$), clone F10.2 was prepared and isolated as described before [35]. Formaldehyde was obtained from Janssen (Geel, Belgium).

## 2.2. Cell culture

The human bronchial epithelial cell line BEAS-2B obtained from Dr J.F. Lechner (National Institutes of Health, Bethesda, MD, USA) was cultured in serum-free keratinocyte medium, supplemented with bovine pituitary extract (25 µg/ml), rhEGF (2.5 ng/ml) and gentamicin (50 µg/ml). In order to obtain an enhanced expression of ICAM-1, BEAS-2B cells were stimulated with IFN-γ (200 U/ml) 24 h prior to use in immunoliposomes binding and internalisation experiments [7]. BEAS-2B cells were cultured at 37°C with 5% CO$_2$ in humidified air.

## 2.3. Preparation of immunoliposomes

Immunoliposomes, bearing covalently coupled F10.2 mAbs on their surface, were prepared as described previously [19]. In short, liposomes were made from PHEPC, EPG, CHOL, and MPB-PE (38.5:4:16:0.06 molar ratio) by lipid film hydration and subsequent extrusion through 0.2 µm polycarbonate filters [36]. When indicated, calcein was incorporated as an aqueous marker at a concentration of 90 mM and/or rhodamine-PE at an amount of 0.1 mol% of total lipid. Freshly thiolated mAb F10.2 (100 µg/ml final concentration) was added to the liposomes (73.3 µmol phospholipid/8 ml) and incubated for 1 h and 45 min at room temperature under constant rotation. The coupling reaction was terminated by adding 50 µl of freshly prepared 8 mM *N*-ethylmaleimide in HEPES buffer (10 mM HEPES; 1 mM EDTA; 135 mM NaCl pH 7.4). Liposomes were separated from unconjugated mAbs by ultracentrifugation (4 runs of 30 min at $60\,000 \times g$) and stored at 4°C. As a control in binding and internalisation experiments, liposomes with the above mentioned composition were used without conjugated F10.2 mAb.

## 2.4. Liposome characterisation

Mean particle size was determined by dynamic light scattering as described before [19]. Mean size of liposomes was 0.2–0.25 µm with a polydispersity index varying from 0.06 to 0.15. Phospholipid concentration was determined by the colorimetric method of Fiske and Subbarow [37]. The amount of conjugated IgG on the liposomal surface was determined with the Bio-Rad *DC* protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with mouse IgG as standard. The protein coupling ratios for the different batches of immunoliposomes were in the range of 2–4 µg IgG/µmol phospholipid, which corresponds to approximately 7–15 IgG molecules per liposome.

## 2.5. Quantification of the degree of cell binding

IFN-γ-activated BEAS-2B cells were detached from culture flasks with trypsin/EDTA (Gibco) in phosphate-buffered salt solution (PBS) for 5 min at 37°C, pelleted by centrifugation (5 min at $750 \times g$) and washed once with immunofluorescence (IF) buffer (1% bovine serum albumin and 0.05% sodium azide in PBS, pH 7.4). A typical washing step involved resuspension of cells into 1 ml of indicated buffer, followed by centrifugation (5 min at $750 \times g$). Cells ($2.5 \times 10^5$ cells for each sample) were resuspended in 300 µl of IF buffer containing varying concentrations of control liposomes or F10.2 immunoliposomes with entrapped calcein as fluorescent label. Cells were incubated in the presence of liposomes for 1 h on ice, washed three times with IF buffer to remove unbound liposomes and resuspended in 300 µl IF buffer before a sample of 50 µl was taken (corresponding to $2.5 \times 10^4$ cells). Samples were diluted with 150 µl of 1% Triton X-100 in PBS and incubated for 15 min at room temperature. Solubilised samples were transferred to a 96-well plate and the fluorescence intensity of the sample was measured with a LS-50B luminescence spectrometer (Perkin Elmer, Beaconsfield, Bucks, UK) with excitation wavelength set at 490 nm and emission at 520 nm. A reference curve was constructed from cal-

cein-containing F10.2 immunoliposomes with a known phospholipid concentration. Background value was obtained from solubilised cells without prior incubation with liposomes.

### 2.6. Cellular internalisation assays

#### 2.6.1. Determination of the amount of cell surface-bound immunoliposomes as a function of incubation time

Cell surface-bound F10.2 immunoliposomes were determined by indirect immunofluorescence analysis as described by Suzuki et al. [38]. In short, IFN-γ-activated BEAS-2B cells ($2 \times 10^5$) were detached from culture flasks using trypsin/EDTA and $2 \times 10^5$ cells were incubated with F10.2 immunoliposomes (0.5 μmol phospholipid/ml) in a total volume of 200 μl keratinocyte medium for 60 min on ice. Cells were washed twice with ice-cold keratinocyte medium, resuspended into 300 μl medium and cultured for 0–3 h at 37°C. Cells were washed once with IF buffer and incubated with goat anti-mouse IgG fluorescently labelled with phycoerythrin (100 fold diluted in IF buffer) for 30 min on ice. After washing twice with IF buffer, cells were analysed by flow cytometry using a FACscan flow cytometer (Becton and Dickinson, Mountain View, CA, USA). The procedure above was also performed on formaldehyde-fixed cells (cells were fixed by incubating with 2% formaldehyde in PBS for 10 min at room temperature).

#### 2.6.2. Determination of intracellular calcein

Detached IFN-γ-stimulated BEAS-2B cells were incubated with calcein containing F10.2 immunoliposomes as described in Section 2.6.1. After two washing steps with ice-cold keratinocyte medium to remove the unbound liposomes, cells were cultured at 37°C for varying time periods (0–2 h). Hereafter, cells were incubated for 10 min in citric acid buffer (40 mM citric acid, 120 mM NaCl, pH 3.0), washed twice with IF buffer and analysed by flow cytometry using a FACscan flow cytometer for cell-associated calcein fluorescence. The mean fluorescence intensity of 10 000 cells was determined for each sample.

#### 2.6.3. Confocal laser microscopy

In these experiments, the F10.2 immunoliposomes

contained encapsulated calcein (90 mM) as a fluorescent aqueous content marker and were labelled with rhodamine-PE as a liposomal lipid marker. Detached, IFN-γ-stimulated BEAS-2B cells were mixed with F10.2 immunoliposomes in keratinocyte medium on ice as described above (Section 2.6.1). Cells were washed once with IF buffer and once with keratinocyte SFM medium and resuspended into 300 μl medium. Subsequently, cells were incubated for varying time periods (0–60 min) at 37°C. Thereafter, cells were either washed twice with IF buffer or three times with citric acid buffer (pH 3.0) and once with IF buffer (pH 7.4). Cells were then fixed with 2% formaldehyde in PBS for 1 h on ice and fixed to slides. Fixed cells were analysed for fluorescence using a laser confocal scanning microscope (Leica TCS NT Laser confocal scanning microscope, Heidelberg, Germany). By using filters of 525–550 nm and >590 nm, rhodamine and calcein fluorescence could be detected separately.

## 3. Results and discussion

### 3.1. Quantification of the degree of cell binding

In a previous study, we have demonstrated that F10.2 immunoliposomes are able to specifically bind to different cell types expressing ICAM-1. The degree of cell binding appeared to be positively cor-
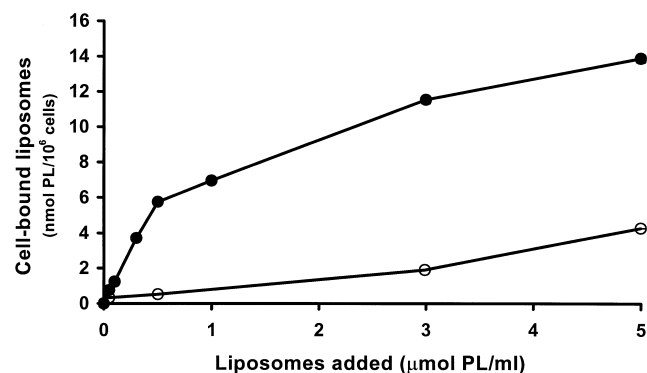


Fig. 1. Effect of liposome concentration on the extent of liposome binding to BEAS-2B cells. INF-γ-stimulated BEAS-2B cells ($1 \times 10^6$ cell/ml) were incubated with F10.2 immunoliposomes (●) or with control liposomes (○) for 1 h on ice. After removal of unbound liposomes, cell-associated calcein fluorescence was determined with a fluorometer ($n = 3$).

related with the degree of ICAM-1 expression [19]. In this study, we have quantified the degree of binding of F10.2 immunoliposomes to IFN-γ-activated BEAS-2B cells. The activated epithelial cells were used as a model system to study targeting efficiency of F10.2 immunoliposomes to cells with an increased expression of ICAM-1 as found at sites of inflammation. The degree of binding of F10.2 immunoliposomes to IFN-γ-stimulated BEAS-2B cells was fluorometrically determined by analysing cell-associated calcein fluorescence after cells were incubated with calcein-containing F10.2 immunoliposomes for 1 h on ice. Fig. 1 shows that the absolute amount of cell-bound immunoliposomes increased with increasing concentrations of calcein-containing F10.2 immunoliposomes. Control liposomes with the same lipid composition but without conjugated F10.2 mAbs showed a much lower degree of cell binding. From the calcein fluorescence values, the number of bound liposomes per cell can be estimated, assuming an average of 1.5 phospholipid bilayers per liposome and an average size of 200 nm (Fig. 2). At the highest concentration of F10.2 immunoliposomes used, approximately 15 400 liposome particles are estimated to be bound per cell. However, at such high concentrations, liposomes without conjugated F10.2 mAbs

also show considerable cell binding (4200 liposomes bound per cell), indicating that at high liposome concentrations part of the cell binding is mediated by other interactions than antibody-antigen interaction. Therefore, the experiments described below were carried out with immunoliposome concentrations of 300–500 nmol phospholipid/ml, at which the binding ratio of F10.2 immunoliposome versus control liposomes is maximal. At these concentrations, approximately 4000–7000 F10.2 immunoliposomal particles were binding per cell as compared to only 200–300 particles in case of control liposomes.

### 3.2. Determination of the amount of cell surface-bound F10.2 immunoliposomes as a function of incubation time

Cell surface-bound F10.2 immunoliposomes were determined by indirect immunofluorescence analysis as a function of incubation time at 37°C. Cells were incubated with F10.2 immunoliposomes for 1 h on ice and after removal of unbound particles, the cells were cultured at 37°C for varying time periods before analysis by flow cytometry. The amount of immunoliposomes that could be detected on the surface of BEAS-2B cells with FITC-conjugated antibodies di-
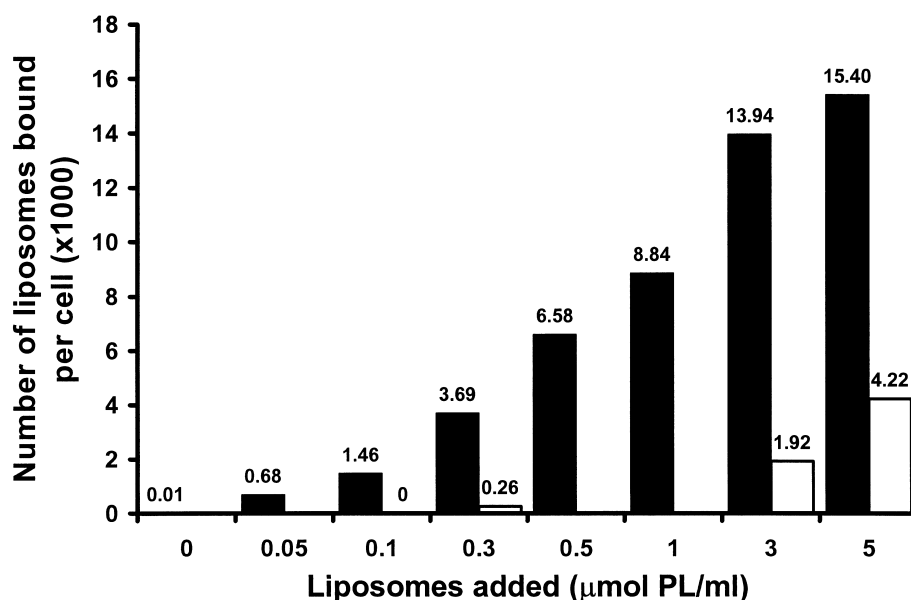


Fig. 2. Effect of liposome concentration on the number of liposomes bound per cell. IFN-γ-stimulated BEAS-2B cells were incubated with F10.2 immunoliposomes (black bars) or with control liposomes (white bars) essentially as described in Fig. 1. The exact values of the number of bound liposomes are depicted above each bar. Results of a typical experiment are shown.
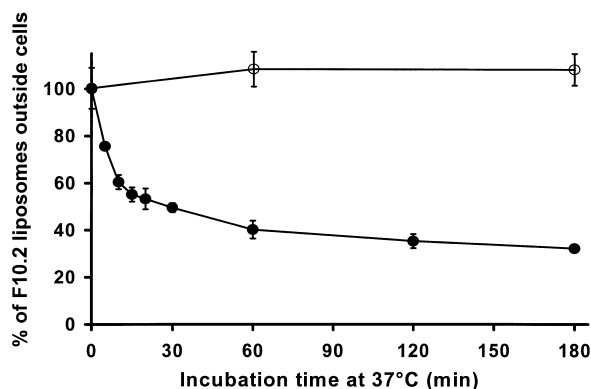
Fig. 3. Determination of cell surface-bound F10.2 immunoliposomes on BEAS-2B cells during prolonged incubation periods at 37°C. Intact (●) or formaldehyde-fixed (○) cells ($1 \times 10^6$/ml) were incubated with F10.2 immunoliposomes (500 nmol phospholipid/ml) for 1 h on ice, washed twice with keratinocyte-SFM medium (without serum), and then cultured at 37°C in SFM medium for the indicated time periods. Subsequently, cells were labelled with phycoerythrin-conjugated goat anti-mouse mAbs and analysed by flow cytometry ($n=4$).
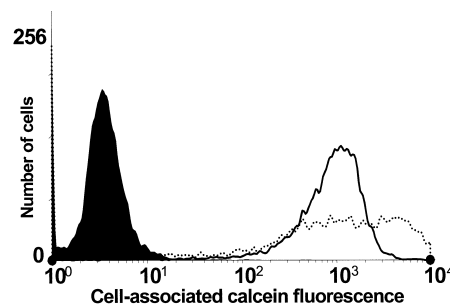


Fig. 4. Flow cytometric analysis of cell-associated calcein fluorescence. BEAS-2B cells were incubated with F10.2 immunoliposomes (500 nmol phospholipid/ml) on ice and unbound liposomes were removed. Thereafter cells were either directly analysed for associated calcein fluorescence (solid line) or after 1 h incubation at 37°C (dotted line). Autofluorescence of BEAS-2B cells is also illustrated (in black).

rected against the liposome conjugated antibodies decreased during prolonged incubation times at 37°C (Fig. 3). After 1 h incubation at 37°C, 60% of the starting amount of cell-bound liposomes had disappeared from the cell surface. Control experiments using cells fixed with formaldehyde prior to liposome binding did not show any reduction in the amount of cell-bound F10.2 immunoliposomes, indicating that the decrease of cell surface-bound immunoliposomes is not caused by dissociation of immunoliposomes from the cell surface, but is related to an active cellular process. This finding strongly suggested that F10.2 immunoliposomes are internalised by BEAS-2B cells.

### 3.3. Calcein release from F10.2 immunoliposomes during cellular internalisation

The next step was to look at the fate of the encapsulated fluorescent marker calcein upon internalisation of F10.2 immunoliposomes by BEAS-2B cells. Cells were incubated with F10.2 immunoliposomes for 1 h on ice and unbound particles were washed away. Hereafter, cell-associated calcein fluorescence was determined by flow cytometry, either directly or after 1 h incubation at 37°C (Fig. 4). The results

show that after 1 h incubation at 37°C, the amount of cell-associated calcein fluoresence had increased in comparison with the cell-associated calcein fluorescence before the 1 h incubation at 37°C. As calcein is encapsulated in the liposomes at a quenching concentration (90 mM), an increase of calcein fluorescence intensity can be explained as a dequenching effect due to calcein release from the liposomes.

In order to demonstrate that dequenching of calcein fluorescence is caused by the intracellular release of calcein from internalised F10.2 immunoliposomes and not merely by extracellular leakage of encapsulated calcein from cell-bound F10.2 immunoliposomes, an attempt was made to remove the cell-bound immunoliposomes by short exposure to low pH (10 min at pH 3.0). It is described in the literature that such exposure to low pH will liberate immunoliposomes from the cell surface, without disrupting the cell membrane [39,40]. However, confocal laser microscopic analysis showed us that acidic exposure of BEAS-2B cells does not result in the detachment of bound F10.2 immunoliposomes from the cell surface, but rather in release of calcein from the cell-bound liposomes (see below, Fig. 6B,D). Nevertheless, acidic treatment of cells can be used to discriminate between immunoliposome-encapsulated calcein located at the cell surface from internalised immunoliposome-encapsulated calcein as this method completely removes immunoliposome-
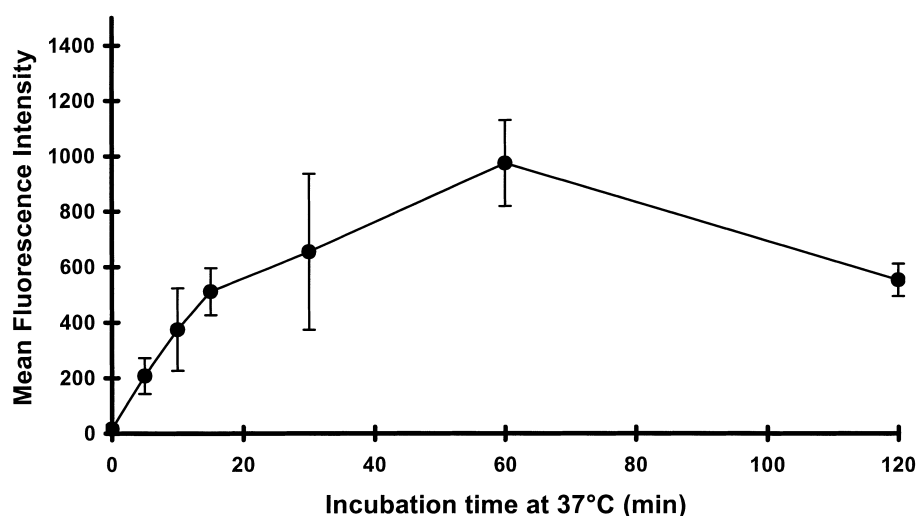
Fig. 5. Flow cytometric analysis of immunoliposome-encapsulated calcein internalisation into BEAS-2B cells. Cells $(1 \times 10^6$ cells/ml) were incubated with F10.2 immunoliposomes at a concentration of 500 nmol phospholipid/ml for 1 h on ice, washed twice with kera-tinocyte-SFM medium and incubated at 37°C in SFM medium for the indicated time periods. Thereafter, cells were exposed to acidic condition (citric acid buffer, pH 3.0) for 10 min and washed twice with IF buffer before flow cytometric analysis $(n = 2)$.

encapsulated calcein fluorescence located at the cell surface. The acid treatment method was used to determine the degree of intracellular calcein-fluorescence as a function of incubation time at 37°C (Fig. 5). For this experiment, cells were pre-incubated with F10.2 immunoliposomes for 1 h on ice to load cells with immunoliposomes. Hereafter, cells were cultured at 37°C for varying time periods followed by acid treatment to remove the calcein from immunoliposomes located at the cell surface. Fig. 5 shows that the amount of intracellular calcein fluorescence increases at increasing incubation times at 37°C. The observation that cell-associated calcein fluorescence decreases after 2 h incubation at 37°C may be caused by intracellular compounds that are able to quench the calcein fluorescence. For example, $Fe^{2+}$ ions present in the cell will quench calcein fluorescence [41]. However, the possibility that calcein that is released intracellularly will subsequently leak out of the cells cannot be excluded.

### 3.4. Confocal laser microscopic analysis of F10.2 immunoliposome internalisation and subsequent intracellular release of calcein

We have further analysed the process of liposome internalisation by confocal laser microscopy (Fig. 6). Cells were incubated with F10.2 immunoliposomes containing two fluorescent markers; rhodamine-PE as a lipid marker and encapsulated calcein as an aqueous marker. After incubation for 1 h at 4°C, both calcein fluorescence and rhodamine fluorescence were observed associated with the cell membrane (Fig. 6A). By superimposing two pictures taken with two different filter settings (525–550 nm and > 590 nm), it can be seen that the liposomal membrane marker rhodamine-PE and the aqueous marker calcein are co-localised as visualised by the yellow fluorescence. Calcein fluorescence, but not rhodamine fluorescence, could be removed from the cell surface by exposing the cells for 10 min to pH 3.0 (Fig. 6B).

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Fig. 6. Confocal laser microscopic analysis of the internalisation of F10.2 immunoliposomes containing calcein and rhodamine-PE as fluorescent tracers into IFN-γ-activated BEAS-2B cells. BEAS-2B cells were incubated with F10.2 immunoliposomes either for 1 h on ice (A,B) or for 1 h on ice and subsequently for 1 h at 37°C (C,D). Thereafter, cells were either directly analysed by confocal laser microscopy (A,C) or exposed to citric acid buffer (pH 3.0) for 10 min (B,D) before analysis. Used filter settings were 525–550 nm (green fluorescence) and > 590 nm (red fluorescence). Superimposed pictures of both filter settings are also illustrated.
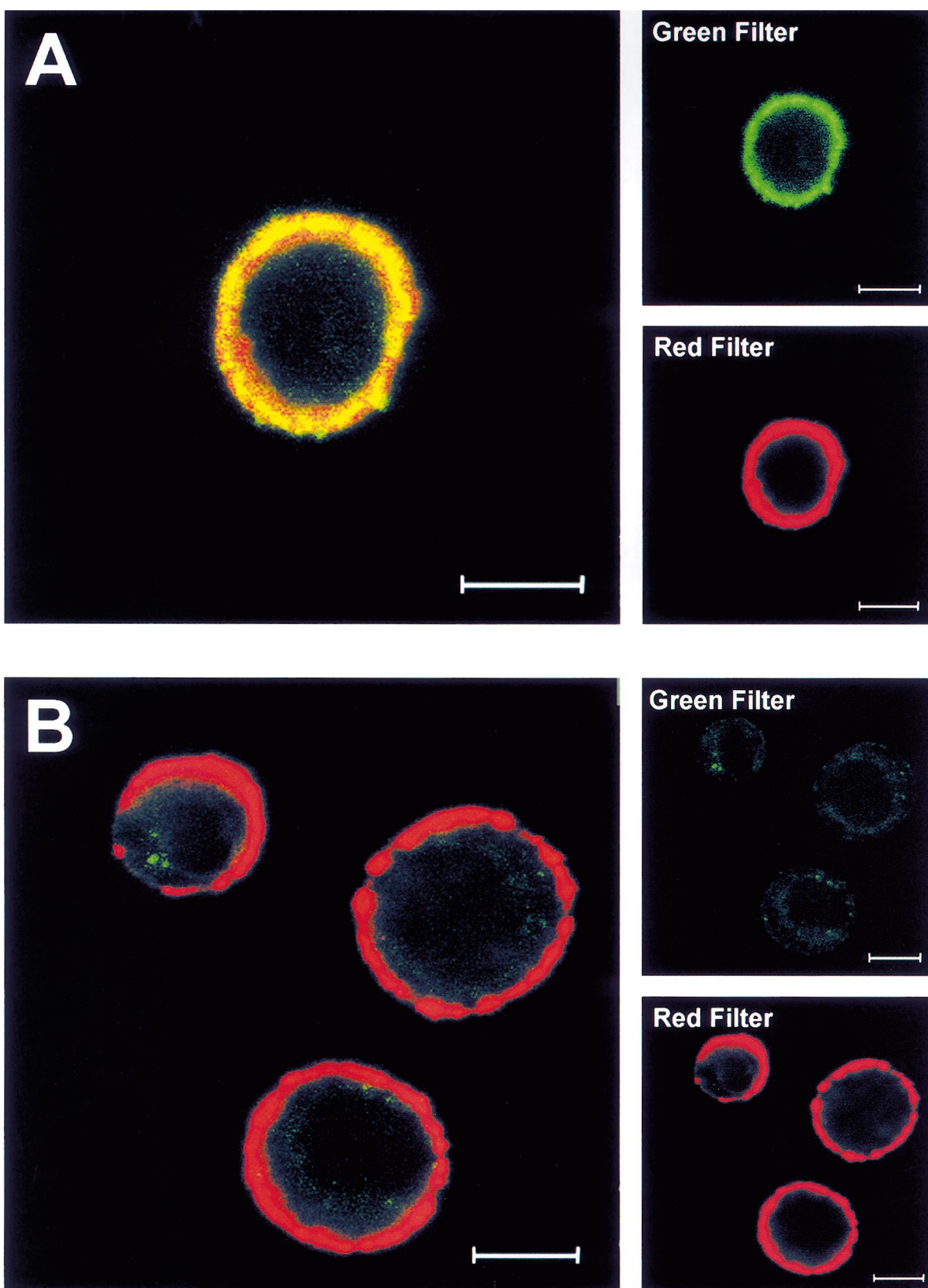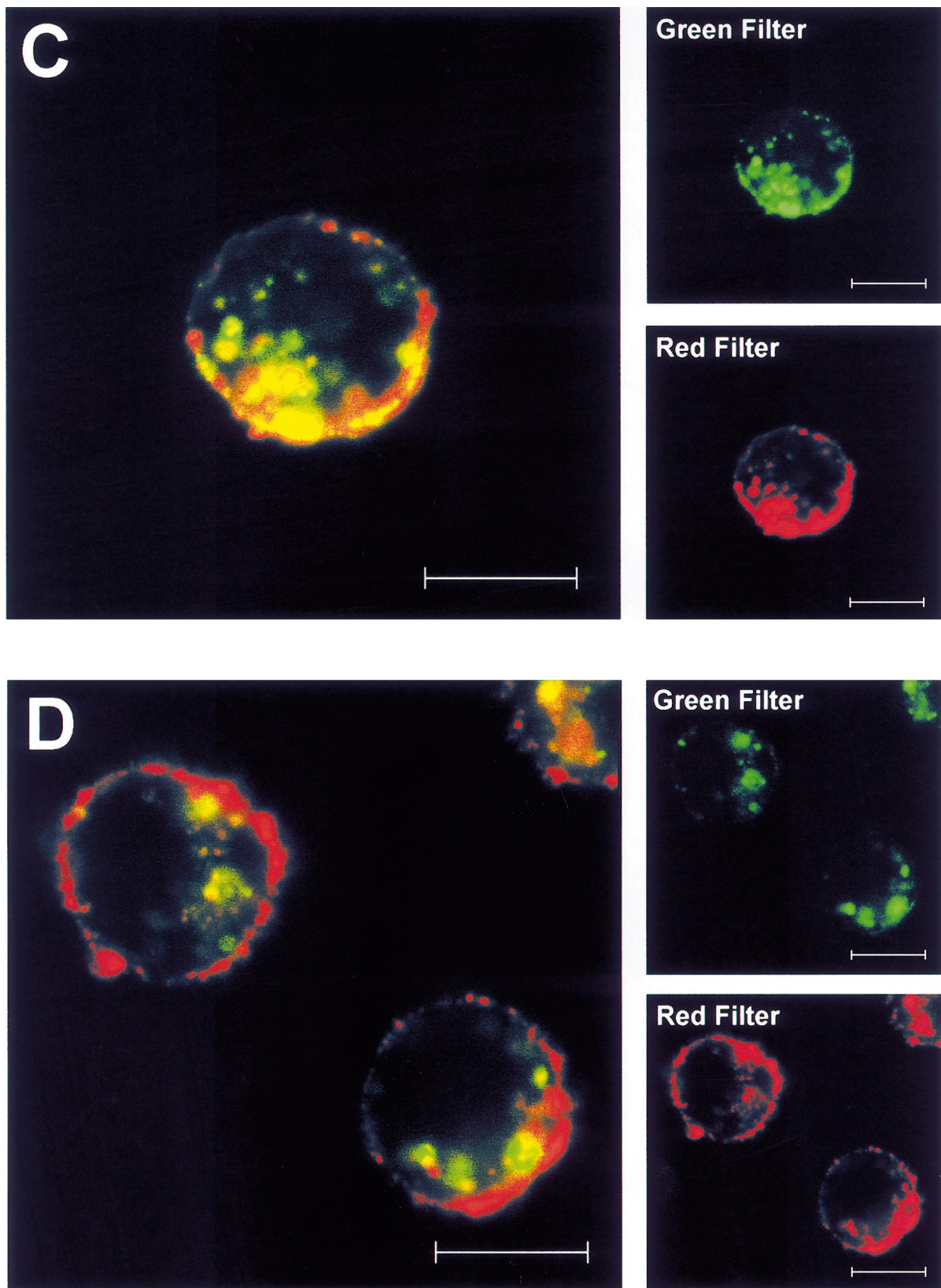
Fig. 6 (*continued*)

Incubation of cells with surface-bound F10.2 immunoliposomes at 37°C resulted in the appearance of bright fluorescent vacuoles in the cell interior, besides the cell membrane-associated fluorescence (Fig. 6C). Exposure to low pH showed the disappearance of surface-bound calcein fluorescence, but not of calcein located inside intracellular vacuoles (Fig. 6D), confirming the evidence that immunoliposomes, together with the encapsulated calcein, are internalised.

### 3.5. Overall conclusions

Taking all results together, we can conclude that immunoliposomes targeted to ICAM-1 on IFN-γ-activated human bronchial epithelial cells are able to bind to these cells. Depending on the concentration of immunoliposomes added, 1–14 nmol liposomal phospholipid appeared to bind per $10^6$ BEAS-2B cells after 1 h incubation on ice, which corresponds to 680–15 400 particles bound per cell. Furthermore, we studied whether ICAM-1-directed immunoliposomes are internalised by BEAS-2B cells. Little is known about the internalisation characteristics of ICAM-1 molecules. ICAM-1 is utilised as a target receptor by the major group of human rhinoviruses. Binding to ICAM-1 results in receptor-mediated endocytosis of the bound virus particles [42]. On the other hand, internalisation of monoclonal antibodies directed against ICAM-1 (mAb RR1/1) on human umbilical vein endothelial cells could not be detected [43]. In this study, it was observed that F10.2 immunoliposomes are rapidly internalised after specific binding to ICAM-1 expressing BEAS-2B cells. After 1 h incubation at 37°C, 60% of the surface-bound immunoliposomes were internalised. These findings have implications for the development of drug-targeting strategies to interfere in inflammatory processes. The rapid and extensive internalisation of ICAM-1-targeted liposomes make them suitable carriers for the intracellular delivery of anti-inflammatory drugs. Also, ICAM-1-directed immunoliposomes may be used to selectively deliver antisense oligonucleotides to endothelial and epithelial cells to down-regulate adhesion molecule expression at sites of inflammation. As ICAM-1 is involved in the migration of inflammatory cells into inflamed tissues, down-regulation of ICAM-1 expression by antisense oligonucleotides will inhibit the migration

of immune cells into inflamed tissues [44], thereby inhibiting the inflammatory response [45,46]. However, it should be realised that the liposomal system needs to be further optimised before application in vivo. The immunoliposome composition used in the present in vitro study may not be optimal in vivo. Also, the presence of whole antibody molecules exposed on the liposome surface may give problems related to immunogenicity when applied repeatedly in vivo [47,48]. In addition, circulating time after intravenous injection might be too short to allow efficient interaction with target epitopes.

Although it was observed that calcein is released from the immunoliposomes upon internalisation, this does not guarantee that other encapsulated compounds will become available to intracellular targets after internalisation of liposomes. The confocal microscopic findings strongly suggest that internalisation occurs via receptor-mediated endocytosis. A large amount of endocytosed liposomes will eventually end up in lysosomal, degradative compartments [22,23,49]. We are currently exploring ways to escape lysosomal degradation by incorporating pH-dependent fusogenic properties into the immunoliposomal carrier, in order to promote delivery over the endosomal membrane and thereby improving the therapeutic availability of the encapsulated compound after cellular internalisation.

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