

# Langerhans-like dendritic cells generated from cord blood progenitors internalize pollen allergens by macropinocytosis, and part of the molecules are processed and can activate autologous naive T lymphocytes

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**Background:** The safety and efficacy of sublingual immunotherapy have been demonstrated in moderate allergic asthma and seasonal rhinitis. However, not much is known about the precise mechanism of action of the allergen when it crosses the oral mucosa.

**Objective:** To define this mechanism, we investigated the role of Langerhans' cells in the capture and internalization of allergens.

**Methods:** We generated dendritic cells in vitro with the phenotypic characteristics of Langerhans-like dendritic cells (LLDCs) from cord blood CD34<sup>+</sup> progenitors. We used two recombinant major allergens: Bet v 1 and Phl p 1 labeled with FITC.

**Results:** Internalization of allergens and control proteins was dose- and time-dependent and related to the immature state of the cells. LLDCs internalized allergens with a high efficiency in comparison with control molecules. Allergens were only internalized by macropinocytosis, as demonstrated by the use of various inhibitors. Addition of intracellular pH-modifying molecules indicated that only a part of the allergens was accumulated in acidic vesicles, whereas the majority remained in other cytoplasmic structures. Pulse-chase experiments calculated a half-life of 4 hours, suggesting that part of the molecules were not metabolized in the lysosome. Allergen internalization by LLDCs might be followed by processing in some experiments, as demonstrated by activation of autologous T lymphocytes in 4 of 9 experiments.

**Conclusion:** These elements showed that Langerhans' cells present in mucosa might play an active role in immune responses to allergens. (*J Allergy Clin Immunol* 2000;105:1194-1201.)

**Key words:** Allergy, antigen-presenting cell, dendritic cells, Langerhans' cells, recombinant Bet v 1, recombinant Phl p 1, internalization, macropinocytosis, primary response

## Abbreviations used

BB: Bandrowski's base

DC: Dendritic cell

LC: Langerhans' cell

LLDC: Langerhans-like dendritic cell

Dendritic cells (DCs) present in lymphoid and non-lymphoid tissues are derived from bone marrow progenitors. Langerhans' cells (LCs) localized in suprabasal layers of the skin and in mucosa represent a subset of DCs that are characterized by the presence of cytoplasmic organelles termed Birbeck granules and by the presence of Lag antigen and Langerin, a new recently described molecule.<sup>1</sup> DCs play a key role in antigen presentation for initiation of the primary T-cell response. In vivo, LCs are able to uptake antigens and subsequently migrate through lymphatics or blood vessels to the T cell-rich zone of secondary lymphoid tissues and are then called interdigitating cells. During migration, they undergo an additional maturation step and become able to present the processed antigen to naive T cells.<sup>2</sup>

The prevalence of type I allergy, such as atopic asthma (a potentially fatal chronic disease),<sup>3</sup> allergic rhinitis, and atopic dermatitis, has increased over the past two generations. Patients are usually desensitized by means of subcutaneous administration of allergens. Over recent years, several trials have been conducted to induce desensitization by oral or sublingual administration of allergens. Most studies were double-blind placebo-controlled trials, and positive results were demonstrated with grass pollen extract,<sup>4-6</sup> house dust mites,<sup>7</sup> and olive pollen extract.<sup>8</sup> However, disappointing results have also been published.<sup>9-11</sup> Oral or sublingual immunotherapy is usually effective, easy to perform, inexpensive, and safe. Because LCs are present at the mucosal site, it seemed interesting to study the potential role of LCs in the uptake of allergens to determine whether they play a role in the induction of allergy or in the mechanism of desensitization.

Isolation of LCs from mucous membranes is tedious, and only a small number of LCs are obtained. We chose to use in vitro-produced LCs (Langerhans-like dendritic

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cells [LLDCs]) because since 1992, human DCs with the characteristics of LCs can be generated in vitro from cord blood CD34<sup>+</sup> progenitors.<sup>12</sup>

In this study we investigated the first contact between LLDCs and allergens. We used recombinant (r)Bet v 1 and recombinant (r)Phl p 1 allergens, which were designated as major allergens frequently involved in respiratory allergy, and investigated allergen uptake and the mechanisms of internalization in comparison with reference molecules, such as FITC-dextran and Lucifer yellow. We showed that recombinant allergens are internalized by macropinocytosis and could induce a proliferative response of naive T lymphocytes in some donors.

## METHODS

### Purification and culture of cord blood CD34<sup>+</sup> hematopoietic cells

Cord blood was collected according to institutional guidelines during normal term deliveries. Mononuclear cells were isolated by flotation on Ficoll (Lymphoprep; Nycomed Pharma AS, Oslo, Norway) and then depleted of adherent cells by overnight culture on a plastic surface. CD34<sup>+</sup> cells were purified by means of immunomagnetic selection with mini-MACS (Miltenyi-Biotec GmbH, Bergisch Gladbach, Germany). This procedure regularly produced a suspension containing more than 95% of CD34<sup>+</sup> cells. Isolated progenitors were cultured in RPMI-1640 containing Glutamax-1 and 25 mmol/L HEPES supplemented with 10% heat-inactivated FCS (FCS Myclone super plus; GIBCO-BRL Life Technologies, Cergy Pontoise, France),  $5 \times 10^{-5}$  mol/L 2-mercaptoethanol (M-6250; Sigma Chemical Co, St Louis, Mo), antibiotics (penicillin-streptomycin-amphotericin B, A-5955, Sigma), 200 U/mL recombinant GM-CSF (specific activity  $2 \times 10^6$  U/mg; kindly provided by Schering Plough Laboratories, Dardilly, France), and 50 U/mL recombinant human TNF- $\alpha$  (specific activity,  $2 \times 10^7$  U/mg; Genzyme Corp, Boston, Mass).

### Phenotypic analysis

Cells were washed with isotonic NaCl/Pi buffer containing 1% BSA, 0.6% acid citrate-dextrose (ACD), and 0.02% sodium azide (PBS-BSA-ACD-azide). Cells ( $1.5 \times 10^5$ ) were incubated with 10  $\mu$ L of labeled mAb for 30 minutes at 4°C. The mAbs used were CD1a (B-B5, IgG1) from Diaclone, CD83 (HB15A, IgG2k) from Immunotech (Marseille, France), HLA-DR (L243, IgG2a) from Becton Dickinson, CD14 (TÜK4, IgG2k) and S-100 protein (polyclonal) from DakoPATTS (Glostrup, Denmark), E-cadherin (HECD-1, IgG1) from Takara (Japan), and Factor XIIIa (polyclonal) from Behring. Anti-Lag mAb (Langerhans-associated granule) was kindly provided by Dr Kashiwara-Sawami<sup>13</sup> (Kyoto, Japan). Anti-Langerin mAb (clone DCGM4) was kindly provided by Dr Sem Saeland<sup>1</sup> (Schering-Plough Research Laboratories, Dardilly, France). Using unlabeled antibodies, we added a second step, including GAMIg-FITC (Zymed Laboratories, San Francisco, Calif). After washes in PBS-BSA-ACD-azide, cells were fixed with 1% formaldehyde in PBS-BSA-ACD-azide. Analysis was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif). Cell size was estimated by means of light diffraction at small angles (FSC).

### Fluorescent markers

FITC-dextran (molecular weight, 40,000 d; reference D-1845) and Lucifer yellow CH (reference L-1177) were purchased from Molecular Probes (Eugene, Ore). rBet v 1 (molecular weight, 17,538 d)<sup>14</sup> and rPhl p 1 (molecular weight, 26,096 d) produced by

*Escherichia coli* were purchased from Advanced Biological Systems (Salzburg, Austria).

rBet v 1 and Phl p 1 were labeled with FITC by using the FluoroTag FITC Conjugation Kit (Sigma). The FITC/protein molar ratio ranged between 0.7 and 1.14 with rBet v 1 and 1.3 and 4.2 with rPhl p 1, according to the preparation.

### Internalization

Samples of  $1.5 \times 10^5$  cells were washed in RPMI-FCS and centrifuged, and 20  $\mu$ L of markers were added to the cell pellet. Incubation was performed at 37°C or 4°C with various time and concentration conditions. After incubation, the cells were washed 3 times with ice-cold PBS containing 1% FCS, 0.01% Na<sub>3</sub>N, and 0.6% ACD.

Inhibition of marker uptake was studied with 25  $\mu$ mol/L monensin<sup>15</sup> (Sigma, M-5273), 250  $\mu$ mol/L chloroquine<sup>16</sup> (Sigma, C-6628), 50  $\mu$ mol/L phenylarsine oxide<sup>17</sup> (Sigma, P-3075), or 5  $\mu$ mol/L cytochalasin D<sup>18</sup> (Sigma, C-8273). Cells were incubated with inhibitors for 1 hour at 37°C before addition of markers; inhibitors were also present during the incubation time with fluorescent markers.

Inhibition of marker release was studied with 1 mmol/L amiloride<sup>19</sup> (Sigma, A-7410). The intracellular pH in endosomes and lysosomes was neutralized by a 30-minute incubation at 4°C after internalization of markers.

Fluorescence analysis was performed on a FACScan flow cytometer (Becton Dickinson). A calibration curve was constructed with DAKO QIFIKIT (reference K-0078, DAKO A/S) to calculate the number of molecules per cell.

### Confocal microscopy

Cells were adhered to glass slides (CML, Nemours, France) previously coated with 0.01% poly-L-lysine (P 8920, Sigma) and then incubated with fluorescent markers. Slides were washed with 10 mmol/L glycine (9582G-2; Research Organics, Inc, Cleveland, Ohio) and fixed in 4% paraformaldehyde. Slides were mounted in Prolong (Molecular Probes, Inc, Eugene, Ore) to minimize photobleaching and analyzed with a confocal laser scanning microscope system (LSM 310, Carl Zeiss). We used an Argon laser ( $\lambda$ 488 nm) with an appropriate filter.

### T cell-proliferative assays

T lymphocytes were isolated from the mononuclear cells remaining after isolation of CD34 progenitors by rosetting with 2-aminoethylisothiuronium bromide-treated (Sigma, A-5879) sheep erythrocytes. Rosettes were recovered by Ficoll-Paque flotation, and red cells were lysed with NH<sub>4</sub>Cl buffer. Dendritic cells were incubated for 48 hours at 37°C with 0.6  $\mu$ g/mL rBet v 1 or 1.25  $\mu$ g/mL rPhl p 1 or, for a positive proliferative control, with 1  $\mu$ g/mL Bandrowski's base (BB; ICN Biomedicals Inc, Aurora, Ohio). Stimulator cells were cocultured with 10<sup>5</sup> autologous responder T cells in round-bottom, 96-well, microtiter plates in RPMI-1640 supplemented with 10% FCS in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. Proliferation was assessed by addition of 1  $\mu$ Ci of tritiated methyl-thymidine per well (2 Ci/mmol, Amersham) 18 hours before harvesting. Incorporated thymidine was quantified in a  $\beta$ -counter with low background (Matrix 96; Packard Instrument, Downers Grove, Ill). Results are expressed as mean disintegrations per minute  $\pm$  SD of sextuplet cultures. The Fisher exact probability test was used for statistical analysis.<sup>20</sup>

## RESULTS

### rBet v 1 and rPhl p 1 were internalized in a time- and dose-dependent manner

Internalization was studied with DCs taken between 9 and 10 days of differentiation by using sequential dilu-

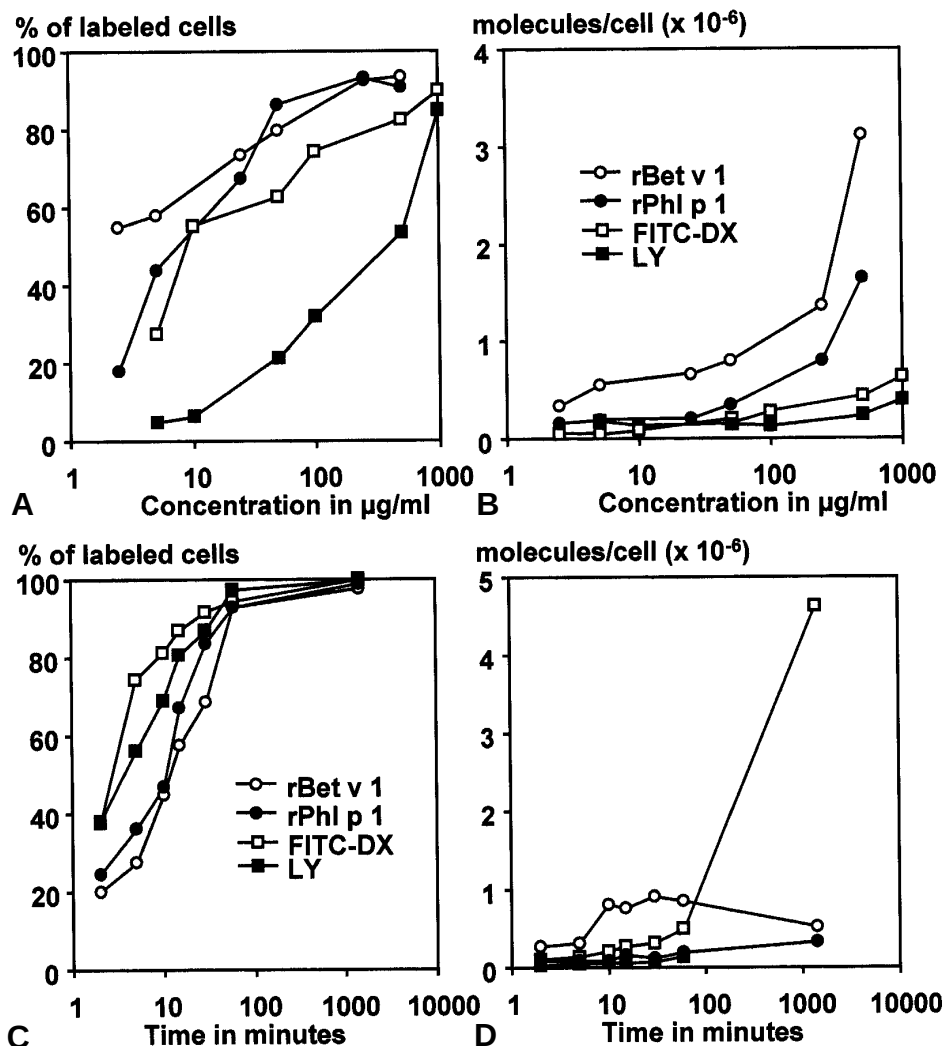


**FIG 1.** Internalization of allergens by LLDCs was dose- and time-dependent. **A** and **B**, LLDCs were incubated at 37°C in the presence of various concentrations of rBet v 1, rPhl p 1, FITC-dextran (FITC-DX), and Lucifer yellow (LY). **C** and **D**, LLDCs were incubated for various times at 37°C with 25 µg/mL rBet v 1 or rPhl p 1 and 1 mg/mL FITC-dextran or Lucifer yellow. One representative experiment from a set of 10 different experiments performed is shown here.

tions of markers and a 15-minute incubation period at 37°C. As a control, the same experiment was performed at 4°C (data not shown). With FITC-dextran, the level of fluorescence at 4°C corresponded to the binding of the molecule on the membrane receptor, whereas with other molecules, the level of fluorescence was close to that of autofluorescence. The percentage of cells that internalize was dose dependent with all markers (Fig 1, A), and calculation of the amounts of markers internalized in molecules per cell indicated a higher efficiency of recombinant allergens in comparison with FITC-dextran and Lucifer yellow (Fig 1, B).

To obtain comparable amounts of internalized molecules, we chose 25 µg/mL for recombinant allergens and 1000 µg/mL for FITC-dextran and Lucifer yellow. All markers were internalized in a time-dependent manner, and

all cells were labeled at 24 hours (Fig 1, C). Calculation of the number of molecules per cell showed that recombinant allergens reached a plateau at 10 to 30 minutes, whereas FITC-dextran accumulated in the cells (Fig 1, D).

### Internalization was correlated with the expression of immature markers

From day 8 to day 12, LLDCs presented the immature phenotype of epidermal LCs as assessed by expression of CD1a and langerin (Fig 2, A). After day 12, these markers decreased, and CD83, a mature marker, became predominant. The percentage of labeled cells reached a maximum at day 6 with recombinant allergens, and Lucifer yellow then decreased regularly until day 19. By contrast, FITC-dextran was more efficient at day 10 (Fig 2, B). The amount of molecules internalized per cell indi-

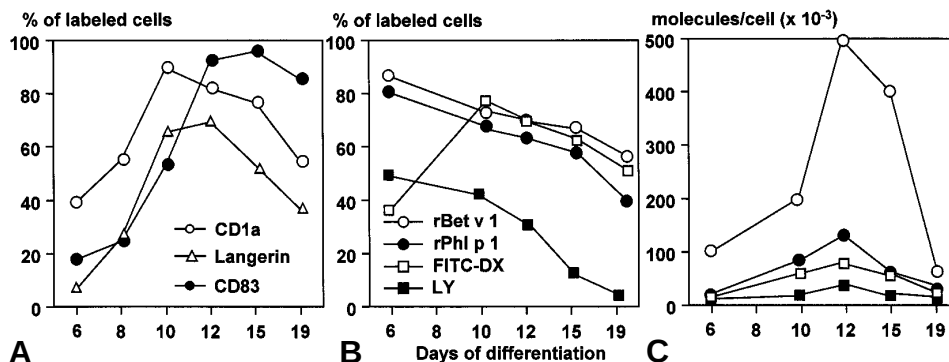


**FIG 2.** Internalization potencies reached a maximum in the premature state of LLDCs. **A**, Phenotypic evolution of DCs between day 6 and day 19. **B**, Modifications of the endocytic capacities expressed as a percentage of labeled cells. **C**, Modifications of the endocytic capacities expressed as molecules per cell. FITC-DX, FITC-dextran; LY, Lucifer yellow.

cated a low efficiency before day 10 and after day 15 (Fig 2, C), suggesting that LLDCs possessed their highest internalization capacity between day 10 and day 12.

### Chase experiment in marker-free medium

Pulse-chase experiments were performed by incubation of LLDCs for 15 minutes with markers; cells were then washed in cold medium and incubated for various periods of time in marker-free medium at 4°C or 37°C (Fig 3).

rBet v 1 and rPhl p 1 presented comparable kinetics, and a 50% decrease occurred within 4 hours. By contrast, after a partial decrease, FITC-dextran was completely retained for long periods of time (up to 48 hours), whereas Lucifer yellow was rapidly lost.

### Recombinant allergens were taken up by macropinocytosis, and parts of the molecules were recovered in acidic vesicles

We used a set of inhibitors to investigate the mechanism of entry of the markers. Phenylarsine oxide, which blocked internalization by receptors, was inefficient on recombinant allergens but blocked internalization of FITC-dextran (Fig 4, A). Monensin and chloroquine blocked the transfer from endosomes to lysosomes in receptor-mediated endocytosis, and monensin also acted in the recycling of pinocytosis vesicles. Both molecules acted on recombinant allergens and FITC-dextran at different degrees. Cytochalasin D, an inhibitor of macropinocytosis, was highly efficient on rBet v 1 and rPhl p 1 but presented a lower efficiency on FITC-dextran.

The effect of amiloride, which inhibits membrane ruffling, was tested in pulse-chase experiments. Incubation at 37°C without inhibitor showed intense release of recombinant allergens and Lucifer yellow but only limited release of FITC-dextran. The inhibitor blocked the release of previously internalized recombinant allergens and Lucifer yellow but was ineffective on FITC-dextran (Fig 4, B). Inhibition was dose dependent within the limit of toxicity of the molecule (data not shown).

The intracellular route of internalized markers was analyzed by means of incubation of cells for 15 minutes at 37°C with fluorescent markers. A first acquisition was performed with FACScan, monensin was then added, and a second acquisition was performed after 30 minutes of incubation at 4°C. Monensin neutralized the pH of acidic vesicles, resulting in suppression of FITC quenching by acidic pH. Addition of monensin enhanced the fluorescence observed with recombinant allergens (Fig 5). This effect was more pronounced during the period of cell differentiation that corresponds to the immature state.

Acidic vesicles were localized by confocal microscopy. Cells were incubated with recombinant allergens, Lucifer yellow, or FITC-dextran and then fixed or treated at 4°C with monensin for 30 minutes. The cell body and dendrites were labeled with rPhl p 1 and rBet v 1, whereas FITC-dextran labeling was concentrated in vesicles (Fig 6). Lucifer yellow did not induce any consistent labeling (data not shown). Addition of monensin revealed an intense perinuclear labeling in FITC-dextran-treated cells, whereas enhancement of fluorescence with allergen-treated cells remained distributed throughout the cell body.

### LLDCs incubated with recombinant protein allergens induced a proliferative response of naive T lymphocytes

LLDCs were pulsed with 0.6 µg/mL rBet v 1 or 1.25 µg/mL rPhl p 1. As a positive control, we used a chemical hapten, BB, at 1 µg/mL. After 48 hours of contact at 37°C, pulsed LLDCs were cocultured with autologous T lymphocytes in a cellular ratio of 1 LLDC for 10 lymphocytes. In a set of 9 experiments with different subjects, BB induced a significant lymphoproliferative response, whereas rBet v 1 induced proliferation in three experiments, and rPhl p 1 induced proliferation in one experiment (Fig 7). Phenotypic analysis of LLDCs was carried out at the time of coculture in some experiments (experiments 2, 3, 8, and 9). Maturation markers, such as CD83 and E-cadherin, were modified on activation with

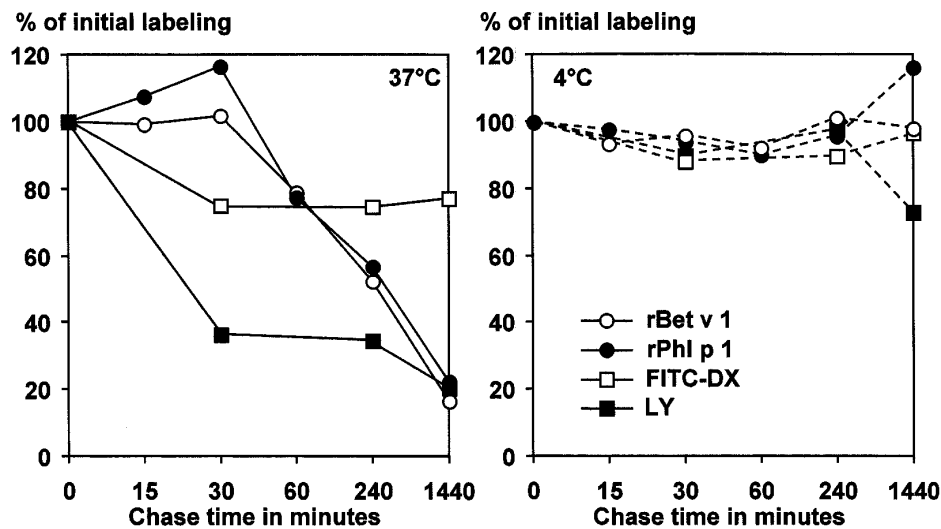


**FIG 3.** Internalized allergens had a 4-hour half-life in LLDCs. Nine-day-old LLDCs were pulsed for 15 minutes with rBet v 1 or rPhl p 1 at 2.5  $\mu$ g/mL or with FITC-dextran (FITC-DX) or Lucifer yellow (LY) at 1 mg/mL, washed with cold medium, and then incubated in culture medium for various times. These data represent one experiment from a set of three.

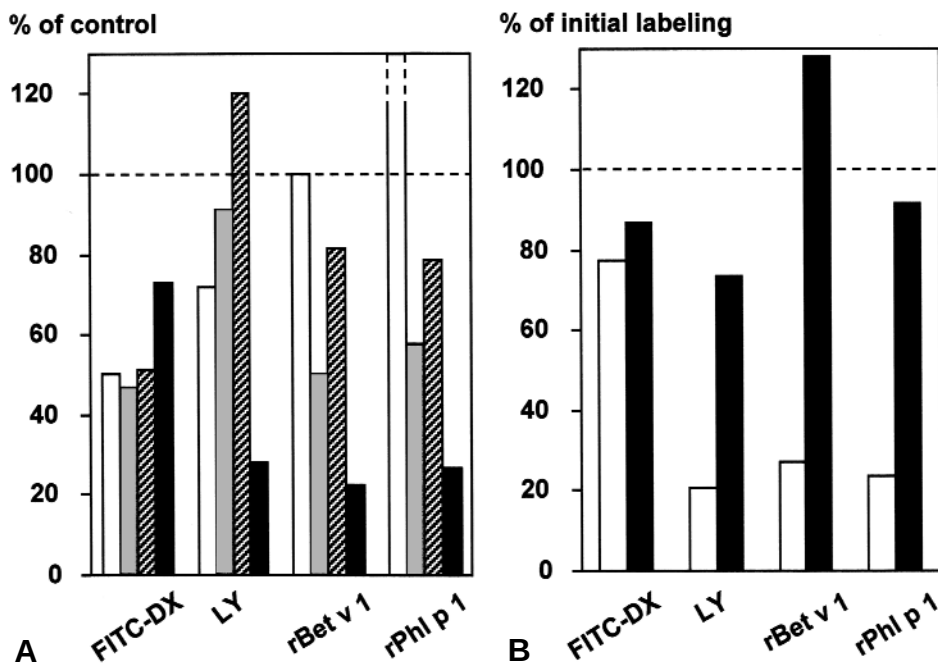


**FIG 4.** Metabolic inhibitors indicated that protein allergen uptake was mediated by macropinocytosis. **A**, Inhibition of marker uptake by phenylarsine oxide (open bars), monensin (shaded bars), chloroquine (hatched bars), or cytochalasin D (filled bars). The drugs were added at 37°C for 1 hour with LLDCs, and then markers were added for 15 minutes at 37°C at the concentration shown in Fig 1, C. Results are expressed as a percentage of uptake without drug. **B**, Markers were internalized for 15 minutes at 37°C, and then LLDCs were washed and incubated with medium alone (open bars) or amiloride (filled bars) for 2 hours. Results are expressed as a percentage of labeling at the beginning of the 2-hour incubation. Data represent one experiment of a set of six. FITC-DX, FITC-dextran; LY, Lucifer yellow.

BB but not after contact with rBet v 1 or rPhl p 1. CD86 was overexpressed (>150%) after contact with BB in all cultures and moderately elevated to 110% in culture 2 with rPhl p 1 and to 130% in culture 8 with rBet v 1.

## DISCUSSION

In this study we demonstrate that LLDCs internalize protein allergens, such as rBet v 1 and rPhl p 1, with a high efficiency. Only part of the intracellular allergens

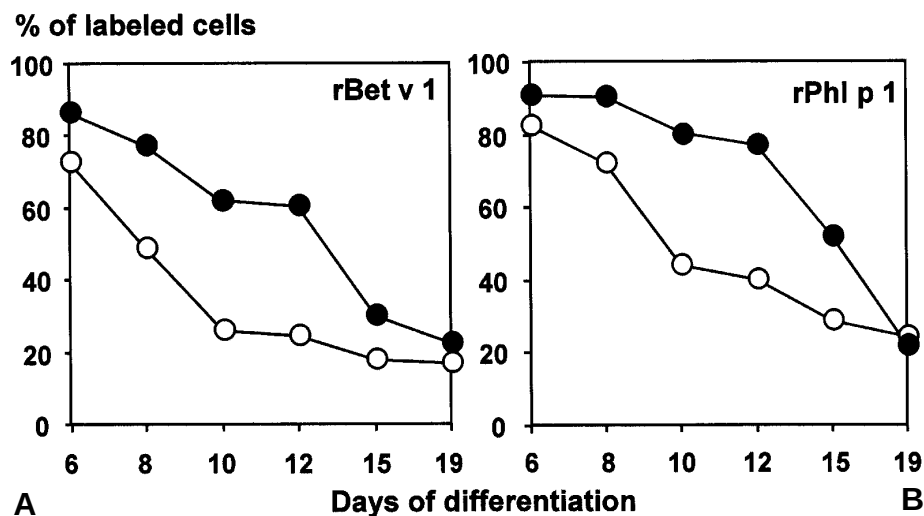


**FIG 5.** The relative amount of allergen in acidic vesicles reached a maximum when LLDCs were in a pre-mature state. *Open circles* represent direct measurement of fluorescent LLDCs after internalization of recombinant allergens tagged with FITC; *filled circles* represent fluorescent measurements of the same cell samples after an additional 30-minute incubation with monensin.

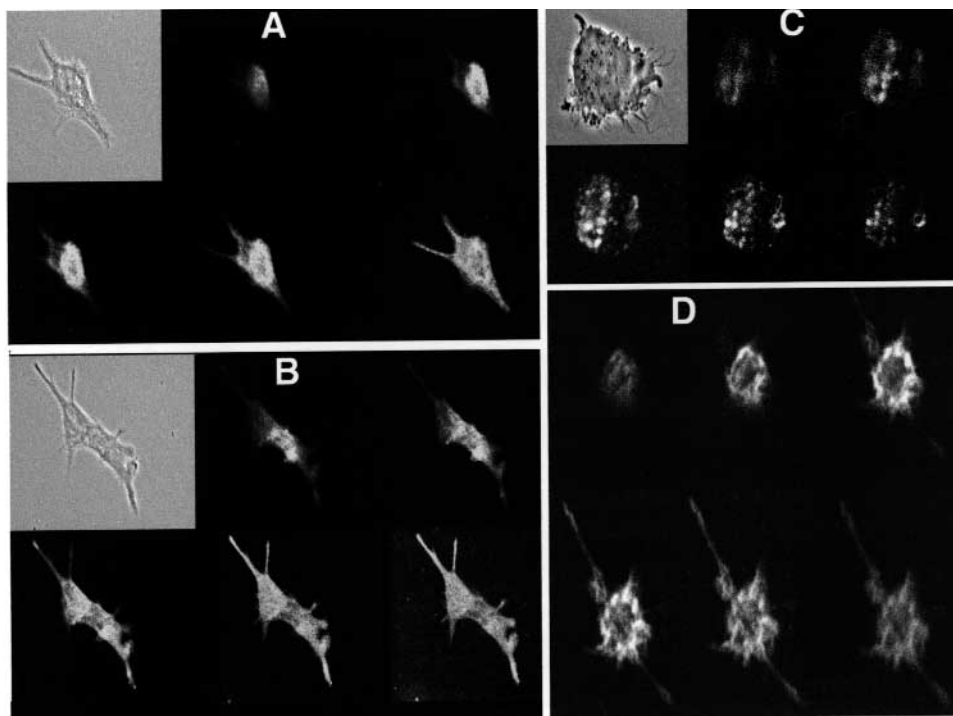


**FIG 6.** Intracytoplasmic localization of rPhl p 1 (**A** and **B**) and FITC-dextran (**C** and **D**) analyzed by means of confocal microscopy. rPhl p 1 was used at 25  $\mu$ g/mL, and FITC-dextran was used at 1 mg/mL. After 30 minutes of contact at 37°C, cells were analyzed directly (**A** and **C**) or after 30 minutes of additional incubation at 4°C with monensin (**B** and **D**). A phase-contrast picture is added as an *inset*, and immunofluorescence was observed at different levels of the cells from the top to the adherent side.

follow the lysosomal pathway. The amount entering this pathway depends on the maturation state of the LLDCs. In some experiments LLDCs processed and presented antigenic peptides to autologous naive T lymphocytes.

We used DCs produced *in vitro* from cord blood CD34<sup>+</sup> progenitors. Our culture conditions led to 60% to 85% LLDCs at days 10 to 12 of differentiation. Generated DCs are considered to be Langerhans-like cells

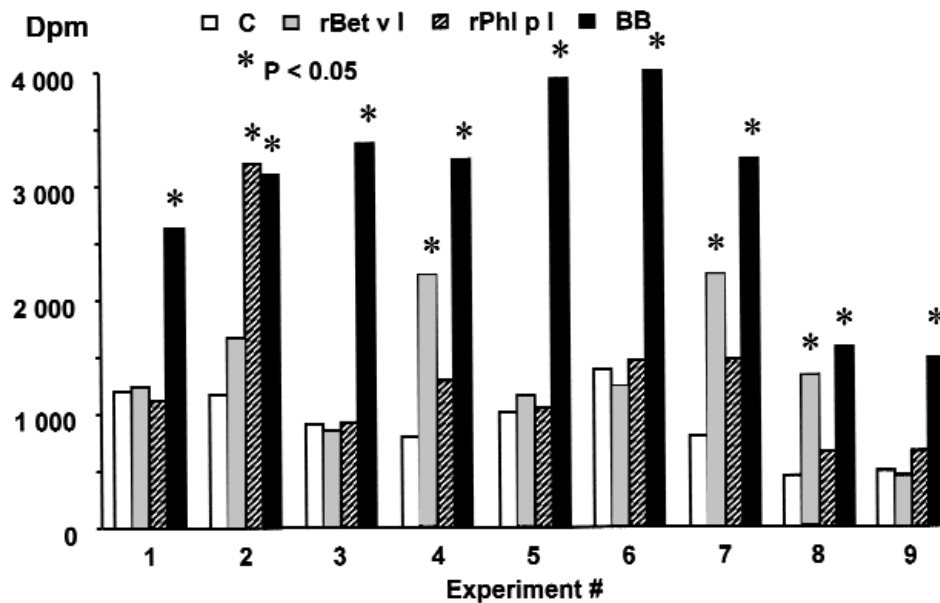


**FIG 7.** LLDCs loaded with allergens and mixed with T lymphocytes induced a lymphoproliferative response in some of the experiments. LLDCs were incubated with medium alone (open bars), rBet v 1 (shaded bars), rPhl p 1 (hatched bars), or BB, a chemical hapten used as positive proliferation control (filled bars). \*Significant difference ( $P < .05$ ) of the corresponding mean in comparison with the control (C) incubated with medium alone.

because they present positive intracellular labeling with Lag antigen and surface labeling with Langerin. They also present LC markers, such as E-cadherin and S-100 protein. Some of the cells in the non-LLDC fraction presented a dermal dendrocyte phenotype with CD1a<sup>+</sup>, E-cadherin<sup>+</sup>, S-100<sup>+</sup>, Lag<sup>+</sup>, Langerin<sup>+</sup>, and F XIIIa<sup>+</sup> markers, whereas most other cells were small round cells devoid of DC markers. The absence or small numbers of CD14<sup>+</sup> cells at day 6 of differentiation confirm the small amount of dermal dendrocytes at days 10 to 12.<sup>12</sup> In addition to their immature phenotype, LLDCs are functionally immature cells. They present the functional capacities of LCs with a period of high uptake and low antigen presentation followed, after antigen priming, by a period of low uptake and high antigen presentation potency.<sup>21</sup>

The uptake potency of DCs differs from that of monocytes. In particular, the phagocytic properties of DCs are reduced. However, DCs are able to phagocytose particles, such as latex beads,<sup>22</sup> apoptotic bodies, viruses, bacteria,<sup>23</sup> and intracellular parasites.<sup>24</sup> Like monocytes, DCs can take up antigens through receptors, such as the mannose receptor<sup>25</sup> or DEC205,<sup>26</sup> as well as FcεRI<sup>27</sup> and FcγR.<sup>28</sup> DCs uptake soluble molecules by means of fluid-phase endocytosis and constitutively use macropinocytosis mechanisms.<sup>25</sup>

Recombinant allergen uptake is a fast and efficient phenomenon despite the use of a concentration 40-fold lower than that of FITC-dextran or Lucifer yellow. The use of a panel of FITC-labeled molecules with various molar masses and devoid of glycoside residues in some cases failed to demonstrate a close relationship between internalization efficiency and molar mass (data not shown).

In our experience allergen uptake cannot be mediated through receptors for glycoproteins because recombinant molecules produced by *E coli* are devoid of glycosylated residues, although native allergens might be internalized by this way. Internalization through other receptors can be excluded because phenylarsine oxide<sup>17</sup> and chloroquine,<sup>16</sup> which inhibit receptor-mediated uptake, are inefficient on allergen internalization. Chloroquine blocks HLA-DR and Langerin mobility on the cell surface, whereas rBet v 1 internalization is only slightly lowered (data not shown). Macropinocytosis, uptake of large vesicles (0.5–3 μm), is mediated by membrane ruffling driven by the active skeleton. Macropinocytosis, which is a constitutive mechanism of DCs,<sup>25</sup> is inhibited by cytochalasin<sup>18</sup> and amiloride,<sup>19</sup> which inhibit membrane ruffling. Cytochalasin D inhibits allergen uptake, whereas FITC-dextran uptake is only slightly reduced, thereby indicating that macropinocytosis is the major mechanism of allergen uptake in the cells. Amiloride blocks allergen loss from DCs, confirming the importance of vesicle recycling for macropinocytosis.

As indicated by pH shift experiments and confocal microscopy data, most of the FITC-dextran molecules are localized in perinuclear acidic vesicles after uptake. By contrast to recombinant allergens, only part of the molecules are in perinuclear acidic vesicles because the fluorescence is disseminated throughout the cytoplasm. Half-life measurement showed a marked loss of allergens in comparison with FITC-dextran. These data, taken together with the need for vesicle recycling to observe a loss of fluorescence, suggest that part of the recombinant allergens do not use the lysosomal pathway but leave cells during vesicle recycling.

The amount of molecules recovered in acidic vesicles is related to the differentiation status of LLDCs. The maximum of lysosomal pathway efficiency is observed between day 10 and day 12, corresponding to the immature state of LLDCs. After day 12, addition of monensin<sup>15</sup> reveals a lower level of fluorescence, showing that only a small part of the molecules use the lysosomal pathway. This may be paralleled with the lower processing capacity of LLDCs, which become efficient in naive T-lymphocyte activation, a stage that corresponds to mature DCs.<sup>28</sup>

Activation of T lymphocytes by LLDCs incubated with rBet v 1 or rPhl p 1 was observed in some of the experiments. The proliferation was low but significant because sextuplets were performed in each case. The low proliferation may be attributed to the primary response situation; the number of T lymphocytes possessing the corresponding CD3-TCR complex is certainly low. In contrast with chemical allergen stimulation,<sup>29</sup> membrane antigen modifications remained limited after contact with pollen allergens, suggesting an incomplete maturation of LLDCs. However, nonactivation of T cells caused by the absence of antigen uptake by LLDCs can be excluded because allergens were always internalized in 45 different experiments.

In conclusion, the large uptake of molecules by macropinocytosis in LLDCs is probably regulated at the intracellular level by bypass mechanisms supplementary to the lysosomal pathway. The mechanism controlling the absence of systematic lymphocyte activation must be studied in the context of allergenic sensitization. Because DCs and LCs play a crucial role in the tissues in contact with the environment, LCs present in the mucosa must play an important role in allergy.<sup>30</sup> It is therefore important to define their potential activity in desensitization through the sublingual or oral route and their interactions with other cells in mucosal sites.

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