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Comparison of the rate of phagocytosis of orthorhombic cyclosporine A (CsA) and latex particles by alveolar macrophages from hamsters

I. Maye[a], A. de Fraissinette[b,*], L. M. Cruz-Orive[c], J. Vonderscher[b], F. Richter[b] and P. Gehr[a]

[a]Institute of Anatomy, University of Bern, Bühlstrasse 26, CH-3012 Bern (Switzerland)
[b]NOVARTIS Pharma AG, CH-4002 Basel (Switzerland), Fax +41 61 324 10 27
[c]Department of Mathematics, University of Cantabria, E-39005 Santander (Spain)

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Abstract. The aim of this study was to develop an in vitro model to estimate the clearance of pulmonary administered cyclosporine A (CsA). To do this we estimated the volume of CsA particles phagocytosed by alveolar macrophages (AM) lavaged from hamsters. AM were cultured with CsA particles at two doses of particles (0.1 mg or 0.5 mg) and at three incubation times (1 h, 6 h or 24 h). The AM were also incubated with or without latex particles. After incubation, AM were processed for light and electron microscopy and the mean volume of phagocytosed particles was estimated stereologically from micrographs of the cells. Here, however, the CsA particles were dissolved during the embedding process and only their negative images (vacuoles) could be detected. An indirect method was therefore developed. The volume of cytoplasmic vacuoles (called 'background' vacuoles) was estimated in control macrophages (without particles or with latex particles and subtracted from the total volume of vacuoles in macrophages incubated with CsA, which gave the volume of phagocytosed CsA. The volume of the 'background' vacuoles remained constant in all study conditions. At a dose of 0.1 mg CsA the volume phagocytosed per macrophage was 13.83 μm^3 at 1 h, 8.43 μm^3 at 6 h and 4.50 μm^3 at 24 h. At a dose of 0.5 mg CsA, the volume phagocytosed varied from 26.59 μm^3 at 1 h, to 4.13 μm^3 at 6 h and 49.10 μm^3 at 24 h. These results show no statistically significant dependence on time for either dose, and a statistically significant dose effect only at 24 h. With latex particles, the phagocytosed volume increased significantly with time and dose and was significantly higher than for CsA particles. This study showed that CsA particles are phagocytosed by AM from hamsters but to a lesser extent than latex particles. This difference could be correlated with physical properties, i.e. a difference between particle size and shape and/or chemical properties, latex particles being inert and CsA particles being peptidic. Moreover, different surface receptors on AM could be involved in the process of phagocytosis of CsA and latex particles.

Key words. Phagocytosis; cyclosporine A particles; morphometry; latex particles.

Cyclosporine A (CsA) is a neutral, lipophilic, cyclic undecapeptide that has been shown to be one of the most effective immunosuppressive drugs used in humans for the treatment of immune-mediated diseases including the prevention of allograft rejection [1]. Its pharmacological mechanism involves interference with helper T cell activation, by blocking the initiation of transcription of the interleukin-2 gene [2–4]. Treatment of pulmonary diseases with CsA is limited by its poor penetration into the lung following oral (in peanut oil) or intravenous (in cremophor vehicle) administration, and by the development of limiting renal and hepatic

* Corresponding author.

toxicity following prolonged administration [5], although in one study CsA at 5 mg/kg per day given orally was reported to be effective in the treatment of asthma [6]. Direct drug delivery into the lung could increase the therapeutic efficiency of CsA for local immunosuppression.

Orthorhombic particles of CsA with a mean diameter of 5 μm were developed as an alternative for local administration to the airways. To understand better the local clearance of these CsA particles, an in vitro model using macrophages from lavaged hamster lungs was developed. Alveolar macrophages are of interest because of their capacity to phagocytize inhaled organic or inorganic particles [7, 8]. Questions remain concerning the clearance of peptidic particles by phagocytosis and the dose and time dependency of this process. We therefore investigated these two aspects by comparing the volume of orthorhombic CsA and latex particles phagocytosed.

Materials and methods

Bronchoalveolar lavage and cell culture

Twelve Syrian golden hamsters with a body weight of approximately 150 g were used according to the ethical principles and guidelines for scientific experiments on animals laid down by the Swiss Academy of Medical Sciences. The animals were anesthetized with an intraperitoneal (i.p.) injection of 0.2 ml ketamine hydrochloride (50 mg/ml) followed by pentobarbital (50 mg/ml). The dose of pentobarbital was adjusted to reach deep anesthesia.

The trachea was exposed and, after tracheotomy, a cannula was introduced and tightly fixed with a thread. A pneumothorax was performed before the bronchoalveolar lavage procedure was started. The bronchoalveolar lavage (BAL) consisted of 10 washes (5 ml per wash) of the lungs with Ca^{2+} and Mg^{2+}-free phosphate buffered saline solution (PBS) [9]. The total recovered lavage fluid (50 ml) was centrifuged, the pellet was resuspended in sterile medium RPMI 1640 without L-glutamine or phenol red (Life Technologies, Basel, Switzerland), and the number of alveolar macrophages (AM) determined using a Neubauer® haemocytometer chamber. AM were easily recognized by shape and size and were known to represent more than 90% of the cells obtained by BAL [10]. AM were further diluted to 0.5×10^6 cells per ml. This cell suspension was distributed (3 ml per dish) in sterile dishes (Nunc Lab-Tek™ Tissue Culture Chamber Slide, Life Technologies, Basel, Switzerland) for incubation at two doses of particles (0.1 mg or 0.5 mg) and three incubation times (1 h, 6 h and 24 h). For one tested dose and one incubation time, 10 dishes were used: 4 dishes for incubation with CsA particles, 3 with latex particles and 3 without particles. To allow the macrophages to adhere, the dishes were incubated for 90 min at 37 °C and 5% CO_2. The dishes were then washed with RPMI 1640 to eliminate nonadherent cells and cellular debris.

CsA particles (SDZ OXL 400) were provided by Sandoz Pharma, Basel, Switzerland. These consist of polydispersed crystalline particles with a geometric diameter of between 1 μm and 10 μm; the mean diameter was 5 μm. They were suspended as particles in RPMI 1640 at a concentration of 1 mg/ml after 15 min ultrasonication. The latex particles (Polybead®, Polysciences, Eppelheim, Germany) were monodispersed particles with a mean diameter of 6 μm and a standard deviation of 0.4 mm. They were resuspended in RPMI 1640 at the same concentration as CsA particles. Then each mother suspension of particles was added to the culture medium in order to obtain final doses of 0.1 mg or 0.5 mg per petri dish. The cell cultures were incubated with the particles for 1 h, 6 h and 24 h at 37 °C, 5% CO_2 and 100% humidity.

Cell viability

Cell viability was tested at 1 h, 24 h, 48 h and 72 h at 37 °C in cell culture plates containing RPMI 1640 cell culture medium supplemented with 5% fetal bovine serum (Gibco, Europe). Cell viability was assessed using Alamar Blue (Biosource International, CA, USA) as a fluorometric/colorimetric growth indicator based on detection of metabolic activity and measured under a reader plate.

Fixation and embedding

After incubation, the cell culture medium was removed and the cells were fixed with 2.5% glutaraldehyde (GA) in PBS. Two dishes were stained with toluidine blue (TB) and the cells were counted under a light microscope. The other dishes were postfixed with 1% osmium tetroxide in cacodylate buffer and contrasted in 0.5% uranyl acetate in maleate buffer. The specimens were then dehydrated in a graded series of ethanol and embedded in Epon.

Stereological procedure

The mean volume of particles per macrophage was estimated by stereology [11, 12] from properly sampled sections and micrographs. A particular problem encountered was the dissolution, after phagocytosis by AM, of the CsA particles during the embedding process. Only their negative images could be detected on micrographs of sections and they were difficult to distinguish from other cytoplasmic vacuoles; vacuoles which contained CsA particles and cytoplasmic vacuoles both looked empty. Various approaches were

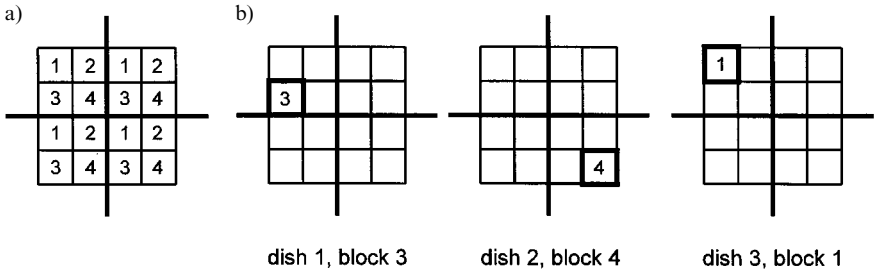

Figure 1. (*a*) Culture dishes were divided into four squares, each of which was subdivided into four smaller squares and labelled with a number from 1 to 4. (*b*) The numbers 1, 2, 3 or 4 were randomly selected. Dishes 2 and 3 were identically randomized.

tried, e.g. immunoradiolabelling of CsA, or different embedding procedures with solutions which would not dissolve CsA, without improving the situation. We therefore developed the stereological procedure described below to overcome this problem of identifying different types of empty vacuoles. In a pilot study we found that the mean volume of (empty) cytoplasmic vacuoles remained the same in all macrophages during the experimental conditions, and referred to them as 'background' vacuoles. The volume of these 'background' vacuoles per macrophage, v(back vac per mac), was determined in macrophages incubated without particles and in macrophages incubated with latex particles (latex particles were not dissolved and could be clearly identified and differentiated from cytoplasmic vacuoles). Therefore the mean volume of phagocytosed CsA, v(CsA per mac), was estimated by subtracting the volume of 'background' vacuoles from the mean total volume of vacuoles in macrophages which were incubated with CsA particles, v(total vac per mac), namely:

$$\text{v(CsA per mac)} = \text{v(total vac per mac)} - \text{v(back vac per mac)} \quad (1)$$

This mean volume of an object (latex particles, CsA particles or vacuoles) per macrophage v(obj per mac) was estimated using the following equation:

$$\text{v(obj per mac)} = \text{v(mac)} \times V_V\text{(obj, mac)} \quad (2)$$

where v(mac) denotes the mean volume of a macrophage (estimated by LM) and V_V(obj, mac) the ratio of total object volume in the macrophages to total macrophage volume (estimated by EM). The abbreviation 'obj' may stand for CsA particles, latex particles, total vacuoles or 'background' vacuoles.

Estimation of mean macrophage volume v(mac)

The estimation of v(mac) was performed in two steps according to Griffiths [13]:

$$\text{v(mac)} = [\text{V(mac)/S(dish)}]/[\text{N(mac)/S(dish)}] \quad (3)$$

where V(mac)/S(dish) represents the mean macrophage volume per surface area of dish and N(mac)/S(dish) represents the mean macrophage number per surface area of dish.

Estimation of the mean number of macrophages per surface area of the dish N(mac)/S(dish). The ratio N(mac)/S(dish) was estimated on light micrographs systematically distributed over the entire bottom area of each dish. Two dishes were analysed per dose and time, and on average 16 systematic micrographs were sampled per dish. A transparent acetate sheet bearing six square sampling frames was superimposed on each micrograph, the upper right corner of each frame representing a test point for this test system. Macrophage projections were counted in an unbiased manner using the forbidden line rule [14, 15] with which double counting of cells is omitted. The relevant ratio was estimated as follows:

$$\text{N(mac)/S(dish)} = (a/p) \times M \times [(\Sigma N)/(\Sigma P)] \quad (4)$$

where a/p denotes the counting area per test point, M the final linear magnification, N the total number of macrophages counted on a micrograph, and P the total number of test points hitting the dish on a micrograph. Summations are over all micrographs and about 200 macrophages were counted per dish.

Estimation of the mean volume of macrophages per surface area of the dish V(mac)/S(dish). The ratio V(mac)/S(dish) was estimated by means of vertical LM sections [16], i.e. sections perpendicular to the dish. Macrophages of three Epon-embedded dishes were analysed per dose and time point. Each dish was split into blocks, and one block per dish was randomly sampled as illustrated in fig. 1. A 1 μm thick vertical section was cut from each block (fig. 2a), perpendicular to the base of the block. The sections were stained with TB, and four or five light micrographs were sub-sampled systematically along each section (fig. 2b). A transparent test system bearing cycloid

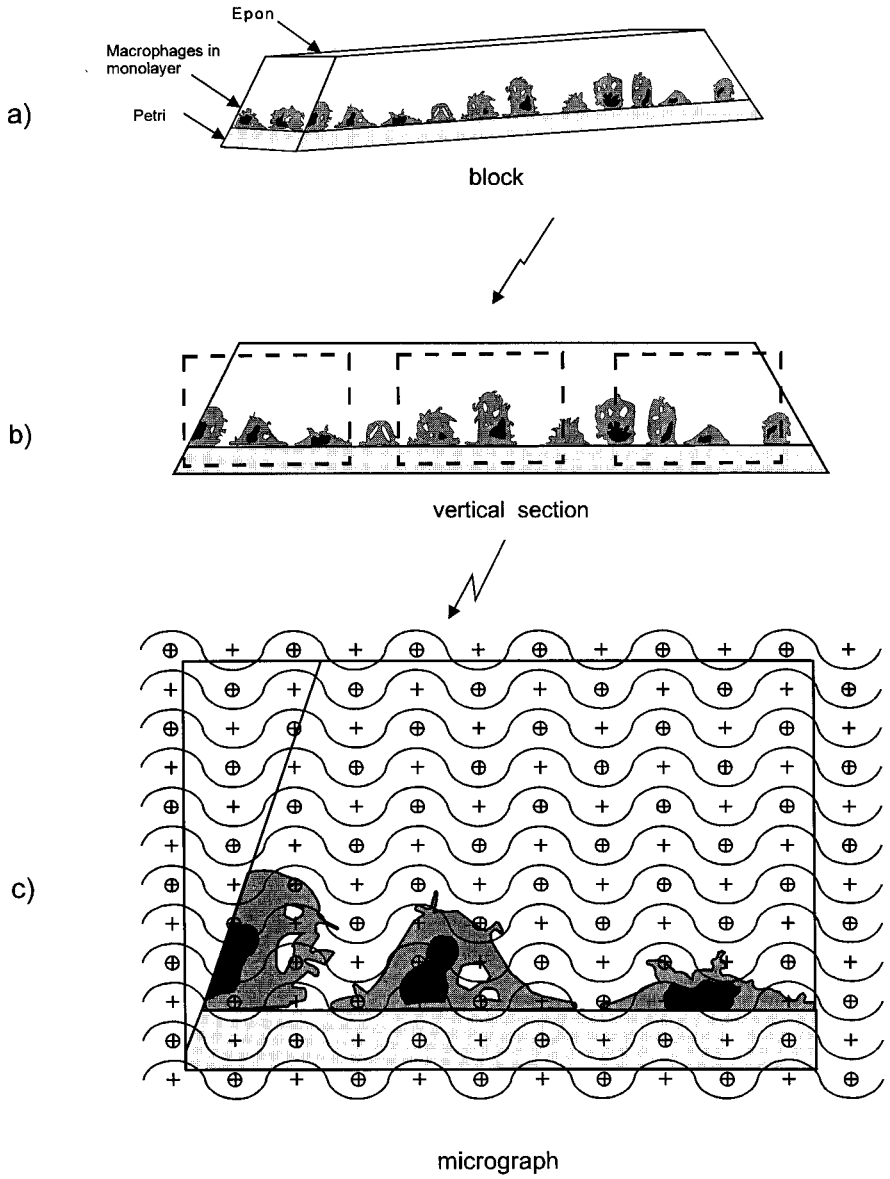

Figure 2. (*a*) The monolayer of macrophages in the culture dish embedded in Epon. (*b*) Vertical section of the Epon block showing three micrographs. (*c*) Micrograph with superimposed cycloid test system used to estimate the volume of macrophages per surface from each culture dish.

test curves and test points [16, 17] was superimposed (fig. 2c) on each micrograph and the relevant ratio was estimated using the following formula:

$$V(mac)/S(dish) = \tfrac{1}{2} \times (\ell/p) \times (1/M) \times [(\Sigma P)/(\Sigma I)] \quad (5)$$

where I denotes the total number of intersections between the cycloid test lines and the horizontal trace of the dish surface in one section, P the corresponding number of test points hitting macrophage profiles, M the final linear magnification, and ℓ/p the ratio of test line length to test point number for the test system used. The summations run over all vertical sections for each dose and time point.

Estimation of the volume ratio V_V(obj, mac)

A vertical ultrathin section was cut from each Epon-embedded block used to estimate the ratio V(mac)/S(dish) (see above) and all macrophage profiles or profile fragments present were photographed at an EM level. On each micrograph a double test system consisting of fine and coarse test points was superimposed and the relevant volume ratio was estimated by counting fine test points falling on the object profiles P(obj) and the coarse test points falling on the macrophage profiles P(mac) (fig. 3) [18, 17] using the following formula:

$$V_V(obj, mac) = [p_1/p_2] \times [\Sigma P(obj)/\Sigma P(mac)] \quad (5)$$

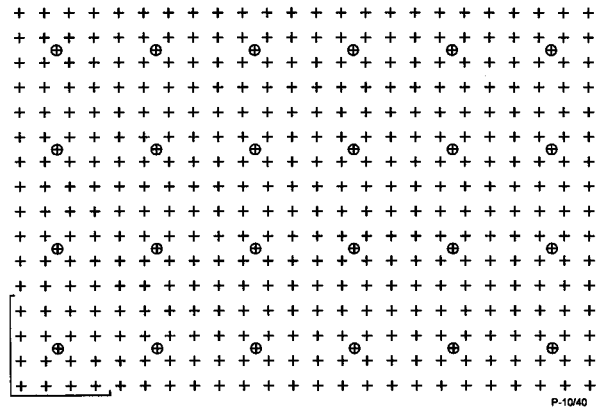

Figure 3. A portion of the test system of points used to estimated V_V(obj, mac). The crosses were used as points for the estimation of the volume of objects (CsA particles, latex particles or vacuoles), circled crosses were used as points for the estimation of the volume of macrophages. The ratio between the crosses and circled crosses test points was $p_2/p_1 = 16$.

where p_1/p_2 denotes the number of fine test points per coarse test point for the test system used (fig. 3). The summations are over all micrographs taken from the EM sections for each dose and time point.

Table 1. Mean volume and standard errors (SE) calculated from three blocks, in μm^3, of 'background' vacuoles per macrophage v(back vac per mac) incubated with latex particles or without particles (control), for two doses of particles and three incubation times. v(back vac per mac) was identical under all experimental conditions. The value 'mean series' was used to determine the CsA volume (equation 1) and is shown in table 2.

	Mean volume (μm^3)		
Incubation time	1 h	6 h	24 h
Latex 0.1 mg vacuoles	19.68 (6.95)	31.63 (9.14)	13.91 (6.40)
Control 0.1 mg vacuoles	26.73 (3.83)	32.88 (7.84)	22.23 (11.01)
Mean series 0.1 mg	22.50	32.13	17.24
Latex 0.5 mg vacuoles	25.98 (5.54)	30.71 (11.61)	19.53 (8.79)
Control 0.5 vacuoles	19.32 (8.01)	25.88 (10.77)	24.26 (6.08)
Mean series 0.5 mg	22.65	28.30	21.89

Statistical analysis

The parameters to be analysed were the mean volume of 'background' vacuoles, of vacuoles representing CsA particles, or of latex particles per macrophage, in μm^3. For the 'background' vacuoles the null hypothesis was that there was no particle, dose or time effect on the volume of vacuoles per macrophage. Three crossed factors, namely the presence or absence of particles, dose (0.1 mg and 0.5 mg) and time (1 h, 6 h and 24 h) were considered and a three-way univariate analysis of vari-

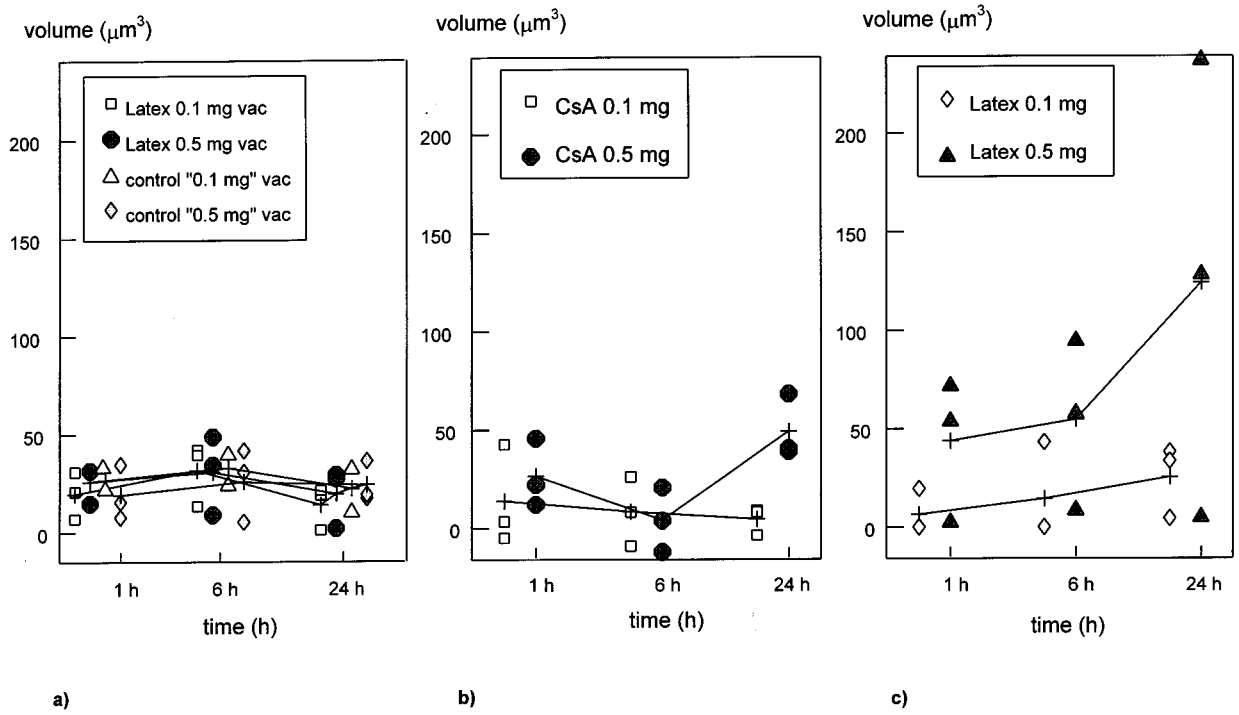

Figure 4. Mean volume of: (*a*) 'background' vacuoles per macrophage v(back vac per mac), (*b*) phagocytosed CsA particles per macrophage v(CsA per mac) and (*c*) phagocytosed latex particles per macrophage v(latex per mac). For each dose of particles or control the values for three blocks plus the mean value (+) are given.

ance (ANOVA) was carried out. To investigate phagocytosis versus time for latex or CsA particles, a linear regression model was chosen, with the volume of particles as the response parameter and time as the controlled variable. The null hypothesis was that the mean volume of particles per macrophage did not vary with time for each dose. For dose-dependency a two-way univariate ANOVA with two crossed factors (dose and time) was applied. The null hypothesis was the coincidence of the two regression lines of the two doses. In order to compare the mean volume of latex versus CsA particles per macrophage, we performed a two-way multivariate (i.e. three-variate in this case, each component corresponding to a time point) analysis of variance (i.e. a 'MANOVA') with two crossed factors (particles and doses) at two levels each, and three replications per combination. The analyses were carried out with the aid of an Splus 3.2 (MathSoft, Inc) statistical package.

Results

The average number of macrophages per dish after incubation was relatively constant with a variability of less than 10% within the different experimental groups. Here, the different treatments did not affect cell viability (which remained constant at 100% and equal to the untreated control cells) or cell adherence in a manner that would otherwise bias our analysis.

The mean volume of 'background'' vacuoles v(back vac per mac) was identical under all experimental conditions (fig. 4a and table 1). Neither the type of particles ($p = 0.79$) nor the dose ($p = 0.96$) or the time ($p = 0.17$) had a significant effect. The average volume of 'background' vacuoles was therefore pooled at each time point and then subtracted from the total mean volume of vacuoles in the macrophages which were incubated with CsA particles v(total vac per mac) in order to determine the mean volume of CsA vacuoles alone, v(CsA per mac) (equation 1).

The mean volume of phagocytosed latex particles v(latex per mac) increased during the incubation time for both doses and was higher for the higher dose (0.5 mg) than for the lower dose (0.1 mg) (fig. 4c and table 1). The variation in the three measurements at each time point was very high. Statistical analysis therefore revealed that the phagocytosis of latex particles versus time was not significantly different from zero for the doses 0.1 mg ($p = 0.24$) and 0.5 mg ($p = 0.17$). On the other hand, a significant dose effect ($p = 0.03$) was found. The two regression lines could be regarded as parallel and horizontal, with the 0.5 mg line lying significantly above the 0.1 mg line.

For the lower dose (0.1 mg) the mean volume of phagocytosed CsA v(CsA per mac) did not change during the incubation time (fig. 4b and table 2); the slope of the regression line was not significantly different from zero ($p = 0.56$). For the higher dose (0.5 mg) the mean volume phagocytosed varied with time. Two linear regression models were considered because the mean responses clearly did not lie on a straight line. The first model used the time points 1 h and 6 h and the slope was found not to be significantly different from zero ($p = 0.18$). The second model used the time points 6 h and 24 h and the slope was found to be significantly different from zero ($p = 0.03$). A significant dose effect was found only at 24 h ($p = 0.048$).

In comparing latex particles with CsA particles and taking the three time points together, the pattern of phagocytosis of the two particle types can be considered to be different. The null hypothesis of no difference between the types of particles was, however, rejected ($p = 0.051$). The null hypothesis of no difference between doses was more clearly rejectable ($p = 0.012$). When separated tests (MANOVA) were performed at each time point, the large variation among replications tended to dominate and only a slight difference between the doses was detected at 24 h.

Table 2. Mean volume and standard errors (SE) calculated from three blocks, in μm^3, of phagocytosed latex particles v(latex per mac) and phagocytosed CsA particles v(CsA per mac) per macrophage incubated with two doses of particles and three incubation times. The mean volume of CsA was estimated by subtracting the mean volume of 'background' vacuoles at each time point and dose from the volume of the total amount of vacuoles from macrophages incubated with CsA particles. Significant dose and time effects were found only at 24 h for the higher dose (0.5 mg) of CsA particles, the other points being not significantly different. At the higher dose (0.5 mg) more latex particles were phagocytosed than at the lower dose (0.1 mg), at each time point. Phagocytosis for both doses versus time was not significantly different.

	Mean volume (μm^3)		
Incubation time	1 h	6 h	24 h
Latex 0.1 mg	6.52 (6.52)	14.38 (14.38)	25.32 (10.54)
Latex 0.5 mg	43.70 (20.77)	54.63 (24.90)	124.19 (66.84)
CsA 0.1 mg	13.83 (14.65)	8.43 (10.13)	4.50 (4.08)
CsA 0.5 mg	26.59 (9.96)	4.13 (9.44)	49.10 (9.51)

Discussion

The aim of this study was to compare the mean volume of latex and CsA particles phagocytosed in vitro by alveolar macrophages. A difference was found based on the characteristics of the particles, latex particles being phagocytosed better than CsA particles.

To determine the mean volume of phagocytosed CsA particles, which were dissolved during the embedding process, we suggested that this volume can be determined by subtracting the mean volume of cytoplasmic vacuoles (called 'background' vacuoles) from the total mean volume of vacuoles in macrophages incubated with CsA according to:

$$v(\text{CsA per mac}) = v(\text{total vac per mac}) - v(\text{back vac per mac}) \quad (1)$$

This mean volume v(back vac per mac) was proposed to remain constant under all study conditions: incubation without particles and incubations with two doses of latex particles at three incubation times (1 h, 6 h and 24 h). Our speculation was confirmed: neither the incubation time nor the dose had any influence on v(back vac per mac). v(back vac per mac) can be assumed to a constant and used therefore in equation 1.

Phagocytosis of particles by pulmonary AM has been described as time-dependent [19]. Our own inhalation studies with latex particles showed that after 24 h essentially all the particles were taken up by the macrophages [20]. We hypothesized therefore that the same would be true in in vitro studies and that the results of experiment with latex particles could serve as an in vitro model for phagocytosis. Furthermore, we wondered whether particle concentration during incubation or incubation time influenced the volume phagocytosed.

We found a tendency towards an increase in the mean volume of phagocytosed latex particles with increasing time and dose. At 24 h, phagocytosis was 3.8 times higher for the lower dose (0.1 mg) and 2.8 times higher for the higher dose (0.5 mg) than after 1 h. Incubated with 0.1 mg, phagocytosis was 6.7, 3.8 and 4.9 times lower at 1 h, 6 h and 24 h respectively than with 0.5 mg. We observed a tendency to increase with time but the mean volume of phagocytosed latex particles did not rise significantly with increasing time. A large variation in the volume of phagocytosed latex per macrophage within a dish and between dishes at each time point was observed and may reflect the nonhomogeneously distributed phagocytic and nonphagocytic macrophages [21, 7]. For the 'background' vacuoles, which are evenly distributed in all macrophages, the variation from one sample to another was low and the statistical test was then significant.

Latex particles appear to be taken up as long as they are available in the medium and at a higher rate at the higher (0.5 mg) than at the lower (0.1 mg) dose. The phagocytosis of CsA particles differed considerably from the phagocytosis of latex particles. CsA particles were taken up to a lesser degree and neither the dose nor the incubation time had any influence on the volume of phagocytosed particles, except for the higher dose (0.5 mg) at 24 h, when the mean volume phagocytosed was 1.8 times higher than at 1 h. The phagocytosis of CsA particles really began to increase between 6 h and 24 h with the highest dose. The uptake of CsA particles by AM in vitro took longer than the uptake of latex particles.

The comparison of CsA with latex particles revealed that for the smaller dose (0.1 mg) at 1 h and 6 h, the mean volumes of phagocytosed particles were not significantly different. At 24 h, however, more latex particles were phagocytosed. At the higher dose (0.5 mg) there were always more latex particles than CsA particles phagocytosed. Alveolar macrophages phagocytosed latex particles at a faster rate than CsA particles. The clearance of inhaled CsA particles is therefore expected to be less than that of latex particles.

Several reasons were considered to explain these differences in the volume phagocytosed between the two types of particles. The receptors on the surface of macrophages play an important role in the mechanism of phagocytosis [22]. Phagocytosis might be different due to a difference in binding of CsA and latex particles by AM. Physical characteristics of the particle types, e.g. their shape and size, might influence the process of phagocytosis. Latex particles are spherical and monodispersed, CsA particles are crystalline, octahedrical and polydispersed. Another factor to be considered is the chemical nature of the two types of particles: latex particles are inert, CsA particles are peptidic.

We conclude that the difference in shape, size and chemical nature of the two types of particles and the difference in the AM surface receptors involved during phagocytosis may influence this process. Further investigations are needed to understand better the role of these factors during phagocytosis of inhaled particles by BAL macrophages.

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