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Comparison of the binding and endocytosis of high-density lipoprotein from healthy (HDL) and inflamed (HDL_{SAA}) donors by murine macrophages of four different mouse strains

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Abstract Serum amyloid A (SAA) is a plasma acute phase protein and the precursor of the AA-fibril protein deposited in AA-amyloidosis. SAA is bound mainly to high-density lipoproteins (HDL_{SAA}). Previous investigations have demonstrated that peritoneal macrophages (mØ) from mice are capable of binding and endocytosing HDL_{SAA}. This observation may indicate a pathway by which SAA enters the mØ and where its intracellular metabolism may be followed by degradation and/or amyloidogenesis. Since binding and internalization defects of lipoproteins may be associated with different diseases, it is possible that mouse strain susceptibility to amyloidosis is associated with qualitative differences in the binding and internalization of HDL_{SAA}. To test this hypothesis a series of binding and internalization experiments was performed in vitro with mØ from four different mouse strains, CD-1, A/J, C57BL/6J and C3H/HeJ, which differ in their susceptibility to AA-amyloidosis. Using colloidal gold-labelled lipoproteins, it was evident by light and electron microscopy that mØ from all four mouse strains are capable of binding and internalizing HDL (without SAA) and HDL_{SAA}. HDL and HDL_{SAA} were found in such compartments of the receptor-mediated pathway as coated pits, coated vesicles, endosomes and multivesicular bodies and in lipid droplets; no qualitative differences were observed. Therefore, it is unlikely that a defect in binding and uptake of HDL_{SAA} is related to the different susceptibilities of these mouse strains to develop AA-amyloidosis. However, the results do not exclude the possibility that differences in the intracellular processing of SAA following endocytosis of HDL_{SAA} is involved in this differing susceptibility.

Key words Amyloid · High-density lipoprotein · Macrophage · Endocytosis

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Introduction

The fibril protein of AA- or reactive-amyloidosis, a fibrillar proteinosis often secondary to chronic inflammatory diseases such as rheumatoid arthritis or tuberculosis, is derived from its autologous plasma precursor serum amyloid A (SAA) [23, 42, 55]. SAA is a type I acute phase protein which, in plasma, is attached to lipoproteins mainly of the high-density fraction (HDL) [23, 42]. SAA bound to HDL is the source of the deposited fibril protein [24, 55]. Intact SAA has been found in AA-amyloid deposits [27, 43, 57] as well as proteolytic fragments, which consist of the aminoterminal two-thirds of SAA [23]. Although the size of the fibril protein is variable, its aminoterminal end rarely differs from that of the precursor protein, and failure of aminoterminal truncation of SAA may be an important step in the pathogenesis of AA-amyloidosis [60]. At the carboxyterminal end 18–30 amino acids are usually cleaved [4, 16, 35, 46] and it is still not known where proteolysis at the carboxyterminal end occurs and at what stage of the disease. It has been suggested that lysosomal proteases and cells of the reticuloendothelial system (RES), mainly macrophages, are involved in this process [3, 12, 13, 15, 43, 51, 54, 58–60]. In addition, amyloid may form intracellularly, indicating a deranged intracellular metabolism of the precursor [51, 54].

SAA's physiological function has not yet been established. It was suggested that SAA may suppress lymphocytic response to antigens [7], may play a part in regulating tissue collagenase gene expression [8], down-regulating oxidative burst in neutrophils [39] and inducing T cell and mast cell adhesion to extracellular matrix [21, 45] and may exert a chemotactic role in inflammation [5,

6]; it may even also reveal antitumour growth properties [17, 44]. SAA appears to have an effect on lecithin cholesterol acyltransferase activity associated with HDL [53], neutral cholesterol ester hydrolase activity of macrophages [38], and on the binding and possible transport of cholesterol [28, 36, 37]. However, at present it is not known whether or how SAA's putative physiological function is linked with the pathogenesis of AA-amyloidosis.

We have recently demonstrated that SAA-bearing high-density lipoproteins (HDL_{SAA}) are bound and internalized by murine peritoneal macrophages (mØ) via endocytosis [26, 49]. This observation may indicate a pathway by which SAA enters the mØ, thus allowing for intracellular metabolism of the precursor protein where aminoterminal truncation would preclude amyloidogenesis and its failure may allow amyloidogenesis to occur. In mice, the susceptibility to reactive amyloidosis varies between different strains [52]. This variability may partly be due to the primary structure of the precursor protein, as in AA-amyloid-resistant CE/J mice, which synthesize nonamyloidogenic acute phase SAA isoforms [52]. However, the primary structure of SAA may not be the only factor contributing to amyloid susceptibility [56]. Differences in the binding and endocytosis of HDL_{SAA} by cells of the RES may also be an influence. To test this hypothesis we performed a series of in vitro experiments with mØ from four different mouse strains which differed in their susceptibility to AA-amyloidosis and atherosclerosis, the latter serving as a control. Using light and electron microscopy we looked for structural differences in the binding and uptake of HDL and HDL_{SAA}.

Materials and methods

CD-1 female mice were purchased from Charles River Laboratories (Montreal, QC, Canada). A/J, C57BL/6J and C3H/HeJ female mice were purchased from Jackson Laboratories (BarHarbor, Me). Retired breeders were used for the preparation of lipoproteins. Mice 6–8 weeks old were used for the preparation of mØ. The animals were fed a normal chow diet (Purina 5001) ad libitum and sacrificed by CO_2 narcosis at the time-points indicated.

Mouse lipoproteins were isolated from pooled plasma by sequential density ultracentrifugation using 10.4 ml polycarbonate centrifuge bottles, a 70.1 Ti fixed angle rotor and an L8-70M ultracentrifuge (all Beckman, Palo Alto, Calif.). High-density lipoproteins without acute phase serum amyloid A (SAA) were recovered from untreated, healthy animals (HDL). For the recovery of acute phase high-density lipoprotein (HDL_{SAA}), mice were injected subcutaneously with 0.5 ml of 2% silver nitrate 18–20 h prior to bleeding by cardiac puncture. The density of the pooled plasma was adjusted by the addition of solid NaBr. Lipoproteins of the density <1.063 g/ml were isolated after centrifugation at 50,000 rpm for 20 h at 4°C. The infranatant was pooled and adjusted to a density of 1.25 g/ml and re-centrifuged at 60,000 rpm for 48 h. The flotate was aspirated and the isolated lipoprotein fractions were dialysed extensively against 10 mM Tris, 0.9% NaCl, 1 mM EDTA (TBE; pH 7.4).

Lipoproteins were oxidized for 12 h at room temperature (rt) in 0.15 M NaCl supplemented with 7.5 μM Cu^{2+} [32]. The oxidation procedure was terminated by dialysing the lipoproteins against TBE (4°C; pH 7.4).

Lipoproteins were labelled with 20 nm colloidal gold probes from Sigma Chemicals (St. Louis, Mo.) according to the technique of Handley et al. [19]. Lipoproteins were dialysed overnight against 0.05 M EDTA (pH 5.5). Approximately 500 μg of lipoproteins (in a volume of 50–100 μl) were mixed rapidly with 15 ml colloidal gold and incubated for 30 min on ice in siliconized Cortex tubes. Unbound lipoproteins were then separated by centrifugation (9,000 rpm, 30 min) against a 35% sucrose cushion and the pellet was resuspended and dialysed against Dulbecco's modified phosphate-buffered saline (D-PBS; 8.00 g NaCl, 0.20 g KCl, 0.10 g $CaCl_2$, 0.10 g $MgCl_2 \cdot 6H_2O$, 1.15 g Na_2HPO_4, $2H_2O$ and 0.20 g KH_2PO_4 with dH_2O to give 1000 ml; pH 7.4).

The successful purification and gold-labelling of the lipoprotein preparations was tested by polyacrylamide gel electrophoresis and negative staining of samples on formvar-coated nickel grids using phosphotungstic acid as a contrasting agent. The apolipoprotein composition of the lipoproteins was examined by urea–SDS–polyacrylamide gel electrophoresis (SDS–PAGE) as described elsewhere [22, 48, 49]. The molecular weights (MW) of the major apolipoproteins (apo) of HDL were consistent with apo A-I (MW 26,000), apo C's (MW 6,000–12,000) and apo A-II (MW 8,000) [33] and of HDL_{SAA} with apo A-I, SAA_2 and SAA_1 (MW 12,600 and MW 11,800), apo C's and apo A-II. Apo E (MW 35,000) was only evident in the lipoprotein fractions of d < 1.063 g/ml.

Protein concentrations were estimated with the BioRad DC Protein assay kit (BioRad Hercules, Calif.). All lipoprotein samples, to be used in cell culture experiments, were used within 48–72 h after their separation by sequential ultracentrifugation and dialysed finally against D-PBS.

For cell culture, mØ were obtained by peritoneal lavage. Ice cold RPMI Medium 1640, 2–3 ml (GibcoBRL, Burlington, Ont., Canada) was rapidly instilled into the peritoneal cavity of ten mice per strain. The aspirate was pooled and centrifuged for 10 min at 1,000 rpm. The supernatant was discarded and the pellet resuspended in RPMI Medium 1640; this step was repeated once. The pellet was finally resuspended in culture medium (RPMI Medium 1640 supplemented with 10% fetal bovine serum and 1% (v/v) antibiotic–antimycotic mix; all GibcoBRL). The number of cells was estimated with a haemacytometer (Neubauer, Germany). The cells were then seeded in four-well *chamber slides* from Nunc (purchased through Life Technologies, Burlington) with an average cell density of 82,000 cells/chamber. Two hours later, nonadherent cells were removed by washing with RPMI Medium 1640. Fresh culture medium was added and the mØ were kept in a tissue culture hood (37°C and 5% CO_2 atmosphere) for 2 days. To load the cells with cholesterol, 24 h before the experiment the culture medium was supplemented with 60 μg/ml oxidized human LDL ($ox\text{-}LDL_h$). In contrast to murine LDL, human LDL did not contain any apolipoprotein other than apo B [14, 48].

Binding and internalization experiments with lipoproteins were performed as described previously [1, 47, 49]. Thirty minutes before the binding, the *chamber slides* were transferred into a cold room (4°C) and the culture medium was exchanged immediately by D-PBS (4°C; pH 7.4) supplemented with 1.0% fatty-acid-free bovine serum albumin (D-PBS/$BSA_{1.0\%}$; Sigma Chemicals, St. Louis, Mo.). D-PBS/$BSA_{1.0\%}$ was changed once after 15 min. Precooled mØ were then incubated on a rocking table for 2 h in D-PBS supplemented with 0.1% fatty-acid free BSA (D-PBS/$BSA_{1.0\%}$) and the indicated amount of lipoproteins. Thereafter, unbound lipoproteins were removed by washing with D-PBS/$BSA_{1.0\%}$ and D-PBS, three times with each. If no further internalization was required, the cells were then fixed.

Internalisation of bound lipoproteins was induced by warming the mØ [1, 47]. D-PBS (4°C) was replaced by warm RPMI Medium 1640 (37°C; pH 7.4) supplemented with additional lipoproteins, and the chamber slides were placed in the tissue culture hood for 60 min. The cells were then washed quickly three times in D-PBS (rt; pH 7.4) and fixed.

For light microscopic examination, the colloidal gold-labelled lipoproteins were visualized by silver enhancement. Following the

experiments described above, the cells were fixed in 3% buffered *p*-formaldehyde (4°C; pH 7.4; 15 min). Subsequently all steps were performed at room temperature. The mØ were washed with PBS (pH 7.4; 3 × 5 min) and treated with 0.2% Triton X-100 in PBS for 2 min, and the slides were washed extensively in PBS (3 × 10 min) and PBS/BSA (PBS supplemented with 1% BSA; 3 × 10 min). Reactive aldehyde groups were eliminated by incubation with 0.05 M glycine for 15 min. Thereafter, the mØ were washed in PBS (3 × 10 min), dH_2O (2 × 10 min) and the colloidal gold was visualized with silver enhancement according to the manufacturers instructions (LM Silver Enhancing Kit [SEKL 15]; Cedarlane Laboratories, Hornby, Ont., Canada). The counterstain was haematoxylin and the cells were dehydrated and covered in Permount (Fisher Scientific, Ottawa, Canada).

MØ dedicated for electron microscopic examination were fixed in Karnovsky's fixative (2% *p*-formaldehyde, 2.5% glutaraldehyde, 0.025% $CaCl_2$, 0.1 M sodium cacodylate; pH 7.4) using a household microwave oven for 10 seconds [40, 41]. Karnovsky's fixative was discarded and replaced by ice-cold 0.1 M sodium cacodylate buffer supplemented with 0.025% $CaCl_2$ (SCB; pH 7.4, 3 × 10 min). The mØ were post-fixed for 2 h in OsO_4 (1.33% OsO_4, 0.067 M *s*-collidine, 0.025% $CaCl_2$). The cells were then rinsed with 0.1 M maleate buffer (pH 5.2; rt; 3 × 10 min), stained with 2% uranylacetate (pH 5.2; rt; 60 min) and again rinsed with 0.1 M maleate (pH 5.2; rt; 3 × 10 min). The cells were dehydrated in ethanol and embedded in LR White using gelatin capsules placed open-faced onto the surface of the slide, covering the cells. Polymerization was initiated with benzoin ethylether (0.075 g/15 g LR White) and UV light overnight. The capsules with the embedded mØ dislodged easily from the slides and semi-thin sections were stained with Toluidine blue. Ultrathin sections of blue and gold sheen were mounted on copper grids and stained with lead citrate for 2–3 min. These were then air-dried and examined using a Hitachi H-500 electron microscope.

Results

Pre-incubation with 60 μg/ml oxidized human LDL converted the mØ of all four mouse strains into foam cells. At the electron microscopic level the lipid droplets showed a translucent matrix with a few irregular cloudy densities, a homogeneous granular matrix of intermediate density, or a fairly dense matrix.

The preceding binding experiments for 2 h at 4°C with 20 μg/ml of AuHDL or $AuHDL_{SAA}$, using mØ from CD 1 mice only, revealed homogeneous staining of the individual cells on the light microscopic level. When the signals were brought into focus, as demonstrated schematically in Fig. 1, it became evident that the staining was limited to the cell surface [26, 49]. The light microscopic staining pattern was confirmed by electron microscopy. Following incubation for 2 h at 4°C gold particles were found only at the cell surface and in coated pits, and none in the cytoplasm (Fig. 2).

No signals were detected above background after competition experiments with 20 μg/ml colloidal gold-labelled lipoproteins and a 25-fold excess of unlabelled lipoproteins, that is either AuHDL (20 μg/ml) and HDL (500 μg/ml) or $AuHDL_{SAA}$ (20 μg/ml) and HDL_{SAA} (500 μg/ml), respectively.

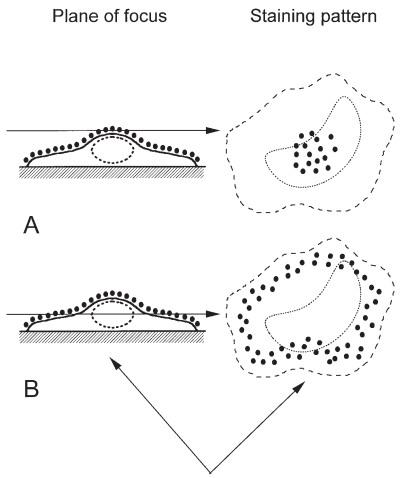


Fig. 1 Schematic demonstration of the light microscopic appearance (*right arrow*) of cell surface staining of a cultured macrophage. According to the plane of focus, signals are visible either in the centre of the cell (see also Fig. 3a, e) projecting onto the nucleus or arranged in circles projecting onto the cytoplasm (see also Fig. 3c, g). (*Left arrow* side elevation)

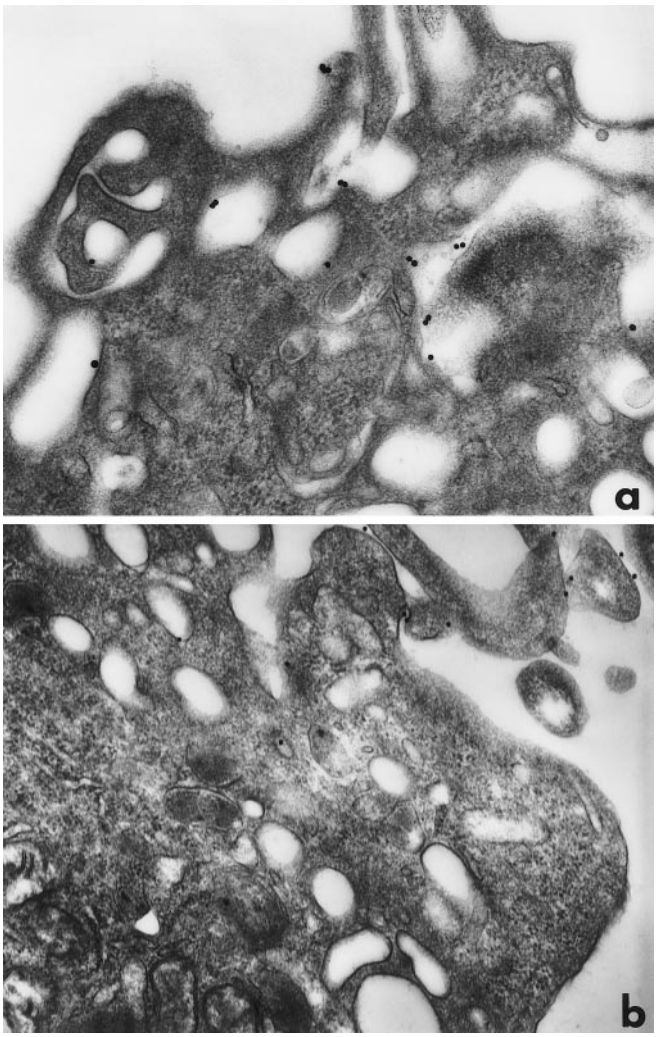
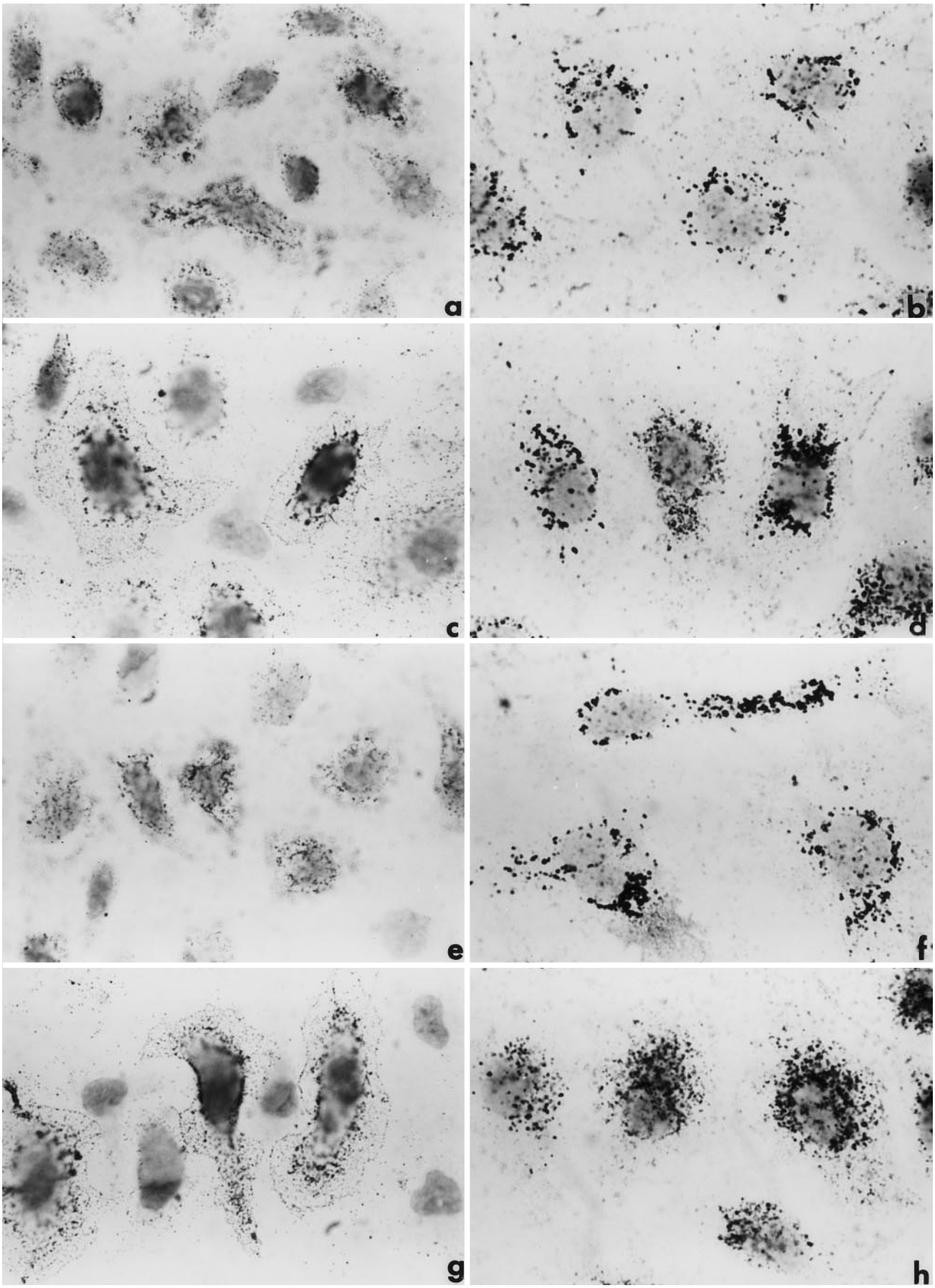


Fig. 2 Binding experiment with 20 μg/ml, with **a** AuHDL and **b** $AuHDL_{SAA}$, as visualized by electron microscopy: following incubation at 4°C for 2 h the mØ were fixed and embedded in LR White. (*CD-1* lead citrate, **a** ×34,000, **b** 26,000)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Binding experiments for 2 h at 4°C with 20 μg/ml of AuHDL or $AuHDL_{SAA}$ revealed a homogeneous staining of the individual cells similar to the pattern observed in the preceding binding experiments (see above; Fig. 3). The staining of the entire cell population varied, with some cells labelled intensely and some scantily. However, the staining pattern of the mØ was similar in all four mouse strains, only quantitative differences were apparent; the mØ from CD-1 and A/J mice were labelled more intensely than those from C57BL/6J and C3H/HeJ mice. Furthermore, binding experiments with HDL, in general, appeared to reveal a more intense staining than those with HDL_{SAA}.

The staining pattern changed following internalization experiments (60 min at 37°C) applying 20 μg/ml of AuHDL or $AuHDL_{SAA}$. The indivdual signals were larger and predominantly localized in close proximity to the nucleus or arranged in circles within the cytoplasm (Fig. 3). The staining pattern of the mØ did not show any significant qualitative or quantitative differences between the different mouse strains.

Electron microscopic examination of the internalization experiments demonstrated gold-labelled lipoprotein particles in such endocytotic compartments as coated pits, coated vesicles, endosomes, and multivesicular bodies as well as in lipid droplets (Fig. 4). Few lysosomes were found and these did not show any gold particles. Regardless of the origin of the mØ, the compartments involved during endocytosis of AuHDL or $AuHDL_{SAA}$ were the same in every mouse strain.

Discussion

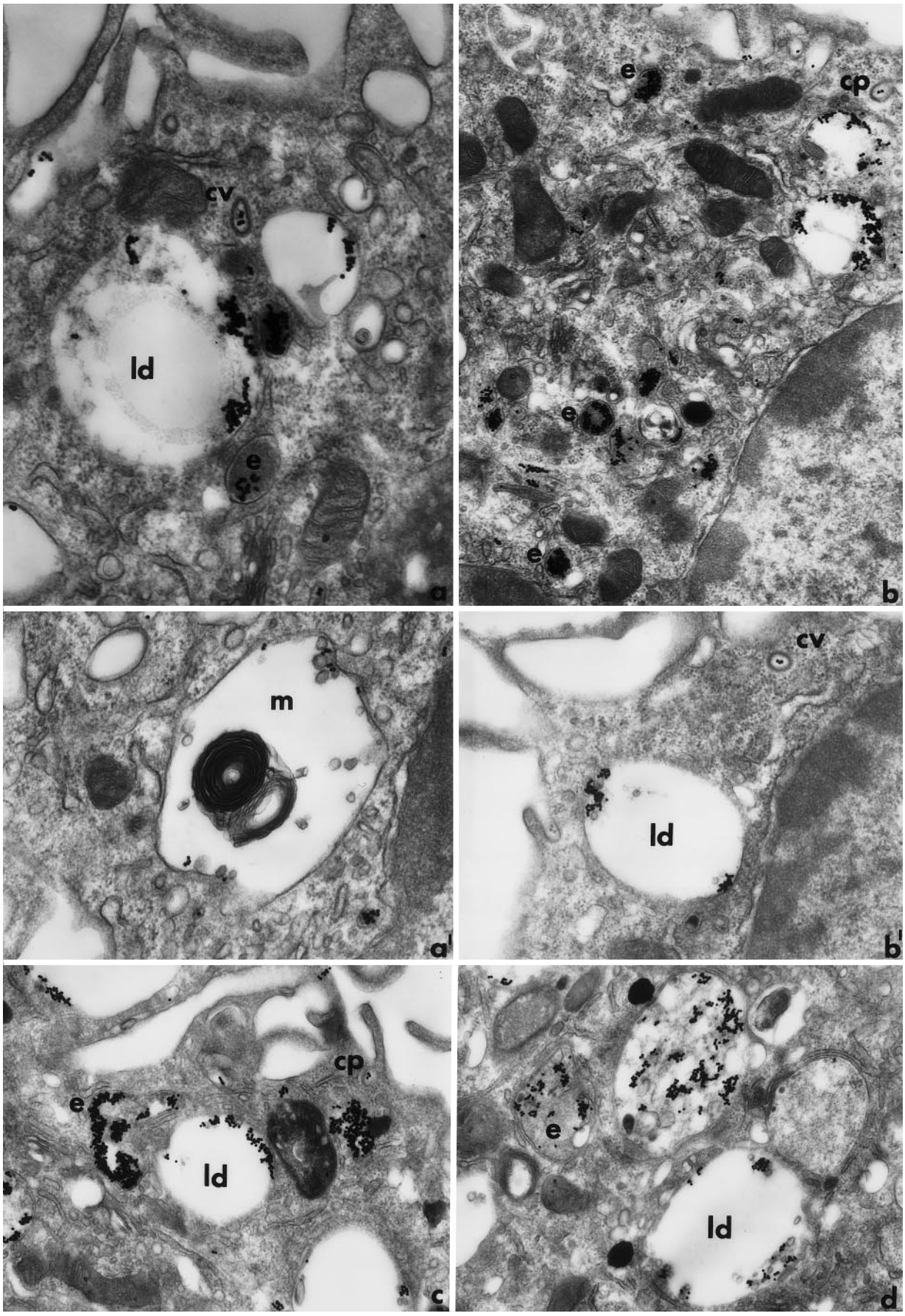
In mice the susceptibility to reactive AA-amyloidosis is defined by the interval between experimental induction of an inflammatory response and the appearance of amyloid deposits as demonstrated by conventional histochemical techniques (apple green birefringence after Congo red staining). In CD-1 mice (amyloid sensitive) this time difference is approximately 9 days, and in A/J mice (partially amyloid resistant) it is approximately 21 days [52]. In C57BL/6J (high atherosclerosis susceptibility) and C3H/HeJ mice (low atherosclerosis susceptibility) amyloid usually occurs 11 and 16 days after onset of an experimentally induced inflammation [52].

The propensity to develop amyloid and amyloidosis probably depends on a variety of factors, which can be divided into non-strain-specific and strain-specific ones. A non-strain-specific factor is the inflammatory stimulus. That produced by silver nitrate is permanent, results in an active local inflammatory reaction at the site of injection and a systemic reaction such as the up- and down-regulation of acute phase and negative acute phase proteins, respectively. A single application of silver nitrate is sufficient to produce an inflammatory response which, in a susceptible strain, inevitably proceeds to the formation and deposition of amyloid. In contrast, casein, another commonly used amyloidogenic stimulus, is active only for a short period of time. Repeated injections on a daily basis are necessary to cause amyloidosis. In general, the non-strain-dependent inflammatory stimulus is a well-recognized variable of AA-amyloidosis, and clinically appropriate management and inhibition of a chronic or recurrent inflammatory disease, such as rheumatoid arthritis or tuberculosis, has become the first choice for treatment and prevention of reactive AA-amyloidosis [25].

Strain-dependent factors that influence amyloid morbidity have been reported as being due to the primary structure [10, 52] or the catabolism of the precursor protein [29]. However, little is known about the amyloidogenic metabolism of SAA. The pathway linking the SAA attached to HDL with the AA-fibril protein assembled to amyloid is still unknown, and multiple strain-specific factors may well be active. It has been demonstrated that HDL_{SAA} is bound and endocytosed by mØ [11, 26, 49] which suggests a receptor-mediated internalization pathway for HDL_{SAA}, that makes it comparable with other lipoproteins, in particular with LDL. Multiple receptor defects were described as being linked to the binding and internalization deficiencies of LDL, causing hypercholesterolaemia and early development of atherosclerosis [9, 34]. Similarly, hypothetically, a defect in binding and internalization of HDL_{SAA} may influence the onset and progress of AA-amyloidosis in a strain-specific manner. However, we have previously investigated only the binding and internalization capabilites of mØ obtained from amyloid-sensitive CD-1 mice [11, 26, 49]. MØ obtained from other strains, in particular from partially amyloid-resistant mice, have not yet been studied, [11, 26, 49].

The experiments reported here did not show any qualitative differences in binding and endocytosis of either HDL or HDL_{SAA} by mØ of four mouse strains with differing amyloid susceptibility. A morphological approach has been successful in the past for demonstrating binding and internalization defects for LDL [2, 9], and the methods applied in the current study followed standard protocols [1, 50]. Therefore, the experimental design should have been sensitive enough to detect an aberrant lipoprotein metabolism, such as the absence of a receptor–ligand interaction (no binding to the cell surface) or a lack of internalization. It is unlikely that nonspecific hydrophobic interaction between the labelled lipoprotein and the cell surface can mask the results of the binding and internalization experiments. In the presence of an excess of unlabelled ligand (competition experiment) the signal was reduced significantly, as evidenced by morphological (see present study and [48]) and biochemical investigations [26]. The data showing binding to clathrin-coated pits also disagree with nonspecific binding (see pres-

◀ **Fig. 3** Binding and internalization experiments with 20 μg/ml AuHDL (**a–d**) and with $AuHDL_{SAA}$ (**e–h**) as visualized by silver enhancement: binding experiments for 2 h at 4°C demonstrated homogeneous staining of individual cells (*left lane*); internalisation experiments for 60 min at 37°C caused a significant change in the staining pattern (*right lane*). No significant differences were found between mØ from different mouse strains, such as CD-1 (**a**, **b**, **e**, **f**) or A/J (**c**, **d**, **g**, **h**). Haematoxylin counterstain, ×793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ent study and [48]). The results presented here still leave open several possibilities, including those listed below:

Binding and internalization of HDL_{SAA} via an endocytotic pathway may not be involved in amyloidogenesis at all. In fact, evidence exists that cell surface proteases may degrade SAA, possibly contributing to the formation of amyloid [30, 31]. However, morphological data support the hypothesis of an intracellular metabolism of the precursor: confocal microscopy localized acute phase SAA exclusively to the endosomes-lysosomes of peritoneal macrophages from alveolar hydatid infected mice [11], and ultrastructural evidence exists for intracellular formation of amyloid fibrils in macrophages [13, 51, 54]. In addition, immunoblotting of macrophage cytoplasmic fractions identified predominantly two, ~5- and 12-kDa, carboxyterminus cleaved acute phase SAA derivatives, which resemble the tissue AA in molecular mass [11], and inhibition of the endosomal/lysosomal enzyme cathepsin D accelerated the progression of murine splenic amyloidosis [60].

Secondly, amyloidogenesis may involve binding and internalization of HDL_{SAA} but does not influence the susceptibility, or finally, amyloidogenesis involves binding and internalization of HDL_{SAA} and does influence the susceptibility, but the method applied was not appropriate to detect the relevant pathophysiological mechanism. In this respect, quantitative rather than qualitative differences may need to be demonstrated. Ham et al. [18] have found that mØ obtained from C57BL/6 mice, in contrast to those obtained from A/J mice, show an intermittent block in the degradation of SAA as analyzed by in vitro degradation experiments with HDL_{SAA}. In contrast, the SAA-degradative capacity of mØ obtained from mice undergoing an amyloidosis induction protocol showed no difference from that of mØ obtained from healthy control animals [20]. Although it appears that the degradative capacity of mØ is influenced by their orgin and not by the amyloid induction protocol, neither of the two studies investigated the pathway by which SAA was degraded and whether mØ from C57BL/6 and A/J mice apply different pathways to degrade SAA [18, 20].

However, a more detailed knowledge about the amyloidogenic metabolism of SAA and of the factors that influence the susceptibility to amyloidosis may help to identify patients at risk before deposition of amyloid complicates a chronic or recurrent inflammatory disease, such as rheumatoid arthritis. The strain-specific differences in mice may help to identify factors that may also contribute to the pathogenesis of human AA-amyloidosis and which, in turn, may be useful for detecting patients at risk as well as for new diagnostic and therapeutic strategies in AA-amyloidosis.

◀ **Fig. 4a–d** Internalisation experiments with 20 μg/ml $AuHDL_{SAA}$ as visualized by electron microscopy: following an internalization period of 60 min at 37°C gold-labelled lipoproteins were found in coated pits (*cp*), coated vesicles (*cv*), endosomes (*e*), multivesicular bodies (*m*) and lipid droplets (*ld*). CD-1 (**a**, **a′**), A/J (**b**, **b′**), C57BL/6J (**c**) and C3H/HeJ (**d**). Lead citrate, **a** ×42,300, **a′** ×34,000, **b** ×30,000, **c** ×26,000, **d** ×22,000

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