Antigen-dependent Internalization is related to Rapid Elimination from Plasma of Humanized Anti-HM1.24 Monoclonal Antibody (AHM)

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Running title page

Pharmacokinetics and Internalization of Anti-HM1.24 Antibody

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List of nonstandard abbreviations

ADCC: antibody-dependent cell-mediated cytotoxicity

AHM: humanized anti-HM1.24 monoclonal antibody

AUC: area under the plasma concentration-time curve

CHO: Chinese hamster ovary

CL: total clearance

ELISA: enzyme linked immunosorbent assay

FBS: fetal bovine serum

MRT: mean retention time

MM: multiple myeloma

PAF: paraformaldehyde

PAO: phenylarsine oxide (arsorosobenzene)

PBMC: peripheral blood mononuclear cell

TCA: trichloroacetic acid

Vss: steady-state distribution volume
Abstract

AHM is a humanized anti-HM1.24 monoclonal antibody that binds to the HM1.24 antigen, a protein which is highly expressed in multiple myeloma cells. Pharmacokinetics of AHM was determined in experiments in which AHM was administered intravenously to cynomolgus monkeys. The area under the plasma concentration-time curve (AUC) increased by more than the dose ratio between 2 and 20 mg/kg, and non-linear pharmacokinetics was observed. The elimination half-life of AHM from the plasma was 7.56 h at 2 mg/kg and 28.6 h at 20 mg/kg, which was shorter than that observed for other therapeutic humanized monoclonal antibodies, such as Trastuzumab and Bevacizumab. Although antibodies to AHM were detected in all monkeys on or after 10 days of administration, there was a temporal disassociation between the rapid elimination of AHM and the appearance of anti-AHM antibodies. HM1.24 antigen-dependent internalization and intracellular metabolism of AHM were investigated in peripheral blood mononuclear cell (PBMC), KPMM2 and U937 cells. In all cases AHM was rapidly internalized from the cell surface, which was significantly prevented by phenylarsine oxide (PAO) in KPMM2 cells, an inhibitor of receptor-mediated endocytosis, and the internalized AHM was subsequently degraded within the cells. Furthermore, immunofluorescence microscopy revealed that the
internalized AHM is delivered to, and degraded in, late endosomes/lysosomes. Taken together, our results suggest that the rapid elimination of AHM from plasma in monkey is due to HM1.24 antigen-dependent internalization followed by delivery to the lysosomes.
Introduction

The HM1.24 antigen (HM1.24) is a transmembrane protein that has unique topology with two membrane anchor domains; an NH$_2$-terminal transmembrane domain and a glycosylphosphatidylinositol (GPI) attached to the COOH terminus. HM1.24 is identical to BST2/CD317 cloned from the bone marrow stroma of patients with multiple myeloma (MM) (Ohtomo et al., 1999). Indeed, it is conceivable that HM1.24 may be a promising target antigen for a cytotoxic antibody in the treatment of MM because HM1.24 is highly expressed on myelocytes and tumor cells derived from B and T cell lymphocytes and is also present in activated lymphocytes (Ohtomo et al., 1999; Ozaki et al., 1997; Ono et al., 1999; Ozaki et al., 1999; Goto et al., 1994). Although HM1.24 is expressed on terminally differentiated B cells, PBMC and some normal cells, its physiological function is unknown. Our recent studies showed that HM1.24 localized not only on the cell surface but also in the trans-Golgi network (TGN) and/or recycling endosomes (Masuyama et al., 2009).

AHM is a humanized anti-HM1.24 monoclonal antibody (IgG1/κ) that binds to the HM1.24. AHM has been humanised by grafting mouse CDRs onto a human IgG, and is produced in Chinese Hamster Ovary (CHO) cells by means of recombinant DNA technology. A humanized antibody is favored because murine antibodies fail to elicit
effector cell activity in experiments with human cells. Moreover, a humanized antibody is expected to be much less antigenic than a murine antibody. Preclinical data and experiments using human cells in vitro suggest that AHM binds with high affinity to myeloma cells. Moreover, in the presence of sufficient numbers of binding sites per cell AHM causes binding of effector cells from the host, which mediates antibody-dependent cell-mediated cytotoxicity (ADCC) activity leading to cell death (Ono et al., 1999). These lines of evidence imply that AHM may have a beneficial effect in disease states in which HM1.24 is over-expressed and there is excessive cell growth, such as MM.

Studies comparing cross-reactivity in vitro in the human and cynomolgus monkey demonstrated binding to similar tissues in both species. These experiments support the idea that the cynomolgus monkey is a relevant experimental animal with human. In the course of the development of AHM, we therefore investigated pharmacokinetics of AHM following single intravenous administration in cynomolgus monkey.

There are two mechanisms in the clearance of humanized antibody from the blood. One is an antigen-independent clearance, which is dependent on a common structure, such as the IgG of a humanized antibody. The other is an antigen-dependent clearance involving binding to the specific antigen of the antibody, saturation of the antigen and
internalization by cells. In fact, it is known that antigen/receptor mediated mechanism mediated by binding to antigens and receptors expressed on the cell surface contribute to the clearance of humanized antibody from the blood, and is largely responsible for the nonlinear pharmacokinetics and increased clearance caused by a low dose of humanized antibody in many therapeutic antibodies (Coffey et al., 2004; Gottlieb et al., 2000; van Der Velden et al., 2001).

Our previous study using HeLa cells suggested that HM1.24 was constitutively internalized from the lipid raft on the cell surface by clathrin-mediated endocytosis (Masuyama et al., 2009). This fact suggests that AHM binds to surface antigens of cells expressing HM1.24, and is followed by internalization into the cells. Therefore, we examined characteristics of the internalization of radiolabeled AHM (125I-AHM) i.e., the saturation of cell surface antigen by AHM, internalization rate and the intracellular degradation rate of antigen-bound AHM, and the intracellular metabolism of AHM using PBMCs and KPMM2 cells expressing the HM1.24. Furthermore, we visually examined the internalization of AHM and the localization of AHM after internalization in U937 cells using immunofluorescence microscopy.
Materials and Methods

Materials

AHM was produced in CHO cells by Chugai Pharmaceutical Co Ltd. (Tokyo, Japan). KPMM2 cells were established from the lymphocytes of patients with multiple myeloma (Goto et al., 1995), HM1.24 was produced in CHO cells using recombinant DNA technology by Chugai Pharmaceutical Co Ltd. PBMC were purified by Ficoll-Paque gradient method from peripheral blood of healthy volunteers. Fetal bovine serum (FBS) and phenylarsine oxide (PAO) were purched from Sigma-Aldrich (St Louis, MO). Mouse monoclonal antibodies to human LAMP-1 were obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa (Iowa City, IA). Mouse monoclonal antibodies to EEA1, GM130, and GGA3 were purchased from BD and Company (Franklin Lakes, NJ). Mouse monoclonal antibodies to TfnR, Alexa488- and Alexa594-labeled secondary antibodies were obtained from Invitrogen Corp. (Carlsbad, CA). Other chemicals and reagents were of the highest grade and purchased from local commercial sources.

Preparation of Radiolabeled AHM

Radiolabeled AHM ($^{125}$I-AHM) was prepared by the Tejedor method (Tejedor and Ballesta, 1982). The specific radioactivity was 0.7 MBq/μg and the average number of
125I atoms introduced per molecule of AHM was 1.3. The radiochemical purity of 125I-AHM determined by gel filtration chromatography was 95.0% or more. The reactivity of the prepared 125I-AHM to the HM1.24 was determined to be 90.6% of that of unlabeled AHM by enzyme linked immunosorbent assay (ELISA).

**Animal Experiments**

AHM was single injected intravenously at a dose of 0.2, 2 or 20 mg/kg into male cynomolgus monkeys (3 animals/group). Blood samples were collected from the brachial vein at pre-dose and at 0.5, 2, 4, 8 and 24 h and 2, 3, 4, 7, 10, 14, 17, 21 and 28 days following administration into a heparinized syringe and plasma was prepared by centrifugation. Urine excreted naturally was collected for up to 28 days at 1 day intervals after administration. Each cage was washed with an appropriate amount of 0.05% Tween 20 solution. The washing fluid was added to the collected urine and the total volume was measured as urine samples. The plasma and urine samples were stored frozen until analysis. The concentrations of AHM and anti-AHM antibody in plasma and AHM concentration in urine samples were determined by ELISA.

125I-AHM was single injected intravenously at a dose of 1 MBq/2 mg/kg into male cynomolgus monkey (3 animals). Urine excreted naturally until 4 days at 1 day intervals after administration was collected. Each cage was washed as described above and the
total volume of urine samples was measured after mixing of the washing fluid and the urine. The total radioactivity and TCA-precipitable radioactivity in urine samples were measured on each day of sample collection.

All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at Chugai Pharmaceutical Co. Ltd.

**Measurement of Radioactivity in Urine Samples**

950 μl of urine sample was transferred to an assay tube containing 50 μl of rat control plasma and the total radioactivity in each assay sample was measured by a γ-counter. Then, 1 ml of 25% trichloroacetic acid (TCA) solution was added to the tube used for the measurement of the total radioactivity described above, and the mixture was centrifuged for 10 min at 3,000 x g and 4°C. The radioactivity in the precipitate obtained was measured as the TCA-precipitable radioactivity after removal of the supernatant by an aspirator.

**Determination of AHM Concentration in Plasma and Urine Samples**

The concentrations of AHM in the plasma and urine samples were quantified by ELISA. Soluble human HM1.24 having HA was immobilized on 96-well microplates via anti-HA. After the addition of assay samples (duplicate) into each well on the microplate, biotinylated goat anti-human IgG, streptavidin-alkaline phosphatase
conjugate and its substrate were added sequentially, and the absorbance at 650 nm was measured. The quantification ranges for AHM in the plasma and urine samples were set at 0.195 - 12.5 μg/ml and 7.81 - 500 ng/ml, respectively.

**Determination of Anti-AHM Antibodies Concentration in Cynomolgus Monkey Plasma**

The anti-AHM antibodies in cynomolgus monkey plasma were measured by ELISA using 96-well microplates onto which was immobilized AHM (Fab). After the addition of assay samples (duplicate) into each well on the microplate, biotinylated AHM (Fab), streptavidin conjugated peroxidase and its substrate were added sequentially. The reaction was stopped by addition of sulfuric acid whereupon absorbance at 492 nm was measured. An affinity purified cynomolgus monkey anti-AHM polyclonal antibody was used as a reference material and the anti-AHM antibody titer was calculated as the concentration converted to the reference material. The quantification range for this method was set at 0.0625 - 4.00 μg/ml.

**Pharmacokinetic Analysis**

Pharmacokinetic analysis software, WinNonlin (Ver. 2.1; Pharsight Corp., Cary, NC), was used to calculate the pharmacokinetic parameters. AUC and the mean retention time (MRT) were calculated using the trapezoid method up to the final measurable point.
After that, AUC was calculated as zero the concentration in the next sampling point of the final measurable point. MRT was calculated by the linear least-squares regression with extrapolation from the measurable points that were selected automatically by the WinNonlin program. Total clearance (CL) and steady-state distribution volume (Vss) was found using equation 1 and 2, respectively. Plasma half-life (t_{1/2}) was calculated using equation 3 after determining λ_Z by least-squares linear regression based on the selected time points as the terminal phase and the corresponding log-transformed plasma concentrations. Plasma concentrations up to the day 4 after dosing were used for calculation of t_{1/2} in two animals (animal number 7 and 9) that were injected with AHM at 20 mg/kg, because the plasma concentrations for these animals decreased in an accelerated manner on and after day 4.

\[
\text{CL} = \text{dose/AUC} \quad \text{Equation 1}
\]

\[
\text{V}_{ss} = \text{CL} \times \text{MRT} \quad \text{Equation 2}
\]

\[
t_{1/2} = \ln 2 / \lambda_Z \quad \text{Equation 3}
\]

**Equilibrium Binding Assay**

KPMM2 cells, U-937 cells and PBMCs were used for the equilibrium binding assay of ^{125}\text{I}-\text{AHM}. KPMM2 and U-937 cells were culture with RPMI1640 medium
containing 20% FBS, 0.1% 2-Mercaptoethanol (ME), 0.1 mg/ml kanamycin sulfate and
40 ng/ml human interleukin-6. PBMCs were cultured with RPMI1640 medium
containing 10% FBS, 0.1% 2-ME and 0.1 mg/ml kanamycin sulfate. The solutions (10
μl each) containing unlabeled AHM at concentrations of 0, 0.5, 1, 2, 4, 8, 16, 32,
64-fold that of $^{125}$I-AHM were added to 10 μl of 2.75 nmol/l $^{125}$I-AHM solution,
respectively. Determination of nonspecific binding was carried out by excess unlabeled
AHM (1024-fold to $^{125}$I-AHM). The prepared samples were mixed with 200 μl of each
cell (2.2 x10$^6$ cells/ml) and incubated at 4°C for 3 h. The studies were conducted in
duplicate. After the cells and medium were separated by centrifugation (2,000 × g, 3
min), the radioactivity of each fraction was measured by a γ-counter. The specific
binding of $^{125}$I-AHM was calculated by subtracting the nonspecific binding from the
total binding. Binding data was analyzed by Scatchard plot analysis to estimate the
dissociation constant ($K_D$), number of binding sites per cell and the maximal binding
site ($B_{max}$).

**Internalization of $^{125}$I-AHM**

PBMCs and KPMM2 cells were used for the study of $^{125}$I-AHM internalization. The
same medium with the equilibrium binding assay was used for culture of PBMCs and
KPMM2 cells. $^{125}$I-AHM of a 10-fold concentration of the $K_D$ value (1.30 nmol/l) for
PBMCs, and 0.1-, 0.5- and 10-fold of the $K_D$ value (0.05, 0.25 and 5.00 nmol/l) for KPMM2 cells was added to medium. Each test was conducted in duplicate.

Cells were collected from the cell suspension by centrifugation ($2,000 \times g$, 5 min) and resuspended in medium containing $^{125}$I-AHM at a given concentration. The control human IgG, which belongs to the same subclass as AHM, was added to medium at 1,000-fold concentration of $^{125}$I-AHM to prevent the nonspecific binding of $^{125}$I-AHM to the cell membrane components other than HM1.24 mediated by constant region. After incubation of cells at 4ºC for 3 h, cells were collected by centrifugation ($2,000 \times g$, 5 min) and washed twice with ice-cold medium. Washed cells were resuspended at a concentration of $1.3 - 9.1 \times 10^6$ cells/ml into medium without $^{125}$I-AHM, aliquoted into test tubes (200 µl each), and then incubated at 4ºC and 37ºC for 0, 0.25, 0.5, 1, 2, 3 and 4 h. After incubation, 800 µl of medium was added to each test tube and supernatant collected as medium fraction after centrifugation ($2,000 \times g$, 1 min). The cell pellet was suspended into 200 µl of medium (dissociation medium) adjusted to pH 3.5 - 3.7 with the appropriate amount of acetic acid, and then incubated at 4ºC for 5 min. Supernatant was then collected by centrifugation ($2,000 \times g$, 1 min). The cell pellet was resuspended into 800 µl of dissociation medium, the operation described above was repeated and supernatant obtained from the two procedures were mixed as the cell surface fraction.
The cell pellet was suspended into 1,000 μl of medium and collected as the intracellular fraction.

KPMM2 cells were used for experiments to inhibit internalization of 125I-AHM. The inhibition experiment was conducted in a manner similar to that described above except for the addition of 0.5 mmol/l PAO to the medium, which is known to inhibit the endocytosis of protein (Kato et al., 1992; Finbloom, 1988).

The radioactivity (total radioactivity) in each fraction was measured by a γ-counter. Then, 500 μl of 25% TCA solution and 200 μl of FBS were added to the tube be used for the measurement of the total radioactivity described above. The mixture was centrifuged for 5 min at 3,000 x g, 4 ºC. The radioactivity in the precipitate obtained was measured as the TCA-precipitable radioactivity after removal of the supernatant by an aspirator.

**Occupancy Rate of Cell Surface HM1.24 by 125I-AHM**

$E_{\text{max}}$ and $E_{\text{C50}}$ were calculated using WinNonlin by applying a ratio of specific binding amount (B) to a maximal binding site ($B_{\text{max}}$) ($B/B_{\text{max}}$) and each AHM concentration (C) of equilibrium binding assay in each cell to equation 4. By applying $E_{\text{max}}$, $E_{\text{C50}}$ and 125I-AHM concentration ($C'$) used for internalization study to equation 5, the occupancy rate of binding site on the cell surface by 125I-AHM was calculated.
B/B_{max} (%) = C \times E_{max}/(C + E_{C50}) \quad \text{Equation 4}

\text{Occancy rate} (%) = C' \times E_{max}/(C' + E_{C50}) \quad \text{Equation 5}

E_{max} \text{ is maximum value of } B/B_{max}(%), \text{ } E_{C50} \text{ is AHM concentration (nmol/l) at 1/2 binding rate of } E_{max} \text{ in equation.}

**Internalization Rate and Degradation Rate of }^{125}\text{I-AHM**}

The internalization rate of complex of }^{125}\text{I-AHM and HM1.24 was represented by equation 6. Equation 7 was obtained by the integration of equation 6 over time from 0 to } t. \text{ The equation for a regression line was obtained by least-squares method from a slope up to 1 h of incubation using } \text{AUC}_{0-t}, (s) \text{ and } [\text{LRi}] \text{ in equation 7, and then, an internalization rate constant (Kint) was calculated with an integration plot.}

\frac{d[LRi]}{dt} = K_{int}[LRs] \quad \text{Equation 6}

[LRi]_t = K_{int} \int_0^t [LRs] \, dt = K_{int} \times \text{AUC}_{0-t}, (s) \quad \text{Equation 7}

\text{In equation, } [\text{LRi}] \text{ is the concentration of }^{125}\text{I-AHM (fmol) internalized in the cell, } [\text{LRs}] \text{ is the concentration of }^{125}\text{I-AHM (fmol) bound to the cell surface, } \text{AUC}_{0-t}, (s) \text{ is the area under the curve of concentration of }^{125}\text{I-AHM (fmol) bound to the cell surface versus time.}

The degradation rate of }^{125}\text{I-AHM in the cell is represented by equation 8. Equation 9 was obtained by the integration of equation 8 over time from 0 to } t. \text{ The equation for a }
regression line was obtained by the least-squares method from a slope from 1 h to 4 h after incubation using AUC<sub>0-4</sub>, (<i>i</i>) and [L<sub>deg</sub>] in equation 9, and then, a degradation rate constant (K<sub>deg</sub>) was calculated with an integration plot. Meanwhile, TCA-soluble radioactivity (total radioactivity – TCA-precipitable radioactivity) was used as a concentration of degraded <sup>125</sup>I-AHM in this study.

\[
d[L_{\text{deg}}]/dt = K_{\text{deg}}[LR_{i}] \quad \text{Equation 8}
\]

\[
[L_{\text{deg}}]_{t} = K_{\text{deg}} \int_{0}^{1} [LR_{i}] dt = K_{\text{deg}} \times \text{AUC}_{0-4}(i) \quad \text{Equation 9}
\]

In equation, [L<sub>deg</sub>] is the concentration of degraded <sup>125</sup>I-AHM (fmol) in medium fraction, AUC<sub>0-4</sub>,(<i>i</i>) is the area under the curve of concentration of internalized <sup>125</sup>I-AHM (fmol) versus time.

**Immunofluorescence Microscopy**

U937 cell was used for immunofluorescence analysis. Immunofluorescence analysis was performed using U937 cells as described previously (Masuyama et al., 2009).

Briefly, cells were fixed in 4% PFA in PBS, pH 7.4, for 30 min at room temperature, and permeabilized with 0.05% saponin in PBS for 15 min. Cells were quenched with 50 mM NH₄Cl in PBS for 15 min and blocked with 1% BSA in PBS for 30 min. The cells were then incubated for 1 h in the primary antibody diluted in blocking solution, as described previously (Hirota et al., 2007). The cells were incubated for 30 min with
Alexa488- or Alexa594-labeled secondary antibodies. Cells were then mounted in Mowiol, and were analyzed by a confocal laser scanning microscope using a ZEISS LMS 510 META equipped with an argon/HeNe laser and a ZEISS 100x/1.4 Plan-Apochromat oil immersion lens. Photographic images were processed using Adobe Photoshop software (Adobe Systems).

**Lysosomal Degradation of Internalized AHM**

U937 cells were incubated with AHM (2 μg/ml; 12.5 nmol/l) for 30 min at 4°C. Cells were washed twice with PBS at 4°C and further incubated in DMEM (Dulbecco’s modified Eagle medium) containing 10% FBS at 37°C for 12 h in the absence or presence of leupeptin and pepstatin. After incubation, cells were fixed, permeabilized, incubated with a LAMP-1 antibody, and the internalized AHM and LAMP-1 antibody were stained with Alexa594- and Alexa488-labeled secondary antibody, respectively.
Results

Pharmacokinetics of AHM after Single Intravenous Dose in Cynomolgus Monkey

The plasma concentration-time curves of AHM and antibodies against AHM intravenously administrated to cynomolgus monkey are shown in Figure 1 and 2, respectively. The pharmacokinetic parameters are presented in Table 1. The plasma concentration of AHM after the lowest dose of 0.2 mg/kg was generally below the limit of detection (0.195 μg/ml) by 2-4 h. However, AHM was detected up to 1 day and 7 days after dosing in every animal given either 2 or 20 mg/kg, respectively. The elimination of AHM from plasma was accelerated on and after day 4 in two of three animals that were injected with AHM at 20 mg/kg. Anti-AHM antibodies were detected in all monkeys containing the accelerated animal on and after day 10, 14 or day 18. There was a temporal dis-association between acceleration of elimination of AHM and the detection of anti-AHM antibodies. Comparison between the two groups given dosages of 2 and 20 mg/kg showed that AUC increased by more than the dose ratio. CL was significantly less in monkeys given 20 mg/kg compared to those given 2 mg/kg, but Vss was not different. The t1/2 prolonged with increasing dose i.e., 7.56 h at 2 mg/kg and 28.6 h at 20 mg/kg.

When cynomolgus monkeys were given a single intravenous injection of 2 mg/kg
$^{125}$I-AHM, 21.4% and 49.9% of the radioactivity administered had been excreted in the urine up to 1 and 4 days after injection, respectively. However, the TCA-precipitable radioactivity recovered in the urine was only 1.5 and 5.7% at 1 day and 4 days after administration, respectively. Furthermore, AHM in the urine was barely detectable by ELISA. These results suggest that most of the radioactivity excreted in the urine was derived from free $^{125}$I ion or low-molecular weight metabolites.

**Equilibrium Binding Assay**

Equilibrium binding assay data was analyzed by Scatchard plot analysis to estimate the dissociation constant ($K_D$), binding sites per cell and the maximal binding site ($B_{\text{max}}$) (Fig 3). The $K_D$, number of binding sites per cell and $B_{\text{max}}$ value in KPMM2 and U-937 cells, PBMCs are shown Table 2.

**Internalization of $^{125}$I-AHM**

The internalization of $^{125}$I-AHM was examined using PBMCs and KPMM2 cells. $^{125}$I-AHM was bound to the cell surface at 4°C for 3 h. After washing the cells, internalization of $^{125}$I-AHM bound to the cell surface was determined by incubating the cells at 37°C or 4°C for up to 4 h.

No variation in radioactivity of the medium or the cells was observed with time upon incubation at 4°C. Furthermore, at 4°C the amount of radioactivity on the cell surface...
remained unchanged. In PBMCs, $^{125}$I-AHM bound to the cell surface was immediately reduced after incubation at 37°C, and decreased to 38.9% and 12.3% of the bound radioactivity after 0.5 and 4 h, respectively (Fig 4). The level of $^{125}$I-AHM inside the cell immediately increased after incubation at 37°C i.e., 39.9% of the radioactivity after 0.5 h of incubation, gradually decreasing to 27.0% by 4 h. Total radioactivity detected in the medium increased over time i.e., 60.7% of radioactivity was detected in the medium after 4 h incubation. The values of total radioactivity and TCA-precipitable radioactivity were consistent with each other at all time points on the cell surface and inside the cell. However, in the medium, percentages of TCA-precipitable radioactivity decreased over time. Specifically, 50.7% of total radioactivity detected in the medium was recovered in TCA insoluble fractions at 4 h after incubation. Similar results were obtained in KPMM2 cells (data not shown).

The inhibitory effect on the internalization of $^{125}$I-AHM was examined by addition of PAO using KPMM2 cells. Total radioactivity detected in the cell after 1-h incubation at 37°C was only 5.5% in the presence of POA. This was significant decrease compared with 24.9% detected in the cells when the experiment was carried out in the absence of PAO. Our results suggest that the internalization of AHM is mediated by binding with HM1.24, rather than by non-specific internalization.
Occupancy Rate of Cell Surface HM1.24 by $^{125}$I-AHM

The occupancy rate of cell surface HM1.24 by $^{125}$I-AHM was determined from the results of equilibrium binding assay and internalization study in KPMM2 cells and PBMCs. When KPMM2 cells were incubated at a concentration of 0.05, 0.25 and 5.00 nmol/l of $^{125}$I-AHM, the occupancy rates were 6.6, 26.3 and 91.1%, respectively (Table 3). For PBMCs, the occupancy rate at a concentration of 1.30 nmol/l of $^{125}$I-AHM was 86.9%. These findings suggest that HM1.24 on the cell surface is nearly saturated in both cell types when $^{125}$I-AHM was added at a 10-fold concentration of the $K_D$ value.

Internalization Rate and Degradation Rate of $^{125}$I-AHM

Based on the results of the internalization study, an internalization rate constant (Kint) and a degradation rate constant (Kdeg) of $^{125}$I-AHM in each cell were calculated (Table 3). Kint in KPMM2 cells was 0.064 h$^{-1}$ (0.05 nmol/l), 0.103 h$^{-1}$ (0.25 nmol/l) and 0.077 h$^{-1}$ (5.00 nmol/l), respectively. Kint in PBMCs was 0.096 h$^{-1}$ at a concentration of 1.30 nmol/l of $^{125}$I-AHM. Very similar values were obtained regardless of the concentration of $^{125}$I-AHM added and cell type used. Kdeg in KPMM2 cells and PBMCs were 0.120 - 0.151 h$^{-1}$ and 0.313 h$^{-1}$, respectively. Because there were few points that a difference was detected between total radioactivity and TCA-precipitable radioactivity in medium fraction, Kdeg at a concentration of 0.25 nmol/l of $^{125}$I-AHM in
KPMM2 cells was not calculated. Kint for KPMM2 cells in the presence of PAO was 0.005 h\(^{-1}\). The internalization rate of \(^{125}\text{I}\)-AHM for PAO-treated cells decreased to 1/21 compared with control cells (0.103 h\(^{-1}\)).

**Intracellular Distribution of HM1.24**

We recently demonstrated that HM1.24 is distributed both on the cell surface and around the perinuclear region in ARH77, U937, HeLa, and A431 cells (Masuyama et al., 2009). In the same publication we also demonstrated that HM1.24 of HeLa cells is localized not only on the cell surface but also in the TGN and/or recycling endosomes. To examine the intracellular localization of HM1.24 in U937 cells, we carried out double staining experiments using several organelle-specific antibodies. The perinuclear localization of HM1.24 partially co-localized with a cis-Golgi marker GM130, TGN marker GGA3, and a recycling endosome marker transferrin receptor (Fig. 5). By contrast, HM1.24 was barely detected in vesicular structures positive for an early endosome marker EEA1 or a late endosome/lysosome marker LAMP-1. These observations indicate that HM1.24 is localized to the TGN and/or recycling endosomes, as well as the cell surface. This dual localization of HM1.24 in U937 cells is entirely consistent with that of HeLa cells (Masuyama et al., 2009).

**Lysosomal Degradation of Internalized AHM**
Although little HM1.24 is localized in the degradative compartments, such as late endosomes/lysosomes, biochemical results mentioned above prompted us to speculate that the internalized AHM may be degraded in lysosomes after endocytosis. To assess this possibility, cells pre-bound with AHM at 4°C were incubated for 12 h in the absence or presence of lysosomal protease inhibitors, leupeptin and pepstatin and the internalized AHM was visualized by a confocal lesser microscopy. As shown in Figure 6, in the absence of leupeptin and pepstatin a fluorescence signal of AHM significantly decreased after the incubation for 12 h. By contrast, addition of leupeptin and pepstatin to the chase medium resulted in a marked accumulation of AHM in vesicular structures, most of which colocalized with late endosome/lysosome marker LAMP-1. Taken together, these results suggest that internalized AHM is delivered to, and degraded in, late endosomes/lysosomes.
Discussion

Our results show that, in contrast to other humanized antibodies, AHM is rapidly cleared from the plasma in cynomolgus monkey (Lin et al., 1999; Tokuda et al., 1999). AHM is a humanized antibody and, therefore, cynomolgus monkey is a heterogeneous animal species for AHM. When humanized antibody is administered to heterogeneous animal species, it is often immediately eliminated from the body through immune-defense mechanisms, including production of antibodies. In this study, it was observed that elimination of AHM from plasma was accelerated from 4 days after administration in two animals administered with 20 mg/kg of AHM. Such phenomena have also been observed in other humanized antibodies and chimeric antibodies (Coffey et al., 2004; Coffey et al., 2005). It is generally thought that this is caused by production of anti-drug antibodies. More specifically, it is known that the cause of the acceleration in clearance is due to anti-drug antibodies forming a complex with the drug antibody in the plasma, leading to immediate elimination by the reticuloendothelial cells. Given this fact, we determined anti-AHM antibody in plasma, which was detected in all animals. However, anti-AHM antibody was detected on or after 18 days post-administration of AHM in animals in which acceleration of elimination was observed, and there was a temporal dis-association as compared with 4 days after administration when elimination
of AHM was accelerated. Similarly, anti-AHM antibody was detected 10 and 14 days or later after administration of 0.2 and 2 mg/kg AHM, even though AHM was no longer detectable in plasma on 4 h and 2 days after the treatment, respectively. These results indicate that the reasons for the acceleration of AHM elimination and the short half-life of AHM are other than the production of anti-AHM antibody. In addition, the characteristics of pharmacokinetics of AHM observed in cynomolgus monkey (short half-life and nonlinear disposition) have also been observed in human patients with MM (unpublished data); therefore, it is obvious that this phenomenon was not specific to cynomolgus monkey administered heteroprotein.

Although not always the case (Lin et al., 1999; Benincosa et al., 2000), it is known that many therapeutic antibodies display nonlinear pharmacokinetics and that clearance is increased in low dosage (Tokuda et al., 1999; Rowinsky et al., 2004; Mould et al., 1999; Bauer et al., 1999). It is believed that nonlinear pharmacokinetics is caused by the saturation of cell surface antigens by antibody and the internalization of antibodies bound to the cell surface antigens in 2F8, anti-EGF receptor monoclonal antibodies (Lammerts et al., 2006); Efalizumab, anti-CD11a monoclonal antibody (Coffey et al., 2004; Coffey et al., 2005). Furthermore, it is now understood that the long half-life of IgG in plasma is caused by the neonatal Fc receptor (FcRN), also known as the IgG
salvage receptor (Ghetie and Ward, 2000). The FcRN is widely expressed in various tissues (Ghetie and Ward, 2002). Endothelial cells located in the skin, muscle and liver are the major sites of FcRN expression and activity (Borvak et al., 1998). FcRN plays a key role in maintaining IgG homeostasis by protecting IgG from catabolism, through transcytosis of IgG from basolateral to apical domains of plasma membranes via endosomes without being destined to the lysosomal degradation pathway. Based on these lines of evidence, our results show that AHM is immediately internalized after binding to HM1.24 on the cell surface, the antibody is then degraded within the cell. The lysosomal delivery of internalized AHM indicates that FcRN is not contributed in maintaining its homeostasis. Such a internalization and lysosomal delivery may also account for the short half-life and nonlinear disposition of AHM.

The occupancy ratio of HM1.24 on the cell surface by \(^{125}\text{I}\)-AHM and the internalization of AHM bound to HM1.24 were studied using KPMM2 cells and PBMCs. Our results indicated that the occupancy ratio of HM1.24 increased with increasing concentration of \(^{125}\text{I}\)-AHM added to medium (KPMM2 cells). Moreover, HM1.24 on the cell surface was nearly saturated by \(^{125}\text{I}\)-AHM at a 10-fold concentration of the \(K_D\) value in both KPMM2 cells and PBMCs. In both cells, \(^{125}\text{I}\)-AHM bound to the cell surface was immediately internalized and the internalization rate constants of
$^{125}$I-AHM (Kint) were 0.077 to 0.103 h$^{-1}$ regardless of cell types. The difference in the occupancy ratio of HM1.24 did not affect the rate of internalization, suggesting that antigen dependent clearance of AHM in vivo depends on total antigen amount in the body ([amount of expressed antigen per cell] × [the number of cells expressing the antigen]). It is known that endocytosis is involved in the internalization mediated by antigens and receptors in various biologically active proteins and therapeutic antibodies (Kato et al., 1996; Kato et al., 1997; Lammerts et al., 2006; Pulczynski et al., 1994). Indeed, internalization of $^{125}$I-AHM by KPMM2 cells in the presence of PAO (Kato et al., 1992; Finbloom et al., 1988), which is an endocytosis inhibitor, was significantly decreased (i.e., 1/21 that in the absence of PAO). Consistent with our recent results (Masuyama et al., 2009), it is evident that at least some $^{125}$I-AHM was internalized by the endocytosis mechanism. The internalized $^{125}$I-AHM was degraded at a degradation rate of 0.120 to 0.313 h$^{-1}$. The ratio of TCA-precipitable radioactivity to the total radioactivity in the medium decreased in both cells with time, suggesting that internalized $^{125}$I-AHM is immediately degraded and released into the medium as $^{125}$I ion or other low-molecular metabolites.

To visually understand the internalization of AHM and localization of AHM in the cell after the internalization, U937 cells were examined using immunofluorescence
microscopy. AHM was initially bound to U937 cells by incubation at 4°C. The cells were then incubated at 37°C for 15 h. The fluorescence signal of AHM at the cell surface significantly decreased after exposure to the higher temperature and no signal was detected in the cell. When leupeptin and pepstatin, which are lysosomal protease inhibitors, were added to the medium, a strong signal of AHM was observed in vesicule-like structures in the cell that co-localized with the late endosome/lysosome marker LAMP-1. This result suggests that AHM bound to HM1.24 on the cell surface is degraded in lysosomes immediately after being internalized into the cell.

Both 2F8 (Lammerts et al., 2006) and Trastuzumab (Austin et al., 2004), which are anti-EGF receptor and anti-HER2 monoclonal antibodies, are degraded in the cell after internalization in a similar way to AHM. However, both these antibodies have a significantly longer half-life than that of AHM i.e., 28.6 h (20 mg/kg). This result indicates that not all antibodies undergoing internalization have a short elimination half-life \textit{in vivo}. It has also been demonstrated that the degradation rate of 2F8 in the cell was much slower than the internalization rate (Lammerts et al., 2006). However, internalized AHM underwent degradation in the cell equivalent to the internalization rate. In addition, it is known that protection through a catabolism mechanism by binding to FcRN is involved in the prolonged elimination half-life of antibodies. It is presumed,
therefore, that the internalized AHM is prevented from this protection mediated by FcRN. This assumption is also supported by the fact that after internalization Trastuzumab is localized to recycling endosomes (Austin et al., 2004), whereas internalized AHM is exclusively delivered to late endosome/lysosome.

In conclusion, our results show that HM1.24 on the cell surface was saturated by AHM in a concentration-dependent manner. This study also revealed that AHM was internalized through HM1.24 by cells, and immediately degraded in the lysosome. Based on these results, we believe that immediate internalization of AHM by HM1.24 expressing cells and its degradation, as well as the saturation of HM1.24 by AHM, is critically associated with rapid elimination from the plasma and nonlinear pharmacokinetics of AHM administered to cynomolgus monkey and humans.
References


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97:3197-3204.
Legends for figures

Fig.1. Plasma concentrations of AHM after single intravenous administration of AHM to cynomolgus monkeys. AHM was single injected intravenously at a dose of 0.2, 2 or 20 mg/kg into male cynomolgus monkey (3 animals/group). Blood samples were collected pre-dose and at 0.5, 2, 4, 8 and 24 h and 2, 3, 4, 7, 10, 14, 17, 21 and 28 days following administration. The concentrations of AHM in plasma were determined by ELISA. Plasma concentrations of AHM were not detected at 2 or 4 h for the 0.2 mg/kg group, on day 2 for the 2 mg/kg group and on day 10 for the 20 mg/kg group. Numbers 1 - 9 in the figure refer to individual monkeys. Limit of detection was 0.195 μg/ml.

Fig.2. Plasma concentrations of anti-AHM antibody after single intravenous administration of AHM to cynomolgus monkeys. AHM was single injected intravenously at a dose of 0.2, 2 or 20 mg/kg into male cynomolgus monkey (3 animals/group), concentrations of anti-AHM antibodies were measured in plasma pre-dose and on days 2, 3, 4, 7, 10, 14, 17, 21 and 28 after administration of AHM. Numbers 1 - 9 in the figure refer to individual monkeys. Limit of detection of anti-AHM antibodies were 0.0625 μg/ml.

Fig.3. Equilibrium binding of 125I-AHM to HM1.24 on cell surface. Each cell was
incubated with $^{125}$I-AHM containing various concentrations of unlabeled AHM at 4°C for 3 h as described in “Materials and Methods.” Determination of nonspecific binding was carried out by adding excess unlabeled AHM (1024-fold to $^{125}$I-AHM). After the cells and medium were separated by centrifugation, the radioactivity of each fraction was measured by $\gamma$-counter. Specific binding to cells calculated from the difference between total binding and nonspecific binding radioactivity (A). The binding data was analyzed by Scatchard plot analysis to estimate the dissociation constant ($K_D$), binding sites per cell and the maximal binding site ($B_{\text{max}}$) (B).

**Fig.4. Internalization fate of $^{125}$I-AHM bound to HM1.24 on PBMC.** PBMC (1.3 x $10^6$ cells/ml) were incubated with $^{125}$I-AHM (1.30 nmol/l) for 3 h at 4°C. The cells were collected by centrifugation and washed to remove $^{125}$I-AHM that had not bound. Cells were then incubated in medium in the absence of $^{125}$I-AHM for 4 h at either 37°C or 4°C. The medium and the cells were separated by centrifugation. The cell pellet was then washed with medium adjusted to pH 3.5 - 3.7 to promote dissociation of the bound radioactivity on cell surface from the radioactivity inside the cells. The total radioactivity ($\Delta$; 37°C, ○; 4°C) and TCA-precipitable radioactivity (▲; 37°C, ●; 4°C) in the medium, cell surface and intracellular fraction was measured using a $\gamma$-counter.
**Fig.5. Intracellular localization of HM1.24.** U937 cells were fixed, permeabilized, and incubated with primary antibodies to HM1.24 (AHM) and EEA1, transferrin receptor (TfnR), GM130, GGA3 or LAMP-1. The primary antibodies were revealed by incubation with either Alexa488-conjugated anti-human immunoglobulin or Alexa594-conjugated anti-mouse immunoglobulin secondary antibodies. Cells were visualized by confocal microscopy. Right columns show the merged images for double staining of AHM (green) and each organelle marker (red).

**Fig.6. Lysosomal localization of internalized AHM.** U937 cells were incubated with AHM at 4°C for 30 min and then immediately fixed (0 h) or incubated at 37°C for 12 h in the absence (DMSO) or presence of leupeptin and pepstatin (+leu, pep). After incubation, cells were fixed, permeabilized, incubated with a LAMP-1 antibody, and the internalized AHM and LAMP-1 antibody were stained with Alexa594- and Alexa488-labeled secondary antibody, respectively. Cells were visualized by confocal microscopy. Right columns show the merged images for double staining of AHM (red) and LAMP-1 (green).
Table 1

**Pharmacokinetic parameters after intravenous administration of AHM into cynomolgus monkeys**

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>C&lt;sub&gt;0.5h&lt;/sub&gt; (μg/ml)</th>
<th>AUC&lt;sub&gt;0-t&lt;/sub&gt; (mg·h/ml)</th>
<th>CL (ml/h/kg)</th>
<th>V&lt;sub&gt;ss&lt;/sub&gt; (ml/kg)</th>
<th>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>0.624 ± 0.428</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>2.0</td>
<td>42.0 ± 3.0</td>
<td>0.507 ± 0.095</td>
<td>4.04 ± 0.74</td>
<td>43.7 ± 6.8</td>
<td>7.56 ± 0.42</td>
</tr>
<tr>
<td>20.0</td>
<td>576 ± 215</td>
<td>18.2 ± 4.7</td>
<td>1.14 ± 0.26</td>
<td>45.4 ± 14.5</td>
<td>28.6 ± 5.9</td>
</tr>
</tbody>
</table>

*Results are the mean ± S.D. of three animals per group.*

*N.D. indicates ‘not determined’ i.e., calculation of PK parameters was not possible.*
Table 2

Characterization of each cell by scatchard plot analysis

<table>
<thead>
<tr>
<th>Cells</th>
<th>$K_D$ (nmol/l)</th>
<th>Binding sites/cell</th>
<th>$B_{max}$ (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KPMM2</td>
<td>0.50</td>
<td>96,000</td>
<td>0.32</td>
</tr>
<tr>
<td>U-937</td>
<td>0.22</td>
<td>19,000</td>
<td>0.06</td>
</tr>
<tr>
<td>PBMC</td>
<td>0.13</td>
<td>2,500</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Table 3

Parameters for binding and internalization of $^{125}\text{I}$-AHM in PBMCs and KPMM2 cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>$^{125}\text{I}$-AHM (nmol/l)</th>
<th>Fold to Kd value</th>
<th>Occupancy rate of cell surface HM1.24</th>
<th>Rate constants</th>
<th>Kint (h$^{-1}$)</th>
<th>Kdeg (h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KPMM2</td>
<td>0.05</td>
<td>0.1</td>
<td>6.6</td>
<td></td>
<td>0.064</td>
<td>0.151</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>0.5</td>
<td>26.3</td>
<td></td>
<td>0.103</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>5.00</td>
<td>10</td>
<td>91.1</td>
<td></td>
<td>0.077</td>
<td>0.120</td>
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<tr>
<td>PBMC</td>
<td>1.30</td>
<td>10</td>
<td>86.9</td>
<td></td>
<td>0.096</td>
<td>0.313</td>
</tr>
</tbody>
</table>

N.D. indicates ‘not determined’ i.e., calculation of Kdeg was not possible.
Fig. 2

Anti-AHM Antibodies Concentration (μg/ml) vs. Time (days)

- 1
- 2
- 3
- 4
- 5
- 6
- 7
- 8
- 9

Dose Levels:
- 0.2 mg/kg
- 2 mg/kg
- 20 mg/kg
Fig. 6

<table>
<thead>
<tr>
<th>Time</th>
<th>AHM</th>
<th>LAMP-1</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr</td>
<td><img src="ahm_0hr.png" alt="Image" /></td>
<td><img src="lamp1_0hr.png" alt="Image" /></td>
<td><img src="merge_0hr.png" alt="Image" /></td>
</tr>
<tr>
<td>12 hr</td>
<td><img src="ahm_12hr.png" alt="Image" /></td>
<td><img src="lamp1_12hr.png" alt="Image" /></td>
<td><img src="merge_12hr.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**DMSO**

**Leup/Pep**