Antigen-Dependent Internalization Is Related to Rapid Elimination from Plasma of Humanized Anti-HM1.24 Monoclonal Antibody

Jun Amano, Naoko Masuyama, Yuko Hirota, Yoshitaka Tanaka, Yuriko Igawa, Rie Shiokawa, Taichi Okutani, Takashi Miyayama, Masahiko Nanami, and Masaki Ishigai

Chugai Pharmaceutical Co., Ltd., Fuji-Gotenba Research Laboratories, Shizuoka, Japan (J.A., Y.I., R.S., T.O., T.M., M.N., M.I.); and Division of Pharmaceutical Cell Biology, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan (N.M., Y.H., Y.T.)

Received July 28, 2010; accepted September 7, 2010

ABSTRACT:

Anti-HM1.24 monoclonal antibody (AHM) is a humanized anti-HM1.24 monoclonal antibody that binds to the HM1.24 antigen, a protein that is highly expressed in multiple myeloma cells. The pharmacokinetics of AHM was determined in experiments in which AHM was administered intravenously to cynomolgus monkeys. The area under the plasma concentration-time curve increased by more than the dose ratio between 2 and 20 mg/kg, and nonlinear 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, KPMM2, and U937 cells. In all cases, AHM was rapidly internalized from the cell surface; this internalization was significantly prevented by phenylarsine oxide 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 NH2-terminal transmembrane domain and a glycosylphosphatidylinositol 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 (Goto et al., 1994; Ozaki et al., 1997, 1999; Ohtomo et al., 1999; Ono et al., 1999). Although HM1.24 is expressed on terminally differentiated B cells, PBMCs, 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 HM1.24. AHM has been humanized by grafting mouse complementarity-determining regions 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 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 overexpressed, 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 for humans. In the course of the development of AHM, we therefore investigated the pharmacokinetics of AHM after a 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 an antigen/receptor mechanism mediated by binding to antigens and receptors expressed on the cell surface contributes 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 (Gottlieb et al., 2000; van Der Velden et al., 2001; Coffey et al., 2004).

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 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 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. PBMCs were purified by the Ficoll-Paque gradient method from peripheral blood of healthy volunteers. Fetal bovine serum (FBS) and phenylarsine oxide (PAO) were purchased from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal antibodies to human LAMP-1 were obtained from Mouse monoclonal antibodies to human LAMP-1 were obtained from 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 (Franklin Lakes, NJ). Mouse monoclonal antibodies to TfnR and Alexa 488- and Alexa 594-labeled secondary antibodies were obtained from Invitrogen (Carlsbad, CA). Other chemicals and reagents were of the highest grade and purchased from local commercial sources.

Preparation of Radiolabeled AHM. Radiolabeled AHM (125I-AHM) was prepared by the method of Tejedor and Ballesta (1982). The specific radiactivity was 0.7 MBq/μg, and the average number of 125I atoms introduced per molecule of AHM was 1.2. 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. A single intravenous injection of AHM at a dose of 0.2, 2, or 20 mg/kg was administered to male cynomolgus monkeys (three animals per group). Blood samples were collected from the brachial vein before the dose and at 0.5, 2, 4, 8, and 24 h and 2, 3, 4, 7, 10, 14, 17, 21, and 28 days after administration into heparinized syringes, 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.

A single intravenous injection of 125I-AHM at a dose of 1 MBq/2 mg/kg was administered to male cynomolgus monkey (three 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 trichloroacetic acid (TCA)-precipitable radioactivity in urine samples were measured on the 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. A 950-μl 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 gamma counter. Then, 1 ml of 25% 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 3000g 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 hemagglutinin, was immobilized on 96-well microplates via anti-hemagglutinin. After the addition of assay samples (duplicate) to each well on the microplate, biotinylated goat anti-human IgG and streptavidin-conjugated alkaline phosphatase conjugate and its substrate were added sequentially, and the absorbance at 492 nm was measured. The quantification ranges for AHM in the plasma and urine samples were set at 0.195 to 12.5 μg/ml and 7.81 to 500 ng/ml, respectively.

Determination of Anti-AHM Antibody 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) to each well on the microplate, biotinylated AHM (Fab) and 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 to 4.00 μg/ml.

Pharmacokinetic Analysis. Pharmacokinetic analysis software, WinNonlin (version 2.1; Pharsight Mountain View, CA), 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 taking zero as the concentration in the next sampling point of the final measurable point. MRT was calculated by 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) were found using eqs. 1 and 2, respectively. Plasma half-life (t1/2) was calculated using eq. 3 after determining t1/2 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 day 4 after dosing were used for calculation of t1/2 in two animals (animals 7 and 9) that were given injections of AHM at 20 mg/kg, because the plasma concentrations for these animals decreased in an accelerated manner on and after day 4.

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

\[
V_{ss} = CL \times \text{MRT} \quad \text{(2)}
\]

\[
t_{1/2} = \ln(2)/\text{MRT} \quad \text{(3)}
\]

Equilibrium Binding Assay. KPMM2 cells, U-937 cells, and PBMCs were used for the equilibrium binding assay of 125I-AHM. KPMM2 and U-937 cells were cultured with RPMI 1640 medium containing 20% FBS, 0.1% 2-mercaptoethanol, 0.1 mg/ml kanamycin sulfate, and 40 ng/ml human interleukin-6. PBMCs were cultured with RPMI 1640 medium containing 10% FBS, 0.1% 2-mercaptoethanol, and 0.1 mg/ml kanamycin sulfate. The solutions (10 μl each) containing unlabeled AHM at concentrations 0, 0.5, 1, 2, 4, 8, 16, 32, and 64-fold that of 125I-AHM were added to 10 μl of 2.75 nM 125I-AHM solution, respectively. Nonspecific binding was determined by excess unlabeled AHM (1024-fold 125I-AHM). The prepared samples were mixed with 200 μl of each cell (2.2 × 107 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 (2000g, 3 min), the radioactivity of each fraction was measured by a gamma counter. The specific binding of 125I-AHM was calculated by subtracting the nonspecific binding from the total binding.
data were analyzed by a Scatchard plot to estimate the dissociation constant (Kd), number of binding sites per cell, and maximal binding site (Bmax).

**Internalization of 125I-AHM.** PBMCs and KPMM2 cells were used for the study of 125I-AHM internalization. The same medium as for the equilibrium binding assay was used for culture of PBMCs and KPMM2 cells. 125I-AHM at a 10-fold concentration of the Kd value (1.30 nM) for PBMCs and at 0.1-, 0.5- and 10-fold concentrations of the Kd value (0.05, 0.25, and 5.00 nM) for KPMM2 cells was added to the medium. Each test was conducted in duplicate.

Cells were collected from the cell suspension by centrifugation (2000g, 5 min) and resuspended in medium containing 125I-AHM at a given concentration. To exclude the nonspecific internalization of 125I-AHM by binding to the cell membrane components other than HM1.24, the control human IgG, which has the same amino acid sequence with AHM except binding site to HM1.24 (complementarity-determining region), was added to the medium at a 1000-fold concentration of 125I-AHM. After incubation of cells at 4°C for 3 h, cells were collected by centrifugation (2000g, 5 min) and washed twice with ice-cold medium. Washed cells were resuspended at a concentration of 1.3 to 9.1 × 10^6 cells/ml in medium without 125I-AHM, aliquoted into test tubes (200 μl each), and then incubated at 4 and 37°C for 0.25, 0.5, 1, 2, 3, and 4 h. After incubation, 800 μl of medium was added to each test tube, and supernatant was collected as medium fraction after centrifugation (2000g, 1 min). The cell pellet was suspended in 200 μl of medium (dissociation medium) adjusted to pH 3.5 to 3.7 with the appropriate amount of acetic acid and then incubated at 4°C for 5 min. Supernatant was then collected by centrifugation (2000g, 1 min). The cell pellet was resuspended in 800 μl of dissociation medium, the operation described above was repeated, and supernatants obtained from the two procedures were mixed as the cell surface fraction. The cell fraction was suspended in 1000 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 mM PAO to the medium, which is known to inhibit the endocytosis of protein (Finbloom, 1988; Kato et al., 1992).

The radioactivity (total radioactivity) in each fraction was measured by a gamma counter. Then, 500 μl of 25% TCA solution and 200 μl of PBS were added to the tube to be used for the measurement of the total radioactivity described above. The mixture was centrifuged for 5 min at 3000g and 4°C. 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.** Emax and EC50 were calculated using WinNonlin by applying a ratio of a specific binding amount (B) to a maximal binding site (Bmax) (B/Bmax) and each AHM concentration (C) of an equilibrium binding assay in each cell to eq. 4. By applying Emax, EC50, and 125I-AHM concentration (C) used for internalization study to eq. 5, the occupancy rate of the binding site on the cell surface by 125I-AHM was calculated.

\[
	ext{Occupancy rate} (\%) = C \cdot E_{\text{max}} / (C + EC_{50})
\]

(5)

Emax is the maximum value of B/Bmax (percent), EC50 is the AHM concentration (nanomoles per liter) at 1/2 the binding rate of Emax in the equation.

**Internalization Rate and Degradation Rate of 125I-AHM.** The internalization rate of the complex of 125I-AHM and HM1.24 is represented by eq. 6. Equation 7 was obtained by the integration of eq. 6 over time from 0 to t. The regression line of a linear plot was obtained by the least-squares method from a slope to 1 h after incubation using AUCOh,r (s), and [LR], in eq. 7, and then an internalization rate constant (Kint) was calculated with an integration plot.

\[
d[L_{\text{deg}}] / dt = K_{\text{deg}} \cdot [LR]
\]

(8)

\[
K_{\text{deg}} = \int_0^t [LR] \, dt = K_{\text{deg}} \times AUC_{\text{deg},r}(s)
\]

(9)

In the equations, [LR] is the concentration of 125I-AHM (femtomoles) internalized in the cell. [LR] is the concentration of 125I-AHM (femtomoles) bound to the cell surface. AUCOh,r (s) is the area under the curve of concentration of 125I-AHM (femtomoles) bound to the cell surface versus time.

The degradation rate of 125I-AHM in the cell is represented by eq. 8. Equation 9 was obtained by the integration of eq. 8 over time from 0 to t. The equation for a regression line was obtained by the least-squares method from a slope to 1 h after incubation using AUCOh,r (i) and [Ldeg] in eq. 9, and then a degradation rate constant (Kdeg) was calculated with an integration plot. Meanwhile, TCA-soluble radioactivity (total radioactivity – TCA-precipitable radioactivity) was used as a concentration of degraded 125I-AHM in this study.

**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 Figs. 1 and 2, respectively. 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 to 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 received injections of AHM at 20 mg/kg. Anti-AHM antibodies were detected in all monkeys showing the accelerated elimination on and after day 10, 14, or 18. There was a temporal disassociation 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 with those given 2 mg/kg, but Vss was not different. The t1/2 was 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 125I-AHM, 21.4 and 49.9% of the radioactivity administered was excreted in the urine up to 1 and 4 days after...
The $K_D$, number of binding sites per cell, and $B_{\text{max}}$ value in KPMM2 and U-937 cells and 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 cells were washed, 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 of 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. In particular, 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 of incubation at 37°C was only 5.5% in the presence of PAO. This was a significant decrease compared with 24.9% detected in the cells when the experiment was performed in the absence of PAO. Our results suggest that the internalization of AHM is mediated by binding with HM1.24, rather than by nonspecific internalization.

**Occupancy Rate of Cell Surface HM1.24 by $^{125}$I-AHM.** The occupancy rate of the cell surface HM1.24 by $^{125}$I-AHM was determined from the results of the 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 nM $^{125}$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 nM $^{125}$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.** On the basis of the results of the internalization study, an internalization rate constant ($K_{\text{int}}$) and a degradation rate constant ($K_{\text{deg}}$) of $^{125}$I-AHM in each cell were calculated (Table 3). $K_{\text{int}}$ in KPMM2 cells was 0.064 h$^{-1}$ (0.05 nM), 0.103 h$^{-1}$ (0.25 nM), and 0.077 h$^{-1}$ (5.00 nM), respectively. $K_{\text{deg}}$ in PBMCs was 0.096 h$^{-1}$ at a concentration of 1.30 nM $^{125}$I-AHM. Very similar values were obtained regardless of the concentration of $^{125}$I-AHM added and cell type used. $K_{\text{deg}}$ in KPMM2 cells and PBMCs was 0.120 to 0.151 and 0.313 h$^{-1}$, respectively.

### Table 1

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

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>$C_{0.5h}$ (µg/ml)</th>
<th>AUC$_{0-24h}$ (mg*h/ml)</th>
<th>CL (ml/h*kg)</th>
<th>$V_m$ (ml/kg)</th>
<th>$t_{1/2}$ (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>4.04 ± 0.74</td>
<td>7.56 ± 0.42</td>
</tr>
<tr>
<td>2.0</td>
<td>42.0 ± 3.0</td>
<td>0.507 ± 0.095</td>
<td>N.D.</td>
<td>43.7 ± 6.8</td>
<td>28.6 ± 5.9</td>
</tr>
<tr>
<td>20.0</td>
<td>576 ± 215</td>
<td>18.2 ± 4.7</td>
<td>N.D.</td>
<td>45.4 ± 14.5</td>
<td></td>
</tr>
</tbody>
</table>

N.D., not determined; i.e., calculation of pharmacokinetic parameters was not possible.

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**Results:**

1. **Equilibrium Binding Assay:** Equilibrium binding assay data were analyzed by a Scatchard plot to estimate the dissociation constant ($K_D$), binding sites per cell, and maximal binding site ($B_{\text{max}}$) (Fig. 3).

2. **Internalization:** 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 cells were washed, 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.

3. **Occupancy Rate:** The occupancy rate of the cell surface HM1.24 by $^{125}$I-AHM was determined from the results of the equilibrium binding assay and internalization study in KPMM2 cells and PBMCs. When KPMM2 cells were incubated at various concentrations, 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 nM $^{125}$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.

4. **Internalization Rate and Degradation:** On the basis of the results of the internalization study, an internalization rate constant ($K_{\text{int}}$) and a degradation rate constant ($K_{\text{deg}}$) of $^{125}$I-AHM were calculated (Table 3). $K_{\text{int}}$ in KPMM2 cells was 0.064 h$^{-1}$ (0.05 nM), 0.103 h$^{-1}$ (0.25 nM), and 0.077 h$^{-1}$ (5.00 nM), respectively. $K_{\text{deg}}$ in PBMCs was 0.096 h$^{-1}$ at a concentration of 1.30 nM $^{125}$I-AHM. Very similar values were obtained regardless of the concentration of $^{125}$I-AHM added and cell type used. $K_{\text{deg}}$ in KPMM2 cells and PBMCs was 0.120 to 0.151 and 0.313 h$^{-1}$, respectively.
Because there were few points for which a difference was detected between total radioactivity and TCA-precipitable radioactivity in the medium fraction, $K_{\text{deg}}$ at a concentration of 0.25 nM $^{125}$I-AHM in KPMM2 cells was not calculated.

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 colocalized with a cis-Golgi marker GM130, TGN marker GGA3, and a recycling endosome marker transferrin receptor (Fig. 5). In 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 to 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, the 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 confocal lesser microscopy. As shown in Fig. 6, in the absence of leupeptin and pepstatin, a fluorescence signal of AHM significantly decreased after incubation for 12 h. In 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 with 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

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>$K_{\text{D}}$ (nmol/l)</th>
<th>Binding Sites/Cell</th>
<th>$B_{\text{max}}$ (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KPMM2 cells</td>
<td>0.50</td>
<td>96,000</td>
<td>0.32</td>
</tr>
<tr>
<td>U-937 cells</td>
<td>0.22</td>
<td>19,000</td>
<td>0.06</td>
</tr>
<tr>
<td>PBMCs</td>
<td>0.13</td>
<td>2500</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Fig. 3. Equilibrium binding of $^{125}$I-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 under Materials and Methods. Nonspecific binding was determined 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. A, specific binding to cells calculated from the difference between total binding and nonspecific binding radioactivity. B, the binding data were analyzed by Scatchard plot analysis to estimate the dissociation constant ($K_{\text{D}}$), binding sites per cell, and maximal binding site ($B_{\text{max}}$).
FIG. 4. Internalization fate of 125I-AHM bound to HM1.24 on PBMCs. PBMCs (1.3 × 10^6 cells/ml) were incubated with 125I-AHM (1.30 nM) for 3 h at 4°C. The cells were collected by centrifugation and washed to remove 125I-AHM that had not bound. Cells were then incubated in medium in the absence of 125I-AHM for 4 h at either 37 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 to 3.7 to promote dissociation of the bound radioactivity on cell surface from the radioactivity inside the cells. Total radioactivity (37°C; E, 4°C) and TCA-precipitable radioactivity (C, 37°C; O, 4°C) in the cell surface (top), and intracellular fraction (middle), and medium (bottom) 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, TfnR, GM130, GGA3, or LAMP-1. The primary antibodies were revealed by incubation with either Alexa 488-conjugated antihuman immunoglobulin or Alexa 594-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 [dimethyl sulfoxide (DMSO)] or presence of leupeptin and pepstatin (Leup/Pep). After incubation, cells were fixed, permeabilized, and incubated with a LAMP-1 antibody, and the internalized AHM and LAMP-1 antibodies were stained with Alexa 594- and Alexa 488-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 3
Parameters for binding and internalization of 125I-AHM in PBMCs and KPMM2 cells

<table>
<thead>
<tr>
<th>125I-AHM</th>
<th>Fold to K_D Value</th>
<th>Occupancy Rate of Cell Surface HM1.24</th>
<th>Rate Constants</th>
<th>K_int</th>
<th>K_deg</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmol/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KPMM2 cells</td>
<td>0.05 0.1</td>
<td>6.6</td>
<td>0.064 0.151</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25 0.5 26.3 0.103 N.D.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.00 10 91.1 0.077 0.120</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBMCs</td>
<td>1.30 10 86.9 0.096 0.313</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N.D., not determined, i.e., calculation of K_deg was not possible.
plasma was accelerated from 4 days after administration in two animals administered 20 mg/kg AHM. Such phenomena have also been observed in other humanized antibodies and chimeric antibodies (Coffey et al., 2004, 2005) and are generally thought to be caused by production of antidrug antibodies. More specifically, it is known that the cause of the acceleration in clearance is antidrug 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 18 days after administration of AHM in animals in which acceleration of elimination was observed, and there was a temporal dissociation compared with 4 days after administration when elimination of AHM was accelerated. Likewise, 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 at 4 h and 2 days after the treatment, respectively. These results indicate that there are reasons for the acceleration of AHM elimination and the short half-life of AHM other than the production of anti-AHM antibody.

Although not always the case (Lin et al., 1999; Benincosa et al., 2000), many therapeutic antibodies display nonlinear pharmacokinetics and clearance is increased in low dosage (Bauer et al., 1999; Mould et al., 1999; Tokuda et al., 1999; Rowinsky et al., 2004). 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, an anti-EGF receptor monoclonal antibody (Lammerts van Bueren et al., 2006) and efalizumab, an anti-CD11a monoclonal antibody (Coffey et al., 2004, 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. On the basis of these lines of evidence, our results show that AHM is immediately internalized after binding to HM1.24 on the cell surface and the antibody is then degraded within the cell. The lysosomal delivery of internalized AHM indicates that FcRN does not contribute to maintaining its homeostasis. Such an 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 125I-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 125I-AHM added to medium (KPMM2 cells). Moreover, HM1.24 on the cell surface was nearly saturated by 125I-AHM at a 10-fold concentration of the KD value in both KPMM2 cells and PBMCs. In both cells, 125I-AHM bound to the cell surface was immediately internalized and the internalization rate constants of 125I-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 (Pulczynski et al., 1994; Kato et al., 1996, 1997; Lammerts van Bueren et al., 2006). Indeed, internalization of 125I-AHM by KPMM2 cells in the presence of PAO (Finbloom, 1988; Kato et al., 1992), 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 125I-AHM was internalized by the endocytosis mechanism. The internalized 125I-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 125I-AHM is immediately degraded and released into the medium as 125I ion or other low-molecular-weight 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 vesicle-like structures in the cell that colocalized 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 van Bueren 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 way similar 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 in vivo. It has also been demonstrated that the degradation rate of 2F8 in the cell was much slower than the internalization rate (Lammerts van Bueren 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. On the basis of 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
Coffey GP, Fox JA, Pippig S, Palmieri S, Reitz B, Gonzales M, Bakshi A, Padilla-Eagar J, and...


