Absorption, Migration and Kinetics in Peripheral Blood of Orally Administered Ovalbumin in a Mouse Model

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Intestinal absorption of food proteins is well known, whereas its physiological significance remains to be investigated. Various amounts (1, 10 and 50 mg) of ovalbumin were orally administered to mice and the blood kinetics were subsequently analyzed by two-site ELISA. The blood ovalbumin concentration consistently reached its maximum (7–90 ng/ml) about 20 min after the oral administration and then gradually decreased in a dose-dependent manner. Only intact (45 kDa) and truncated (40 kDa) ovalbumins were always detected in the blood independently of the administration site, intra-stomach or intra-intestine, while various fragments of the protein were observed in the gastrointestinal lumen after the oral administration. Recognition by a specific monoclonal antibody and an acidic shift of its pI value suggested that the 40-kDa truncated ovalbumin was produced by intracellular limited proteolysis at its C-terminus. Such stable absorption and blood kinetics of undigested ovalbumin in normal mice suggest some sort of physiological significance for the intestinal uptake of intact food proteins.

Key words: ovalbumin; intestinal protein absorption; blood kinetics; biochemical characterization

Intestinal absorption of a small proportion, but non-negligible amount, of intact food proteins has been known for 20 years or more.1) Special attention to such protein absorption has recently been paid from the viewpoint of food allergy. In healthy humans and rats, respective orally administered egg white lysozyme and ovalbumin (OVA) were detected at a nanogram per milliliter level in peripheral blood.2–4) Furthermore, a soybean allergen (a seed oil body protein)5) and rice 14–16-kDa allergens (α-amylase inhibitors)6) were also detected at a similar level in the blood of mice after an oral administration. Co-administration with some other food components has been reported to affect the protein intestinal absorption, i.e., corn oil increased the intestinal absorption of the soybean allergen,5) whereas fermented food (kefir) decreased the absorption of OVA.7) On the other hand, increased luminal mucin induced by the ingestion of dietary fiber did not affect the intestinal absorption of OVA.8) In case of abnormal conditions, Candida albicans colonization in the mouse gastrointestinal increased the intestinal absorption of OVA,8) and enforced exercise to mice after an oral administration of the allergens, egg white lysozyme9) and wheat gliadin,10) also promoted their intestinal absorption and accumulation in the liver.

Several studies have also been reported on the biochemical nature of proteins that were orally administered and migrated into the blood circulation. An earlier study has demonstrated that orally ingested OVA could be detected as several protein bands in the peripheral blood of mice by an immunoblotting analysis although purity of the OVA preparation used was low.11) Similarly, the soybean allergen (30 kDa) and its fragments (20 kDa) were detected in mouse peripheral blood,5) while intact OVA was occasionally found in mouse portal blood.12)

Thus, substantial experimental evidence has been accumulated on intestinal absorption of many proteins. Such intestinal absorption of food proteins is known to result in the induction of systemic immunological unresponsiveness, termed oral tolerance,13) and the
mucosal local immune response as represented by IgA secretion. On the other hand, in hypersensitive individuals with food allergy as well as in a few special model animals, systemic immune responses including IgE antibody production were induced by absorbed proteins. Such allergic responses might not be objectives but consequential events of protein absorption. The aims of the present study are to characterize the absorption and migration of OVA in peripheral blood in more detail as an initial step to determine whether such absorption is done by active uptake or passive leakage and to elucidate why and how food proteins are absorbed from the digestive tract. Some results were obtained which could not be simply explained by passive leakage from the intestinal tract.

Materials and Methods

Materials. OVA (Grade V) was purchased from Sigma (St Louis, MO, USA) and used in all experiments, except for immunization of rabbit to generate the specific antiserum. Bovine serum albumin (BSA; Cohn fraction V) was also purchased from Sigma (St Louis, MO, USA). Biotinylated rabbit anti-OVA IgG and horseradish peroxidase (POD)-conjugated streptavidin were purchased from Rockland Immunochemicals (Gilbertsville, PA, USA) and GE Healthcare Life Sciences (Piscataway, NJ, USA), respectively. The POD-conjugated goat affinity purified antibody to mouse IgG F(ab')2 was obtained from MP Biomedicals, LLC-Cappel Products (Irvine, CA, USA). An Affi-Gel Hz Immunoaffinity kit, peptide N-glycanase F (PNGase F) and Biotinylated concanavalin A (Con-A) were purchased from Bio-Rad (Hercules, CA, USA), TaKaRa (Kyoto, Japan) and J-Oil Mills (Tokyo, Japan), respectively.

Animals. All experiments were carried out with 6–7-week-old female ddY mice (24–29 g). The mice were purchased from SLC (Shizuoka, Japan) and maintained on an OVA-free commercial diet (CE-2; CLEA Japan) in a room with controlled temperature (25 ± 2°C) and lighting (lights on 8:00–20:00), according to the Nagoya University guidelines for animal studies. OVA was administered at fixed times (between 10:00 and 12:00 in the morning) to mice maintained by ad libitum feeding without fasting to analyze under natural conditions. The research plan for the present study was approved by the Committee on Animal Experiments of the Graduate School of Bioagricultural Sciences, Nagoya University (authorization number: 2007060801).

Continuous blood collection after the intragastric administration of OVA. Mice were intragastrically administered with OVA (1, 10, and 50 mg) or OVA (10 mg) with BSA (10 and 40 mg) dissolved in 0.2 ml of PBS by using a 20-gauge stainless steel feeding tube with a rounded tip (Fuchigami Instrument, Kyoto, Japan). Peripheral blood (15 μl) was collected from the tail vein at pre-administration and 5, 10, 15, 20, 30, 60, 90 and 120 min after administration, diluted 10 times with PBS, and immediately vortexed for several seconds. Diluted blood samples were centrifuged at 17,500 x g for 10 min at 4°C, and each resulting supernatant was stored at −30°C until needed for analysis.

Gastrointestinal administration of OVA and collection of blood. To characterize OVA transported from the gastrointestinal lumen to the blood circulation, mice were intrastrangally administered with 50 mg of OVA as just described, and peripheral and portal blood samples were collected 20 min after the administration. The peripheral blood samples (0.8 ml) were collected from the orbital veins of the mice by using capillary glass pipettes under diethyl ether anesthesia. To collect the portal blood samples, the mice were intraperitoneally injected with pentobarbital (Dinabot) 5 min after the oral administration. Further 10 min later, the abdominal cavities of the mice were opened and blood samples (0.4 ml) were drawn from the portal veins by using 25-gauge winged needles (TERUMO, Japan). Upon infusion into the duodenum, jejunum or ileum, 10 mg of OVA dissolved in 0.2 ml of PBS and pre-warmed to 37°C was given to the mice by using 27-gauge stainless steel needles (TERUMO, Japan) via laparotomy under pentobarbital anesthesia. After the infusion, the abdominal wall and skin were carried back, and the abdominal part of the mice was covered with PBS-soaked gauze. Peripheral blood samples were collected as already described 40 min after the infusion. Serum samples were prepared from the blood samples and stored at −30°C until needed for analysis.

Preparation of the rabbit anti-OVA IgG fraction. OVA was prepared for immunization by an isolectric ammonium sulfate crystallization method from fresh egg white. The antiserum against OVA was raised in rabbits by subcutaneously immunizing with Freund’s adjuvant. The IgG fraction of the antiserum was prepared by ammonium sulfate precipitation and subsequent anion-exchange chromatography in a column of DEAE-Sephael (GE Healthcare Life Sciences).

Monoclonal antibodies. The mouse hybridoma clone, 1B2/5E5, secreting an OVA-specific monoclonal antibody (mAB) was prepared from spleen cells of a BALB/c mouse, which had been immunized with OVA, according to the established method. An epitope recognized by the mAB, 1B2/5E5, was identified to be within 11 amino acid residues (Ile335-Ser345) of OVA.

Deglycosylation of OVA with PNGase. OVA was digested with PNGase under denaturing conditions. One microgram of OVA was dissolved in 5 μl of 500 mM Tris–HCl (pH 8.6) containing 0.5% SDS and heated at
coated with 1 ml of 1.25% Nonidet P-40 including 1 milli-unit of peptide N-glycanase F (PNGase F) was added to the mixture and incubated at 37 °C for 20 h.

OVA determination by two-site ELISA. ELISA plates (Nunc-Immuno™ plate, Nalge Nunc International) were coated with 1 μg/ml of the rabbit anti-OVA IgG fraction in PBS at 4 °C for 16 h. After being washed with PBS containing 0.05% Tween 20 (PBST), the plates were blocked with 1% BSA in PBST at 37 °C for 1 h. Standard OVA was diluted at 0–4 ng/ml with PBS. Mouse normal serum or plasma was added as necessary to each standard solution to equalize the serum concentration between the samples. The standard solutions and diluted blood samples were applied to antibody-coated plates and the plates were incubated at 37 °C for 1.5 h. The plates were then washed with PBST and incubated at 37 °C for 1 h with the biotinylated rabbit anti-OVA IgG fraction diluted with 1% BSA in PBST. After washing, the plates were finally incubated with POD-conjugated streptavidin diluted with 1% BSA in PBST at 37 °C for 1 h and the POD activity was measured by using o-phenylenediamine as a substrate.

Preparation of the luminal contents of the mice administered with OVA. Mice were intragastrically administrated with 10 mg of OVA in 200 μl of PBS or with the same volume of PBS for a mock administration. Twenty minutes later, the stomach and small intestine were removed, and the small intestine was cut into six portions. The stomach and the portions of the small intestine were transferred to microtubes and immersed in 0.5 ml of PBS containing protease inhibitors, 1 mM PMSF and 10 μg/ml of leupeptin, before the luminal contents were washed out by mechanical mincing. Each solution containing the luminal contents was centrifuged twice at 17,500 x g for 15 min at 4 °C, and the resulting supernatant was used for subsequent assays.

Immunoprecipitation and immunoblotting of OVA. Rabbit anti-OVA IgG prepared as already described was coupled to Affi-Gel Hb hydrazide gel (Bio-Rad) according to the manufacturer’s instruction. Mouse serum (500 μl) containing OVA or a supernatant of the luminal contents diluted with 500 μl of normal mouse serum was incubated with the antibody-coupled gel at 4 °C for 16 h. Each gel was washed three times with PBS and once with distilled water, mixed with the SDS-PAGE sample buffer, and boiled for 5 min. Proteins in the sample were separated by SDS-PAGE and electrophoretically blotted onto a PVDF membrane (Immobilon, Millipore). After blocking with 2.5% gelatin, the membrane was incubated with biotinylated rabbit anti-OVA IgG, and then incubated with POD-conjugated streptavidin. In the case of immunoblotting when using 1B2/5E5, POD-conjugated anti-mouse IgG F(ab')2 was employed as the 2nd antibody. The protein bands were visualized with the enhanced chemiluminescence Western Blotting Detection Reagents (GE Healthcare Life Sciences) and AE6962 light capture system (ATTO, Japan).

Two-dimensional IEF/SDS gel-electrophoresis. OVA (75 ng) in the peripheral blood was immunoprecipitated as just described. The immunoprecipitated OVA was released from the insolubilized antibody by incubating in 20 mM glycine/HCl (pH 2.5), and the recovered sample was neutralized with 100 mM NaHCO3 (pH 10). The resulting OVA sample was mixed with Kunitz soybean trypsin inhibitor (KSTI; 25 ng) as an internal control for IEF and dissolved in a rehydration buffer (8 M urea, 4% CHAPS, 50 mM DTT, and 0.001% (w/v) bromophenol blue). The sample was loaded on ReadyStrip™ (11-cm-long, linear pH 3–6, Bio-Rad) by in-gel rehydration. First-dimensional IEF was performed using the PROTEAN IEF Cell (Bio-Rad) according to the manufacturer’s instructions. Prior to the second-dimensional separation, the focused IEF strips were incubated for 10 min with 130 mM DTT in an equilibration buffer (6 M urea, 2% SDS, 375 mM Tris–HCl, pH 8.8, and 20% v/v glycerol), before being incubated again with 135 mM iodoacetamide in the equilibration buffer. After mounting the strips on 12.5% acrylamide gel without a stacking gel, the second-dimensional SDS-PAGE was carried out. The separated proteins were detected by immunoblotting as already described.

Statistical analysis. Each data value was expressed as the mean ± SD. Differences in the blood OVA concentration among groups were analyzed by the Kruskal-Wallis test and the Scheffe’s multiple-comparison test using Microsoft Excel with Add-in statistical software (Social Survey Research Information Co., Ltd., Tokyo, Japan). Differences were considered significant at p < 0.05.

Results

Absorption and blood kinetics of intragastrically administered OVA

Protein absorption from the digestive tract and transport into the blood circulation was first quantitatively investigated by determining the absorbed protein in the peripheral blood. OVA was orally administered into the stomach of mice, and the time-course characteristics of appearance and disappearance of blood OVA were monitored by using two-site ELISA (Fig. 1A). When 1 mg of OVA was given to the mice, OVA was detectable within 5 min of its administration at a level of a few nanograms per milliliter in the peripheral blood, reached its maximum level of 7 ng/ml 20 min after the administration, and gradually decreased with an apparent half-life of about 80 min. Such blood kinetics (the increase and decrease profiles in the blood) of OVA...
were well conserved among the three experimental groups with different dosages (1, 10 and 50 mg per mouse). The overall concentration of OVA in the blood increased in a dose-dependent manner, but not in a linear fashion, e.g., an increase in dose by 10- and 50-fold (from 1 to 10 and then to 50 mg) resulted in a 5- and 13-fold increase (from 7 to 35 and then 90 ng/ml) in the maximum concentration of OVA in the blood. To examine the effect of the total protein amount on the OVA absorption, 10 or 40 mg of another protein, BSA, was co-administered with 10 mg of OVA (Fig. 1B). Co-administration with BSA markedly reduced the intestinal absorption of OVA; i.e., the maximum OVA concentration in the blood decreased to 30–40% of the level without BSA. The mathematical sum of the blood OVA concentration obtained at 10 time-points during 120 min following the administration, that is, the area under the curve (AUC) of the OVA blood kinetics, is shown for each experimental group (Fig. 1C). An increase in dose from 10 to 50 mg (5 times) resulted in only a 2.5-fold increase in AUC. The relationship between the OVA dosage and AUC was almost the same as that between the dosage and the maximum OVA concentration in the blood.

**Molecular size of OVA intragastrically administered and migrating into peripheral blood**

To determine whether intact OVA molecules and/or its proteolytic fragments were absorbed and transported, the transported OVA was recovered 20 min after its oral administration from the portal and peripheral blood samples by immunoprecipitation, and then analyzed by SDS–PAGE/immunoblotting (Fig. 2). A 45-kDa band corresponding to intact OVA was clearly detected as the major band in both blood samples. In addition, 40-kDa truncated OVA was also detected in both samples, although no other fragments with lower molecular mass were visible. The proportions of the 40-kDa to the 45-kDa band intensity in both the portal and peripheral blood samples from all of the 10 mice were almost constant and estimated to be 0.46 ± 0.07 and 0.43 ± 0.11, respectively, based on a densitometric analysis. Only intact OVA, but neither the truncated 40-kDa band nor the lower molecular mass fragments, could be detected when OVA (30 ng) was added to a control mouse serum with subsequent immunoprecipitation in the same manner (Fig. 2A and B). No degradation of OVA was apparent even when OVA was incubated in mouse blood in vitro (data not shown).

**Absorption and molecular size of OVA infused intra-intestinally**

Pylorus ligation reduced the absorption of OVA administered intragastrically, suggesting a contribution of the small intestine to protein absorption from the digestive tract. Therefore, to determine whether OVA was absorbed directly from the small intestine without passing through the stomach, OVA (10 mg) was infused...
into the lumen of three sections of the small intestine (duodenum, jejunum and ileum) of anesthetized mice. OVA transported into the peripheral blood was then collected 40 min after this infusion because preliminary experiments indicated that the intestinal protein absorption was delayed under anesthetized conditions (data not shown). The blood OVA concentrations 40 min after the intestinal infusion into the duodenum, jejunum and ileum, as estimated by ELISA, were 173 ± 20, 172 ± 153, and 492 ± 221 ng/ml, respectively. These values were even higher than that from intragastric administration (5–100 ng/ml, see Fig. 1). Immunoblotting re-

evealed the presence of intact and truncated OVA (45-
and 40-kDa bands) and the absence of digested frag-
ments with a lower molecular mass (Fig. 3), identical

to the case of intragastric administration. The proportion

of the truncated form to intact one was almost constant

among the three mice tested, while a certain degree

of individual variability was seen in the quantity of

blood OVA.

Molecular size of OVA intragastrically administered

and remaining in the gastrointestinal lumen

To determine whether intragastrically administered

OVA remained intact in the lumen of the digestive tract,

the luminal contents were collected from the stomach

and small intestines 20 min after administration when

the blood OVA concentration had reached its maximum

(Fig. 1), and OVA and its fragments were separated and

detected by SDS–PAGE/immunoblotting (Fig. 4A).

Intact OVA as well as its fragments with relatively

higher molecular mass (30–40 kDa) were detected
clearly in the gastric contents, whereas several bands

with relatively lower molecular mass (40-, 30-, 21-, 16-, 12- and 9-kDa) were dominant and intact OVA was

detected below the detectable level in the luminal contents of the

upper sections (duodenum and jejunum) of the small

intestine. Hardly any immunoreactive bands were
detected in the lower sections (ileum).

Unlike the lower molecular mass fragments re-

maining in the intestinal lumen, intestinally absorbed

OVA and its fragments in the blood samples were

only in the intact form and 40-kDa truncated proteins

which had been recovered and concentrated from

the blood samples by immunoprecipitation (Figs. 2

and 3). To rule out the possibility of selective concen-

tration of the intact protein and loss of the low molecular

mass fragments by the immunoprecipitation procedure,

the luminal contents of the third intestinal section

(number 3 in Fig. 4A) were subjected to immunopreci-

pitation under conditions identical to those used for the

blood samples. As shown by the results of the

immunoblotting analysis in Fig. 4B, most of the major

OVA fragments (40-, 30-, 21-, 16- and 9-kDa) detected

in the intestinal luminal contents were recovered by

immunoprecipitation.

Immunochemical characterization of 40-kDa OVA in

the blood and gastrointestinal lumen

Truncated OVA of about 40 kDa was detected by the

immunoblotting analysis in the luminal contents of the

stomach and small intestine (Fig. 4), as well as in the

portal and peripheral blood samples (Fig. 2). The

molecular size of this truncated OVA was compared in
detail by using SDS–PAGE gel with a lower acrylamide

concentration and with subsequent immunoblotting

(Fig. 5). The electrophoretic migration of truncated

OVA from the small intestine (intestinal section no. 3)

was found to be slightly but definitely faster than that

from the stomach and blood (Fig. 5C).
To further characterize the truncation of OVA, the reactivity of the OVA samples was examined by immunoblotting to mAb (1B2/5E5) which is known to recognize an epitope within the 11 residues of Ile335-Ser345 near the OVA C-terminus (Fig. 5A). As shown in Fig. 5B, 40-kDa OVA from the stomach and blood was reactive to this mAb, whereas almost no immunoreactive bands could be detected in the samples from the small intestine. Most of the OVA fragments from the small intestine were immunostained strongly by anti-OVA polyclonal antibodies (panel C) but hardly detected by the mAb (panel B). In contrast, 45-kDa and 40-kDa OVA from stomach and blood showed immunoreactivity to the mAb (panel B), comparable to the polyclonal antibodies (panel C). A 28-kDa band seen in the OVA samples from the blood in panel B would not have been an OVA fragment, but rather the light chain of contaminating mouse serum IgG which was recognized by the secondary antibody used, the POD-conjugated goat antibody to mouse IgG F(ab’)_2.

Fig. 3. Immunoblot Analysis of Peripheral Blood OVA Immunoprecipitated Following the Intra-Intestinal Infusion.
OVA (10 mg) was infused at laparotomy into the lumen of the duodenum (lanes 1–3), jejunum (lanes 4–6), and ileum (lanes 7–9) of anesthetized mice. Forty minutes later, peripheral blood was collected from each mouse. The serum concentration of OVA was determined by ELISA, and serum samples (an appropriate volume equivalent to 25 ng of OVA) were subjected to immunoprecipitation and processed as described in the legend to Fig. 2. Lane numbers 1 to 9 represent the individual numbers for 9 mice.

Fig. 4. Immunoblot Analysis of the Luminal Contents Recovered from the Digestive Tract Following the Intragastrical Administration of OVA.
Mice were intragastrically administered with 10 mg of OVA, and 20 min later, the stomach and small intestine were removed. The luminal contents recovered from the stomach (S) and from six portions of the small intestine (numbers 1, duodenum; 2–4, jejunum; 5 and 6, ileum) were subjected to immunoblotting with the anti-OVA antibody (A). Centesimal volumes of the luminal contents of the number 3 portion from the small intestine prepared from three mice following OVA (lanes b and c) or PBS administration (lane a) were directly subjected to SDS–PAGE (IP+) or to immunoprecipitation (IP+) (B). For immunoprecipitation, the luminal contents were mixed with 500 μl of normal serum to make the experimental conditions identical to those for the OVA-containing serum, and then analyzed as described in the legend to Fig. 2. Standard OVA (Std) was also analyzed as a positive control (A and B).
Biochemical characterization of 45- and 40-kDa OVA in the blood

To evaluate any electrostatic changes in OVA through possible modification by intraluminal and/or intracellular hydrolytic enzymes, OVA recovered from the mouse blood samples was analyzed by 2D-PAGE and subsequent immunoblotting (Fig. 6A). The 45-kDa blood OVA was focused as several major spots almost identical to those of the control native OVA, except that these spots were shifted slightly to the basic side. On the other hand, the 40-kDa OVA was focused as several spots with a more acidic pI value than those of the 45-kDa OVA. Possible modification to an N-linked carbohydrate chain of OVA was examined by a lectin-blotting analysis using Con-A, which recognizes high-mannose-type carbohydrate chains. The 45-kDa OVA in the blood, as well as the standard native OVA, were clearly positive to Con-A staining, although unknown ConA-positive contaminants were also present in the immunoprecipitate from each mouse blood sample (Fig. 6B). On the other hand, the 40-kDa OVA, which was detected by immunoblotting using anti-OVA antibodies, was estimated to be weakly positive to Con-A staining, based on a comparison between the immunostaining and Con-A staining intensities (Fig. 6B). The control OVA, which had been treated with PNGase, was negative to ConA-staining as expected, and migrated as a 43-kDa band between those of the blood 45-kDa and 40-kDa bands in SDS–PAGE.

Discussion

The absorption and blood kinetics of intragastrically administered OVA were quantitatively and qualitatively reproducible in the mouse model used in the present study, supporting the idea that the absorption of intact protein across intestinal epithelium is not a pathological accident, but rather a normal physiological event. Intestinally absorbed OVA was qualitatively invariable; that is, only the intact (45 kDa) and truncated (40 kDa) forms of OVA were always detected in the blood (Figs. 2 and 3), and such blood kinetics as the time required to reach the maximum blood concentration were well conserved within each group and among the groups with different OVA dosages (Fig. 1). In contrast, various low
molecular mass fragments of 10–40 kDa were detected in the lumen of the small intestine (Fig. 4), and most of these intestinal OVA fragments had lost the mAB (1B2/5E5) epitope, which was retained in the blood 40-kDa OVA fragment (Fig. 5). Thus, there was a large difference in the population of OVA fragments between the blood and the intestinal lumen. These results suggest the restricted migration of intact and slightly truncated OVAs from the intestinal lumen to peripheral blood, and the presence of some selective mechanisms for cellular absorption and/or intracellular degradation during passage across the intestinal epithelial cell layer. A simple paracellular diffusion theory could not well explain such preferential absorption of intact and truncated OVAs from the digestive tract, the non-linear dose dependence of OVA concentration in the peripheral blood, or the suppressive effect of BSA co-administration on OVA absorption (Fig. 1). The maximum OVA concentration in the peripheral blood was estimated to be 35 ng/ml (20 min after administration) when 10 mg of OVA had been given to a mouse intragastrically (Fig. 1). Determining the total amount of intestinally absorbed OVA would not be easy, because OVA that had migrated into the circulating blood was immediately eliminated by the blood clearance mechanism, and because part of absorbed OVA migrated into the lymph circulation. Nevertheless, about 100 ng, i.e., 1/100,000 of the 10 mg of given OVA, would have been absorbed as the lowest estimate under our experimental conditions, assuming that the total volume of blood in a mouse is 2 ml and 30% of absorbed OVA had already been eliminated by blood clearance.

OVA fragments of about 40 kDa were clearly detected as major components in the intestinal lumen by the polyclonal antibodies, but hardly by the mAB (1B2/5E5), which recognizes a sequential epitope within the 11-residue peptide corresponding to Ile335-Ser345 of OVA (Fig. 5), indicating that the intestinal 40-kDa fragment had lost the C-terminal 42 residues (Ser345-Pro386) or more. On the other hand, blood 40-kDa OVA was estimated to have suffered C-terminal truncation to shorter than 41 residues or N-terminal truncation. The OVA fragment of 40 kDa was also detected in the stomach lumen and showed reactivity to the mAB (1B2/5E5) antibodies, indicating that this 40-kDa fragment still retained the mAB epitope. After being sent from the stomach to small intestine, this OVA fragment would have been further trimmed by pancreatic proteases, resulting in the loss of the mAB epitope in the sequence of Ile335-Ser345. Taking these propositions together, it could be speculated that the 40-kDa truncated OVA detected in the blood was not derived from the intestinal OVA fragments of about 40 kDa, but was produced by the truncation of intact OVA during or before passing through the epithelial cell layer.

As well as by intragastric administration (Fig. 2), 40-kDa truncated OVA was also detected in the peripheral...
blood after the direct infusion of OVA into the small intestine (Fig. 3), indicating that blood 40-kDa OVA was not derived from stomach 40-kDa OVA. Furthermore, an intra-intestinal administration of OVA produced this truncated OVA in the blood with a truncated/ intact proportion a little higher than that resulting from the intra-gastric administration (Figs. 2 and 3). These results support the speculation just made for the origin of truncated OVA in the blood and suggest the contribution to OVA truncation of intracellular proteolysis rather than intraluminal digestion. OVA belonging to the family of serine-protease inhibitors (serpins) is known to be resistant to proteolytic degradation, unless it has been denatured. A nicked form of OVA, plakalbumin, produced by limited proteolysis near the C-terminus with subtilisin was little different in its three-dimensional conformation from intact OVA. It would therefore be likely that some intact OVA molecules, which would have escaped from proteolytic attack by digestive enzymes and have been absorbed by intestinal epithelial cells, were truncated to become a relatively stable intermediate by cellular proteases in the endosomal/lysosomal compartment of the cells, and that, in contrast, its low-molecular-mass fragments produced by intraluminal digestion were easily degraded by the proteases. Thus, the presence of intact and truncated OVA and the absence of low molecular mass fragments in the blood circulation might be explained by the difference in susceptibility to proteolytic degradation between intact molecules and fragments rather than by a difference in cellular absorbability. Low molecular mass fragments equally absorbed by the epithelial cells might have been rapidly degraded further to smaller peptides which could not be detected by immunoblotting. It is unlikely that the disappearance of low molecular mass fragments from circulation was due to the blood clearance mechanism, because the fragments could not be detected even in the portal vein before passing through the liver.

The focused spots of blood 40-kDa OVA exhibited a slightly acidic pI value compared with those of 45-kDa OVA (Fig. 6A). This pI shift to the acidic side (a decrease and increase in positive and negative charges, respectively) by truncation would have been, at least in part, due to peptide deletion corresponding to 5 kDa at the N- or C-terminus. The theoretical pI values calculated from the pK values of their constituent charged amino acids of intact (2–386), N-terminal (41–386) and C-terminal (2–347) truncated OVA are 5.07, 5.04 and 4.98, respectively. The experimental value of the pI shift was estimated to be at least 0.05 based on the 2D-PAGE profile shown in Fig. 6A and, moreover, three known cleavage sites on OVA by such proteases as subtilisin, bromelain and papain are located within the sequence from Ala346 to Val355 which is conserved as a limited-proteolysis target region among the serpin-family proteins. Therefore, it is most likely that intact OVA was absorbed by intestinal epithelial cells and suffered limited hydrolysis by endosomal/lysosomal proteases within the conserved protease-target region near the C-terminus, resulting in the production of 40-kDa truncated OVA.

The slight pI shift to the basic side of 45-kDa OVA would have been due to partial dephosphorylation by intestinal alkaline phosphatase and/or lysosomal acid phosphatase, because in vitro dephosphorylation by the alkaline phosphatase induced a similar pI shift for intact OVA (Matsubara et al., in submitting). The molecular mass of OVA that have been enzymatically deglycosylated in vitro was estimated to be 43 kDa (Fig. 6B). This molecular mass indicates that 40-kDa OVA in the blood was produced merely by removal of its N-linked carbohydrate chain, although incomplete deglycosilation could not be ruled out.

It has been suggested in this decade that intestinal epithelial cells can function as non-professional APCs because of their constitutive expression of MHC class II on their basolateral membranes and their ability to present soluble antigens to primed CD4(+)-T cells, leading to oral tolerance. For antigen presentation, antigen peptides need to be loaded on MHC class II molecules in the endosomal compartment of the cells. It would therefore be likely that both qualitative and quantitative susceptibility to the intracellular proteolysis of each protein antigen taken up from the intestinal lumen affect the loading efficiency to MHC class II molecules, thus leading to oral tolerance induction. It would be of interest to analyze low molecular mass peptides from OVA in both the epithelial cells and blood, although technical problems remain for the recovery and concentration of such peptides. Meanwhile, intact OVA infused into the portal vein has been reported to induce antigen-specific T cell tolerance and, furthermore, antigen presentation to CD8(+)-T cells by liver endothelial cells has been shown to result in antigen-specific T-cell tolerance. Taken together, it might be concluded that the physiological significance of appropriately controlled protein absorption from the digestive tract is, at least in part, to induce oral immunological tolerance by making both intestinal epithelial and liver endothelial cells present protein antigens to specific T cells.

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