# Reveromycin A Inhibits Antigen Receptor-mediated Antigen Presentation by

# B Lymphoma Cells *via* Its Effect on Intracellular Trafficking

# of the Antigen

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Reveromycin A (Rev.A) is an inhibitor of epidermal growth factor-dependent cell growth that is produced from the culture broth of an actinomycete strain. Rev.A was assessed for its ability to regulate antigen (Ag) presentation by A20-HL B lymphoma cells bearing trinitrophenyl (TNP)-specific surface IgM (sIgM) to cloned T cells specific for $OVA_{323\sim339}$/I-A$^d$. Rev.A-treatment inhibited the presentation of Ag internalized *via* sIgM, but not of Ag *via* fluid-phase pinocytosis. Rev.A-treatment decreased protein synthesis, but a similar decrease in protein synthesis by cycloheximide induced much less inhibition of sIgM-mediated Ag presentation. Rev.A-treatment decreased the rate of re-expression of sIgM in A20-HL cells, the amount of Ag internalized *via* sIgM during 3 hours of incubation, and the generation of antigenic peptides from TNP-OVA internalized *via* sIgM. Rev.A-treatment was suggested to affect intracellular trafficking from early endosomes into late endocytic compartments of Ag internalized *via* sIgM. Rev.A might provide a useful tool for studying intracellular transport of Ag, especially Ag internalized *via* sIgM.

Antigen (Ag)-specific B cells are capable of binding and internalizing Ag upon introduction of Ag into the body *via* their cell surface B cell receptor (BCR) molecules. Ag-specific B cells process Ag internalized *via* BCR and express complexes of Ag-derived peptides and major histocompatibility complex (MHC) class II molecules on their cell surface to present Ag to T helper cells[1,2]. The BCR-mediated Ag presentation by B cells plays a critical role in Ag-specific and T-dependent B cell activation and differentiation[3~7]. BCR-mediated Ag internalization has several characteristic features. First, BCR-binding Ag is efficiently taken up at a low concentration, and the presentation of Ag taken up through BCR works at some $10^6$-fold lower concentrations when compared with Ag

taken up by fluid-phase pinocytosis[8,9]. Secondly, Ag internalized *via* BCR efficiently targets into MHC class II peptide-loading compartment[10~12]. Intracellular targeting is accelerated by BCR cross-linking, which is dependent on the cytoplasmic domains of the Ig$\alpha$ and Ig$\beta$ components of the BCR[13~17]. Thirdly, B cells have been suggested to possess a mechanism for prolonging the intracellular persistence of Ag-BCR complexes within nonterminal late endocytic compartments to allow prolonged cell surface expression of complexes of Ag-derived peptides and MHC class II molecules and the interaction of Ag-specific B cells with T helper cells[18]. In addition, presentation of Ag internalized through BCR requires newly synthesized MHC class II molecules enroute to the cell surface from the

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endoplasmic reticulum[19~22], whereas peptides generated from Ag taken up via fluid-phase pinocytosis are capable of forming complexes with MHC class II molecules recycled from the cell surface[19~23]. However, these findings have not fully revealed the mechanisms underlying intracellular trafficking of Ag internalized via BCR or fluid-phase pinocytosis.

Pharmacological reagents have been useful tools for investigating the intracellular events in Ag presentation. The use of inhibitors for protein synthesis, emetine and cycloheximide (CHX), or a blockade of the egress of proteins from the endoplasmic reticulum, brefeldin A, has elucidated that Ag internalized via BCR is presented in the context of newly synthesized MHC class II molecules, whereas Ag taken up through fluid-phase pinocytosis is presented with MHC class II molecules recycled from the surface of APC[19~23]. Investigation with an inhibitor of protein tyrosine phosphorylation, genistein or staurosporin, has revealed that BCR signaling regulates MHC class II containing late endosomes[25]. The present study used reveromycin A (Rev.A) to analyze intracellular trafficking of Ag internalized via BCR into B cells. Rev.A is an inhibitor of epidermal cell growth factor-dependent cell proliferation produced by Streptomyces spp.[26] that shows antitumor activity against transforming growth factor-$\alpha$-dependent growth of ovarian carcinoma cells[27]. Results obtained suggested that the treatment of B lymphoma cells with Rev.A inhibited the presentation of Ag internalized via BCR, but not of Ag taken up through fluid-phase pinocytosis, and further that treatment induced a prolonged co-localization of Ag internalized via BCR with transferrin internalized via its receptor in the endocytic compartments.

## Materials and Methods

### Reagents

Rev.A was prepared from the culture broth of an actinomycete strain belonging to the genus Streptomyces, as described[26]. Following reagents were used; chicken ovalbumin (OVA), CHX, and fluorescein isothiocyanate (FITC) from Sigma (St. Louis, MO, USA); $CH_3NH_2$ and $NaN_3$ from Wako Pure Chemical Industries Ltd. (Tokyo, Japan); Bovine serum albumin (BSA) from Serological Proteins, Inc. (Kankakee, IL, USA); trinitrophenyl (TNP)$_{3.9}$-OVA prepared as described[28]; anti-I-A$^{b,d,k}$, I-E$^{dk}$ mAb, M5/114 (rat IgG2b[29]), and anti-CD4 mAb, GK1.5 (rat IgG2b[30]), from the American Type Culture Collection (Rockville, MD, USA); FITC-conjugated anti-mouse IgM goat IgG F(ab')$_2$ from Organon Teknika Corp. (Durham,

NC, USA); anti-mouse LAMP-1 (CD107a) mAb, 1D4B (rat IgG2a), and monoclonal rat IgG2a from BD PharMingen (San Diego, CA, USA); Biotinylated anti-rat $\kappa$ mAb, MARK-1 (mouse IgG1), and FITC-conjugated streptavidin from Zymed Laboratories (San Francisco, CA, USA); Cy3-conjugated streptavidin from Jackson Immuno Research Laboratories, Inc. (West Grove, PA, USA); FITC-conjugated anti-rabbit IgM+IgG Ab from Southern Biotechnology Associates, Inc. (Birmingham, AL, USA); anti-mouse IgM mAb, Bet 2, in the form of culture supernatant of the hybridoma; Lyso-Sensor Green DND-189 with a pH range of 4.5~6.0, a pH indicator reagent, and Alexa™594-conjugated transferrin from Molecular Probes, Inc. (Eugene, OR, USA); FITC-TNP-OVA prepared as described previously[31]. Rabbit antibody against C-terminal of OVA$_{321~336}$ peptide was prepared by immunization with keyhole limpet hemocyanin (KLH)-conjugated to cysteine added to the N-terminal of OVA$_{329~336}$. This antibody positively reacted with OVA$_{321~336}$ peptide, but not with OVA$_{323~339}$ peptide nor with native OVA, on enzyme-linked immunosorbent assay.

### Cells and Culture Conditions

A20-HL cell expressing TNP-specific IgM was generated as described[32], and kindly provided by Dr. M. WATANABE, Mitsubishi Chemical Co., Ltd. (Tokyo, Japan). A T cell clone specific for OVA$_{323~339}$/I-A$^d$, 42-6A, was established in our laboratory[33]. RPMI 1640 (Sigma Chemical Co.) supplemented with 10% fetal calf serum (Summit Biotechnology, Greeley, CO, USA), $5\times10^{-5}$ M 2-mercapto-ethanol, and $100\,\mu g/ml$ kanamycin, was used as culture medium. Cells were cultured and maintained at 37°C in a humidified atmosphere of 5% $CO_2$.

### Ag Presentation Assays

To examine the ability of Rev.A to regulate APC function of A20-HL cells, they were incubated for 18 hours with Rev.A at $20\,\mu g/ml$ or with CHX at $1.28\,\mu g/ml$, unless otherwise stated. TNP-OVA or OVA was added to the cells for 3 additional hours. After washing, the Ag-pulsed cells ($1\times10^5$) were incubated with $1\times10^4$ 42-6A cells for 20 hours in $250\,\mu l$ culture medium on a 96-well culture plate. Then, to assess IL-2 activity, culture supernatant was harvested and added to CTLL-2 cells, an IL-2-dependent T cell line[33]. CTLL-2 cells were pulsed with $0.25\,\mu Ci$ [$^3$H]thymidine during the last 6 hours of 20 hour-incubation. Incorporation of [$^3$H]thymidine was measured on a Matrix 96 direct beta counter, Packard Instrument Co. (Meriden, CT, USA). The treatment of A20-HL cells with Rev.A or CHX at indicated concentrations did not alter their viability.

## Flowcytometric Analysis

For the analysis of cell surface molecules, A20-HL cells were stained with an appropriate antibody and examined on a FACSCalibur flowcytometer (BD Biosciences, Mountain View, CA, USA). For the analysis of internalization of Ag, Rev.A- or CHX-treated A20-HL cells were incubated with 3.2 $\mu$g/ml FITC-TNP-OVA or 10 $\mu$g/ml FITC-F(ab')$_2$ from anti-mouse IgM goat IgG Ab for the indicated hours in the presence of Rev.A or CHX, respectively. Removing FITC-TNP-OVA or FITC-anti-IgM IgG F(ab')$_2$ remaining on the cell surface by incubating in 150 mM NaCl containing 20 mM HCl for 15 minutes on ice, the cells were analyzed on a FACSCalibur flowcytometer.

To examine restoration of sIgM expression, A20-HL cells were incubated with anti-IgM IgG F(ab')$_2$ for 30 minutes on ice, washed, and incubated at 37°C for the indicated hours. They were then stained with FITC-anti-IgM IgG F(ab')$_2$, and analyzed on a FACSCalibur flowcytometer. $\Delta$MFI was calculated for each sample. $\Delta$MFI=(mean channel obtained with an appropriate Ab)−(mean channel obtained with a control Ab), where mean channel was calculated using CELLQuest™ software (BD Biosciences).

## Protein Synthesis Assay and Measurement of Intracellular pH

Protein synthesis and intracellular pH in A20-HL cells were determined as described previously[34].

## Analysis Under Confocal Laser Microscopy

To analyze generation of antigenic peptides, A20-HL cells were pulsed with 3.12 $\mu$g/ml TNP-OVA or 0.8 mg/ml OVA at 37°C for the indicated duration, and were stained by sequential treatment with rabbit antibody against the C-terminal of OVA$_{321\sim336}$ peptide, biotinylated anti-rat $\kappa$ mAb, and Cy3-streptavidin. They were then analyzed under a confocal laser scanning microscope system.

To compare intracellular trafficking of anti-IgM IgG F(ab')$_2$ and transferrin, A20-HL cells were pretreated with 2% BSA in RPMI 1640 medium for 1 hour at 37°C, and incubated with FITC-anti-IgM IgG F(ab')$_2$ or Alexa594-transferrin for 30 minutes on ice. After washing, they were incubated at 37°C for the indicated hours, washed again, fixed with 3.5% paraformaldehyde, and imaged on a MRC-600 Confocal Imaging System (Bio-Rad Laboratories, Richmond, CA, USA).

For comparison of intracellular trafficking of anti-IgM IgG F(ab')$_2$ with LAMP-1$^+$ vesicles, A20-HL cells were incubated with FITC-anti-IgM IgG F(ab')$_2$ for 30 minutes on ice, washed, and incubated for the indicated hours at 37°C. The cells were then fixed with 3.5% paraformaldehyde, permeabilized with PBS containing 0.1% saponin and 2% BSA, and stained by sequential treatment with anti-LAMP-1 mAb, biotinylated anti-rat $\kappa$ mAb, and Cy3-streptoavidin. They were imaged on a MRC-600 Confocal Imaging System.

# Results

## Rev.A Inhibited BCR-mediated Ag-presentation

To analyze the regulation of Ag-presentation by Rev.A, A20-HL B cells were treated with Rev.A at 20 $\mu$g/ml, pulsed with 0.8 $\mu$g/ml TNP-OVA or 0.8 mg/ml OVA, and examined for their ability to stimulate 42-6A T cells to secrete IL-2. As shown in Fig. 1-A, treatment with Rev.A dramatically decreased the function of TNP-OVA-pulsed A20-HL cells to induce IL-2 production by 42-6A cells, whereas treatment did not affect the ability of OVA-pulsed A20-HL cells. Inhibition was dependent on the dose of Rev.A. When A20-HL cells were treated with 10$\sim$30 $\mu$g/ml Rev.A, similar results were obtained, but the treatment with 3 $\mu$g/ml Rev.A resulted in the decrease in the inhibition of the presentation of TNP-OVA (data not shown). These results suggested that Rev.A-treatment inhibited presentation of Ag internalized via BCR, but not of Ag taken up through fluid-phase pinocytosis.

Inhibition of protein synthesis has been shown to selectively inhibit BCR-mediated Ag presentation[19]. When protein synthesis was examined in A20-HL cells, treatment with Rev.A at 20 $\mu$g/ml induced approximately 60% inhibition, which was similar to the inhibition obtained with CHX at 1.28 $\mu$g/ml (Fig. 1-B). A20-HL cells treated with Rev.A at 20 $\mu$g/ml were compared with those treated with CHX at 1.28 $\mu$g/ml for their ability to present Ag internalized via BCR. CHX-treatment mildly decreased Ag presentation, whereas Rev.A-treatment thoroughly inhibited this ability (Fig. 1-C), suggesting that Rev.A-treatment inhibited BCR-mediated Ag presentation not only by the partial inhibition of protein synthesis but also via some other mechanisms. Then, Rev.A-treated A20-HL cells were compared with the CHX-treated cells in order to analyze the mechanisms underlying Rev.A inhibition of BCR-mediated Ag-presentation.

## Internalization of Ag via BCR into Rev.A-treated A20-HL Cells

BCR-mediated internalization of Ag in Rev.A-treated A20-HL cells was analyzed. TNP-OVA was internalized

Fig. 1. Rev.A-treatment inhibited BCR-mediated Ag presentation by A20-HL cells.
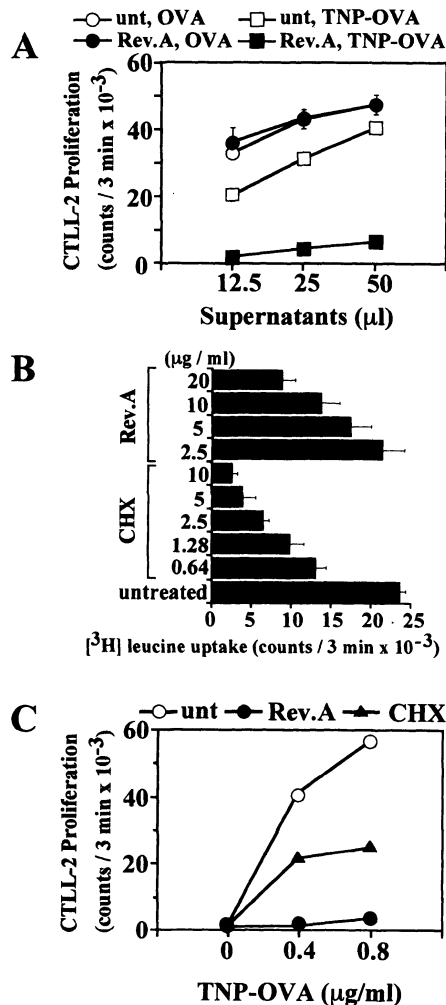


(A) A20-HL cells were incubated for 18 hours at 37°C in the presence of 20 μg/ml Rev.A. TNP-OVA (0.8 μg/ml) or OVA (0.8 mg/ml) was added during the final 3 hours. After washing, the treated cells ($1 \times 10^5$) were incubated with 42-6A cells ($1 \times 10^4$) for a further 20 hours. IL-2 activity in the culture supernatant (12.5, 25, or 50 μl) was assessed using IL-2 dependent CTLL-2 cell proliferation. Results are shown as mean of triplicate assay.

(B) Protein synthesis was determined in Rev.A-treated A20-HL cells. A20-HL cells were treated with Rev.A or CHX at indicated doses and incubated in the presence of [³H]leucine for 1 hour at 37°C. [³H]leucine incorporated into the 5% trichloroacetic acid insoluble fraction of the cell lysates was counted.

(C) A20-HL cells were incubated for 18 hours in the presence of Rev.A (20 μg/ml) or CHX (1.28 μg/ml). TNP-OVA at indicated doses was added during the final 3 hours of incubation. Treated cells ($1 \times 10^5$) were washed, and incubated with 42-6A cells ($1 \times 10^4$) for 20 hours. IL-2 activity in the 50 μl culture supernatant was assessed by proliferation of IL-2-dependent CTLL-2 cells.
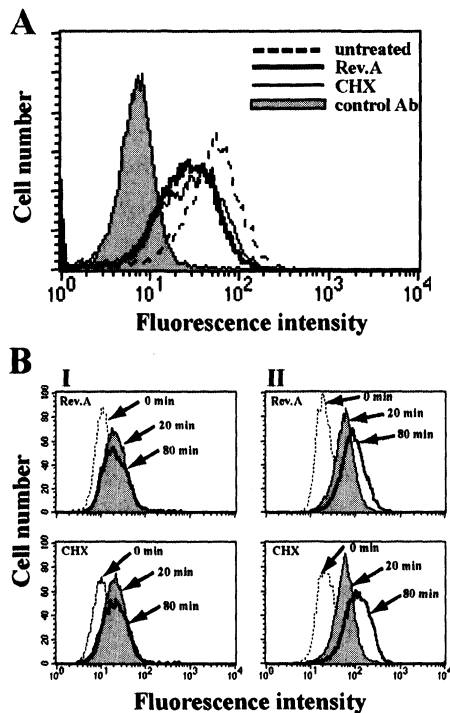
into A20-HL cells *via* BCR, which was TNP-specific sIgM in these cells. sIgM expression on the Rev.A-treated A20-HL cells was decreased relative to untreated A20-HL cells, but indistinguishable from that on cells treated with CHX (Fig. 2-A). Internalization of FITC-labeled TNP-OVA *via* BCR into Rev.A-treated A20-HL cells appeared to be slightly less than that observed into the CHX-treated cells (Fig. 2-B-I), although the detectable amount was small. To confirm these findings, FITC-anti-mouse IgM goat IgG F(ab')₂ was used as a model for TNP-OVA. A20-HL cells were incubated with FITC-anti-IgM IgG F(ab')₂ for 20 or 80 minutes at 37°C, and analyzed for intracellular FITC after stripping the remaining FITC-anti-IgM IgG F(ab')₂ on the cell surface using treatment with acidic buffer. As shown in Fig. 2-B-II, the intracellular FITC detected in Rev.A-treated A20-HL cells after 20 minutes incubation with FITC-anti-IgM IgG F(ab')₂ was quite similar to that in the CHX-treated cells (ΔMFI for the former=33.1, ΔMFI for the latter=31.44). However, the fluorescent intensity detected in Rev.A-treated A20-HL cells after 80 minutes incubation was lower than that in the CHX-treated cells. ΔMFIs were 59.04 for the former, and 78.34 for the latter. When the internalization of BCR was inhibited by the addition of NaN₃, the cell-associated FITC was decreased to the control level (data not shown), suggesting that FITC-anti-IgM IgG F(ab')₂ remaining on the cell surface was almost completely removed by the acid treatment.

Re-expression of BCR on the cell surface in Rev.A-treated A20-HL cells was compared with cells treated with CHX. After treating with unlabelled anti-IgM IgG F(ab')₂, A20-HL cells were incubated for 60 or 180 minutes at 37°C and stained with FITC-anti-IgM IgG F(ab')₂. As shown in Fig. 3, re-expression at 60 or 180 minutes was less intense in Rev.A-treated A20-HL cells than in the cells treated with CHX. ΔMFI for 60 and 180 minutes in the Rev.A-treated cells were 4.67 and 11.15, respectively, and those in the CHX-treated cells were 13.56 and 19.17, respectively. These results suggested that the rate of re-expression of IgM on the cell surface in the Rev.A-treated cells was slower than in the CHX-treated cells.

## Analysis of Intracellular Trafficking of Ag Internalized *via* BCR

Intracellular trafficking of FITC-anti-IgM IgG F(ab')₂ was analyzed in Rev.A-treated A20-HL cells. First, A20-HL cells were allowed to bind Alexa594-transferrin and FITC-anti-IgM IgG F(ab')₂ on ice to their surface transferrin receptors and BCR, respectively, incubated at 37°C for 0~30 minutes, and examined under confocal laser

Fig. 2. Expression of sIgM on and internalization of Ag *via* BCR into Rev.A-treated A20-HL cells.
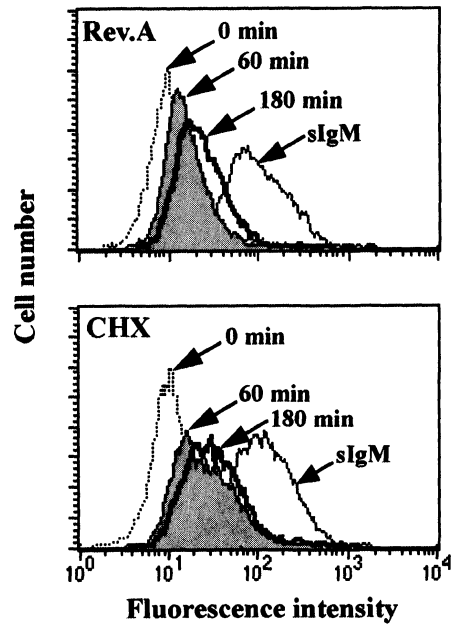


Fig. 3. Restration of sIgM on Rev.A-treated A20-HL cells.



Rev.A- or CHX-treated A20-HL cells were incubated with unlabelled anti-IgM F(ab')$_2$ on ice, washed, and incubated at 37°C for 0, 60, or 180 minutes in the presence of Rev.A or CHX, respectively. The cells were stained with FITC-anti-IgM F(ab')$_2$ and analyzed on a flowcytometer. Expression of sIgM was also analyzed before treatment and shown by a thin line indicated as sIgM.

(A) A20-HL cells were treated for 18 hours in the absence or presence of Rev.A (20 μg/ml) or CHX (1.28 μg/ml), and stained by sequential treatment with anti-mouse IgM mAb (Bet 2), biotinylated anti-rat k mAb (MARK 1), FITC-streptavidin. Cells were then analyzed on a flowcytometer. Polyclonal rat IgG was used as control antibody. Staining profiles with rat IgG were quite similar among untreated, Rev.A-treated, and CHX-treated A20-HL cells, and were almost superimposable.

(B) Rev.A- or CHX-treated A20-HL cells were incubated at 37°C for 0, 20, or 80 minutes in the presence of FITC-TNP-OVA (I) or FITC-anti-IgM F(ab')$_2$ (II), and analyzed on a flowcytometer after acid treatment to remove reagent from the cell surface.

microscopy. Transferrin was a representative marker of the early endosomes. As shown in Fig. 4-A, transferrin was rapidly internalized as early as 5 minutes incubation, and had mostly disappeared at 10 minutes in CHX-treated A20-HL cells. FITC-anti-IgM IgG F(ab')$_2$ was transiently co-localized with transferrin at 5 minutes and mostly dissociated thereafter. In contrast, transferrin was similarly internalized rapidly but mostly stayed in the Rev.A-treated cells for at least 30 minutes. FITC-anti-IgM IgG F(ab')$_2$ was mostly co-localized with transferrin for at least 30 minutes. Thus, Rev.A-treatment seemed to affect

intracellular trafficking of the early endosomes.

Then, A20-HL cells were incubated on ice with FITC-anti-IgM IgG F(ab')$_2$, incubated at 37°C for 0~60 minutes, and analyzed for co-localization with lysosomal associated membrane glycoprotein-1 (LAMP-1), which was a representative marker for the late endosomes and lysosomes[35]. As shown in Fig. 4-B, in CHX-treated A20-HL cells, FITC-anti-IgM IgG F(ab')$_2$ co-localized with LAMP-1 as early as 10 minutes after incubation, and increased at 30 minutes. In 20 μg/ml Rev.A-treated A20-HL cells, co-localization was observed marginally at 10 minutes after incubation and remained at a much lower level than in the CHX-treated cells. Thus, Rev.A-treatment decreased the co-localization of FITC-anti-IgM IgG F(ab')$_2$ and LAMP-1, suggesting that traffic from the early endosomes into the late endosomes was inhibited. This inhibition was Rev.A-dose dependent. When A20-HL cells were treated with 0.5 μg/ml Rev.A, the co-localization of

Fig. 4.    Intracellular transport of anti-IgM F(ab′)$_2$ in Rev.A-treated A20-HL cells.
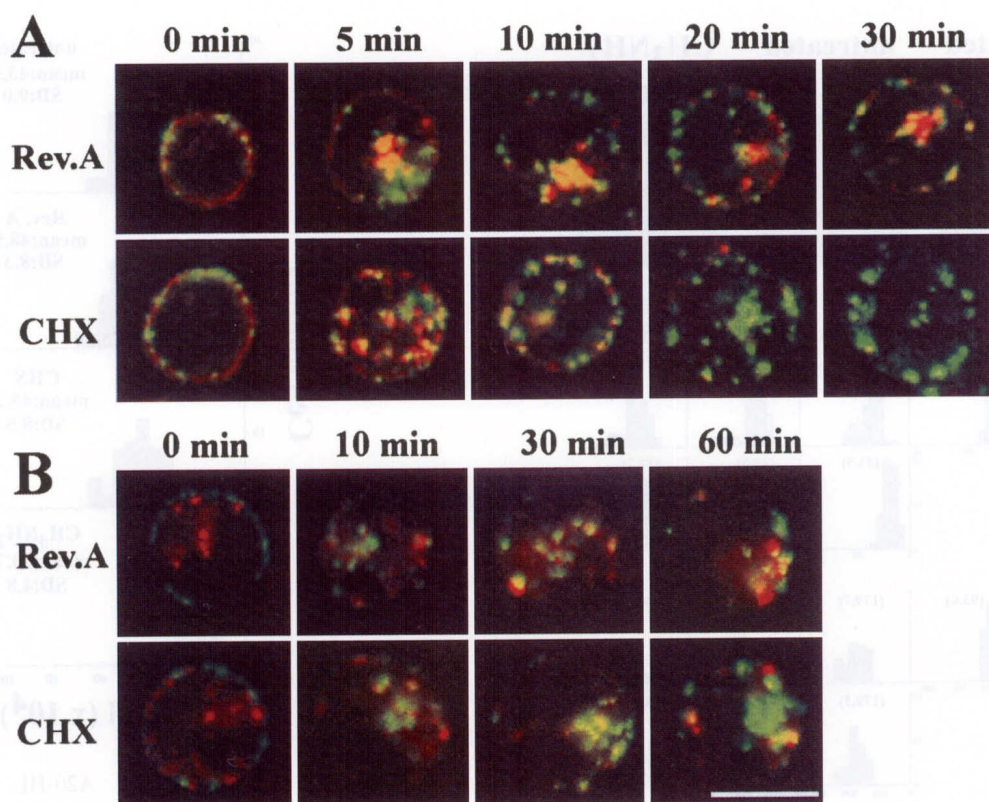


(A) Rev.A- or CHX-treated A20-HL cells were incubated with FITC-anti-IgM F(ab′)$_2$ (green) and Alexa594-transferrin (red) for 30 minutes on ice. Treated cells were washed, incubated at 37°C for the indicated duration (minute) in the presence of Rev.A or CHX, and analyzed on a confocal laser scanning microscope system.

(B) Rev.A- or CHX-treated A20-HL cells were incubated with FITC-anti-IgM F(ab′)$_2$ for 30 minutes on ice, washed, and incubated at 37°C for indicated min in the presence of Rev.A or CHX. Then, they were fixed with paraformaldehyde, permeabilized, and stained by sequential treatment with anti-LAMP-1 mAb (1D4B), biotinylated anti-rat k mAb, and Cy3-streptavidin. These cells were analyzed on a confocal laser scanning microscope system.

FITC-anti-IgM IgG F(ab′)$_2$ and LAMP-1 was similarly observed to that in untreated A20-HL cells (data not shown).

### Ag Processing in Rev.A-treated A20-HL Cells

Analysis of the antigenic peptide generated in A20-HL cells from OVA and recognized in the context of I-A$^d$ by 42-6A cells strongly suggested it to be OVA$_{321\sim336}$ (manuscript in preparation). To examine the amount of peptide generated in Rev.A-treated A20-HL cells, rabbit antiserum specific for C-terminal of OVA$_{321\sim336}$ peptide was prepared. The specificity of this antiserum was supported by findings that it stained A20-HL cells only when pulsed with OVA, which was inhibited by the

treatment with methylamine, a reagent that increases pH in the acidic intracellular compartments (Fig. 5-A). Using the antiserum, the amount of antigenic peptides was analyzed in Rev.A-treated A20-HL cells and shown as pixels/cell. As shown in Fig. 5-B, the antigenic peptides were detected in CHX-treated A20-HL cells at as early as 45 minutes incubation with TNP-OVA, whereas the peptides were not detected in the Rev.A-treated cells at this time point. The peptides were detected in Rev.A-treated A20-HL cells at 90 minutes and 180 minutes, but the amounts were much smaller than those in the CHX-treated cells, suggesting that the generation of the antigenic peptides from TNP-OVA internalized via BCR was delayed and smaller in Rev.A-treated A20-HL cells than in the cells treated with CHX. Generation of the peptides from OVA taken up through

Fig. 5.  Analysis of the generation of antigenic peptides in Rev.A-treated A20-HL cells.
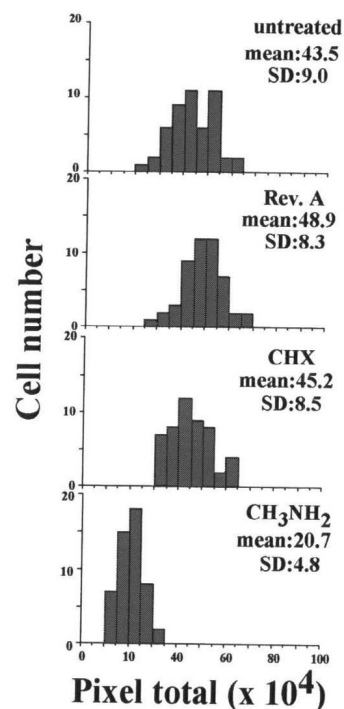


Fig. 6.  Analysis of intracellular pH in Rev.A-treated A20-HL cells.
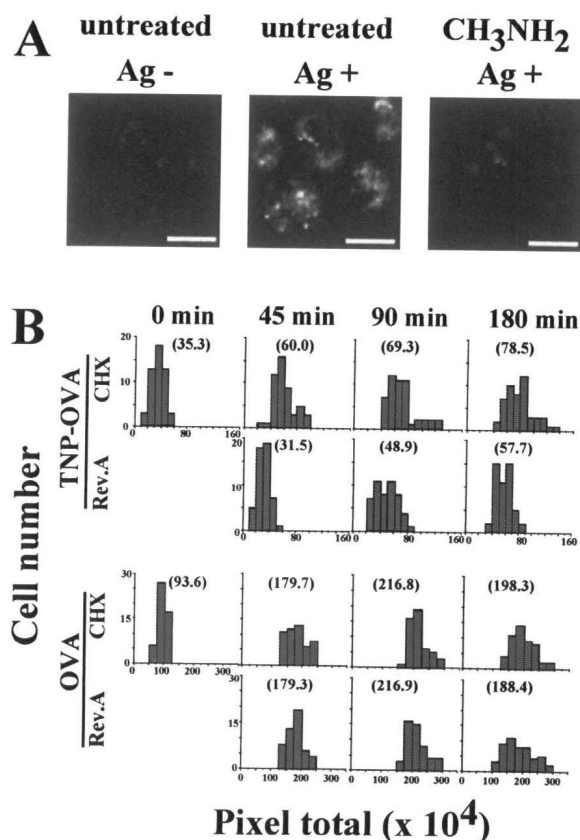


(A) A20-HL cells were pulsed with or without OVA at 0.8 mg/ml at 37°C for 3 hours in the presence or absence of $CH_3NH_2$. The cells were fixed with paraformaldehyde, permeabilized, treated with rabbit Ab against C-terminal of $OVA_{321\sim336}$ peptide, and stained with FITC-anti-rabbit IgG+IgM. They were then analyzed on a confocal laser scanning microscope system.

(B) Rev.A- or CHX-treated A20-HL cells were pulsed with TNP-OVA at 3.12 $\mu$g/ml or OVA at 0.8 mg/ml at 37°C for the indicated duration (minute). The cells were washed, fixed with paraformaldehyde, and treated with rabbit Ab against C-terminal of $OVA_{321\sim336}$ peptide. They were then stained with FITC-anti-rabbit IgG+IgM and analyzed on a confocal laser scanning microscope system. The brightness of each cell was expressed as pixels/cell. The mean pixels/cell in each group is shown in parenthesis.

Rev.A- or CHX-treated A20-HL cells were incubated with a pH indicator reagent, Lyso-Sensor Green DND-189 with a pH range 4.5~6.0, and analyzed on a confocal laser scanning microscope system. A20-HL cells treated with $CH_3NH_2$, a reagent which increases pH in the acidic intracellular compartments, were used as a control.

Rev.A-treated A20-HL cells. As shown in Fig. 6, the intracellular pH in A20-HL cells was not affected by treatment with Rev.A.

## Discussion

The present study analyzed the mechanisms underlying Rev.A-induced inhibition of BCR-mediated Ag-presentation. Inhibition was selective in that Rev.A-treatment did not affect the presentation of OVA taken up through fluid-phase pinocytosis. Rev.A-treatment appeared to affect a process characteristic for BCR-mediated antigen presentation. Although protein-synthesis inhibition selectively inhibits BCR-mediated Ag-presentation[19], partial inhibition of protein synthesis in Rev.A-treated A20-HL cells did not fully account for the severe inhibition of

fluid-phase pinocytosis was quite similar in Rev.A-treated A20-HL cells and those treated with CHX (Fig. 5-B).

An acidic pH was required for the generation of antigenic peptides from protein Ag in the endocytic compartments[36]. Intracellular pH was also examined in

BCR-mediated Ag-presentation. Expression of sIgM on Rev.A-treated A20-HL cells was indistinguishable from that on the CHX-treated cells. However, the amount of internalized TNP-OVA or anti-IgM IgG F(ab')$_2$ via BCR into Rev.A-treated A20-HL cells during incubation with these reagents was decreased relative to that into the CHX-treated cells. This decrease might be due in part to the decreased rate of re-expression of sIgM. Consistent with these findings, the generation of the antigenic peptide from TNP-OVA internalized via BCR, which was recognized by 42-6A T cells in the context of I-A$^d$, was decreased in Rev.A-treated A20-HL cells, whereas the generation from OVA taken up via fluid-phase pinocytosis was not affected.

Rev.A-induced disturbance of intracellular trafficking of Ag internalized via BCR might also contribute to the decrease in the generation of antigenic peptide. In the Rev.A-treated A20-HL cells, anti-IgM IgG F(ab')$_2$ internalized via BCR seemed to co-localize with transferrin for a longer period and with LAMP-1 at lower levels when compared with co-localization in the CHX-treated cells, suggesting that Rev.A-treatment might disturb the trafficking of early endosomes. Ag internalized via BCR is effectively targeted into MHC class II peptide loading compartments, where antigenic peptides are generated from the Ag under optimal acidic environments for degradation by proteases, and formed complexes with MHC class II molecules en route to the cell surface[19~22]. Rev.A-treatment of A20-HL cells did not affect intracellular pH, although the pH in intracellular vesicles could not be precisely determined. These findings suggested that TNP-OVA internalized via BCR was not transported into appropriate compartments in the Rev.A-treated A20-HL cells, which in turn resulted in a decrease in the generation of antigenic peptides.

Ag taken up via fluid-phase pinocytosis into Rev.A-treated A20-HL cells was similarly presented to T cells to that into CHX-treated cells. Thus, the intracellular transport of OVA internalized via fluid-phase pinocytosis appeared to be differently regulated from that of TNP-OVA via BCR. Consistently, antigenic peptides from OVA internalized via fluid-phase pinocytosis, but not those from Ag via BCR, are capable of forming complexes with MHC class II molecules recycled from the cell surface[19~23].

The mechanisms for intracellular transport of Ag internalized via BCR or fluid-phase pinocytosis are presently unknown. Actin polymerization is required for endocytosis of BCR[35,37]. The BCR cross-linking contributes to the actin polymerization and acceleration of Ag targeting to the peptide loading compartments[12~17,38]. Actin microfilaments control the MHC class II Ag

presentation pathway in B cells[39]. Cytochalasin D-treatment markedly reduced the rate of Ag internalization via BCR[35]. However, Rev.A-treatment appears not to affect actin polymerization, based on findings wherein Rev.A-treatment did not inhibit the internalization of Ag via BCR. Bafilomycin A1 is produced from Streptomyces and interferes with the intracellular transport from early to late endosomes by inhibiting vacuolar H$^+$ ATPases[40]. Rev.A prepared also from Streptomyces appeared to differently interfere the intracellular trafficking from bafilomycin A1. Treatment with Rev.A did not affect intracellular pH, whereas inhibition of vacuolar H$^+$ ATPases increases intracellular pH[41]. BCR reportedly signals vesicular coalescence, which is the primary target for endocytosed BCR and blocked by the tyrosine kinase inhibitor genistein[25]. In our preliminary experiments, genistein treatment of A20-HL cells affected the intracellular trafficking of TNP-OVA internalized via BCR and of transferrin. These findings were similar to those obtained in the Rev.A-treated cells. Rev.A-treatment might alter the BCR-mediated signals.

B cells play a critical role as APC in vivo[3~7]. Thus, it will be useful to elucidate the mechanisms underlying intracellular trafficking of Ag internalized via BCR into B cells for the regulation of in vivo Ag presentation and the resultant immune response. Rev.A might provide a valuable tool for the analysis of intracellular trafficking of Ag internalized via BCR.

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