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WEAK BASE AMINES CAN INHIBIT CLASS I MHC-RESTRICTED ANTIGEN PRESENTATION[1]

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This report describes the effects of NH_4Cl, CH_3NH_2, and chloroquine on class I and II MHC-restricted Ag presentation. OVA-specific T-T hybridomas were used to detect processed OVA in association with class I, H-2K^b, and class II, I-A$^{d/b}$, molecules on a B lymphoblastoid APC. OVA, internalized by APC under hypertonic conditions, was presented in association with class I and II MHC molecules. Treating the APC with NH_4Cl or CH_3NH_2 inhibited class I- and II-restricted Ag presentation. In contrast, chloroquine markedly inhibited class II, but not class I-restricted Ag presentation. Controls indicated that drug-treated APC were fully competent to interact with T cells and present processing-independent antigenic peptides in association with both class I and II MHC molecules. NH_4Cl and CH_3NH_2 did not inhibit the uptake of radiolabeled Ag by the APC. After the proteolytic removal of H-2K^b from the surface of APC, NH_4Cl and CH_3NH_2-treated and control APC regenerated identical amounts of surface H-2K^b and this regeneration required de novo protein synthesis. These latter results indicate that NH_4Cl and CH_3NH_2 can inhibit Ag presentation without affecting the synthesis, transport, or surface expression of H-2K^b. Also, NH_4Cl did not affect the transport of H-2D^b to the surface of mutant RMA-S cells that were cultured with exogenous peptides. Taken together these results strongly suggest that NH_4Cl and CH_3NH_2 but not chloroquine can inhibit a critical and early intracellular step in class I-restricted Ag presentation while simultaneously inhibiting class II-restricted Ag presentation.

T lymphocytes recognize protein Ag in association with class I or class II MHC molecules on the surface of APC (1, 2). APC must convert a native, nonstimulatory protein to an immunogenic form before T cell recognition can occur. This conversion is commonly referred to as antigen processing.

For class II MHC-restricted T cells, several observations suggest that Ag processing involves denaturation and/or degradation of Ag in endocytic compartments. First, elevating the pH of acidic intracellular compartments by treating the APC with lysosomotropic drugs can block Ag processing without affecting the function of class II MHC molecules on the APC surface (3, 4). Defective Ag presentation has also been demonstrated in mutant Chinese hamster ovary cells that express temperature sensitive defects in the acidification of their early endosomes (5). Second, specific protease inhibitors at non-cytotoxic concentrations can also block Ag processing (6, 7). Third, in vitro proteolytic or chemical cleavage of proteins can generate peptide fragments that function as Ag with processing-inactive APC (6, 8). Fourth, immunogenic peptides from in vivo processed Ag have been eluted from purified class II MHC molecules (9). Recently, analysis of endosomes by immunoelectron microscopy has shown the co-localization of endocytosed Ag, class II MHC molecules, invariant chain, and proteolytic enzymes (10).

Similar to class II MHC-restricted T lymphocytes, class I MHC-restricted T lymphocytes also recognize degraded forms of protein Ag. Expressed products of truncated viral genes as well as synthetic peptides are capable of sensitizing target cells for class I restricted CTL killing (11–13). Purified class I MHC molecules appear to bind immunogenic peptides (14) and an unknown electron dense material has been observed in the putative peptide-binding groove of a class I MHC molecule (15).

Evidence presented by Morrison et al. (16) suggests that the pathways for class I and II MHC-restricted Ag presentation may have distinct and separable cellular mechanisms. In particular, the lysosomotropic drug chloroquine did not inhibit the presentation of infectious influenza A virus to class I-restricted T lymphocytes but the same concentration of chloroquine did inhibit presentation to class II-restricted T lymphocytes. There are additional reports that lysosomotropic drugs do not inhibit the processing of Ag for presentation by class I MHC molecules (17–19). Endogenously synthesized Ag or soluble Ag introduced into the cytoplasm is efficiently presented by class I MHC molecules whereas exogenous Ag in an endocytic compartment is not (16, 20). Taken together, these results have suggested that class I and class II MHC-restricted Ag processing occur in different intracellular compartments. The proteolytic system(s) responsible for processing of Ag for presentation by class I MHC molecules has not been characterized.

We have recently generated OVA-specific, class I-restricted, T cell hybridomas that produce IL-2 in an Ag-specific manner upon incubation with live or paraformaldehyde-fixed APC. Since these characteristics provide distinct advantages for studying Ag processing and presentation, we have initiated the search for compounds

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that affect class I MHC-restricted Ag presentation. Herein we describe the effects of three weak base amines/lysosomotropic drugs, NH_4Cl, CH_3NH_2, and chloroquine.

MATERIALS AND METHODS

Reagents. Crystallized chicken OVA grade VII was purchased from Sigma Chemical Co., St. Louis, MO, and dissolved in PBS at 10 mg/ml. OVA was digested with trypsin or cyanogen bromide as described (8). OVA was radioiodinated to a sp. act. of 5.0×10^6 cpm/μg by using chloramine T (Sigma) and carrier-free ^{125}I sodium iodide (New England Nuclear, Boston, MA). NH_4Cl was purchased from Fisher Scientific, Fair Lawn, NJ. CH_3NH_2, chloroquine, cycloheximide, and ricin communis (ricin) were purchased from Sigma. One molar stock solutions of NH_4Cl and CH_3NH_2 were made in RPMI 1640, filter sterilized, and stored at 4°C. Chloroquine was dissolved in PBS and freshly prepared for each experiment. Polyethylene glycol 1450 (m.w. 1300 to 1600) was purchased from American Type Culture Collection (ATCC), Rockville, MD. Protease from *Streptomyces griseus* type XIV (pronase) was purchased from Sigma and dissolved immediately before use at 4 mg/ml in RPMI 1640. AF6-88.5.3 (anti-H-2K^b) (21) was purified from hybridoma culture supernatant by protein-A Sepharose. One mg of AF6-88.5.3 (1 mg/ml) in 0.1 M $NaHCO_3$, pH 8.4, was biotinylated by incubating with 0.125 ml N-hydroxy-succinimidobiotin (1 mg/ml in DMSO) (Sigma) for 4 h at room temperature. The biotinylated antibody was extensively dialyzed against PBS and then stored at 4°C. Phycoprobe PE-streptavidin was purchased from Biomeda Corp., Foster City, CA and used according to the manufacturers directions. The synthetic peptide NP(1968)[3] 365–380 representing the sequence between residues 365 and 380 of the 1968 influenza nucleoprotein was prepared at the Dana Farber Cancer Institute's Molecular Biology Core Facility by Merrifield's solid-phase method using t-Boc suitably protected amino acids. The NP(1968) 365–380 was dissolved in DMSO at 10 mg/ml. Hybridoma culture supernatants containing the mAb AF6-88.5.3, 28-11-5S (anti-H-2D^b) (22), and 7D4 (anti-murine IL-2R) (23) were prepared as previously described (24). 28-11-5S and 7D4 are both IgM antibodies.

Cell lines. The T cell hybridoma DO-11.10.S4.4 (DO-11.10; BALB/c (anti-OVA-I-A$^{d/b}$)-BW5147 thymic lymphoma) was provided by Drs. J. Kappler and P. Marrack (National Jewish Hospital, Denver, CO) and has been described (8). The T cell hybridoma RF33.70 (C57BL/6 (anti-OVA-K^b)-BW.CD8.7 thymic lymphoma) was produced in our laboratory (25). These T cell hybridomas are stimulated to produce the lymphokine IL-2 upon recognition of the appropriate antigen and MHC molecule on the surface of APC. The LB27.4 cell line was obtained from ATCC. LB27.4 was produced by fusing the BALB/c lymphoma line A20.2J (I-A^d) to T cell-depleted spleen cells of a C57BL/10 mouse (H-2K^b) (26). The T cell hybridomas are passaged in vitro in DMEM with 4.5 g of glucose per liter supplemented with 10% (v/v) heat-inactivated formula fed bovine calf serum (iron supplemented), 4 mM L-glutamine, 100 U of penicillin, 100 μg of streptomycin, and 0.25 μg of fungizone per ml. The RMA-S cell line (27, 28) was made available by Dr. Klas Karre (Karolinska Institutet, Stockholm, Sweden) and was obtained from Dr. Gib Otten (National Institutes of Health, Bethesda, MD). It is passaged in vitro in RPMI 1640 with 10% (v/v) heat-inactivated FCS and supplements as described above. LB27.4 is passaged in vitro in RPMI 1640 with 10% (v/v) heat-inactivated FCS, 50 μM 2-ME, and supplements as described above. Media, supplements, and formula fed bovine calf serum were purchased from Irvine Scientific, Santa Ana, CA. FCS was purchased from Sigma. RPMI 1640 containing 10% (v/v) heat-inactivated formula fed bovine serum, 4 mM L-glutamine, and antibiotics was used for incubations in the various assay systems described below.

Cell culture. Culture medium was RPMI 1640 with 10% (v/v) FCS and was supplemented as indicated above along with 50 μM 2-ME, 20 mM HEPES (Irvine Scientific) and 1 mM nonessential amino acids (Irvine Scientific). Cultures containing 10^5 T-T hybridomas per well with or without a source of APC in the presence or absence of Ag were brought to a final volume of 200 μl in flat-bottom microculture plates. Before addition to culture, the APC were extensively washed with ice-cold PBS and then metabolically inactivated by incubation with 1% paraformaldehyde for 10 min at room temperature. The fixed cells were washed free of fixative with ice-cold PBS. The T cell hybridoma cultures were incubated at 37°C for 18 to 24 h, after which time 100 μl of supernatant was removed, frozen, and then assayed for IL-2 content. The IL-2-dependent cell line HT-2 was used to assay for IL-2 as previously described (24).

Drug treatment and OVA pulsing of LB27.4 cells. LB27.4 cells (2×10^6/ml) were incubated in the presence (drug-treated cells) or absence (control cells) of NH_4Cl, CH_3NH_2, chloroquine, cycloheximide, or ricin for 1 h at 37°C. The precise concentrations of these drugs are indicated in the respective experimental protocols.

For incubating APC with exogenous OVA, OVA was added to the extracellular medium of control or drug-treated LB27.4 cells to a final concentration of 1 mg/ml. The cells were then incubated in the continued presence or absence of drug for 3 h at 37°C.

For introducing soluble OVA into the cytoplasm of LB27.4, control or drug-treated cells (as described above) were pelleted then incubated with OVA in hypertonic media as described by Moore et al. (20). Throughout this report the following procedure will be referred to as the hypertonic/hypotonic pulsing procedure. Briefly, OVA was dissolved in a hypertonic media composed of 0.5 M sucrose, 10% w/v polyethylene glycol 1450, 10 mM HEPES in RPMI 1640, pH 7.2. The cells were pelleted, resuspended at 1×10^7 cells/ml in prewarmed OVA-containing hypertonic media, and then incubated for 10 min at 37°C. The cell suspension was diluted 15-fold with prewarmed hypotonic media (60% RPMI 1640, 40% water), incubated for 3 min at 37°C, and then centrifuged for 5 min at room temperature. In early experiments, drug was added to the hypertonic and hypotonic medias. However, we subsequently determined that drug could be omitted from this step without influencing the results and data are displayed from experiments utilizing both of these conditions. Residual exogenous OVA was removed by washing the cells three times with ice-cold serum-free RPMI 1640. The cells were resuspended at 2×10^6/ml and then incubated in the continued presence or absence of the appropriate drug for 3 h at 37°C. In some experiments, LB27.4 cells were not treated with drugs until after the hypertonic/hypotonic pulsing procedure.

Removal of H-2K^b from surface of LB27.4 cells with pronase. Control or drug-treated cells (prepared as described above) were subjected to the hypertonic/hypotonic pulsing procedure as described above except that OVA was omitted from the hypertonic media. After the washes with RPMI 1640, the cells were resuspended in RPMI 1640 at 1×10^7/ml with pronase (2 mg/ml) in the continued presence or absence of the appropriate drug and incubated for 1 h at 37°C. Ice-cold serum free RPMI 1640 was added to stop the reaction and the cells were pelleted by centrifugation at 4°C. The cells were then washed an additional three times with ice-cold serum free RPMI 1640. After each centrifugation, the cell pellet was resuspended by gently mixing with a 10-ml pipet and cell debris was removed with a Nytex mesh membrane (Tetko, Inc., Elmsford, NY). After the three washes, the pronase-treated cells were split into two separate groups. One group was immediately assayed for the presence of surface H-2K^b by indirect immunofluorescence whereas the other group was incubated in the continued presence or absence of the appropriate drug for 3 h at 37°C. After the 3-h incubation, the second group was also assayed for the presence of surface H-2K^b by indirect immunofluorescence as described below.

Expression of H-2D^b on surface of RMA-S cells. RMA-S cells (7.5×10^5/ml in passage media) were incubated in the presence or absence of 20 mM NH_4Cl for 1 h at 37°C. After the 1-h incubation, the cells were pelleted and then resuspended at 7.5×10^5 cells/ml in passage media containing 20 mM NH_4Cl plus 100 ug/ml NP(1968) 365–380 peptide, 100 μg/ml NP(1968) 365–380 peptide only, or 20 mM NH_4Cl plus the appropriate volume of DMSO. RMA-S cells incubated with NH_4Cl and DMSO were used to determine the baseline levels of H-2D^b and H-2K^b. The cells were then incubated for 20 h at 37°C in a humidified incubator with 5% CO_2. After the 20-h incubation, the cells were assayed for the presence of surface H-2D^b and H-2K^b by indirect immunofluorescence as described below.

Immunofluorescence. Staining of cells for the removal and regeneration of surface H-2K^b by indirect immunofluorescence was performed as follows: 1×10^6 cells treated as described above were incubated with a saturating amount of biotinylated AF6-88.5.3 in 1% (w/v) BSA/PBS, pH 7.2. After three washes with ice-cold BSA/PBS, the cells were incubated with a saturating amount of Pycoprobe PE-streptavidin and then washed three times with ice-cold BSA/PBS. Background staining by Phycoprobe PE-streptavidin was determined by omitting the biotinylated AF6-88.5.3 from the first incubation. All incubations were carried out for 30 min at 4°C with shaking every 5 to 10 min. After the final wash, cells were fixed in 1% paraformaldehyde and 5×10^3 cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA).

Staining of RMA-S cells for surface H-2D^b and H-2K^b was performed essentially as described above except the cells were incubated with hybridoma culture supernatant containing mAb and bound mAb was detected by incubating the cells with a saturating

[3] Abbreviations used in this paper: NP(1968) 365–380, the sequence between the residues 365 and 380 of the 1968 influenza nucleoprotein; FITC-RAMG, rabbit anti-mouse Ig conjugated to FITC; PE, phycoerythrin; T-OVA, OVA digested with trypsin; CNBR-OVA, OVA digested with cyanogen bromide.

amount of FITC-RAMG. Background staining was determined by incubating with FITC-RAMG only. Cells were analyzed on a FACScan as described above.

Uptake of ^{125}I-labeled OVA by LB27.4 cells. Control or drug-treated cells (prepared as described above) were pelleted and then resuspended in hypertonic media preequilibrated at either 4 or 37°C and containing 2.5 μg of ^{125}I-OVA. The incubation in hypertonic media, subsequent dilution with hypotonic media, and washing with ice-cold, serum free RPMI 1640 were performed as described above for pulsing with non-radioactive OVA. However, the hypotonic media was preequilibrated at 4 or 37°C and drug was present in the hypertonic and hypotonic medias for the drug-treated cell groups. After the final wash with RPMI 1640, the cells were transferred to new tubes, pelleted, and the supernatant removed before cell-associated radioactivity was measured.

RESULTS

Effect of weak base amines on Ag presentation. Recently, our laboratory has generated a number of cloned, OVA-specific, class I MHC-restricted, T cell hybridomas. One of these T cell hybridomas, RF33.70, has been described (25). RF33.70 produces IL-2 in response to stimulation by EG7 (a clone of EL-4 transfected with the OVA gene) and by a tryptic or cyanogen bromide digest of OVA in the presence of EL-4 cells. Fibroblasts (WOP3027) transfected with the K^b gene present in vitro processed OVA to RF33.70, whereas the untransfected control cells do not. Based on these and other criteria, RF33.70 recognizes an OVA peptide in association with the class I MHC molecule, K^b.

In the present study, we have used the RF33.70 and DO-11.10 T cell hybridomas to assay for the presence of processed OVA in association with K^b and I-$A^{d/b}$, respectively. As a means for comparing class I- and II-restricted Ag presentation, LB27.4 cells, which express both K^b and I-$A^{d/b}$, have been used as APC.

APC were treated with drug for 1 h before pulsing with OVA. APC were pulsed by adding OVA to the extracellular medium (exogenous OVA) or by incubating with OVA in hypertonic media (hypertonic-pulsed OVA). The enclosed contents of pinosomes formed in the presence of hypertonic media are released into the cytoplasm by osmotic lysis (29). After a 3-h incubation to allow for the expression of processed Ag on the cell surface, the APC were fixed with 1% paraformaldehyde to prevent subsequent recovery and/or further Ag processing. The fixed cells were then added to culture with RF33.70 or DO-11.10.

Exogenous OVA was not presented to the class I MHC-restricted, T cell hybridoma RF33.70 (Fig. 1*A*) but was presented to the class II MHC-restricted, T cell hybridoma DO-11.10 (Fig. 1*B*). Exogenous OVA was not presented to RF33.70 even if the APC were incubated with OVA for 18 h (data not shown). These results are consistent with other studies of class I-restricted Ag presentation using both the identical and different Ag systems (16, 20). The separation of class I- and II-restricted Ag presentation for exogenous Ag presumably occurs because pinocytosis under normal isotonic conditions does not deliver Ag to the cytoplasm. As expected, NH_4Cl (20 mM) and CH_3NH_2 (5 mM) inhibited the presentation of exogenous OVA to DO-11.10 (Fig. 1*B*).

APC incubated with OVA in hypertonic media stimulated RF33.70 (Fig. 1*C*). The osmotic lysis of hypertonic pinosomes is thought to introduce Ag into the class I pathway of Ag presentation by releasing Ag into the cytoplasm (20). Unexpectedly, APC incubated with OVA in hypertonic media also stimulated DO-11.10 (Fig. 1*D*).

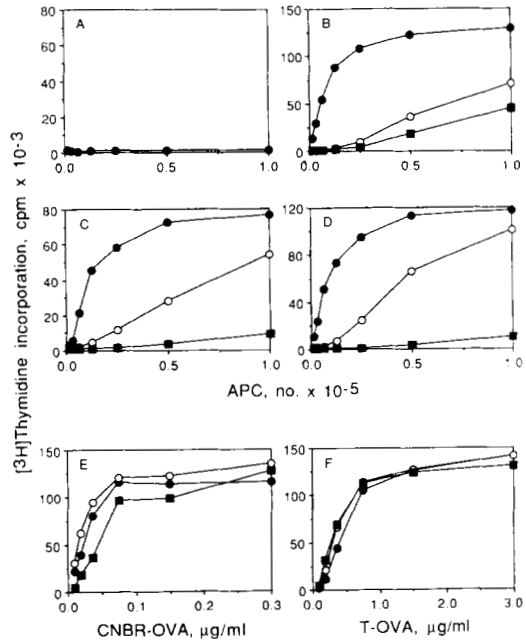

Figure 1. NH_4Cl and CH_3NH_2 inhibit class I and II MHC-restricted presentation of OVA. Duplicate microcultures (200 μl) were prepared with the T cell hybridomas (10^5 cells) RF33.70 (*A*, *C*, and *E*) or DO-11.10 (*B*, *D*, and *F*) and the indicated number of control (●), NH_4Cl-treated (20 mM) (■), or CH_3NH_2-treated (5 mM) (○) LB27.4 B-lymphoblastoid cells. LB27.4 cells were pulsed with 1 mg/ml exogenous OVA (*A* and *B*), pulsed with 7.5 mg/ml OVA in hypertonic media (*C* and *D*), or not pulsed with OVA (*E* and *F*). Ag pulsing and drug incubations were performed as described in *Materials and Methods* after which time the APC were fixed with paraformaldehyde. LB27.4 cells (5×10^4) not pulsed with OVA were treated with or without drug for 4 h at 37°C, fixed, and then incubated with DO-11.10 and RF33.70 and the indicated concentration of T-OVA or CNBR-OVA. Cultures were incubated for 18 to 24 h at 37°C, after which 100 μl of supernatant was removed, frozen, and assayed for IL-2 content by measuring the incorporation of [^{3}H]thymidine by IL-2-requiring HT-2 cells. The results are representative of more than three separate experiments.

This result may indicate that some of the hypertonic pinosomes were not lysed and thus provided a source of OVA for class II restricted Ag presentation. Although this finding was unexpected, it provided a means to determine whether the drugs could simultaneously affect class I- and II-restricted Ag presentation. Surprisingly, both NH_4Cl and CH_3NH_2 inhibited the presentation of hypertonic-pulsed OVA to RF33.70 (Fig. 1*C*). Both drugs also inhibited the presentation of hypertonic-pulsed OVA to DO-11.10 (Fig. 1*D*). It should be noted that the data in Figure 1 cannot be used to compare the potencies of both drugs because their molar concentrations are not identical. No attempt was made to increase the concentration of CH_3NH_2 because of its effects at higher concentrations on the expression of class I MHC molecules (see below). We consistently observed that both drugs inhibited the presentation of hypertonic-pulsed OVA to RF33.70, although the level of inhibition in some experiments was somewhat less complete than that obtained for DO-11.10. Drug-treated and control APC that were not pulsed with OVA were equivalent in their ability to present a processing independent form of OVA to RF33.70 (Fig. 1*E*) and DO-11.10 (Fig. 1*F*). These results demonstrate that NH_4CL and CH_3NH_2 do not affect the presentation of processed Ag at the APC surface but rather inhibit an earlier event of Ag handling by the APC.

Figure 2 shows the results of a representative experiment in which the effects of chloroquine and NH_4Cl on

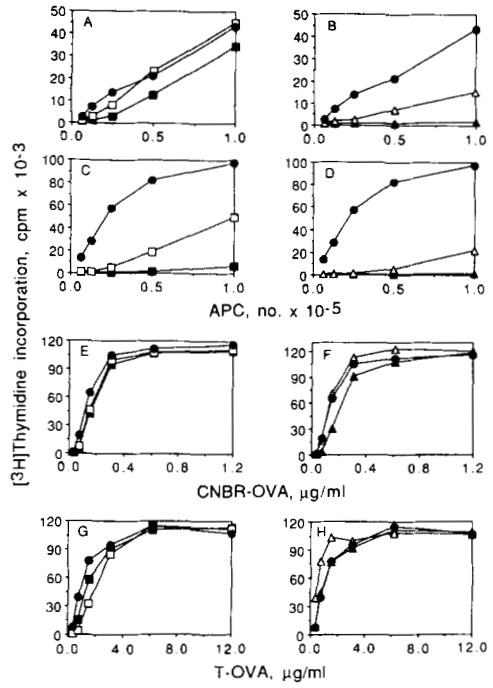

Figure 2. The effect of chloroquine and NH_4Cl on class I and class II MHC-restricted presentation of OVA. Duplicate microcultures (200 μl) were prepared with the T cell hybridomas (10^5 cells) RF33.70 (*A*, *B*, *E*, and *F*) or DO-11.10 (*C*, *D*, *G*, and *H*) and the indicated number of control (●), 10 μM (□) and 20 μM (■) chloroquine-treated, or 10 mM (△) and 20 mM (▲) NH_4Cl-treated, LB27.4 B lymphoblastoid cells. LB27.4 cells were either pulsed with 7.5 mg/ml OVA in hypertonic media (*A*, *B*, *C*, and *D*) or not pulsed with OVA (*E*, *F*, *G*, and *H*). Ag pulsing and drug incubations were performed as described in *Materials and Methods* after which time the APC were fixed with paraformaldehyde. LB27.4 cells (5×10^4) not pulsed with OVA were treated with or without drug for 4 h at 37°C, fixed, and then incubated with DO-11.10 and RF33.70 and the indicated concentration of T-OVA or CNBR-OVA. Cultures were incubated and assayed for IL-2 as described in the legend to Figure 1. The results are representative of three separate experiments.

class I- and II-restricted Ag presentation were determined simultaneously. A total of 10 μM chloroquine did not inhibit the presentation of hypertonic-pulsed OVA to RF33.70 (Fig. 2*A*). Minimal inhibition by 20 μM chloroquine (Fig. 2*A*) was observed in a majority but not all of our experiments and may be a result of its effects on the expression of class I molecules (see below). In contrast, both concentrations of chloroquine markedly inhibited the presentation of hypertonic-pulsed OVA to DO-11.10 (Fig. 2*C*). As previously demonstrated, NH_4Cl inhibits both class I (Fig. 2*B*) and class II- (Fig. 2*D*) restricted presentation of hypertonic-pulsed OVA. NH_4Cl and chloroquine treatment did not affect the ability of APC to present a processing independent form of OVA to RF33.70 (Fig. 2 *E* and *F*) and DO-11.10 (Fig. 2 *G* and *H*). This latter result demonstrates that both drugs do not affect the presentation of processed Ag at the APC surface. By comparing 10 μm chloroquine with 10 mM NH_4Cl and 20 μM chloroquine with 20 mM NH_4Cl, the results in Figure 2 show that, when both drugs have similar quantitative effects on class II-restricted Ag presentation, chloroquine minimally inhibits, if at all, class I-restricted Ag presentation when compared to NH_4Cl. These results suggest that neutralizing the pH of intracellular acidic compartments is not the mechanism by which NH_4Cl and CH_3NH_2 inhibit class I-restricted Ag presentation.

We next determined whether it was necessary to treat the APC with drug before pulsing with OVA to obtain inhibition of class I-restricted Ag presentation. For this purpose, APC were first subjected to the hypertonic/hypotonic pulsing procedure and then incubated in the presence or absence of NH_4Cl. When NH_4Cl was present only after Ag pulsing, it still caused marked inhibition of class I-restricted Ag presentation (Fig. 3*A*). Slightly more inhibition was observed when APC were also preincubated in NH_4Cl (Fig. 3*A*). It is likely that the intracellular accumulation of drug to an inhibitory level is time dependent. As previously demonstrated, drug treatment does not affect the presentation of processed Ag at the APC surface (Fig. 3*B*). Most of the pinocytic vesicles formed in the presence of hypertonic media are lysed during the 3-min incubation with hypotonic media (29). Therefore, the above results indicate that NH_4Cl inhibits a step in class I-restricted Ag presentation which follows and is distinct from the introduction of OVA into the cytoplasm.

Effect of weak base amines and protein synthesis inhibitors on regeneration of surface H-2K^b. It is conceivable that the presentation of OVA by the class I MHC molecule H-2K^b is affected because the synthesis of H-2K^b or its transport from the endoplasmic reticulum to

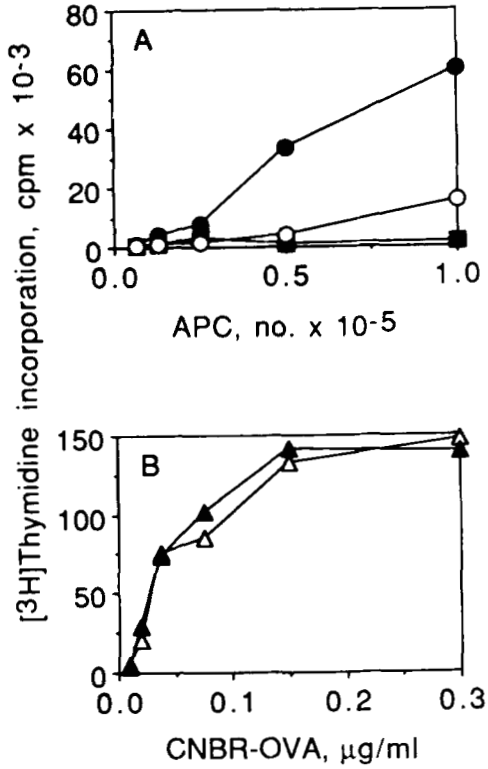

Figure 3. Treating APC with NH_4Cl after pulsing with OVA is sufficient to inhibit class I MHC-restricted Ag presentation. Duplicate microcultures (200 μl) were prepared with the T cell hybridoma RF33.70 (10^5 cells) and the indicated number of LB27.4 B lymphoblastoid cells pulsed with 7.5 mg/ml OVA in hypertonic media (*A*). LB27.4 cells pulsed with OVA were not treated with NH_4Cl (●), treated with 20 mM NH_4Cl after the hypertonic/hypotonic pulsing procedure (○), or treated with 20 mM NH_4Cl before and after the hypertonic/hypotonic pulsing procedure (■). After Ag pulsing, LB27.4 cells were incubated for 3 h at 37°C to allow for the expression of processed Ag on the cell surface. LB27.4 cells (5×10^4) not pulsed with OVA (*B*) were treated (▲) or not treated (△) with 20 mM NH_4Cl for 4 h at 37°C and then incubated with RF33.70 (10^5 cells) and the indicated concentration of CNBR-OVA. Drug treatment before and after Ag pulsing and pulsing with OVA in hypertonic media were performed as described in *Materials and Methods*. APC were fixed with paraformaldehyde before addition to culture and the cultures were incubated and assayed for IL-2 as described in the legend to Figure 1. The results are representative of three separate experiments.

the cell surface is inhibited. We therefore determined whether drug-treated APC could regenerate H-2K^b on the cell surface after removing the preexisting H-2K^b molecules. For this purpose, APC were treated with or without drug and subjected to the hypertonic/hypotonic pulsing procedure as previously described for pulsing with Ag. The pretreated APC were subsequently incubated with pronase and then stained for surface H-2K^b either immediately or after a 3-h incubation in the continued presence or absence of drug. Pronase treatment removed more than 90% of the H-2K^b molecules from the cell surface (Fig. 4 *A* and *C*, and data not shown). NH_4Cl-(Fig. 4*A*) and CH_3NH_2-(Fig. 4C) treated APC stained immediately after pronase treatment lost almost as much H-2K^b from their cell surface as control APC. Thus, the H-2K^b molecule on drug-treated cells was not refractory to the proteolytic activity of pronase. When the pronase-treated control cells were allowed to incubate at 37°C for 3 h, the level of H-2K^b expressed on the cell surface increased by a factor of 4 to 10-fold (Fig. 4 compare *A* with *E* and *C* with *G*). Typically, pronase-treated APC regenerated 25 to 50% of the steady-state level of H-2K^b over this time period. After the 3-h incubation period, APC treated with NH_4Cl (Fig. 4*E*) or CH_3NH_2 (Fig. 4*G*) regenerated the same amount of surface H-2K^b as the control APC. Higher concentrations of CH_3NH_2 inhibited the regeneration of H-2K^b and H-2D^b (data not shown). Non-specific binding of PE-streptavidin (Fig. 4 *B, D, F,* and *H*) for each cell group was low and did not account for the amount of fluorescence in the presence of specific antibody. We conclude from these results that the concentrations of NH_4Cl and CH_3NH_2 used to inhibit class I-restricted Ag presentation do not inhibit the synthesis, transport, or surface expression of the H-2K^b molecule. Identical results have also been obtained for the H-2D^b molecule (data not shown).

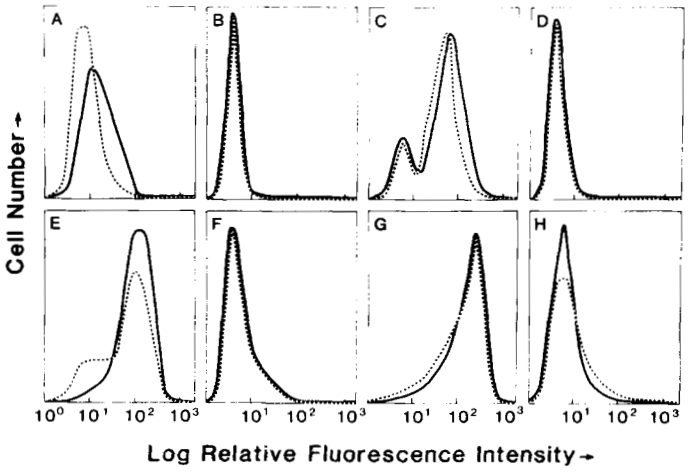

Figure 4. NH_4Cl and CH_3NH_2 do not inhibit the regeneration of surface H-2K^b. LB27.4 treated with or without drug and hypertonic medium as described in *Materials and Methods* were incubated with pronase (2 mg/ml) for 1 h at 37°C in the continued presence or absence of the appropriate drug. The cells were stained immediately after pronase treatment (*A, B, C,* and *D*) or after a 3-h incubation at 37°C (*E, F, G,* and *H*) in the continued presence or absence of the appropriate drug. APC treated with 20 mM NH_4Cl (*dark lines* in *A, B, E,* and *F*) or 5 mM CH_3NH_2 (*dark lines* in *C, D, G,* and *H*) and control APC (*dotted lines*) were analyzed by indirect immunofluorescence and flow fluorocytometry as described in *Materials and Methods*. APC were incubated with biotinylated-mAb AF6-88.5.3 (anti-K^b) and PE-streptavidin (*A, C, E,* and *G*) or PE-streptavidin alone (*B, D, F,* and *H*). Fluorescence was quantitated on a Becton Dickinson FACScan and the data are displayed as histograms of log relative fluorescence intensity (*x axis*) versus linear cell number (*y axis*). The results for NH_4Cl-treated and CH_3NH_2-treated APC were obtained in two separate experiments. NH_4Cl was present in the hypertonic and hypotonic medias for the NH_4Cl-treated cells whereas CH_3NH_2 was absent from the hypertonic and hypotonic medias for the CH_3NH_2-treated cells. The results for each drug treatment are representative of three separate experiments.

The effect of chloroquine on the regeneration of surface H-2K^b was also determined with the experimental procedure described above. APC incubated with 10 μM (Fig. 5*A*) or 20 μM (Fig. 5*C*) chloroquine and stained immediately after pronase treatment lost as much surface H-2K^b as control APC. After the 3-h incubation period, the level of H-2K^b on the surface of pronase-treated control cells increased by a factor of 10-fold (Fig. 5 compare *A* with *E* and *C* with *G*). The amount of surface H-2K^b regenerated in the presence of 10 μM (Fig. 5*E*) and 20 μM (Fig. 5*G*) chloroquine was 75 and 45%, respectively, of the control cells not exposed to this drug. Identical results have also been obtained for the H-2D^b molecule (data not shown). The degree to which 20 μM chloroquine inhibited the regeneration of surface H-2K^b could account for the small amount of inhibition of class I-restricted Ag presentation that we have observed with this concentration of drug. Non-specific binding of PE-streptavidin (Fig. 5 *B, D, F,* and *H*) for each cell group was low and did not account for the amount of fluorescence in the presence of specific antibody.

The protein synthesis inhibitors cycloheximide and ricin were used in the experimental procedure described above to determine whether the regeneration of surface H-2K^b requires de novo protein synthesis. APC incubated with 10^{-4} cycloheximide (Fig. 6*A*) or 2×10^{-9} ricin (Fig. 6*C*) and stained immediately after pronase treatment lost as much H-2K^b as control APC. After the 3-h incubation period, the level of H-2K^b on the surface of pronase-treated control cells increased by a factor of 10-fold (Fig. 6 compare *A* with *E* and *C* with *G*), whereas both protein synthesis inhibitors completely inhibited the regeneration of surface H-2K^b (Fig. 6 *E* and *G*). These results show that de novo protein synthesis is required for the regeneration of surface H-2K^b. Identical results have also been obtained for the H-2D^b molecule (data not shown).

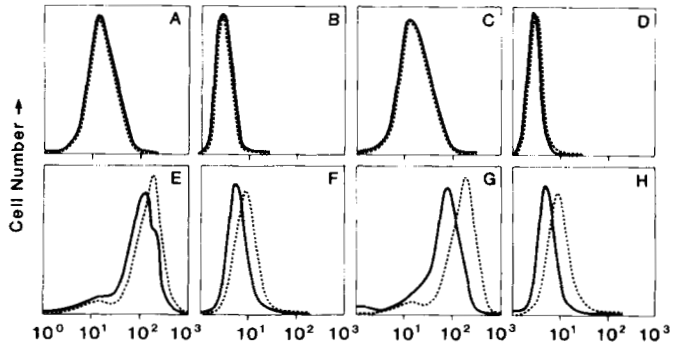

Figure 5. The effect of chloroquine on the regeneration of surface H-2K^b. LB27.4 cells treated with or without chloroquine and pronase were prepared as described in the legend to Figure 4. The cells were stained immediately after pronase treatment (*A, B, C,* and *D*) or after a 3-h incubation at 37°C (*E, F, G,* and *H*) in the continued presence or absence of chloroquine. APC treated with 10 μM (*dark lines* in *A, B, E,* and *F*) or 20 μm (*dark lines* in *C, D, G,* and *H*) chloroquine and control APC (*dotted lines*) were analyzed by indirect immunofluorescence and flow fluorocytometry as described in *Materials and Methods*. APC were incubated with biotinylated-mAb AF6-88.5.3 (anti-K^b) and PE-streptavidin (*A, C, E,* and *G*) or PE-streptavidin alone (*B, D, F,* and *H*). Fluorescence was quantitated and the data is displayed as described in the legend to Figure 4. The results are representative of three separate experiments.

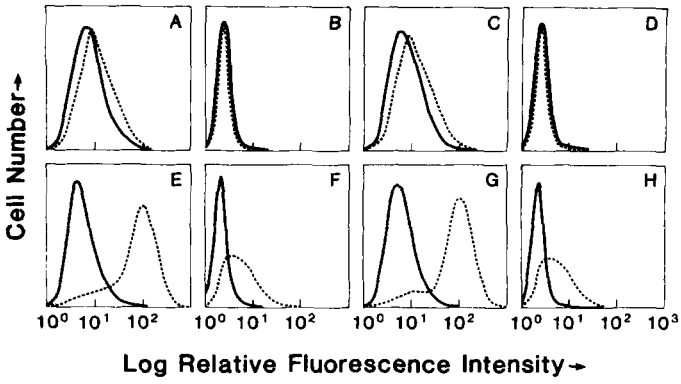

Figure 6. Regeneration of surface H-2K^b requires de novo protein synthesis. LB27.4 cells treated with or without protein synthesis inhibitors and pronase were prepared as described in the legend to Figure 4. The cells were stained immediately after pronase treatment (*A, B, C,* and *D*) or after a 3-h incubation at 37°C (*E, F, G,* and *H*) in the continued presence or absence of inhibitor. APC treated with 10^{-4} M cycloheximide (*dark lines* in *A, B, E,* and *F*) or 2×10^{-9} M ricin (*dark lines* in *C, D, G,* and *H*) and control APC (*dotted lines*) were analyzed by indirect immunofluorescence and flow fluorocytometry as described in *Materials and Methods.* APC were incubated with biotinylated-mAb AF6-88.5.3 (anti-K^b) and PE-streptavidin (*A, C, E,* and *G*) or PE-streptavidin alone (*B, D, F,* and *H*). Fluorescence was quantitated and the data are displayed as described in the legend to Figure 3. The results are representative of two separate experiments. The difference in background staining observed in the hisograms in *B* vs *F* and *D* vs *H* reflects a pronase-mediated decrease and subsequent regeneration in the background binding of PE-streptavidin to treated cells (see also Figs. 4 and 5); the latter regeneration requires de novo protein synthesis (see *F* and *H*). However, the magnitude of this effect is minor and does not significantly affect the measurement of class I MHC molecules on these treated cells.

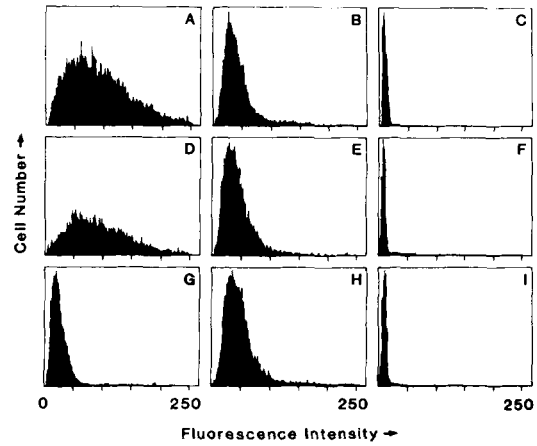

Figure 7. Expression of H-2D^b on RMA-S cells in the presence of NH_4Cl. RMA-S cells incubated in the presence or absence of 20 mM NH_4Cl for 1 h at 37°C were transferred to new media containing 20 mM NH_4Cl plus 100 μg/ml NP(1968) 365–380 (*A* to *C*), 100 μg/ml NP (1968) 365–380 alone (*D* to *F*), or 20 mM NH_4Cl plus DMSO (*G* to *I*) and incubated further for 20 h at 37°C. After the 20-h incubation, the cells were analyzed by indirect immunofluorescence and flow cytometry using the following mAb: 28.11.5S (anti-H-2D^b) (*A, D,* and *G*); AF6-88.5.3 (anti-H-2K^b) (*B, E,* and *H*); 7D4 (anti-IL-2R) (*C, F,* and *I*). RAMG conjugated to FITC was used as a second step reagent. Fluorescence was quantitated on a Becton Dickinson FACScan and the data are displayed as histograms of linear relative fluorescence intensity (*x axis*) vs linear cell number (*y axis*). The results are representative of three separate experiments.

Expression of H-2D^b on RMA-S cells in presence of NH_4Cl. The mutant cell line RMA-S is markedly deficient in its surface expression of H-2D^b and H-2K^b. The surface expression of H-2D^b can be increased by incubating the cells with antigenic peptides that are known to bind to H-2D^b (27). The original interpretation of this finding was that the exogenous peptides were transported to the endoplasmic reticulum where they induced the assembly of class I H and L chains. The assembled class I heterodimers could then be transported to the plasma membrane. However, subsequent studies with RMA-S cells suggest that exogenous peptides may increase class I expression by binding to and stabilizing class I heterodimers at the cell surface (30–32). Class I molecules expressed on the surface of RMA-S cells are conformationaly unstable presumeably because they lack peptide in the peptide binding groove. Regardless of the mechanism by which exogenous peptides increase class I expression, the RMA-S cells do provide a means for testing whether class I heterodimers are being transported from the endoplasmic reticulum to the cell surface. Therefore, we used RMA-S cells to determine whether NH_4Cl could inhibit the increased surface expression of H-2D^b. The NP(1968) 365–380 peptide was used to increase the expression of H-2D^b (27). RMA-S cells were first incubated in the presence or absence of NH_4Cl for 1 h at 37°C. The cells were then transferred to new media containing NH_4Cl plus NP(1968) 365–380 (Fig. 7 *A* to *C*), NP(1968) 365–380 alone (Fig. 7 *D* to *F*), or NH_4Cl plus DMSO (Fig. 7 *G* to *I*). After a 20-h incubation at 37°C, the cells were stained for the presence of surface H-2D^b and H-2K^b. RMA-S cells incubated with NH_4Cl but without NP(1968) 365–380 were used to determine the baseline levels of H-2D^b and H-2K^b. RMA-S cells incubated with NP(1968) 365–380 in the presence (Fig. 7*A*) or absence (*D*) of NH_4Cl had identical amounts of surface H-2D^b as detected by the mAb 28.11.5S. When compared to the baseline level of H-2D^b on RMA-S cells (Fig. 7*G*), incubation with the peptide increased the surface expression of H-2D^b by a factor of 2.5 to 10-fold. Binding of the isotype-matched control mAb 7D4 (Fig. 7 *C, F,* and *I*) was low and identical to the binding of FITC-RAMG alone (data not shown). This later result indicates that the binding of 28.11.5S was specific. The effect of NP(1968) 365–380 on the RMA-S cell was H-2 allele-specific since incubation with the peptide did not increase the expression of surface H-2K^b (Fig. 7 compare *B* and *E* with *H*). Taken together these results show that NH_4Cl does not inhibit the increased expression of H-2D^b on RMA-S induced by an antigenic peptide. However, the presentation of OVA by the wild type RMA cells to RF33.70 is inhibited in the same manner as described for LB27.4 cells (data not shown).

Uptake of OVA by APC. Inhibition of class I restricted Ag presentation might occur if NH_4Cl and CH_3NH_2 were interfering with the uptake of Ag. To test this possibility, the uptake of radiolabeled OVA was measured at 37 and 4°C in drug-treated and control APC. APC were incubated in hypertonic media containing ^{125}I-labeled OVA and cell-associated radioactivity was measured after extensive washing. The amount of cell-associated radioactivity with drug-treated APC at 37°C was higher than that associated with control APC at 37°C (Table I). There was no difference between drug-treated and control APC incubated at 4°C. Control and drug-treated cells incubated at 37°C had a two- to fourfold higher amount of cell-associated radioactivity than their respective counterparts incubated at 4°C. This temperature dependent effect is consistent with the presence of active membrane processes at 37°C since low temperature is an efficient inhibitor of endocytosis and pinocytosis (33). We conclude from these results that NH_4Cl and CH_3NH_2 do not inhibit the uptake of fluid-phase Ag during the hypertonic/hypotonic pulsing procedure.

TABLE I
Uptake of OVA by LB27.4 cells[a]

Expt.	Temperature (°C)	Drug Treatment	Cell-Associated ^{125}I-OVA cpm
1	37	NH_4Cl	1401[b]
	37		911
	4	NH_4Cl	325
	4		387
2	37	CH_3NH_2	1274
	37		890
	4	CH_3NH_2	497
	4		411

[a] Control, NH_4CL-treated, and CH_3NH_2-treated LB27.4 cells (1×10^7/group) were incubated at 37 or 4°C for 10 min in hypertonic media containing 2.5 μg of ^{125}I-OVA. The subsequent dilution with hypotonic media and cell washes were performed as described in *Materials and Methods*. The results for each drug treatment are representative of three separate experiments.
[b] All values are the mean of duplicate determinations.

DISCUSSION

This report describes the effects of NH_4Cl, CH_3NH_2, and chloroquine on class I and class II MHC-restricted Ag presentation. Osmotic lysis of hypertonic pinosomes provided a source of OVA for class I, H-2K^b, restricted Ag presentation. The hypertonic/hypotonic pulsing procedure also provided a source of OVA for class II-, I-A$^{d/b}$-, restricted Ag presentation. We do not know the mechanism by which OVA entered the class II-restricted pathway of Ag presentation, but it provided a means for examining the effects of inhibitors on both class I- and II-restricted Ag presentation under identical conditions of Ag pulsing.

Herein we present evidence that NH_4Cl and CH_3NH_2 but not chloroquine can markedly inhibit class I-restricted presentation of OVA. All three weak base amines inhibit class II-restricted presentation of OVA. Presentation of in vitro processed OVA by H-2K^b and I-A$^{d/b}$ on drug-treated APC and control APC was very similar if not identical. This latter result indicates that drug-treated APC were fully competent to present peptides in association with both class I and class II MHC molecules. Treating APC with NH_4Cl after Ag pulsing was sufficient to inhibit class I restricted Ag presentation. Furthermore, NH_4Cl and CH_3NH_2 did not inhibit the uptake of OVA during the hypertonic/hypotonic pulsing procedure, as evidenced by the amount of radiolabeled OVA associated with the drug-treated and control APC. These latter results indicate that NH_4Cl and CH_3NH_2 inhibit a step in class I-restricted presentation of OVA that follows and is distinct from the introduction of Ag into the cytoplasm. After proteolytic removal of H-2K^b from the APC surface, NH_4Cl- and CH_3NH_2-treated APC regenerated the same amount of surface H-2K^b as control APC. The protein synthesis inhibitors cycloheximide and ricin, however, did inhibit the regeneration of H-2K^b. Therefore, the regeneration of H-2K^b in our experimental system requires de novo protein synthesis. We conclude from the above results that the concentrations of NH_4Cl and CH_3NH_2 that inhibit Ag presentation do not affect the synthesis, transport, or surface expression of H-2K^b. This conclusion is also supported by the observation that NH_4Cl did not interfere with the transport of a second class I MHC molecule, H-2D^b, to the surface of RMA-S cells that were incubated with NP peptide. Taken together these results strongly suggest that NH_4Cl and CH_3NH_2 but not chloroquine affect class I-restricted Ag presentation by selectively interfering with a critical intracellular step in Ag processing or transport of the antigenic peptide.

A considerable part of the cellular uptake of weak base amines (i.e., NH_4Cl and CH_3NH_2) probably occurs through simple diffusion of the unprotonated amine across the plasma membrane (34). These weak base amines are lysosomotropic in the sense that they selectively accumulate in the lysosome and other acidic compartments. Accumulation in the lysosome occurs because the unprotonated amine is converted to the protonated form by the initially low pH and the continuous pumping of protons. Thus, the active (unprotonated) form of the weak base amine is simultaneously present in the cytoplasm and acidic compartments. High amine concentrations rapidly decrease the concentration of free protons and elevate the lysosomal pH to a new equilibrium. Although elevating the pH of acidic compartments is a direct effect of weak base amines, other cellular processes can also be affected (34).

For characterizing the events of class I MHC-restricted Ag presentation, there are several features of our experimental system that provide advantages over the use of CTL with cytolytic assays. First, the production of IL-2 by T cell hybridomas can be measured by a sensitive and quantitative assay. These T cell hybridomas provide an efficient means for detecting the presence of Ag-MHC complexes on the APC surface. Second, before incubation with the T cell hybridomas, the APC can be fixed with paraformaldehyde to prevent subsequent recovery and/or further Ag processing. This is a distinct advantage in drug/inhibitor studies because the T cell hybridomas are never exposed to drug and the APC can be exposed to the highest possible concentration of drug during the whole period of Ag processing. In contrast, CTL generally require intact APC for Ag-specific activation and, therefore, drugs may need to be continually present in the T cell-APC coculture. Secondary effects of a drug on T cell function may limit the concentration of a drug that can be used. Third, our system does not use an endogenously synthesized protein as a source of Ag for class I-restricted Ag presentation. In viral-CTL systems, pharmacologic agents could potentially inhibit viral replication and/or synthesis of viral antigens. Also, the amount of viral Ag that is produced is difficult to control and it is possible that sufficient Ag could be produced to overcome any inhibitory effects of a particular drug.

Our observation that chloroquine does not inhibit class I-restricted Ag presentation is consistent with the results of previous investigators and with the idea that class I and II MHC-restricted Ag processing occur in different intracellular compartments (16–18). We are not aware of any studies that have analyzed the effect of CH_3NH_2 and only one report that has analyzed the effect of NH_4Cl on class I-restricted Ag presentation. In this latter case, NH_4Cl inhibited the sensitization of target cells for recognition by class I-restricted anti-influenza CTL (19). By neutralizing the endosomal pH, NH_4Cl inhibited the fusion of viral and cellular membranes and prevented the entry of viral Ag into the cytoplasm. Therefore, it was difficult to determine whether NH_4Cl was also inhibiting the processing of viral Ag for class I-restricted Ag presentation.

Our results do not define the mechanism by which NH_4Cl and CH_3NH_2 inhibit class I MHC-restricted pres-

entation of OVA. We have shown that these weak base amines do not inhibit the synthesis, transport, or expression of H-2K^b or the uptake of OVA. Inasmuch as chloroquine has little if any effect on class I-restricted Ag presentation although inhibiting class II-restricted Ag presentation to the same degree as NH_4Cl, there is a distinct possibility that NH_4Cl and CH_3NH_2 inhibit class I-restricted Ag presentation through a nonlysosomotropic effect. In this context it is interesting to note that NH_4Cl and CH_3NH_2 as aliphatic alkylamines are structurally very different than chloroquine that is an aromatic alkylamine. The identification of inhibitors, such as NH_4Cl and CH_3NH_2, should provide useful reagents for the study of class I MHC-restricted Ag presentation.

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