is expected. In contrast, in protein association reactions (insulin dimerization, for example), such a polar to nonpolar mutation can play an important role due to solvation effects; an example is given by sickle cell hemoglobin [Glu A3(6)β→Val], where the mutated residue is exposed in the monomer and partly buried in the dimer (*44*) so that the complex is calculated to be stabilized about 15 kcal/mole per interface (*45, 46*).

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26. The molecules were partitioned into MD and Langevin regions with radii of 10 and 15 Å, respectively; the regions were centered on the center-of-mass coordinates of the Asp G1(99)β side chain in the two x-ray structures. The space inside the sphere was filled with water by overlaying a previously equilibrated box of TIP3P water molecules (*27*); the radius for the water sphere was taken to be 14 Å, slightly smaller than the protein sphere, to prevent water molecules from penetrating into the protein core at the boundary region. Water molecules within 2.5 Å of any protein atom were removed from the system. After a 5-ps equilibration of the water structure in the presence of the fixed protein molecule, a second TIP3P overlay was made to fill any voids in the solvent. The calculation of $\langle \Delta V \rangle_\lambda$ for a given λ consisted of a 5-ps equilibration (except for the initial and final integration points, λ = 0.1 and 0.9, where 10-ps equilibrations were used), followed by an averaging period of 5 ps; the coordinates from every fifth time-step were used in the evaluation of $\langle \Delta V \rangle_\lambda$. The simulations were done at 25°C. On a Cray XMP/48, a 5-ps simulation required about 40 min of CPU time with a 1-fs integration step. The variation of $\langle \Delta V \rangle_{\lambda_i}$ with λ_i was monotonic (*28*) and approximately linear. Calculations that used the exponential formula (*4–7*) for ΔG give almost identical values; examination of 1-ps intervals in the 5-ps averages and test studies with smaller λ intervals suggest that the standard deviations of the integrations are ±2 kcal/mole, corresponding to those of other free energy simulations (*4–6*).
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42. Neglect of the internal energy contributions to the free energy difference, as was done, for example, in (*6*), yields a calculated ΔΔG value of −3.3 kcal/mole, in even better agreement with the measured value (−3.4 kcal/mole).
43. Although statistical error estimates for free energy simulation can be made, it is not yet possible to determine the systematic errors; see C. L. Brooks III *et al.*, *Proteins: A Theoretical Perspective of Dynamics, Structure and Thermodynamics* (Wiley, New York, 1988).
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Brefeldin A Specifically Inhibits Presentation of Protein Antigens to Cytotoxic T Lymphocytes

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Cytotoxic T lymphocytes (CTLs) recognize foreign antigens, including viral proteins, in association with major histocompatibility complex (MHC) class I molecules. Brefeldin A, a specific inhibitor of exocytosis, completely and reversibly inhibited the presentation of viral proteins, but not exogenous peptides, to MHC class I–restricted CTLs directed against influenza virus antigens. The effect of brefeldin A on antigen presentation correlated with its inhibition of intracellular transport of newly synthesized class I molecules. Brefeldin A is thus a specific inhibitor of antigen processing for class I–restricted T cell recognition. Its effect on antigen presentation supports the idea that exogenous peptide antigens associate with cell surface class I molecules, whereas protein antigens processed via the cytosolic route associate with nascent class I molecules before they leave the trans-Golgi complex.

CYTOTOXIC T LYMPHOCYTES (CTLs) are a critical component of the immune response to infectious agents and neoplasms. Some of the general features of CTL recognition of foreign antigens are understood: (i) epitopes recognized by CTLs are linear determinants that can be replaced by short synthetic peptides (*1*); (ii) nonfragmented proteins must be delivered to the cytosol of target cells to be processed for presentation to CTLs (*2, 3*); and (iii) foreign proteins are recognized by CTLs only in association with class I molecules of the major histocompatibility complex (MHC) (*4*).

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MHC class I molecules are typical integral membrane proteins that are co-translationally translocated into the endoplasmic reticulum (ER) and subsequently transported to the plasma membrane via the Golgi complex and post-Golgi transport vesicles. Understanding how antigens are processed by cells for CTL recognition requires the identification of the subcellular compartment in which the interaction of class I molecules with antigens occurs. We demonstrated earlier that sensitization of target cells with noninfectious influenza virus occurs in the presence of protein synthesis inhibitors (*2*). This indicates that antigen association does not occur co-translationally with class I biosynthesis. We have now used the compound brefeldin A to investigate whether the class I–antigen complex is formed during the intracellular transport of newly synthesized class I molecules between the ER and the cell surface. Unlike other inhibitors of intracellular transport that affect many other cellular functions (for example, monensin), brefeldin A is thought to specifically inhibit exocytosis of integral membrane and secretory proteins. It was originally proposed that brefeldin A blocks the egress of proteins from the ER (*5*). Recent work confirmed this idea and further demonstrated that brefeldin A causes disassembly of the Golgi complex and the redistribution of resident and itinerant medial- and cis-Golgi complex proteins to the ER (*6*).

We examined the effect of brefeldin A on the presentation of influenza virus antigens to mouse H-2^k–restricted anti-influenza virus CTLs. These CTLs recognize four viral gene products biosynthesized by influenza virus–infected L929 cells (H-2^k): hemagglutinin (HA), nucleoprotein (NP), basic polymerase 1 (PB1), and nonstructural 1 protein (NS1) (*7*). HA, NP, and PB1 can also be processed for CTL recognition from noninfectious virus (*2, 8*).

In preliminary experiments, we showed that brefeldin A completely blocked the surface expression of newly synthesized K^k and D^k class I molecules in L929 cells. Continuous incubation of influenza virus–infected L929 cells with brefeldin A completely inhibited the presentation of viral antigens to CTLs specific for NP, PB1, HA, and NS1 (Table 1, experiment A). This effect is not due to brefeldin A inhibition of viral entry or protein synthesis, since (i) the same target cells were heavily stained in an indirect immunoperoxidase assay in which monoclonal antibodies specific for HA, NP, or NS1 were used; and (ii) removal of brefeldin A during the CTL assay and concomitant addition of cycloheximide (15 μg/ml) to prevent additional viral protein synthesis resulted in the complete recovery of CTL recognition. Furthermore, brefeldin A also inhibited the presentation of HA, NP, and PB1 proteins derived from noninfectious virus (Table 1, experiment B).

The effect of brefeldin A on CTL recognition cannot be attributed to inhibition of either CTL function or target cell susceptibility to CTL-mediated lysis, since specific release values were not diminished by including brefeldin A during the coincubation of CTLs and non-brefeldin A–treated target cells. This finding suggested that brefeldin A does not affect the presentation of previously existing class I molecule–antigen complexes. In keeping with this conclusion, when transfected L929 cells constitutively expressing the HA gene were incubated with brefeldin A for 6 hours, their recognition by HA-specific CTLs was unaltered. An additional experiment further demonstrates the specificity of brefeldin A for nascent determinants; brefeldin A inhibited the recognition of influenza virus–infected HA-transfected L929 cells by CTLs specific for NP, PB1, and NS1, but did not inhibit recognition by CTLs specific for HA (Table 1, experiment C).

The brefeldin A blockade was reversible, since antigen presentation was inhibited only if brefeldin A was present during the coincubation of CTLs with target cells. In addition, we found that the brefeldin A blockade can be bypassed rapidly at 37°C; incubation of virus-infected cells for just 7.5 min without brefeldin A partially restored antigen presentation, and complete recovery was obtained after 15 to 30 min of incubation without brefeldin A. Recovery of antigen presentation after the withdrawal of brefeldin A was temperature-dependent: a sharp break in recovery occurred between 20°C and 25°C when brefeldin A was removed for 90 min from noninfectious virus-sensitized cells (Fig. 1). Using L929 cells infected with a recombinant vaccinia virus containing the cloned H-$2K^d$ gene, we found that reversal of the brefeldin A blockade of K^d transport to the cell surface had an

Table 1. Effect of brefeldin A on presentation of antigens to CTLs. L929 cells were sensitized with infectious or noninfectious neuraminidase-inactivated A/Puerto Rico/8/34 (PR8) (H1N1) influenza virus for 3 to 5 hours at 37°C and labeled with $Na^{51}CrO_4$ for 1 hour at 37°C as described (*2*). Brefeldin A–treated cells were incubated continuously with the drug at 3 to 6 μg/ml before the ^{51}Cr release assay and at 0.2 to 0.5 μg/ml during the assay (*17*). For peptide sensitization, virus-infected or uninfected cells were incubated for 135 min at 37°C, with and without brefeldin A, and suspended in 100 μl of Iscove's modified Dulbecco's modified Eagle's medium supplemented with 7.5% fetal bovine serum (IDMEM+) and the NP(50–63) peptide at 100 μg/ml. After 1 hour of incubation at 37°C, 100 μCi of $Na^{51}CrO_4$ was added, and cells were incubated an additional hour, washed, and used as target cells in the ^{51}Cr release assay. An L929 cell line constitutively expressing the PR8 HA gene was produced by infection with a defective retrovirus containing the PR8 HA gene (*18*). Transfected cells were infected with PR8 in exactly the same manner as nontransfected cells. Effector cells were splenocytes from CBA mice, primed with vaccinia virus recombinants containing cloned PR8 genes encoding HA, NP, PB1, or NS1, stimulated in vitro for 6 days with autologous PR8-infected splenocytes. Splenocytes derived from recombinant vaccinia virus–primed mice stimulated in this manner demonstrate exclusive anti-influenza CTL specificity for the influenza gene product expressed by the vaccinia virus recombinant. Effector cells were incubated for 4 hours at 37°C with target cells at ratios ranging from 3:1 to 60:1, and the amount of ^{51}Cr released into the supernatant was determined by gamma counting. Specific ^{51}Cr release is defined as experimental release minus spontaneous release (no CTL) divided by total release (in the presence of detergent) minus spontaneous release. In experiments A and B, background specific release values obtained with noninfected cells were subtracted from all values. All three experiments were performed with effector-to-target cell ratios comprising a 27-fold range. Similar results were obtained at all effector-to-target cell ratios. For each of the experimental conditions, similar results were obtained in at least two independent experiments.

L929 target cells	Brefeldin A	Percent specific ^{51}Cr release by CTLs specific for			
		NP	PB1	HA	NS1
		Experiment A			
Infectious virus	−	67	70	64	70
Infectious virus	+	2	0	0	1
NP peptide	−	53	0		
NP peptide	+	54	0		
Infectious virus plus NP peptide	+	58	2		
		Experiment B			
Noninfectious virus	−	43	76	53	0
Noninfectious virus	+	3	1	3	0
		Experiment C			
HA-transfected	−	7	2	67	5
HA-transfected plus infectious virus	−	35	66	66	47
HA-transfected plus infectious virus	+	17	7	67	9

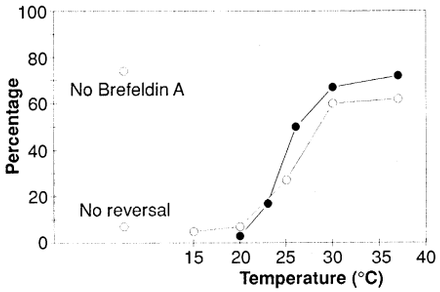

Fig. 1. Temperature dependence of brefeldin A reversal. Cells sensitized with noninfectious neuraminidase-inactivated influenza virus (*2*) and labeled with $Na^{51}CrO_4$ in the continuous presence of brefeldin A (3 μg/ml) were washed twice and suspended in IDMEM+. After a 90-min incubation at various temperatures, cells were suspended in IDMEM+ containing brefeldin A at 0.4 μl/ml and incubated for 1 hour at room temperature. Cells were then tested in a ^{51}Cr release assay with secondary PB1-specific CTLs (stimulated in vitro) at a ratio of 30:1 in the presence of brefeldin A at 0.2 μg/ml. Cells were continuously incubated with protein synthesis inhibitors to prevent the expression of noninactivated viral genes during their preparation and during the ^{51}Cr release assay. Similar results were obtained (i) in the same experiment with effector cells specific for other viral components and (ii) in two additional experiments. To examine the surface expression of class I molecules, we infected L929 cells with a recombinant vaccinia virus containing the K^d gene (*19*) at a multiplicity of 10 and incubated them for 5.5 hours at 37°C in the presence of brefeldin A at 6 μg/ml. Cells were washed and incubated for 1 hour at various temperatures with anisomycin (26 μg/ml) to prevent additional protein synthesis. Cells were then incubated for an additional hour at 37°C with brefeldin A (6 μg/ml) and anisomycin (26 μg/ml) to allow for surface expression of intracellular K^d that bypassed the brefeldin blockade. The cell surface expression of K^d was determined by indirect immunofluorescence with monoclonal antibody HB-159 (American Type Culture Collection) and fluorescein-conjugated rabbit antibody to mouse immunoglobulin. Similar results were obtained in four additional experiments. (●) K^d-positive surface fluorescence; (○) specific ^{51}Cr release.

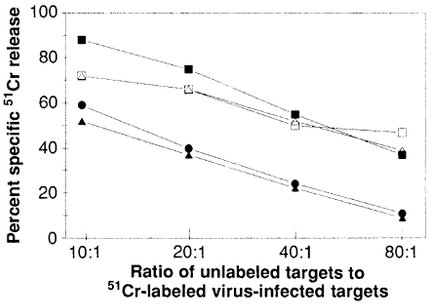

Fig. 2. Presentation of NP peptide by paraformaldehyde-fixed L929 cells. (△) Paraformaldehyde-fixed cells pulsed with peptide, (○) unfixed cells pulsed with peptide, and (□) paraformaldehyde-fixed cells without peptide. Filled symbols, anti-NP CTLs; empty symbols, anti-PB1 CTLs. L929 cells were fixed with 0.5% freshly prepared paraformaldehyde for 15 min at room temperature and washed extensively (*20*). Fixed and unfixed cells were incubated with NP(50–63) peptide (100 μg/ml) for 3 hours at 37°C and washed. Cells were added at various concentrations to wells containing NP- or PB1-specific secondary CTL populations and ^{51}Cr-labeled influenza virus–infected cells (effector-to-target ratio 9:1). A standard 4-hour ^{51}Cr release assay was performed. All experimental values represent the average of triplicates; all data points had a standard error of the mean of less than 4% specific release. Similar results were obtained with cells at an effector-to-target ratio of 3:1.

identical temperature dependence (Fig. 1). Similar findings were made regarding the cell surface expression of the two influenza virus glycoproteins, HA and neuraminidase, synthesized in the presence of brefeldin A. These data are consistent with the possibility, but of course do not prove, that brefeldin A blocks antigen presentation through its effect on the exocytosis of newly synthesized integral membrane proteins.

In contrast to nonfragmented proteins, which must be deliberately introduced into the cytoplasm of antigen presenting cells in order to sensitize the cells for CTL recognition, target cells are sensitized with oligopeptides merely by co-incubation. It was therefore of interest to determine whether brefeldin A affected CTL recognition of cells sensitized with a synthetic peptide corresponding to residues 50 to 63 of NP, which constitutes an epitope recognized by H-2^k–restricted, NP-specific CTLs (*9*). Preincubation of L929 cells with brefeldin A for 2 hours did not diminish the ability of this peptide to sensitize cells for lysis by NP-specific CTLs (Table 1, experiment A) (*10*). To exclude the possibility that the discrepancy between the effect of brefeldin A on virus-infected versus peptide-sensitized cells was due to virus-induced alterations in the target cells, we added the NP peptide to virus-infected cells that had been incubated continuously for 2 hours with brefeldin A. These cells were lysed by NP-specific CTLs at levels identical to those obtained with untreated peptide-sensitized target cells, whereas CTL recognition of the other viral proteins remained at background levels (Table 1, experiment A).

The ability of peptide to bypass the brefeldin A blockade suggested that the peptide associates with class I molecules at the cell surface. To test this idea, we examined the ability of metabolically inert paraformaldehyde-fixed cells to present the NP peptide to CTLs. We found no difference between efficiencies of live and paraformaldehyde-fixed cells to inhibit recognition of ^{51}Cr-labeled virus-infected cells by NP-specific CTLs when used as unlabeled inhibitors (Fig. 2). The specificity of inhibition mediated by fixed peptide-sensitized cells is demonstrated in two ways: (i) fixed cells not incubated with peptide failed to specifically inhibit recognition by anti-NP CTLs; and (ii) fixed cells sensitized with peptide failed to specifically inhibit recognition by CTLs directed against PB1.

We also found that the effects of brefeldin A were identical when influenza virus proteins and a different NP peptide were presented to H-2^d–restricted anti-influenza CTLs. The effects of brefeldin A are not limited to the presentation of influenza virus antigens, since presentation of vaccinia virus antigens to H-2^k– and H-2^d–restricted vaccinia-specific CTLs was also completely inhibited by brefeldin A.

Thus, brefeldin A specifically inhibits the processing of antigens for class I–restricted T cell recognition. This is important because (i) brefeldin A can be used to determine whether presentation of a given antigen requires processing via the cytosolic route; (ii) brefeldin A can be used to identify and characterize other agents that inhibit antigen processing; and (iii) the action of brefeldin A demonstrates that protein antigens and exogenous peptides are presented to CTLs by fundamentally different processes.

Understanding the differential effects of brefeldin A on the presentation of protein versus exogenous peptide antigens requires identifying the most distal compartment in the exocytic pathway affected by the drug. In parallel studies, we investigated the effect of brefeldin A on the transport of HA in influenza virus–infected L929 cells. The biosynthesis, assembly, and transport of the HA have been extensively characterized (*11*). In agreement with reported effects of brefeldin A on exocytosis, HA synthesized in the continuous presence of brefeldin A is arrested in the ER. Brefeldin A also blocks exocytosis of HA transported to the trans-Golgi complex in the absence of brefeldin A. Brefeldin A does not, however, appear to block exocytosis of HA that has reached an intracellular post–trans-Golgi compartment (*12*). These findings place the most distal block of exocytosis mediated by brefeldin A at the trans-Golgi complex.

Accordingly, the failure of brefeldin A to affect the presentation of exogenous peptides to CTLs indicates that peptides can associate with class I molecules after they have exited the trans-Golgi complex. Transport of H-2^k class I molecules between the Golgi complex and the cell surface is believed to occur rapidly, with a half-time on the order of 5 to 10 min (*13*). The fact that incubation of cells for 5 hours with brefeldin A does not affect the presentation of peptides suggests that exogenous peptides associate with class I molecules present at the cell surface. This conclusion is supported by the

ability of fixed cells to present peptides to CTLs and complements the extensive findings regarding the association of antigenic peptides with class II molecules (*14*).

By contrast, the inhibitory effect of brefeldin A on the presentation of viral proteins suggests that antigens processed by the cytosolic route associate with newly synthesized class I molecules prior to their exit from the trans-Golgi complex. To establish this point more firmly, it will be necessary to examine whether brefeldin A inhibits other processes potentially involved in antigen presentation, such as proteolysis, translocation of antigen into an exocytic compartment, and exocytosis of antigen not mediated by class I molecules. The idea that processed antigens associate with newly synthesized class I molecules is attractive, however, as it is consistent with (i) the parallel effects of brefeldin A on class I molecule transport and antigen presentation in the temperature reversal experiments, (ii) the inhibition of antigen presentation mediated by adenovirus E3/19K glycoprotein, which specifically interacts with newly synthesized class I molecules and blocks their intracellular transport (*15*), and (iii) our recent findings that prolonged incubation of cells with protein synthesis inhibitors (4 to 8 hours) prior to their sensitization with noninfectious influenza virus inhibits the K^k-associated presentation of HA and NP [but not NP(50–63) peptide] without affecting the amount of K^k present on the cell surface (*16*).

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Involvement of a Leukocyte Adhesion Receptor (LFA-1) in HIV-Induced Syncytium Formation

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Cell fusion (syncytium formation) is a major cytopathic effect of infection by human immunodeficiency virus (HIV) and may also represent an important mechanism of $CD4^+$ T-cell depletion in individuals infected with HIV. Syncytium formation requires the interaction of CD4 on the surface of uninfected cells with HIV envelope glycoprotein gp120 expressed on HIV-infected cells. However, several observations suggest that molecules other than CD4 play a role in HIV-induced cell fusion. The leukocyte adhesion receptor LFA-1 is involved in a broad range of leukocyte interactions mediated by diverse receptor-ligand systems including CD4–class II major histocompatibility complex (MHC) molecules. Possible mimicry of class II MHC molecules by gp120 in its interaction with CD4 prompted an examination of the role of LFA-1 in HIV-induced cell fusion. A monoclonal antibody against LFA-1 completely inhibited HIV-induced syncytium formation. The antibody did not block binding of gp120 to CD4. This demonstrates that a molecule other than CD4 is also involved in cell fusion mediated by HIV.

HUMAN IMMUNODEFICIENCY VIRUS (HIV) is the etiologic agent of acquired immunodeficiency syndrome (AIDS), a fatal disease characterized by profound immunosuppression, opportunistic infections, and neuropathies (*1*). Although only a small fraction of circulating lymphocytes are infected with the virus (*2*), there is a marked loss of T cells bearing the virus receptor CD4 (*3, 4*). The depletion of $CD4^+$ T cells appears to contribute significantly to the immunosuppression associated with AIDS. Syncytium formation resulting from HIV-induced cell fusion has been shown to be the primary cytopathic effect of the virus in vitro (*5*) and has been postulated to account for the loss of $CD4^+$ T cells in vivo (*6–8*).

CD4, through its interaction with the HIV envelope glycoprotein gp120, plays an important role in syncytium formation (*6, 9–12*). However, several observations suggest that molecules on the surface of uninfected cells other than CD4 are also involved in HIV-induced cell fusion. First, fusion of HIV-infected cells to uninfected cells does not correlate with CD4 density on the surface of the uninfected cells (*13, 14*). In addition, whereas transfection of nonlymphoid human cells with CD4 renders such cells capable of fusion to HIV-infected cells, this is not true for CD4-transfected mouse cells (*15*). Finally, there is a disparity in the capacity of sera from AIDS patients to block binding of HIV particles to $CD4^+$ cells and the capacity of the same sera to block fusion of HIV-infected cells to $CD4^+$ uninfected cells (*11*).

CD4 interacts directly with class II major histocompatibility complex (MHC) molecules in class II MHC–restricted T helper cell responses (*16, 17*). The involvement of the leukocyte adhesion receptor (LAR) LFA-1 in such responses has been demonstrated with monoclonal antibodies (MAbs)

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