

Inactivated Influenza Virus, when Presented on Dendritic Cells, Elicits Human CD8⁺ Cytolytic T Cell Responses

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Summary

Inactivated or subunit virus preparations have been excellent vaccines for inducing antibody responses. Generation of cytolytic T cell responses, however, is thought to require replicating virus, primarily to provide sufficiently large amounts of cytoplasmic proteins for processing and presentation on major histocompatibility complex class I molecules by antigen-presenting cells. Potent human CD8⁺ cytolytic T cell responses to live replicating influenza A virus are generated when dendritic cells are used as the antigen-presenting cells. Here, we demonstrate that dendritic cells pulsed with poorly replicating, heat- or ultraviolet-inactivated influenza virus, induce equally strong CD8⁺ cytolytic T lymphocyte responses. The cytotoxic T lymphocytes are generated in the apparent absence of CD4⁺ helper cells or exogenous cytokines. Active viral protein synthesis is not required to charge class I molecules on dendritic cells. When pulsed with inactivated virus, <1% of dendritic cells express nonstructural protein 1, which is only synthesized in the infectious cycle. To be optimally effective, however, the inactivated virus must retain its fusogenic activity, and presumably access the cytoplasm of dendritic cells. The data indicate, therefore, that dendritic cells require only small amounts of viral protein to charge class I molecules, most likely via traditional class I processing pathways. These results reopen the potential use of inactivated virus preparations as immunogens for cytotoxic T lymphocyte responses.

CD8⁺ CTLs are considered to be important mediators of resistance against malignancies (1) and infections (2–7). CTLs recognize antigens that are synthesized within the cytoplasm of an APC, e.g., during viral infection (8) or that access the cytoplasm by other means, e.g., via liposomes (9, 10). After specific proteolytic steps, peptides from antigenic proteins are conveyed to the rough endoplasmic reticulum under the influence of the TAP gene products, where they bind to newly synthesized MHC class I molecules (11). The MHC class I-peptide complexes are then transported to the cell surface where only small amounts (~200) are sufficient to be recognized by antigen-specific CTLs (12). There is evidence to suggest that the processing of proteins to form MHC class I-peptide complexes is quantitatively inefficient. Target cells infected with UV-inactivated influenza virus, which accesses the cytoplasm but does not replicate, are not lysed by active CTLs (8, 13–15). Instead, active viral protein synthesis seems necessary for the requisite small number of MHC-peptide complexes to form. These findings apply to fully formed or effector CTLs, but a similar inefficiency seems to apply during the

generation of CTLs from quiescent CD8⁺ precursors. Repeated stimulation with antigen and exogenous addition of IL-2 are often required to produce effector CTLs (3, 16–18). As a result, inactivated or nonreplicating virus vaccines have not been used to generate CTL-mediated immunity.

The immune system, however, does have a specialized pathway to elicit CTLs with great efficiency. When antigen is presented on dendritic cells, CTLs recognizing transplantation antigens, tumor antigens, H-Y, and viral antigens, are proficiently generated (for review, see reference 19). In all instances, dendritic cells are more potent in eliciting the CTL responses than other APCs. In a recent example, we have demonstrated that human dendritic cells elicit vigorous CTL responses to influenza A virus from primed donors (20). The responses can be elicited with a few dendritic cells (APC/T cell ratio of 1:50–1:200), and develop in the absence of CD4⁺ helper T cells. Whereas both dendritic cells and monocytes can be infected with influenza virus, only the former serve as effective APCs for CTL induction. The latter can act as targets for the CTLs once induced. This proficiency to induce CTLs on the part of dendritic cells has been ascribed to several features. Influenza virus infects most dendritic cells from human blood. The infection appears to be nonproductive and nontoxic since the cells are not lysed, and the cells remain viable for

A preliminary report of this work was presented in abstract form at the Keystone Symposium on Dendritic Cells in Taos, NM, 11–15 March 1995.

>2 d. Thus influenza virus serves to target dendritic cell class I molecules for long periods. Nevertheless, the efficacy with which dendritic cells process proteins onto MHC class I molecules is unclear, i.e., how much native antigen must be taken up by dendritic cells for their specialized presenting activities to become manifest? Here we report that dendritic cells successfully present nonreplicating influenza virus to CD8⁺ T cells. For optimal CTL induction, the nonreplicating virus needs to access the cytoplasm, implying that dendritic cells efficiently process proteins for presentation on MHC class I molecules. These results reopen the possible use of nonreplicating or inactivated virus as an immunogen for generating CTLs, as long as the antigen is presented by dendritic cells.

Materials and Methods

Culture Medium

RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 20 µg/ml gentamicin, 5% human serum, and 10 mM Hepes buffer was used.

Blood Mononuclear Cells

PBMCs were obtained from buffy coats by density gradient centrifugation over Ficoll Hypaque and separated into T cell-enriched and T cell-depleted (ER⁻)¹ fractions by sheep erythrocyte rosetting. T cells were purified by removal of monocytes, NK cells, and MHC class II⁺ cells as previously described (20). T cell subsets (CD4⁺ and CD8⁺) were prepared by incubation with Leu 2 or Leu 3 mAbs, followed by panning onto plastic plates coated with goat anti-mouse IgG. Alternatively, T cells were sorted into CD4⁺ and CD8⁺ subsets on a FACSORTER[®] after staining with Simultest CD4-FITC/CD8-PE-conjugated mAbs (Becton Dickinson and Co., Mountain View, CA). Monocytes were obtained from ER⁻ cells by plastic adherence. Nonadherent ER⁻ cells were depleted of residual monocytes by panning on γ-globulin-coated dishes. The remaining cells (primarily B cells and dendritic cells) were adequately enriched for dendritic cells to induce strong CTL responses. B cells are not infected with influenza A virus and therefore do not elicit CTL responses (20). Highly purified dendritic cells were obtained as low density cells by metrizamide gradient centrifugation, as previously described (20).

Virus Preparation

Influenza A virus (PR8, Puerto Rico/8/34, source: allantoic fluid; Spafas Inc., Storrs, CT) was either live, inactivated at 56°C for 30 min in a water bath, or exposed on ice to shortwave UV radiation at 254 nm (Mineralight UV lamp, UVGL58; Ultra-Violet Products, San Gabriel, CA) for 40 min at a distance of 4 cm. Influenza virus purified on sucrose density gradients (Spafas Inc.) was also used in some experiments.

Assays for Viral Infectivity, Titer, and Fusogenic Capacity

Infectivity. A modified plaque assay was performed using trypsin-resistant Madin-Darby canine kidney-II cells (21). Cells were

grown in six-well plates to monolayer density, washed once with RPMI 1640, and then inoculated with 0.33 ml of serially diluted virus preparations. After a 1-h incubation at 37°C, the cells were washed, and overlaid with 0.6% agarose solution (type L; Behringwerke, Marburg, Germany) in RPMI 1640 with freshly added trypsin (2.5–5.0 µg/ml). After 3 d, cultures were stained with 0.2% crystal violet solution containing 4% formaldehyde. Plaques were visualized and enumerated as plaque-forming units calculated per milliliter. An EID₅₀ assay was done by injecting serially diluted virus into embryonated hen eggs. The EID₅₀/ml for live virus was >10⁴ greater than for heat-treated virus.

Titer. A standard hemagglutination assay with chicken RBC (CRBC) was used for quantitation of virus in hemagglutinating units (HAU) per milliliter (22).

Fusogenic capacity. Viral fusion activity was determined using an assay that relies on the hemolysis of CRBC by virus at low pH (23, 24). CRBC were preincubated with virus at room temperature for 15 min to permit binding. pH was then adjusted to 5 and the virus-CRBC preparations were incubated at 37°C for 30 min to induce fusion and subsequently hemolysis. The amount of hemoglobin released was determined by absorption at 410 nm. Fusogenic capacity was eliminated by exposure of virus to citrate buffer at pH 5 for 30 min. This did not significantly alter binding (hemagglutinating) capacity, but resulted in loss of fusogenic activity and a substantial decrease in infectivity (>4 × 10⁴-fold).

Viral Adsorption. Influenza virus in allantoic fluid was adsorbed to CRBC to remove intact virions. CRBC were resuspended to 20% vol/vol in RPMI 1640 and mixed with an equal volume of allantoic fluid. After 20 min of incubation on ice, cells and bound virus were spun down at "low speed" (1,000 rpm, 5 min, 4°C). Supernatants were adsorbed two more times with CRBC as above. The final supernatant was considered to be a 1:8 dilution of the starting preparation.

Induction of CTL Responses

Dendritic cells were infected with 1,000 HAU/ml (multiplicity of infection [MOI] of 2–4 for live virus) of different forms of virus for 1 h at 37°C in serum-free medium, washed extensively, and added to bulk cultures of purified syngeneic T cells in 24-well plastic dishes. After 7 d, influenza-specific effector cells were harvested and distributed in varying numbers in 100-µl vol to 96-well microtiter plates. CTL activity in cell cultures was measured using a standard ⁵¹Cr-release assay with infected or uninfected macrophages, as previously described (20). In brief, macrophages were brought up to 10⁷/ml in serum-free medium, and infected with 1,000 HAU/ml of influenza virus. They were simultaneously labeled with ⁵¹Cr by the addition of 400 µCi of Na⁵¹CrO₄ (1 mCi/ml, sterile stock; New England Nuclear, Boston, MA) per ml. Targets were then washed four times, resuspended to 2 × 10⁵/ml, and aliquoted in 50-µl vol to 96-well round-bottomed dishes containing effector cells. Percent specific ⁵¹Cr release was calculated from the following formula: 100 × [(release by CTL – spontaneous release)/(total release – spontaneous release)]. Spontaneous release was generally 15% of the total release.

FACS[®] Analysis of Cell Populations

Cells were fixed with 4% paraformaldehyde, washed, and permeabilized with 1% saponin for 30 min at 4°C (25). Anti-NS1 (kindly provided by Dr. J. Yewdell, National Institutes of Health, Bethesda, MD) or isotype control (anti-CD8) mAbs were added for 30 min. Cells were washed in a solution of PBS containing 0.1% saponin, 0.1% azide, and 1% FCS and human serum, incu-

¹Abbreviations used in this paper: ER⁻, T cell depleted; HA, hemagglutinin; HAU, hemagglutinating units; MOI, multiplicity of infection; NA, neuraminidase; NP, nucleoprotein; NS-1, nonstructural protein 1.

bated with 1:200 dilution of PE-conjugated goat anti-mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN), washed, and analyzed on a FACScan® (Becton Dickinson and Co.).

Detection of Viral Proteins by Immunohistochemistry

Cytospins of dendritic cell populations were fixed in acetone for 5 min, and incubated for 45 min with anti-NS1 or anti-NP mAbs (H16-L10-4R5; American Type Culture Collection HB65, provided by Dr. J. Yewdell) as described (20). After washing, slides were incubated with biotinylated goat anti-mouse Ig for 45 min, followed by a horseradish peroxidase biotin-avidin complex for 30 min. The HRP reaction product was developed with H₂O₂ and diaminobenzidine tetrahydrochloride.

Results

Live and Inactivated Influenza Viruses Are Presented by Dendritic Cells to CTL Precursors. Different forms of influenza A virus (PR8) were used to generate secondary virus-specific CTL responses from several blood donors. As shown previously (20), when dendritic cells were pulsed with replicating ("live") virus (MOI of 2–4), CTLs were generated that recognize virus-infected but not uninfected targets (Table 1, top row). Remarkably, when dendritic cells were pulsed with equivalent intact virion doses of nonreplicating influenza virus, comparable CTL responses were induced. Influenza virus could be inactivated either by heating for 30 min at 56°C, or by exposure to UV light (Table 1, rows 2 and 3). These procedures reduced virus infectivity by >10⁴-fold (Table 1, middle column). Heat inactivation, in particular, reliably reduced viral infectivity >10⁶-fold. Through its envelope hemagglutinin, influenza virus binds to sialic acid residues on cell surfaces, permitting adsorptive endocytosis of virus into cells. In acidic endosomes, the hemagglutinin undergoes a pH-dependent conformational change that leads to fusion and delivery of viral nucleocapsid into the cytoplasm (26, 27). The binding and fusogenic functions of inactivated influenza virus were intact as assessed by standard hemagglutinating (binding) and hemolytic (fusion) assays, and confirmed earlier studies (13, 28). The importance of an intact hemagglutinin was further demonstrated by exposing virus to 60°C for 30 min (which partly dena-

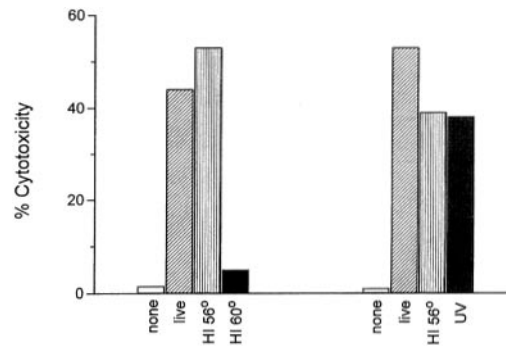


Figure 1. Ability of heat-inactivated virus to generate CTL responses is dependent on temperature of inactivation. Live (replicating), heat-inactivated [56°C vs 60°C], and UV-inactivated forms of influenza virus were compared in their ability to generate CTL responses from blood donors. Target cells were syngeneic uninfected and live influenza virus-infected macrophages. E/T ratio was 50:1. Lysis of uninfected targets was <5% (data not shown).

tures the hemagglutinin [29]). This abolished the hemagglutinating and hemolytic properties of the virus as well as the capacity to elicit CTL responses (Fig. 1). Treatment at higher temperatures (100°C) also destroyed CTL-inducing ability (data not shown). Therefore inactivated virus is presented to CTL precursors by dendritic cells, but the virus must retain binding and fusogenic activity.

The ability of dendritic cells to induce cytolytic responses to nonreplicating, heat-inactivated, and UV-irradiated virus was observed in >20 experiments. This finding stands in striking contrast to the inefficacy of inactivated influenza virus to charge class I molecules when it has been applied to target cells (8, 13–15). These earlier studies showed that CTL-mediated lysis of influenza virus-infected target cells required new viral protein synthesis. To investigate the phenomenon in more detail, we performed dose-response titrations where graded doses of inactivated virus were pulsed onto dendritic cells, and the ability to induce CTL responses was evaluated. Both UV- and heat-inactivated viruses were comparable to replicating virus in successfully charging dendritic cells with antigen for CTL generation (compare closed and open symbols in Fig. 2, A and

Table 1. Poorly Infectious Forms of Influenza Virus Induce Human CTL Responses

	CTL response			Viral titers PFU/ml	Hemagglutination HAU/ml	Hemolysis
	50:1	15:1	5:1			
Influenza virus						
Replicating	31	21	8	$6.4 \pm 2.2 \times 10^8$	$1-2 \times 10^4$	+
Heat-inactivated, 56°C	44	25	9	$\leq 3 \times 10^2$	$0.5-2 \times 10^4$	+
UV-inactivated	42	24	9	$1.3 \pm 1.3 \times 10^4$	$0.5-2 \times 10^4$	+

Live (replicating), heat-inactivated, and UV-inactivated forms of influenza virus were compared in their ability to generate CTL responses from blood donors. CTL responses are representative of >20 experiments. *E/T ratio. Target cells were syngeneic uninfected and live influenza virus-infected macrophages. Lysis of uninfected targets was <5%. For each virus form, the average viral and hemagglutinating titers are listed in terms of plaque-forming units per milliliter and hemagglutinating units per milliliter, respectively. Hemolysis reflects the fusogenic capacity of virus to lyse chicken erythrocytes (CRBC) at acidic pH (see Materials and Methods).

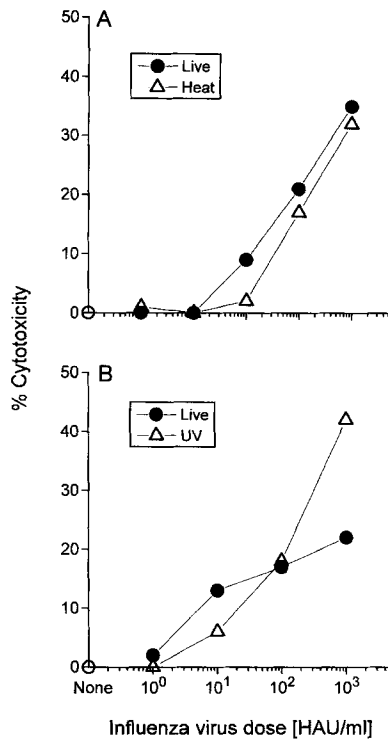


Figure 2. Dose-response titration of live virus inactivated virus. Partially enriched preparations of dendritic cells were pulsed with several dilutions of live, heat-inactivated or UV-irradiated virus, washed, and added to syngeneic donor T cells at a 3:1 T:APC ratio (20). CTL activity was assayed on infected syngeneic monocyte targets, as described in Table 1. E/T ratios: 60:1 (A), 50:1 (B). Lysis of uninfected targets was <5% (data not shown).

B). Thus, despite the substantial reduction in infectious titers of the heated and UV-treated viruses (Table 1), they retained the ability to induce CTL responses even at low virion doses (e.g., 10–100 HAU/ml) similar to live virus. We considered the possibility that live virus infection might compromise antigen presentation, as an explanation for the overlapping dose-response curves for live vs inactivated virus. However, we have previously shown that influenza virus-infected APCs could present purified protein derivative to *Mycobacterium tuberculosis*-reactive T cell clones as well as uninfected APCs (20).

Absence of New Viral Protein Synthesis in Dendritic Cells Infected with Inactivated Influenza Virus. Unlike live virus, heat-inactivated virus was deficient in synthesizing new viral proteins in dendritic cells (see below) and monocytes (data not shown). This was evident by (a) cytofluorography (in-

tracellular staining with an mAb to nonstructural protein 1, or NS1, Fig. 3), and (b) by immunocytochemistry for newly synthesized viral NS1 and nucleoprotein antigens (Fig. 4). The advantage of using NS1 is that it is synthesized only in the infectious cycle and is not present in mature virions. With nonreplicating virus, synthesis of new viral antigen was rarely detectable. Fewer than 1% of the dendritic cells showed staining for viral antigens like NS-1, compared with >60–80% of the dendritic cells that had been pulsed with live virus. Our findings confirm other studies in the murine system demonstrating the lack of new viral protein synthesis by heat-treated or UV-irradiated virus (13, 14). Human CTL responses to live influenza virus are generally obtained with T:dendritic cell ratios of 30:1–200:1. For heat-inactivated virus, this corresponds to a T:dendritic cell ratio of 3,000:1–20,000:1, if one considers that only dendritic cells in which viral protein synthesis is occurring are capable of inducing CTL responses. At these T:dendritic cell ratios, however, CTL responses cannot be generated (20). Thus it seems that active viral protein synthesis is not required to charge class I molecules on dendritic cells. Instead, the small amounts of protein brought into dendritic cells by noninfectious particles suffice to induce CTL responses.

Free viral proteins or peptides in allantoic fluid did not account for the immunogenicity of the live or inactivated virus preparations. Adsorption of intact virions with CRBC removed all sensitizing activity from the allantoic fluid for dendritic cells and monocytes at the induction (Fig. 5, top) and target level, respectively (Fig. 5, bottom). The CRBC-adsorbed fractions demonstrated virus activity in a tissue culture infectious dose (TCID)₅₀ assay (data not shown). Therefore antigens presented within inactivated virus preparations are only associated with virus that can bind to CRBC, rather than free proteins or peptides.

CD4⁺ and CD8⁺ T Cells Respond to Inactivated Virus-pulsed Dendritic Cells. We next verified that the CTLs that had been induced by dendritic cells pulsed with inactivated virus were CD8⁺ T lymphocytes. T cells were purified into respective CD8⁺ and CD4⁺ subsets, either after or before culture with virus-pulsed dendritic cells. When the T cells were sorted after killer activity had been induced by dendritic cells, all the lytic activity was in the CD8⁺ fraction (Fig. 6 A), as reported previously with live virus (20). When the T cells were purified before stimulation with virus-pulsed dendritic cells, the bulk of the cytolytic response was induced from the CD8⁺ subset, but killer activity could now also be detected in the purified CD4⁺ subset

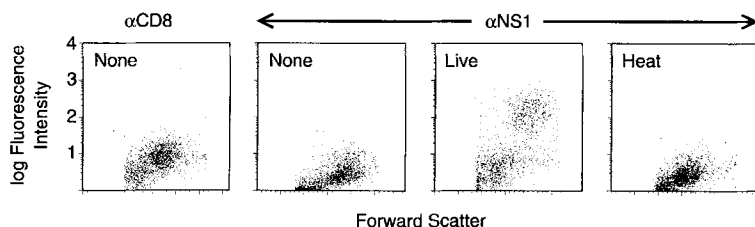


Figure 3. Cytofluorography of dendritic cells exposed to heat-inactivated virus. Enriched populations of dendritic cells were uninfected or pulsed with live or heat-inactivated virus and cultured for 16 h. Cells were fixed, permeabilized with saponin (25), and stained with anti-NS1 mAb or isotype control mAb (anti-CD8), followed by FITC-conjugated goat anti-mouse IgG. Dot plots were gated to remove debris and are of forward scatter versus anti-CD8 (left) or anti-NS1 (remaining panels). Dendritic cells are the large cell population and comprise ~65% of the preparation.

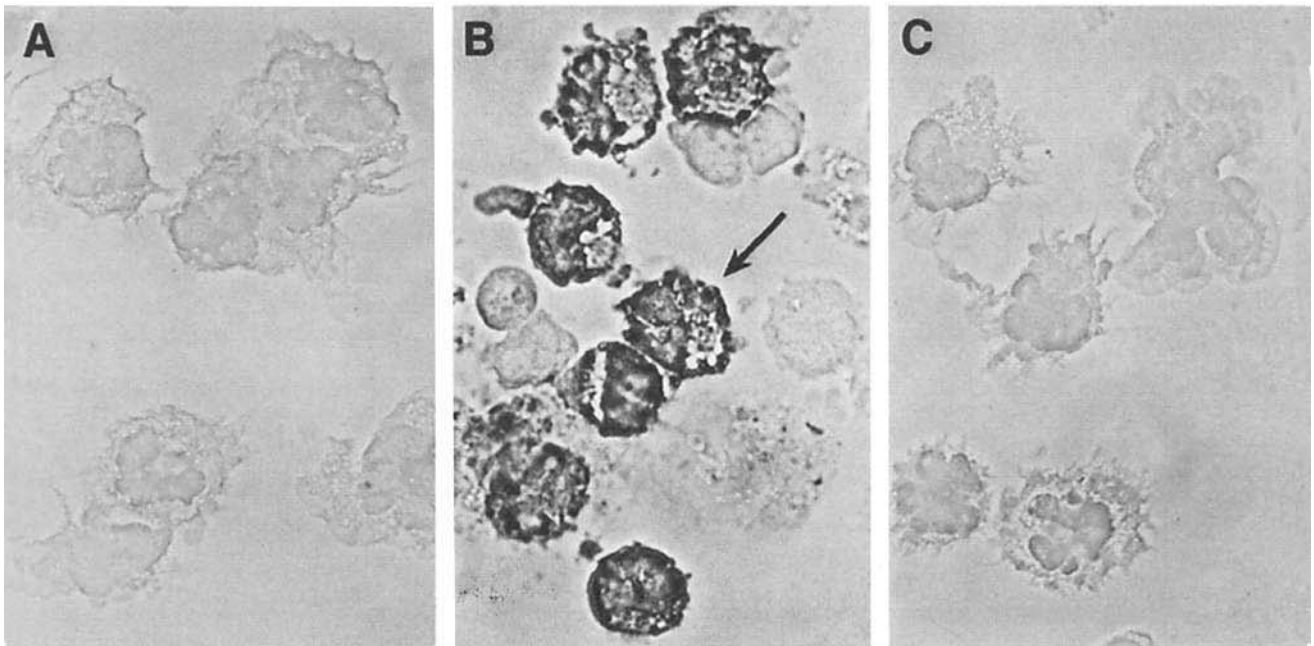
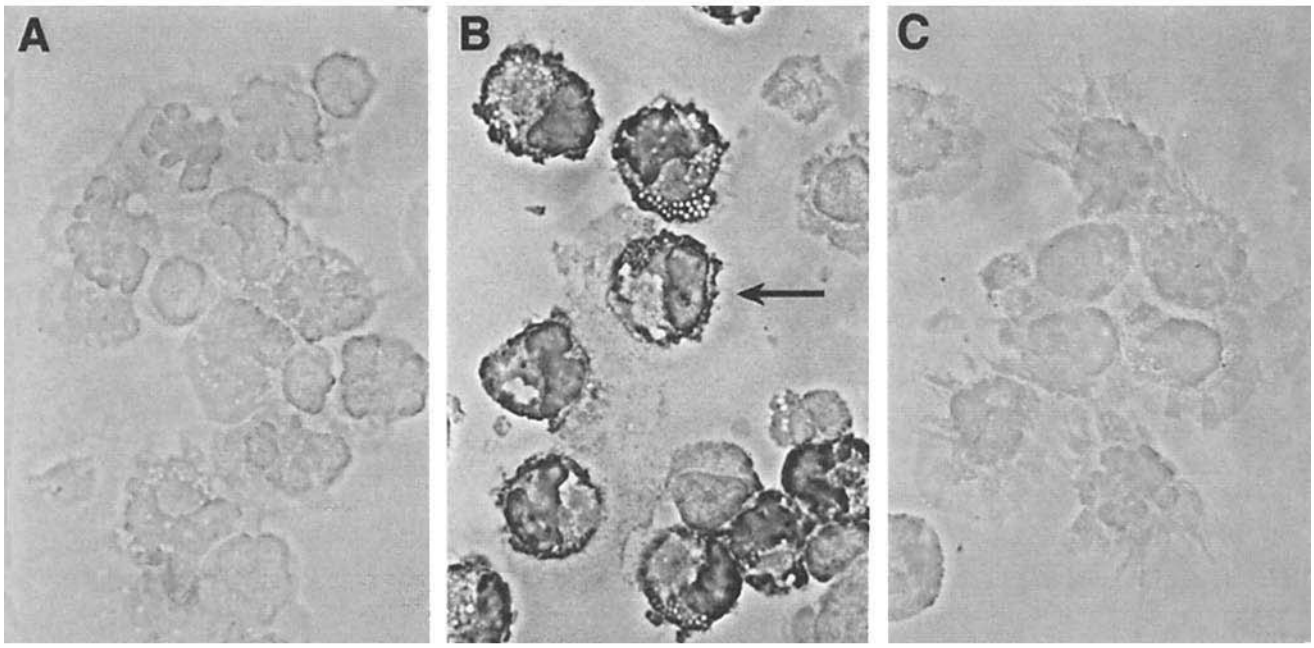


Figure 4. Expression of NS1 and NP protein in dendritic cells. Dendritic cells were uninfected (A), pulsed with live (B) or heat-inactivated virus (C) as in Fig. 3 above, cytospun onto glass slides, and stained with mAbs to NS1 (*top*) or NP (*bottom*), as previously described (20). The arrows point to cells expressing high levels of NS1 and NP.

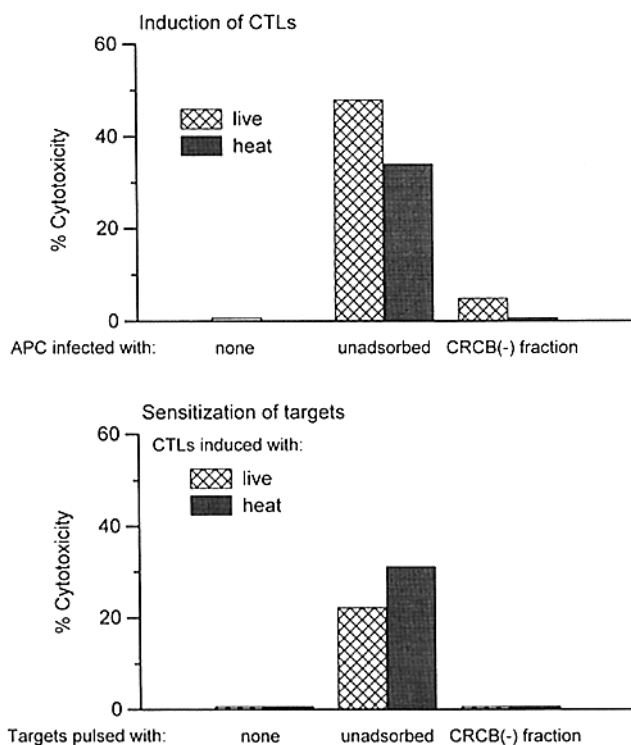


Figure 5. Adsorption of virus with CRBC removes CTL-inducing activity and target cell sensitization. Partially enriched preparations of dendritic cells were pulsed with live or heat-inactivated virus before or after adsorption to CRBC and then tested for their ability to stimulate CTL responses (*top*). Percent CTL activity is shown on infected macrophage targets. E/T ratio was 50:1. CRBC adsorbed or unadsorbed preparations of allantoic fluid-derived virus were also tested at the target level for sensitization of macrophages (*bottom*). CRBC (-) refers to fractions of virus after adsorption to CRBC. Percent CTL activity is shown on infected macrophage targets. E/T ratio was 40:1. Lysis of uninfected targets was <5% in all experiments (data not shown).

(Fig. 6 B). Thus, as for live virus, CD8⁺ T cells acquire the capacity to become CTLs when few or no CD4⁺ T cells are present. In all respects, therefore, noninfectious forms of influenza virus are as efficient as live virus in inducing human CTL responses, provided they are presented by dendritic cells.

Loss of Fusogenic Activity Decreases Virus Presentation. To determine whether live and heat-inactivated influenza virus must access the cytoplasm to be presented, we pretreated both forms of virus at pH 5. This destroys fusogenic capacity, and therefore delivery of nucleocapsid to the cytoplasm, but not hemagglutinating activity (24). Treatment at pH 5 rendered the virus less able to sensitize dendritic cells for inducing CTL responses from purified CD8⁺ T cells (Fig. 7). We substantiated that low pH treatment of live virus reduced infectivity by >4 × 10⁴-fold in standard viral plaque assays (data not shown). Thus it is likely that access to the cytoplasm was significantly diminished with this treatment. Another approach to block viral fusion, e.g., NH₄Cl or chloroquine treatment of APCs, which neutralizes endosomal pH, also interferes with antigen processing and therefore is not as selective.

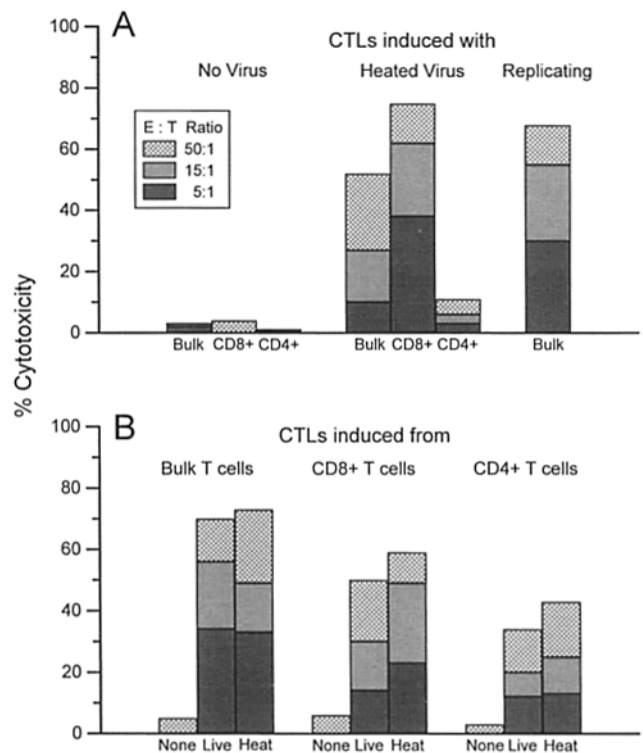


Figure 6. (A) Influenza virus-specific CTLs generated to heat-inactivated virus are CD8⁺. After 7 d of culture, T cells stimulated with uninfected or heat-inactivated influenza-infected APCs were stained with CD4-FITC and CD8-PE mAbs and sorted on a FACStar[®] Plus (Becton Dickinson and Co.). The CD4⁺ and CD8⁺ populations were >98% pure. Bulk refers to unsorted T cells. Lytic activity of each population was measured on infected syngeneic macrophage targets. Results are representative of three experiments. (B) Heat-inactivated influenza virus stimulates the development of both CD4⁺ and CD8⁺ CTLs. Bulk and purified T cell subsets (CD4⁺ or CD8⁺) were stimulated with live or heat-inactivated virus-pulsed APCs for 7 d. Cytolytic activity was measured as in (A). Results are representative of four experiments. Lysis of uninfected targets in A or B was <5% (data not shown).

Discussion

Inactivated Influenza Virus Elicits Potent Human CTL Responses. Our data show that nonreplicating influenza virus is comparably effective to live virus in generating CD8⁺ CTL responses, as long as the virus is delivered to dendritic cells. Prior work has shown that UV-inactivated influenza virus does not charge various target cells for effector cell recognition (8, 13–15). In these studies, CTL-mediated lysis of influenza virus-infected target cells presumably required new viral protein synthesis. Heat inactivated influenza virus sensitized L929 target cells for CTL recognition by murine T cells (14, 30). In our hands, however, human monocytes were poor targets for CTLs with this form of virus at standard doses of 1,000 HAU/ml (unpublished data). The important differences in our studies are the use of specialized APCs, dendritic cells. These APCs can induce CTLs from resting precursors and probably require very low levels of processed antigen to do so.

The mechanism by which the influenza virus is rendered

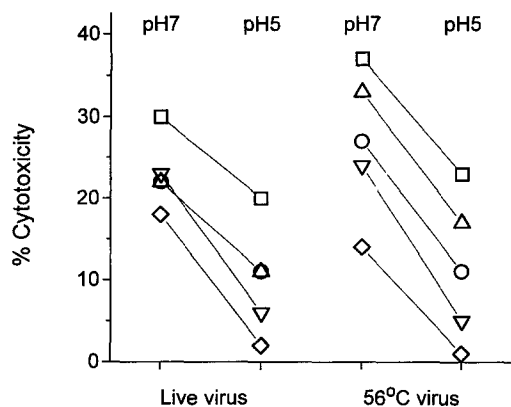


Figure 7. Induction of influenza virus-specific CD8⁺ CTLs is dependent upon its fusogenic activity. Live or heat-inactivated influenza virus (sucrose density gradient purified) was incubated in citrate buffer at pH 5 or pH 7 for 30 min at 37°C (see Materials and Methods). APCs were pulsed with 1,000 HAU/ml of the different virus preparations and added to purified syngeneic CD8⁺ T cells. Cytolytic activity was measured after 6–7 d. Each different symbol represents a separate experiment. E/T ratio = 50–40:1. Lysis of uninfected targets was <5% (data not shown).

nonreplicating has been ascribed to cross-linking of viral nucleic acid in the case of UV treatment, and possibly inactivation of viral polymerases (28, 31) in the case of heat treatment. Short courses of heat treatment (1–3 min) destroy the neuraminidase activity (13), which is thought to be required for normal viral replication and release from infected cells (32). From the data presented here, we presume that the inactivated influenza virus gains access to the now classical, cytoplasmic machinery for processing and presentation on MHC class I molecules. pH 5 treatment of heat-inactivated virus substantially reduced the ability to induce CTL responses from purified CD8⁺ T cells (Fig. 7). In contrast, the ability to generate CTL responses from CD4⁺ T cells was unaffected (data not shown). The alternative, that a specialized endocytic compartment for processing antigens onto class I is used (the so-called exogenous pathway that has been proffered in several studies [33–35]), remains a possibility since pH 5 inactivation of virus did not completely ablate CTL induction (Fig. 7).

Dendritic Cells Are Specialized APCs for Inducing Human CTL Responses. The efficacy of dendritic cells as APCs for CD8⁺ killer cell responses may be explained by three critical functional properties. The first is that only small amounts of MHC-associated ligand need to be presented on the surface of the dendritic cell. This became evident initially in studies of MHC class II presentation of superantigens (36). Only a few hundred MHC–superantigen complexes suffice to elicit a CD4⁺ T cell proliferative response from naive human T cells. In the current study with CD8⁺ T cells, MOIs of 2–4 to 0.02–0.04 (1,000–10 HAU/ml) for live virus are sufficient to induce strong CTL responses (Fig. 2). For heat-inactivated virus where infectivity is reduced >10⁶ fold (Table 1), the MOI with “infectious virus” is considerably lower, <2–4 × 10^{–6}–10^{–8}. At these doses, infectious live virus is ineffective in inducing CTL

responses. Thus dendritic cells do not require actively replicating virus to charge their class I molecules. The proteins brought in by incoming heat-inactivated virions are sufficient to elicit CTLs. This is supported by data obtained with cytochemistry and cytofluorography (Figs. 3 and 4) showing little or no expression of NP or NS1 in dendritic cells pulsed with heat inactivated virus. Whether dendritic cells use exogenous pathways for processing MHC class I molecules, as demonstrated for macrophages (35), remains to be determined. Preliminary studies with influenza virus expressing uncleaved HA, which lacks fusogenic capacity, indicate that access to the cytoplasm is necessary for optimal induction of CTL responses.

The second important feature of dendritic cells that has bearing on the generation of CD8⁺ T cells is their expression of known accessory or costimulatory molecules for T cell stimulation. These include CD80, CD86, LFA1 and 3, intracellular adhesion molecule-1 and 3, and CD40 (37–40).

The third feature is that dendritic cells are distributed in situ in such a way that they can pick up foreign antigens and then migrate to appropriate sites to interact with both memory and naive T cells. For example, dendritic cells are found in airway epithelium, the entry point for influenza virus in mammals. After exposure to antigens, dendritic cells can migrate from the airway to the T cell areas of the draining lymph nodes where T cells are then sensitized to the corresponding antigens (41, 42).

Inactivated Virus As An Immunogen for CTL Responses. Subunit and split influenza virus preparations that are currently used in vaccine formulations preferentially induce humoral rather than cytolytic responses in man. The viral antigens, e.g., HA, to which antibody responses are boosted undergo frequent antigenic shift and drift, and consequently yearly vaccination with new circulating virus preparations is needed. The appearance of influenza virus-specific CTLs is associated with a more rapid clearance of virus from nasal washings (2). The advantage of CTLs is that they are directed towards conserved internal viral proteins, e.g., NP, and therefore are cross-reactive for several virus subtypes (2).

Our findings are important for the development of vaccines and therapies that will elicit the generation of CD8⁺ CTLs from quiescent human T cells. In animal models, live viral vectors engineered to express foreign antigens have been used to induce immunity against many pathogens (43–47). Influenza virus that is both heat-treated and UV-irradiated primes mice for secondary CTL responses in vitro (30). Nonreplicative recombinant Semliki Forest virus expressing the NP influenza gene induces specific CD8⁺ CTL responses in mice (48). However, the potential usefulness of these vectors as vaccine delivery systems remains to be established in humans.

The use of nonreplicating influenza virus, together with dendritic cells, offers certain advantages over subunit and split virus preparations. First, influenza virus would be expected to have little pathogenicity in the blood stream, and therefore could be delivered in inactivated form on dendritic cells to prime or expand CTL function in vivo. Second, influenza virus can be engineered to express foreign

antigenic epitopes into HA and neuraminidase (49). These have been demonstrated to contribute to the development of CD8⁺ T cell-protective responses in mice against malaria

(46, 47). By virtue of its capacity to target dendritic cells, influenza virus, even nonreplicating virus, may be superior to strategies that do not target these specialized APCs.

We thank R. M. Steinman for advice and support, J. Yewdell for generous supplies of reagents, J. Adams for graphics, and S. Gezelter for assistance with sorting.

This work was supported by grants from the National Institutes of Health (AR-39552, AR-42557), the Irma T. Hirsch Trust, and the New York Community Trust to N. Bhardwaj, and the Deutsche Forschungsgemeinschaft (Be 1069/1-1) to A. Bender.

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Received for publication 7 June 1995 and in revised form 15 July 1995.

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