

## ROLE OF VIRAL INFECTIVITY IN THE INDUCTION OF INFLUENZA VIRUS-SPECIFIC CYTOTOXIC T CELLS

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Cellular immune responses have been shown to play an important role in determining the outcome of virus infection in several experimental models (1, 2). Among the manifestations of cellular immunity in viral infection the capacity of specifically sensitized thymus-derived (T)<sup>1</sup> lymphocytes to destroy virus-infected target cells in vitro has been demonstrated to correlate with elimination of infectious virus in vivo (3, 4) and hence suggests a direct role for cytotoxic T lymphocytes (CTL) in recovery from virus infection. Since the functional activity of CTL raised against viruses (5-8) as well as minor histocompatibility antigens (9, 10) and chemically modified cells (11) has also been shown to be under the control of genes in the major histocompatibility complex (MHC), the specificity of CTL for both the gene products of the MHC and the foreign determinant on target cell surfaces has been analyzed in great detail. On the other hand, much less information is presently available on the requirements for induction of virus-specific CTL. An issue which is presently controversial is the requirement for infectious virus in the induction of virus-specific CTL. Several laboratories have reported that CTL responses to a diverse group of viruses can be readily stimulated with inactivated virion preparations (12-15), whereas infectious virus is required for CTL induction in other hands (16). Also inactivated virus (12, 13) or indeed virion subunit preparations (17) have been reported to sensitize target cells for CTL-mediated lysis in the absence of nascent viral protein in the target cell (12, 17), whereas infectious virus and nascent viral protein synthesis is required for lysis of target cells in other circumstances (18). The resolution of this issue would appear to have direct implications with respect to viral vaccines, particularly in assessing the efficacy of live virus and killed virus vaccines. Furthermore, an understanding of the role of viral infectivity both in the induction of CTL and target cell sensitization may be of value in understanding the mechanism of CTL recognition.

This report examines the capacity of infectious and inactivated influenza virus to stimulate virus-specific CTL responses in vivo and in vitro. We have

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<sup>1</sup> *Abbreviations used in this paper:* CTL, cytotoxic thymus-derived lymphocyte(s); EID<sub>50</sub>, egg infectivity dose yielding 50% positive response; HAU, hemagglutination units of virus; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; T cell, thymus-derived lymphocyte; UV, ultra-violet.

observed that while infectious influenza virus was capable of stimulating both CTL responses and humoral immune responses in vivo over a broad immunizing dose range, ultra-violet inactivated influenza virus neither induced CTL responses in vivo nor sensitized putative target cells for lysis by influenza-specific CTL in spite of its capacity to stimulate a comparable in vivo humoral immune response. Inactivated influenza virus could, however, stimulate a CTL response in vitro. These results are discussed in the light of the observations outlined above. Possible implications for virus-specific CTL induction are also considered.

### Materials and Methods

*General.* Male BALB/C mice (7–12 wk of age) bred at the John Curtin School were used throughout. P815 mastocytoma cells, maintained in tissue culture, were used as target cells in all experiments (19). Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, N.Y. catalogue no. F-15), supplemented with 10% heat-inactivated fetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia) was used as the medium in all cytotoxicity assay.

*Viruses.* Influenza virus strains A/WSN (H0/N1), A/JAP/305 (H2/N2), and B/LEE were grown in the allantoic cavity of embryonated eggs and stored as infectious allantoic fluid as described previously (19).

*Virus Purification and Inactivation.* Before inactivation, virus, as infectious allantoic fluid, was concentrated and purified according to standard procedures (20). Influenza A/WSN was concentrated by adsorption-elution from fowl erythrocytes followed by ultracentrifugation. Influenza A/JAP was similarly concentrated and further purified by velocity centrifugation over sucrose gradients (20). Purified virus was diluted to a concentration of  $10^8$  hemagglutination units (HAU)/ml in sterile phosphate-buffered saline (PBS) and exposed to a 25-watt ultraviolet (UV) light source at a distance of 20 cm for 7 min in 9 cm glass Petri dishes containing 5–6 ml of virus suspension. The virus suspension was stirred constantly during exposure to the UV source. The infectious viruses had a titer of  $5 \times 10^8$ – $10^9$  EID<sub>50</sub> U/ml and  $1.5$ – $3.0 \times 10^3$  HAU/ml. Egg infectivity titers were determined by the modified Spearman-Käber method (21). Hemagglutination titrations were performed according to Fazekas de St. Groth and Webster (22). No residual infectious virus was detectable in inactivated virus preparations as measured by egg infectivity. No loss of viral hemagglutinating activity was observed after virus inactivation. Inactivated virus was stored at 4°C.

*Immunization.* Mice were inoculated with virus by the intravenous route. Infectious virus, as allantoic fluid was diluted in PBS and administered in quantities as indicated in the text. UV-inactivated virus was administered in a similar fashion. For in vivo primary cytotoxic responses, spleens from three donor mice were removed 6 days after immunization and a spleen cell suspension was prepared as previously described (8). For in vivo secondary responses, mice primed previously with 100 HAU of infectious virus were inoculated 3–4 wk later with infectious or inactivated virus as indicated in the text. 5 days later spleens from three donors were removed and processed as above. For adoptive in vivo secondary responses  $80$ – $100 \times 10^6$  spleen cells from donors primed 3–4 wk previously with infectious virus were transferred intravenously into age and sex matched recipient mice which had received 450 rads of total body  $\gamma$ -irradiation from a radioactive cobalt source. Recipient mice were inoculated with virus immediately after cell transfer. Recipient spleens were removed 5 days later and tested for cytotoxicity.

*In Vitro Secondary Responses.* Cytotoxic T lymphocytes were generated in vitro essentially as described previously (8, 19). Briefly,  $40 \times 10^6$  spleen cells from mice primed 3–8 wk previously with 100 HAU of infectious influenza virus were cultured with "stimulator" cells in 25 cm<sup>2</sup> Falcon tissue culture flasks (Becton, Dickinson and Co., Oxnard, Calif.) containing 15 ml of medium (8, 19). The responder cell to stimulator cell ratio was 10:1. Stimulator cells consisted of normal syngeneic spleen cells either infected with 5 EID<sub>50</sub> U of infectious virus per nucleated cell (60 HAU of infectious virus per  $4 \times 10^6$  cells) or treated with various quantities of inactivated influenza virus in an identical fashion. Stimulator cells were washed twice before culturing with

responder cells to remove unadsorbed virus. Viable cells were tested for cytotoxic activity after 5 days of culture. Recovery of viable cells was 20–40% at that time.

**Assay for Cell-Mediated Cytotoxicity.** The  $^{51}\text{Cr}$  release cytotoxicity assay was carried out as described in previous reports (8, 19).  $^{51}\text{Cr}$ -labeled P815 target cells were infected in suspension with 10  $\text{EID}_{50}$  U of infectious virus per cell (30 HAU of infectious virus per  $10^6$  target cells) as described (19). Target cells were treated with various quantities of UV-inactivated influenza virus in an identical fashion. Unless otherwise indicated in the text, assay times were 8–8.5 h for primary effector activity and 6.5 h for secondary effectors. Spontaneous  $^{51}\text{Cr}$  release from target cells incubated with medium only usually ranged from 5 to 15% and was always less than 18%. Percent specific  $^{51}\text{Cr}$  release was obtained from the formula:

$$\frac{\text{test counts} - \text{spontaneous release}}{\text{water lysis counts} - \text{spontaneous release}} \times 100.$$

All values represent the mean percent specific  $^{51}\text{Cr}$  release of four replicate wells.

**Assay for Anti-Hemagglutinin Antibody.** Serum anti-hemagglutinin antibody was quantitated by the microtitration hemagglutination inhibition test (23). 4 HAU of virus in a vol 0.025 ml were added to serial twofold dilutions of serum in a final vol of 0.025 ml of PBS. After 35 min of incubation, 0.025 ml of a 1% suspension of fowl erythrocytes was added to each well. After 30 min of incubation, the hemagglutination-inhibition endpoint was determined. All sera were treated with *Vibrio cholera* receptor-destroying enzyme (Center for Disease Control, Atlanta, Ga.) and heated to 56°C for 30 min to remove nonspecific inhibitors. Preimmune sera from immune sera donors served as controls.

## Results

In a series of preliminary experiments the capacity of influenza virus strain A/WSN to induce CTL was assessed after inactivation of the virus by several different methods. In contrast to results obtained with infectious influenza virus (8, 19) no influenza-specific CTL activity was detectable in the spleens of mice after intravenous inoculation of A/WSN virus inactivated either by UV irradiation, sodium deoxycholate disruption, or heat treatment (data not shown). Because UV irradiation was considered to have the least detrimental effect on both virion architecture and viral antigen stability, this method of virus inactivation was used in subsequent experiments.

**Antigen Dose Dependence of the *in Vivo* Primary CTL Response to Infectious Influenza Virus.** Fig. 1 shows the cytotoxic response from the spleens of mice 6 days after administration of the indicated doses of infectious influenza A/WSN. Cytotoxic activity was detectable with infectious virus doses as low as  $10^{-3}$  HAU ( $10^2$   $\text{EID}_{50}$  U). The magnitude of lytic activity was directly proportional to the concentration of infectious virus in the immunizing inoculum over a range of antigen doses. This direct relationship between immunizing virus dose and splenic CTL activity was consistently observed in a series of experiments. An analysis of the kinetics of the cytotoxic response (not shown) indicated that as demonstrated previously (8, 19, 24) optimal cytotoxic activity was maximum at 5–7 days postinoculation. Thus, the difference in magnitude of the cytotoxic response with different virus doses was not attributable to differences in the kinetics of appearance of cytotoxic activity. The T-cell origin of the cytotoxic cell activity has been demonstrated previously (8, 19).

**Absence of an *in Vivo* Primary CTL Response with UV-Inactivated Influenza Virus.** Table I shows a comparison CTL response of mice 6 days after *i.v.* inoculation with various concentrations of infectious or UV-inactivated influenza A/WSN. In contrast to infectious virus, UV-inactivated A/WSN virus did

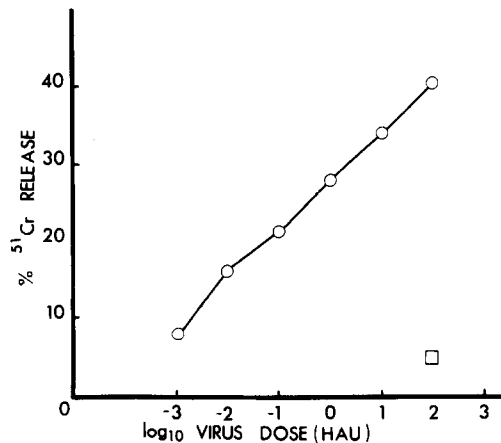


FIG. 1. Antigen dose dependence of the in vivo primary cytotoxic T-cell response to infectious influenza virus A/WSN. Spleen cells from pools of three mice were obtained 6 days after primary immunization with the indicated virus dose (abscissa) and tested for cytotoxicity on <sup>51</sup>Cr-labeled A/WSN infected target cells (○—○). The cytotoxic activity of normal spleen cells is also included (□). The effector cell: target cell ratio is 50:1. Values are the mean of four individual wells. Standard errors less than ± 3% in all cases have been omitted.

not generate a significant CTL response in vivo with virus doses as high as  $10^4$  HAU. The low level of cytotoxicity observed in the assay at the highest effector to target ratio is comparable to the background cytotoxicity observable with normal spleen cells and probably does not reflect low level specific cytotoxic activity. Splens from mice immunized with  $10^3$  HAU of inactivated virus were also examined for CTL activity at 2-day intervals up to 10 days after immunization. No cytotoxic activity was detectable during this period at a time when optimal CTL responses are detectable in a variety of diverse viral systems (25). Although these results were obtained with influenza strain A/WSN, we have obtained similar results with two other influenza strains: A/JAP/305 (H2N2) and B/LEE.

*Humoral Immune Response to Infectious and Inactivated Influenza Virus.* Since the above results indicated a marked disparity between infectious and UV-inactivated virus in their respective capacities to induce CTL responses in vivo, the humoral immune response to various doses of these virus preparations was examined (Table II). Both virus preparations induced significant levels of anti-viral antibody as measured by hemagglutination inhibition. Likewise for both virus preparations the magnitude of the response was proportional to the immunizing antigen dose. Although the humoral response to infectious virus was greater at lower immunizing doses, similar antibody levels were achieved upon immunization with higher doses of UV-inactivated virus, i.e.,  $10^2$ – $10^4$  HAU.

*Absence of an in Vivo Secondary CTL Response on Challenge with Inactivated Influenza Virus.* Secondary CTL responses to infectious influenza virus have been demonstrated both in vivo (24) and in vitro (8, 19) after primary immunization with infectious influenza virus. Since the above results indicated that UV-inactivated influenza was a poor stimulator of a primary CTL response,

TABLE I  
 Comparison of the *in Vivo* Primary Cell-Mediated Cytotoxic Response to Infectious and Inactivated Influenza Virus\*

Virus dose‡	Effector cell to target cell ratio§	% Specific <sup>51</sup> Cr release on A/WSN infected P815 target cells¶ Immunization	
		Infectious virus	Inactivated virus
10 <sup>-2</sup>	25:1	19.5 ± 0.4**	NT‡‡
	50:1	43.4 ± 1.9	"
	100:1	48.4 ± 2.7	"
10 <sup>-1</sup>	25:1	43.3 ± 1.5	0.1
	50:1	59.5 ± 0.5	1.0 ± 0.1
	100:1	57.5 ± 2.9	3.1 ± 0.1
10 <sup>0</sup>	25:1	47.0 ± 1.8	0.6
	50:1	62.5 ± 1.2	2.0 ± 0.1
	100:1	74.6 ± 0.6	3.8 ± 0.1
10 <sup>1</sup>	25:1	60.1 ± 1.2	0.7
	50:1	69.5 ± 2.5	2.7 ± 0.1
	100:1	79.3 ± 4.6	6.3 ± 0.2
10 <sup>2</sup>	25:1	64.0 ± 1.1	1.7 ± 0.1
	50:1	73.8 ± 1.2	3.6 ± 0.3
	100:1	81.7 ± 1.9	7.3 ± 0.3
10 <sup>3</sup>	25:1	NT	1.5 ± 0.1
	50:1	"	2.9 ± 0.1
	100:1	"	5.2 ± 0.3
10 <sup>4</sup>	25:1	NT	3.0 ± 0.1
	50:1	"	6.0 ± 0.2
	100:1	"	8.5 ± 0.2

\* BALB/c mice were immunized i.v. with the indicated dose of infectious or UV-inactivated influenza A/WSN. 6 days later spleen cells from pools of three mice were assayed for cytotoxicity.

‡ Virus dose employed in primary immunization measured in HAU. 1 HAU of infectious virus contains 1.7-3.0 × 10<sup>5</sup> EID<sub>50</sub> U of infectious virus.

§ 2 × 10<sup>4</sup> <sup>51</sup>Cr-labeled P815 cells/well.

¶ All effector populations were simultaneously examined on target cells infected with A/WSN and B/LEE viruses. <sup>51</sup>Cr release values on B/LEE infected targets were <10% and <2% for cells obtained from donors receiving infectious and inactivated virus respectively.

|| Indicates source of spleen cells, i.e. from donors receiving infectious or inactivated virus.

\*\* Means ± standard errors of the mean from four wells with spontaneous release subtracted.

‡‡ Not tested.

TABLE II  
*Primary Serum Anti-Hemagglutinin Antibody Response to  
 Infectious and Inactivated Influenza Virus\**

Virus dose‡	Anti-hemagglutinin antibody titer§ Immunization	
	Infectious virus	Inactivated virus
10 <sup>4</sup>	NT	160 ± 0¶
10 <sup>3</sup>	160 ± 0	NT
10 <sup>2</sup>	160 ± 0	80 ± 0
10 <sup>1</sup>	113 ± 11	NT
10 <sup>0</sup>	95 ± 10	16 ± 11
10 <sup>-1</sup>	73 ± 11	NT
10 <sup>-2</sup>	20 ± 11	<10

\* Groups of four mice were immunized i. v. with the indicated dose of infectious or UV-inactivated influenza A/WSN. 10 days later, individual mice were bled from the tail vein and the serum hemagglutination inhibiting antibody titer determined.

‡ As in Table I.

§ Hemagglutination inhibiting antibody titer as measured in the microtitration hemagglutination-inhibition test.

|| Indicates source of serum, i.e. from mice receiving infectious or inactivated virus.

¶ Values are the reciprocals of means ± standard errors of the highest serum dilution giving complete inhibition of hemagglutination from four individual mice.

it was of interest to examine the capacity of inactivated virus to induce a CTL response in mice previously primed with infectious influenza virus. Mice immunized 3–4 wk previously with 100 HAU of infectious influenza A/WSN or A/JAP were challenged with 1,000 HAU of UV-inactivated A/WSN or A/JAP virus. Control mice received no further treatment beyond primary immunization. After 5 days the cytotoxic activity of spleen cells from the various groups was examined on target cells infected with A/WSN, A/JAP, B/LEE, or uninfected target cells (Table III). In no instance did the cytotoxic activity of spleen cells from mice receiving secondary challenge with inactivated influenza virus exceed the background cytotoxicity of cells from control mice. On the other hand, spleen cells from mice previously primed with infectious A/WSN virus showed significant cytotoxic activity on both A/WSN and A/JAP infected target cells when secondarily stimulated with infectious A/JAP virus (Table III).

*Generation of an in Vitro Secondary Response to Inactivated Influenza Virus.* Although the above results would indicate that inactivated influenza is also an inefficient stimulator of secondary CTL responses, the presence of circulating anti-viral antibody in the primed recipients could alter the secondary CTL response upon challenge with inactivated virus. Indeed, it has been demonstrated that poor secondary CTL responses are observed when mice, previously primed with a given infectious type A influenza strain are challenged with the homologous infectious virus (24), whereas secondary stimulation with an infectious type A influenza strain of a different subtype generates good CTL responses in the primed recipients (Table III and [24]). This poor cytotoxic T-cell response on secondary stimulation with homologous virus appears to be due to

TABLE III  
*In Vivo Secondary Cell-Mediated Cytotoxic Response to Inactivated Influenza Virus*

Immunization*		% Specific <sup>51</sup> Cr release from target cells†			
Primary	Secondary	Uninfected	A/WSN	A/JAP	B/LEE
A/WSN	A/WSN (Inactivated)	2.5 ± 0.1	13.5 ± 0.7	9.3 ± 0.3	0
A/WSN	A/JAP (Inactivated)	2.4 ± 0.1	18.4 ± 0.9	12.0 ± 1.0	2.5 ± 0.2
A/WSN	None	0	17.3 ± 0.8	14.7 ± 0.4	0.7
A/JAP	A/WSN (Inactivated)	3.9 ± 0.2	9.8 ± 0.3	11.5 ± 0.5	0.8
A/JAP	A/JAP (Inactivated)	0	7.6 ± 0.1	13.3 ± 0.3	0.3
A/JAP	None	0	12.5 ± 0.6	10.3 ± 0.4	0.7
A/WSN	A/JAP (Infectious)	0	60.4 ± 1.4	54.3 ± 1.5	0.3

\* 3-4 wk after primary immunization with infectious A/WSN or A/JAP virus mice were challenged with either inactivated A/WSN or A/JAP virus as indicated in the text. Controls consisted of mice receiving no secondary immunization and A/WSN primed mice challenged with 100 HAU of infectious A/JAP virus. 5 days later, spleen cells from pools of three mice were assayed for cytotoxic activity on the indicated target cells. Effector cell:target cell ratios were 100:1.

† Values are the means ± standard errors of the mean from four wells with spontaneous release subtracted.

neutralization of the infectious virus inoculum by circulating antibody in the primed recipient. Since highly potent secondary CTL responses to homologous type A influenza virus can be obtained by in vitro secondary stimulation of primed cells (8, 19), we examined the capacity of inactivated virus to stimulate a secondary CTL response in vitro where the problem of circulating anti-viral antibody could be circumvented. Spleen cells from mice previously immunized with infectious A/JAP virus were cultured with normal spleen cell stimulators which had been treated with either inactivated A/JAP virus, inactivated A/WSN virus, or infectious A/JAP, A/WSN, or B/LEE viruses. After 5 days the cytotoxic activity of the cultured cells was examined (Table IV). In contrast to the results obtained above, spleen cells from A/JAP-primed mice generated potent cytotoxic effector cells when stimulated in vitro with UV-inactivated A/JAP virus. Furthermore, the magnitude of the response was dependent upon the dose of stimulating antigen. Also no response was observed when normal spleen cells, treated with inactivated A/WSN virus, were used for in vitro stimulation. It is of interest to note the cytotoxic activity generated by A/JAP primed cells after stimulation with inactivated A/JAP was directed exclusively to A/JAP-infected target cells. There was no lysis of A/WSN infected targets above the background seen with uninfected target cells or target cells infected with the serologically unrelated B/LEE influenza virus. This observation is in contrast to the finding with infectious virus where, as has been previously shown (19), stimulation of A/JAP primed cells with stimulator cells treated (infected) with infectious A/JAP or A/WSN virus generates CTL which can efficiently lyse both A/WSN and A/JAP infected targets. Finally, there was no

TABLE IV  
*In Vitro Secondary Cell-Mediated Cytotoxic Response to Inactivated Influenza Virus*

In vitro stimulation*	Effector cell to target cell ratio <sup>§</sup>	% Specific <sup>51</sup> Cr release from target cells <sup>‡</sup>			
		Uninfected	A/WSN	A/JAP	B/LEE
A/JAP-Inactivated (25 HAU) <sup>  </sup>	1:1	0.4	1.3 ± 0.1	3.4 ± 0.3	0
	2.5:1	0.5	1.8 ± 0.1	9.8 ± 0.3	1.6 ± 0.1
	5:1	2.2 ± 0.1	3.3 ± 0.1	18.3 ± 0.5	4.3 ± 0.2
A/JAP-Inactivated (250 HAU)	1:1	0	1.1 ± 0.1	18.8 ± 0.1	1.1 ± 0.1
	2.5:1	1.4 ± 0.1	3.5 ± 0.2	43.0 ± 1.3	2.4 ± 0.1
	5:1	3.2 ± 0.1	5.8 ± 0.2	61.9 ± 1.3	4.8 ± 0.2
A/JAP-Inactivated (2,500 HAU)	1:1	2.0 ± 0.1	3.4 ± 0.1	41.5 ± 1.5	2.2 ± 0.1
	2.5:1	5.4 ± 0.2	8.8 ± 0.4	66.5 ± 1.7	6.9 ± 0.2
	5:1	10.5 ± 0.2	15.2 ± 0.3	79.7 ± 1.5	14.3 ± 0.4
A/WSN-Inactivated (2,500 HAU)	1:1	0	0	1.3 ± 0.1	1.0
	2.5:1	2.2 ± 0.1	2.6 ± 0.1	3.1 ± 0.1	2.3 ± 0.1
	5:1	4.1 ± 0.2	5.2 ± 0.1	4.3 ± 0.1	4.5 ± 0.2
A/JAP-Infectious	1:1	4.5 ± 0.2	42.6 ± 1.2	60.2 ± 1.5	4.8 ± 0.1
	2.5:1	10.3 ± 0.5	58.2 ± 1.2	73.2 ± 2.7	9.7 ± 0.3
	5:1	16.5 ± 0.4	66.2 ± 2.1	78.6 ± 2.4	18.7 ± 0.2
A/WSN-Infectious	1:1	1.7 ± 0.1	30.5 ± 1.1	34.3 ± 1.9	1.5 ± 0.1
	2.5:1	4.1 ± 0.3	55.4 ± 1.8	59.8 ± 1.3	3.4 ± 0.1
	5:1	8.0 ± 0.6	62.0 ± 1.0	80.2 ± 0.6	7.2 ± 0.2
B/LEE-Infectious	1:1	2.1 ± 0.1	3.2 ± 0.1	2.6 ± 0.1	2.3 ± 0.1
	2.5:1	4.6 ± 0.3	4.9 ± 0.1	4.5 ± 0.3	5.2 ± 0.1
	5:1	6.9 ± 0.3	7.3 ± 0.1	7.7 ± 0.3	7.3 ± 0.4
-¶	1:1	3.4 ± 0.2	2.0 ± 0.1	0.7	1.7 ± 0.1
	2.5:1	4.5 ± 0.3	2.3 ± 0.1	1.7 ± 0.1	2.1 ± 0.1
	5:1	4.2 ± 0.2	3.9 ± 0.1	3.6 ± 0.1	2.9 ± 0.2

\* Spleen cells from A/JAP-immune mice were cultured in vitro with stimulator spleen cells treated with infectious or inactivated influenza virus as described (Materials and Methods). After 5 days of culture, the cytotoxic activity of the responder cells was examined.

‡ As in Table III.

§ As in Table I.

|| Parentheses indicate dose of inactivated virus incubated with stimulator spleen cells.

¶ Indicates normal stimulator spleen cells not exposed to virus.

cytotoxic activity detectable in cultures stimulated with untreated or influenza B/LEE infected stimulators.

*Absence of an in Vivo Secondary CTL Response to Inactivated Virus after Adoptive Transfer.* Two points emerge from the results obtained with secondary stimulation in vitro: (a) inactivated virus is capable of stimulating a secondary CTL response in vitro; (b) the response is observed only when in vitro stimulation is carried out with the homologous virus strain used in primary immunization and the cytotoxic activity is directed exclusively to the target cells infected with the homologous virus strain. These in vitro observa-



TABLE V  
*Adoptive in Vivo Secondary Cytotoxic Response to Infectious and Inactivated Influenza Virus*

Immune cells transferred*	Secondary stimulation†	Effector cell to target cell ratio‡	% Specific <sup>51</sup> Cr release from target cells			
			Uninfected	A/WSN	A/JAP	B/LEE
A/WSN	A/WSN-Inactivated	25:1	1.5 ± 0.1	5.0 ± 0.2	3.7 ± 0.1	4.6 ± 0.1
		50:1	1.3 ± 0.1	6.8 ± 0.2	4.4 ± 0.1	4.3 ± 0.1
A/WSN	A/JAP-Inactivated	25:1	1.0	2.5 ± 0.1	1.6 ± 0.1	3.9 ± 0.1
		50:1	2.0 ± 0.1	5.4 ± 0.1	3.9 ± 0.1	4.8 ± 0.3
A/WSN	A/WSN-Infectious	25:1	2.5 ± 0.1	69.4 ± 1.9	32.7 ± 1.9	4.5 ± 0.1
		50:1	3.9 ± 0.1	83.6 ± 3.1	50.4 ± 2.9	6.8 ± 0.2
A/WSN	None	25:1	1.7 ± 0.1	1.7 ± 0.1	2.0 ± 0.1	2.7 ± 0.1
		50:1	2.1 ± 0.1	4.7 ± 0.1	3.8 ± 0.1	4.2 ± 0.1
A/JAP	A/WSN-Inactivated	25:1	3.7 ± 0.1	3.5 ± 0.1	6.3 ± 0.1	6.1 ± 0.2
		50:1	4.0 ± 0.1	6.6 ± 0.4	8.1 ± 0.1	6.7 ± 0.2
A/JAP	A/JAP-Inactivated	25:1	4.4 ± 0.2	5.8 ± 0.2	8.1 ± 0.2	6.5 ± 0.5
		50:1	5.9 ± 0.1	6.7 ± 0.3	10.5 ± 0.5	7.1 ± 0.3
A/JAP	A/JAP-Infectious	25:1	6.1 ± 0.3	45.7 ± 2.0	47.2 ± 0.5	8.5 ± 0.6
		50:1	8.4 ± 0.3	63.2 ± 2.6	63.5 ± 1.7	12.2 ± 0.6
A/JAP	None	25:1	6.2 ± 0.2	5.5 ± 0.2	8.1 ± 0.1	8.0 ± 0.4
		50:1	8.5 ± 0.1	10.8 ± 0.2	12.2 ± 0.1	10.3 ± 0.1

\* Spleen cells from mice previously primed with the indicated infectious virus were transferred into sublethally irradiated (450 rads) mice. Recipient mice were immediately challenged with the indicated virus as described (Materials and Methods). Recipient spleens were examined for cytotoxic activity 5 days later on the indicated target cells.

† i.v. inoculation of 1,000 HAU of inactivated virus or 100 HAU of infectious virus.

‡ As in Table I.

|| Values are the means ± standard errors of the mean from four wells with spontaneous release subtracted.

tions prompted a re-examination of the capacity of UV-inactivated virus to stimulate in vivo secondary CTL responses under conditions where circulating anti-viral antibody was eliminated. This situation was achieved by adoptive transfer of spleen cells from mice primed with infectious A/WSN or A/JAP viruses into sublethally irradiated syngeneic recipients. Recipient mice were then challenged with either inactivated A/WSN, inactivated A/JAP, or the infectious homologous virus used in primary immunization. 5 days later, the spleens of these recipients were examined for cytotoxic activity (Table V). In contrast to the results obtained in vitro, recipients of A/JAP-primed spleen cells failed to respond either to inactivated A/JAP or A/WSN virus. Likewise, recipients of A/WSN primed spleen cells failed to generate cytotoxic responses when secondarily stimulated with either inactivated virus preparation. On the other hand, recipients of virus-primed cells, when inoculated with infectious homologous virus, generated significant CTL responses. Thus, in the absence of

TABLE VI  
Sensitization of Target Cells for Cell-Mediated Cytotoxicity by  
Inactivated Influenza Virus

Target cell treatment*	Relative virus concentration‡	% Specific <sup>51</sup> Cr release from treated target cells§
A/JAP-Inactivated	2	23.7 ± 0.3
	20	20.7 ± 1.1
	200	19.9 ± 1.0
A/JAP-Infectious	1	86.2 ± 0.7
A/WSN-Inactivated	2	17.3 ± 0.3
	20	23.3 ± 0.8
	200	21.9 ± 0.7
A/WSN-Infectious	1	97.2 ± 1.1
B/LEE-Infectious	1	20.4 ± 0.6
None	—	16.5 ± 0.6

\* <sup>51</sup>Cr-labeled P815 cells were incubated with the indicated virus preparation as described (Materials and Methods).

‡ Target cells were incubated either with infectious A/WSN, A/JAP, or B/LEE virus at a concentration of 30 HAU per 10<sup>6</sup> cells (10 EID<sub>50</sub> U/cell) or with 2, 20, or 200-fold higher concentrations of inactivated virus.

§ Target cells were exposed to potent secondary cytotoxic effectors for 8 h at an effector:target ratio of 5:1. Cytotoxic effectors directed to influenza A/JAP were generated in vitro as described (Materials and Methods).

circulating anti-viral antibody, a potent CTL response can be obtained on secondary stimulation with homologous infectious virus. However, neither homologous virus nor heterologous type A influenza virus, when inactivated, stimulated a cytotoxic response under these conditions.

*Lack of Target Cell Sensitization with Inactivated Influenza Virus.* Several laboratories have reported that treatment of uninfected cells with inactivated paramyxoviruses (12, 13) or paramyxovirus subviral components (17) rendered these cells susceptible to specific lysis by cytotoxic T cells directed to these viruses. Because of the disparity between the in vivo and in vitro results described above, it was of interest to determine if inactivated influenza virus could sensitize putative target cells for lysis by influenza specific CTL in vitro. <sup>51</sup>Cr-labeled uninfected P815 mastocytoma cells were incubated under standard conditions (see Materials and Methods) with infectious A/WSN, A/JAP, or B/LEE viruses or with various concentrations of UV-inactivated A/WSN or A/JAP viruses and exposed in a standard cytotoxicity assay to highly potent influenza A/JAP-specific secondary effectors generated in vitro (Table VI). As demonstrated previously (Table IV and [19]), target cells infected with either A/WSN or A/JAP infectious virus were highly susceptible to lysis by these effector cells. On the other hand, target cells treated with inactivated A/WSN or A/JAP at concentrations up to 200-fold higher than the concentration of infectious virus

needed to sensitize target cells, showed no lysis above background observed on B/LEE-infected or uninfected target cells. Identical results were obtained with secondary effectors specific for influenza A/WSN (not shown). The high degree of background lysis demonstrable on influenza B/LEE-infected or uninfected target cells has been previously observed with virus-specific cytotoxic T cells generated *in vitro* (3, 8) and was somewhat magnified by the relatively high effector to target ratio (5:1) and the longer incubation time (8 h) employed in the assay. These assay conditions were chosen to increase the possibility of detecting sensitization of target cells by inactivated virus.

### Discussion

In this report, we have examined the issue of whether infectious virus is necessary both for the induction of CTL responses (i.e., stimulator cell sensitization) and for target cell sensitization or alternatively, whether induction and target cell sensitization can be achieved with noninfectious virus preparations. We have observed that while infectious influenza virus was highly efficient at inducing both primary and secondary influenza-specific CTL responses, noninfectious (UV-inactivated) influenza virus failed to stimulate detectable primary or secondary CTL responses *in vivo*. Similarly, noninfectious virus failed to sensitize target cells for lysis by influenza-specific cytotoxic T cells *in vitro*. However, inactivated virus could stimulate an influenza-specific secondary CTL response *in vitro*.

Before considering possible interpretations and implications of these results, two critical issues pertinent to our *in vivo* observations must be considered. The first issue is whether the inactivation procedure itself rendered the virus immunologically inactive. This possibility is unlikely since inactivated virus was capable of stimulating an adequate humoral immune response *in vivo* and could *in vitro* stimulate a specific cell-mediated cytotoxic response. The second issue is whether the parenteral administration of infectious influenza virus generates a sufficient antigen dose, as a result of replication *in vivo*, to induce a CTL response, whereas noninfectious (inactivated) virus fails to achieve such stimulatory antigen concentrations. We have attempted to approach this issue by examining the antigen dose dependence of CTL generation with both infectious and inactivated virus. Although low doses of infectious influenza virus ( $10^{-3}$ - $10^{-2}$  HAU) induced detectable cytotoxic T-cell responses *in vivo*, no specific cytotoxic activity was detectable *in vivo* with  $10^5$ - $10^6$ -fold higher concentrations of inactivated virus (Fig. 1, Table I). Furthermore, since the humoral immune response to both infectious and inactivated virus was proportional to the immunizing virus dose and similar in magnitude, it is unlikely that extensive virus replication occurs *in vivo* after intravenous inoculation of infectious influenza virus. Also, current evidence indicates that the relevant target organs, presumably involved in the clearance of the parenterally administered virus inoculum (e.g., liver, spleen, lymph nodes), are not productively infected with influenza virus (26, 27). Taken together, these observations suggest that the difference between infectious and noninfectious influenza viruses in their respective capacities induce CTL responses *in vivo* is not purely a function of antigen dose *in vivo*.

A number of laboratories have recently reported results different from those

reported here (12–15, 17). The results which are perhaps most germane to the present discussion involve the paramyxovirus model where inactivated virus preparations have been shown to both induce virus-specific CTL responses (12, 13) and sensitize target cells for T-cell-mediated lysis *in vitro* (12, 13, 17). The difference between these observations and those reported here, we believe, lies in the fact that the paramyxovirus virion possesses a specific fusion protein (28) which allows for the efficient integration of virion surface antigens into the cell cytoplasmic membrane (28) and also promotes cell-to-cell fusion (29). Such fusion activity has not been demonstrated in influenza viruses (29, 30). Furthermore, it has been recently reported that a functionally active fusion protein is necessary for the sensitization of target cells by a UV-inactivated paramyxovirus (31). In the light of these findings and our inability to sensitize target cells with inactivated influenza viruses (Table VI), we propose that both for the induction of virus-specific CTL responses and for the expression of the effector activity of CTL, the relevant viral antigens must be presented on the surface of the putative stimulator or target cell as integral membrane components, *i.e.*, inserted into the membrane lipid bilayer. Such a situation could be readily achieved either by direct integration of the virion antigens into the cell membrane through fusion, as in the case of paramyxoviruses and other viruses which possess efficient fusion capacity or as exemplified by viruses such as influenza, which lack such efficient fusion activity, by incorporation of nascent antigens into the cell membrane during the course of virus infection.

Although our results on the induction of CTL responses *in vivo* and target cell sensitization *in vitro* with inactivated influenza virus are consistent with the above hypothesis, the induction of a secondary cytotoxic response to inactivated virus *in vitro* is in apparent disagreement. This result is open to two interpretations: first, it is possible that there is a qualitative difference in the requirements for CTL induction under *in vitro* conditions of stimulation, *i.e.*, precursors of cytotoxic T cells can be directly stimulated by free virus or virus adsorbed to the stimulator cell surface *in vitro* but not *in vivo*. Second, it is possible that this difference is quantitative, *i.e.*, inactivated influenza virus is capable of sensitizing stimulator cells by integration of virion antigens into the cell cytoplasmic membrane but with an efficiency too low to be detectable either at the level of target cell sensitization *in vitro* or under *in vivo* conditions of stimulation. *In vitro* conditions of stimulation, on the other hand, would favor the detection of a response to the small number of sensitized stimulator cells generated by inactivated virus. A resolution of this point may come from experiments with purified influenza virus antigens which have recently been shown to stimulate a specific CTL response from primed cells *in vitro* (32).<sup>2</sup> Such studies are now in progress.

At least two distinct subpopulations of cytotoxic T cells are generated in response to infectious type A influenza virus (19, 24), one of which is specific for the immunizing virus strain (virus-strain-specific), the other of which exhibits a high degree of crossreactivity for target cells infected with type A influenza viruses of any subtype (19, 24, 33). Current evidence suggests that the target antigens for these two cytotoxic subpopulations are the influenza virion surface

<sup>2</sup> T. J. Braciale, and T. J. Higgins. Manuscript in preparation.

glycoproteins (hemagglutinin and possibly neuraminidase) and the internal virion antigen matrix protein, respectively (34). Since inactivated influenza A/JAP stimulated a cytotoxic response which was specific for A/JAP targets (Table IV), only the virus-strain-specific CTL subpopulation appears to have been generated in response to this virus preparation.<sup>3</sup> This result implies that the *in vitro* response to inactivated A/JAP virus was not due to a low level of infectious virus in the virion preparation since infectious A/JAP virus also stimulates a response in the crossreactive cytotoxic subpopulation (Table IV). Zweerink et al. (32), however, have recently reported the induction of a CTL response to UV-inactivated influenza virus which was highly cross-reactive. The most likely cause for the discrepancy between our results and those of Zweerink et al. is the difference in the extent of virus inactivation: 7 min vs. 1 min UV exposure, respectively. Since the infectivity of an influenza virus preparation is lost more rapidly during inactivation than the capacity of the virions to direct the synthesis of specific viral antigens (35), it is possible that a partially inactivated virus preparation, although incapable of producing infectious virions, is capable of directing the synthesis and expression of relevant viral antigens on the stimulator cell surface during an abortive cycle of replication. Consistent with this concept is the observation that the putative target antigen for cross-reactive cytotoxic subpopulation, influenza matrix protein (34) is expressed on the cell surface during the course of infection (34, 36, 37) but is internally located in the influenza virion (30). Two other reports of CTL responses to inactivated virus (14, 15) might be explained on a similar basis, i.e., synthesis and expression of the relevant viral antigens in the absence of infectious virus production. Since, in these reports, the capacity of inactivated virus to sensitize target cells or to direct nascent viral protein synthesis was not examined, the discrepancy between these observations and those reported here, remains to be resolved.

An observation reported here which warrants further discussion is the capacity of inactivated influenza virus to stimulate an *in vivo* primary humoral immune response in the absence of a detectable CTL response *in vivo*. Since the induction of an *in vivo* primary humoral immune response to influenza virus has been shown to be thymus-dependent (38-40), it is likely that helper T cells can be activated by either infectious or inactivated influenza virus *in vivo*. However, only infectious virus stimulates a detectable CTL response *in vivo*. One possible interpretation of this observation is that helper T cells and cytotoxic T-cell precursors differ in their requirements for induction with respect to mode of antigen presentation. According to the hypothesis outlined above, the induction of a CTL response to specific viral antigens would require their presentation on the stimulator cell as integral membrane components, whereas the activation of helper T cells directed to these antigens could be achieved in a manner analogous to that suggested for soluble antigens (41). In this connection, it should be noted that precursors of helper T cells and precursors of cytotoxic T cells also differ in their requirements for induction

<sup>3</sup> By using appropriate recombinant influenza virus strains, the specificity of the cytotoxic cells for influenza A/JAP hemagglutinin has been demonstrated (T. J. Braciale, unpublished observations).

with respect to genes in the MHC (42-44). The question of whether this genetic difference in the requirement for helper and cytotoxic T-cell induction is a reflection of the difference in the mode of antigen presentation will require further analysis of these two T-cell subsets.

The requirement for viral infectivity in the induction of virus-specific cytotoxic T cells remains to be fully elucidated. Factors which should be considered in assessing this requirement include: (a) whether the virus employed has efficient fusion activity which could promote efficient integration of virion antigens into the cell cytoplasmic membrane; (b) whether an inactivated virus preparation which fails to undergo a complete cycle of replication, i.e., produce infectious virus, is also incapable of inducing nascent viral protein synthesis during an abortive cycle of replication; (c) whether the analysis is undertaken *in vivo* or *in vitro*. Based on our own observations and those of other investigators we have proposed that both the induction of virus-specific cytotoxic T cells and the expression of their lytic activity requires the presentation of the relevant viral antigens on the surface of the stimulator or target cell as integral membrane components. Experiments are now in progress to test this proposal.

### Summary

This report examines the requirement for infectious virus in the induction of influenza virus-specific cytotoxic T cells. Infectious influenza virus was found to be highly efficient at generating both primary and secondary cytotoxic T-cell response *in vivo*. Inactivated influenza virus however, failed to stimulate a detectable cytotoxic T-cell response *in vivo* even at immunizing doses  $10^5$ - $10^6$ -fold higher than the minimum stimulatory dose of infectious virus. Likewise inactivated virus failed to sensitize target cells for T-cell-mediated lysis *in vitro* but could stimulate a specific cytotoxic response from primed cells *in vitro*. Possible requirements for the induction of virus-specific cytotoxic T-cell responses are discussed in light of these observations and those of other investigators.

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