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Influence of One Virus Infection on a Second Concurrent Primary In Vivo Antiviral Cytotoxic T-Cell Response

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The influence of one virus on the in vivo cytotoxic T-cell response to a different concurrent viral infection was analyzed. Lymphocytic choriomeningitis and Newcastle disease viruses, known to induce high interferon titers, and the synthetic interferon inducer polyriboinosinic acid-polyribocytidylic acid inhibited the cytotoxic T-cell response against the second virus. In contrast, vaccinia and vesicular stomatitis viruses failed to induce inhibition. Inhibition directly correlated with the interferon titers; similarly, the interferon titers directly correlated with macrophage and natural killer cell activation. The involvement in vivo of interferon in macrophage and natural killer cell activation and the possible mechanisms of inhibition of the cytotoxic responses are shown by the inhibition of the effect by antibodies against interferon.

Induction of cell-mediated immunity to foreign cell surface antigens such as those induced by viruses is controlled partly by immune response genes mapping in the major histocompatibility complex (23). Cell-mediated immunity plays a major role in recovery from viral infections. Studies in mice have shown that viral infections themselves can impair these cellular immune responses (13, 15). This impairement can result from (i) changes in lymphocyte or macrophage circulation altering the availability of the cells required for immune responsiveness, (ii) induction of cell-mediated suppressor mechanisms, or (iii) induction of nonspecific mediators such as interferon which have a wide variety of immunoregulatory functions (or from a combination of these). The relative contribution of these different mechanisms may be dependent on the type and conditions of the viral infection.

Recently the nonspecific immunoregulatory properties of interferons have been stressed, in particular, the activation of macrophages (18) and natural killer cells (NK cells) (21). Many have observed that immune reactions are impaired in vivo when sick mice are used or in vitro when lymphoid cells from such mice are used. In this report we attempt to analyze the nonspecific modulation of immune responses induced by one viral infection on a second, concurrent primary antiviral cytotoxic T-cell response in vivo. We demonstrate that interferon may influence cytotoxic T-cell responses.

MATERIALS AND METHODS

Mice. C57BL/6 (H-2^b) mice were purchased from Olac Ltd., Bicester, Oxon, England, and from the Institut für Zuchthygiene, Universität Zürich.

Viruses. Lymphocytic choriomeningitis virus Armstrong (LCMV) and vesicular stomatitis virus (VSV) Indiana strain were grown and titrated as described previously (17, 22). Lyophilized vaccinia virus "Lancy Vaccinia" was purchased from Schweizerisches. Serum- und Impfinstitut, Bern. Newcastle disease virus (NDV) propagated in the allantoic cavity was kindly given by O. Haller (University of Zürich).

Immune cytotoxic T-cells were generated by immunizing mice intravenously (i.v.) with 5×10^6 PFU of vaccinia virus, 5×10^5 PFU of LCMV or VSV, or 10^7 50% egg infectivity doses of NDV on the days indicated below.

Poly(I)(C). Polyriboinosinic acid-polyribocytidylic acid [poly(I)(C)] was purchased from Serva Feinbiochemica. Mice were immunized with 100 μg i.v.

Bacteria. *Listeria monocytogenes* organisms were prepared from overnight cultures grown in Trypticase soy broth (BBL Microbiology Systems) as described previously (3). Mice were immunized with 5×10^3 bacteria i.v.

Target cells. MC57 (H-2^b) and L929 (H-2^k) fibroblasts and YAC (H-2^k) lymphoma cells were infected with 0.1 to 0.5 PFU per cell for LCMV and 10 to 100 PFU per cell for the other viruses used. LCMV was added to the target cell cultures 24 to 48 h before labeling, and vaccinia virus and VSV were added at the time of labeling.

Cytotoxic assay. The cytotoxic assay method has been described previously in detail (4). Briefly, target cells were labeled with 100 μg of sodium [^{51}Cr]chromate for 2 h. Effector virus-immune spleen cells were generated as stated in each experiment. Dilutions of single-cell suspensions of effector cells in Iscove modified minimal essential medium supplemented with 10% fetal calf serum were dispensed in triplicate into flat-bottomed microtiter plates, and 5×10^4 target cells were added. Maximum release was determined by adding 100 μl of 1 N hydrochloric acid, and spontaneous release was determined by adding 100 μl of supplemented Iscove modified minimal essential medium to target cell replicates. The duration of the assays

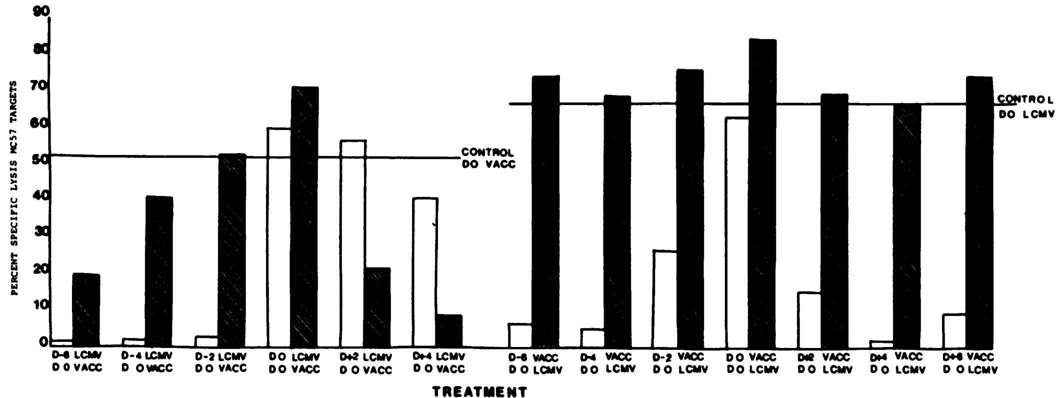

FIG. 1. (A) Kinetic influence of LCMV on the primary in vivo day 0 vaccinia virus cytotoxic response. (B) Kinetic influence of vaccinia virus on the primary in vivo day 0 LCMV cytotoxic response. The columns represent percent specific lysis of vaccinia virus-infected (□) or LCMV-infected (▧) MC57 tumor target cells at a killer to target (K/T) cell ration of 30:1. The percent specific lysis of uninfected MC57 tumor target cells was 5% in the experiment shown. Spontaneous release ranged from 10 to 15%.

was 6 h. The percent specific lysis was calculated by the formula: percent specific lysis = [(experimental release) − (medium release × 100)]/(maximum release − medium release). The spontaneous release ranged from 6 to 20%. Standard errors of the mean of triplicates were less than 3% in the results shown.

Mouse interferon assay. Triplicate samples (50 μl) of serial twofold dilutions of standard and test interferon samples were made in 96-well, flat-bottomed microtiter plates to which 5×10^4 L929 fibroblast cells in 50 μl were added. The plates were incubated at 37°C overnight, and 10^4 PFU of VSV in 100 μl were added to each well and incubated for a further 24 to 48 h. The plates were stained with crystal violet.

Test samples were obtained from groups of primed mice that were tail bled. Samples were centrifuged at 3,000 rpm, and the sera were harvested and stored at −70°C for testing.

Listeria assay. Groups of five mice were injected with poly(I)(C) and 48 h later challenged with 5×10^3 bacteria. Spleens were removed 48 h later, and the number of viable bacteria was determined by the method of Mackaness (11). Briefly, spleens were removed asceptically and homogenized individually in 2 ml of nutrient broth with Teflon pestles in glass tubes. Tenfold dilutions were plated on tryptose blood agar (no. 0232-01; Difco Laboratories, Detroit, Mich.) and incubated at 37°C overnight. Colonies were counted. The results are expressed as the mean ± the standard error of the mean of groups of five mice.

Sheep anti-α,β-interferon antiserum. Sheep anti-α,β-interferon antiserum and normal sheep control serum were generously donated by I. Gresser, Institut de Recherches Scientifiques sur le Cancer, Villejuif, France. Each mouse was injected i.v. with 33 μl in 200 μl of Iscove modified minimal essential medium. The antiserum had a neutralizing titer of $1:3 \times 10^6$ against 8 IU of mouse interferon per ml. Its characterization was previously described by Gresser et al. (8, 9).

RESULTS

Kinetics of inhibition of antiviral cytotoxic responsiveness. Different viral combinations were tested to analyze the effect of one virus on the primary viral immune cytotoxic T-cell response to another. Test groups of three mice were primed twice in vivo. In each experiment a specified virus was injected at the time intervals stated above and after induction of a 6-day vaccinia virus or VSV or a 7-day LCMV primary immune response. Viral immune spleen cells from individual mice were tested on uninfected and virus-infected target cells after 6 or 7 days respectively.

Figure 1A shows a representative example of the effect of LCMV on the in vivo 6-day primary anti-vaccinia virus cytotoxic T-cell response. LCMV given on day -6, -4, or -2 (before priming to vaccinia virus) inhibited the vaccinia virus response. In contrast, LCMV given at the same time as vaccinia or on day 2 or 4 (after priming to vaccinia virus) produced slight or no inhibition compared with the vaccinia virus control.

Figure 1B shows the results of the reverse experiment. Vaccinia virus given before or after priming to LCMV produced no inhibition of the LCMV cytotoxic response.

Comparable inhibition was measured with LCMV given before priming to VSV (Table 1) or NDV (data not shown).

Influence of interferon on cytotoxic responsiveness. The possibility that inhibition of the primary cytotoxic response was due to interferon induced by the first virus was tested by measuring interferon production at different times in primed mice (Table 2). The interferon titer in the sera from groups of mice bled before immunization and at days 1 and 5 after immunization with LCMV, VSV, or vaccinia virus were measured.

Table 2 shows that the interferon titer in each serum sample was higher at day 1 compared with that on day 5. Comparison of the interferon

TABLE 1. Kinetic influence of LCMV on the primary in vivo anti-VSV cytotoxic T-cell response in C57BL/6 (H-2^b) mice

Treatment	Day	K/T cell ratio	% Specific lysis of MC57 (H-2^b) target cells[a]		
			Uninfected	VSV	LCMV
VSV	0	30:1	7	84	4
		10:1	5	51	4
		3:1	1	28	2
		1:1	<1	11	<1
LCMV	−6	30:1	<1	6	6
VSV	0	10:1	<1	1	2
		3:1	<1	<1	1
		1:1		<1	1
LCMV	0	30:1	5	76	27
VSV	0	10:1	1	36	14
		3:1	<1	21	11
		1:1	<1	12	6
LCMV	+5	30:1	8	77	9
VSV	0	10:1	1	36	5
		3:1	<1	20	3
		1:1	<1	9	1

[a] Spontaneous release ranged from 6 to 13%.

TABLE 2. Kinetics of interferon titers in the sera of mice primed with LCMV, VSV, or vaccinia virus (VACC)

Serum samples	Day	Interferon titer (U/ml)[a]
C57BL/6 anti-LCMV	Control before immunization	0
	+1	1,500
	+5	0
C57BL/6 anti VSV	Control before immunization	0
	+1	40
	+5	0
C57BL/6 anti VACC	Control before immunization	0
	+1	5
	+5	0

[a] A standard interferon sample contained 1,500 U/ml.

titers at day 1 shows that LCMV induced the highest and vaccinia virus induced the lowest. These results directly correlated with the degree of inhibition produced by these viruses (Fig. 1 and Table 1). In each group the level of serum interferon before priming to virus was negligible.

The influence of interferon on the cytotoxic response was further investigated by measuring the effect of NDV and poly(I)(C) (high interferon inducers) given on days -6, -1, 0, and +1 on the primary vaccinia cytotoxic T-cell response. In these experiments YAC target cells were included as indicators of NK cell activity.

NDV and poly(I)(C), when given before priming with vaccinia virus, inhibited the primary in vivo vaccinia virus response maximally; in contrast, when given simultaneously or after priming to vaccinia virus the degree of inhibition was reduced (Tables 3 and 4). Vaccinia did not inhibit the NDV response (data not shown). In all experimental groups YAC cells were killed.

Influence of macrophage activation on cytotoxic responsiveness. It has been reported previously that interferon activates macrophages (19); therefore, it was of interest to measure macrophage activation and compare this with the inhibition of the primary vaccinia virus cytotoxic T-cell response.

Macrophage activation by the interferon inducer poly(I)(C) was measured by *L. monocytogenes* clearance. Mice treated with poly(I)(C) reduced the colonies 10-fold compared with control mice injected with *L. monocytogenes* organisms alone (Table 5).

The possibility that activated macrophages were involved in the inhibition of cytotoxic T-cell responses was tested by injecting *L. monocytogenes* organisms before and after eliciting a 6-day vaccinia virus cytotoxic response. Only *L. monocytogenes* organisms injected before priming to vaccinia virus inhibited the response (Table 6).

Anti-interferon antiserum neutralize effects of poly(I)(C) on anti-vaccinia virus T-cell response.

TABLE 3. Kinetic influence of NDV on the primary in vivo anti-vaccinia virus (VACC) cytotoxic T-cell response in C57BL/6 mice

Effector	Day	K/T cell ratio	% Specific lysis of target cells[a]		
			MC57		YAC
			Uninfected	VACC	Uninfected
VACC	0	30:1	5	51	8
		10:1	6	35	9
		3:1	5	17	<1
		1:1	2	7	1
NDV	−6	30:1	6	19	28
VACC	0	10:1	5	5	14
		3:1	4	2	5
		1:1	3	2	3
NDV	−1	30:1	3	7	30
VACC	0	10:1	5	4	6
		3:1	4	<1	<1
		1:1	3	<1	<1
NDV	0	30:1	3	22	25
VACC	0	10:1	3	10	10
		3:1	2	4	<1
		1:1	2	3	<1

[a] Spontaneous release ranged from 8 to 16%.

TABLE 4. Influence of poly(I)(C) on the primary in vivo anti-vaccinia virus (VACC) cytotoxic T-cell response in C57BL/6 mice

Effector	Day	K/T cell ratio	% Specific lysis of target cells[a]		
			MC57		YAC
			Uninfected	VACC	Uninfected
Poly(I)(C)	0	30:1	7	6	27
		10:1	6	2	13
		3:1	5	<1	5
		1:1	3	<1	1
VACC	0	30:1	10	64	12
		10:1	5	43	7
		3:1	5	24	7
		1:1	3	9	3
Poly(I)(C)	−6	30:1	7	15	27
VACC	0	10:1	5	7	9
		3:1	5	2	3
		1:1	5	1	1
Poly(I)(C)	−1	30:1	6	<1	18
VACC	0	10:1	4	1	11
		3:1	3	1	5
		1:1	1	2	2
Poly(I)(C)	0	30:1	9	34	30
VACC	0	10:1	8	15	13
		3:1	6	8	5
		1:1	7	4	2
Poly(I)(C)	+1	30:1	5	26	17
VACC	0	10:1	5	10	7
		3:1	2	5	2
		1:1	2	2	<1

[a] Spontaneous release ranged from 11 to 20%.

Since most of the evidence gathered suggested that interferon played a major role in the effects seen, the effect of anti-α,β-interferon antiserum was evaluated (Table 7). When anti-interferon antiserum was injected i.v. 6 h before the injection of poly(I)(C), the drastic inhibition of the induction of a primary anti-vaccinia virus cytotoxic T-cell response was neutralized to a great extent (group B) when compared with the effect of poly(I)(C) alone (group A) or the small effect seen with normal goat serum (group C). A small neutralizing effect was observed in one example (group D1) when anti-interferon antiserum was injected 12 h after poly(I)(C). Anti-interferon antiserum or normal goat serum alone injected at −24 or −6 h (before vaccinia virus) had no significant effect on the anti-vaccinia virus T-cell response (compare groups F, G, H, and I with group K).

DISCUSSION

In the experiments presented in this paper we attempted to analyze the nonspecific mechanisms induced in the inhibition of a primary in vivo viral immune cytotoxic T-cell response during a concurrent viral infection. Most viruses are good interferon inducers; since interferon is capable of non-specifically inhibiting cell-mediated immunity (18), the possibility of interferon-mediated inhibition of cytotoxic responsiveness was investigated. The following results are in accordance with a role for interferon.

(i) Cytotoxic responses were inhibited non-specifically by different viruses. The vaccinia virus response was inhibited by LCMV (Fig. 1A) and NDV (Table 3). Similarly, LCMV inhibited the VSV (Table 1) and NDV (data not shown) cytotoxic responses.

(ii) There was a significant positive correlation between the interferon titers and the degree of inhibition of the vaccinia virus response—the highest interferon titers induced the most complete inhibition (Table 2).

(iii) The synthetic interferon inducer poly(I)(C) inhibited the vaccinia virus response (Table 4).

TABLE 5. Protection against *L. monocytogenes* in C57BL/6 mice injected with poly(I)(C)

Treatment of effectors	Day	Log_{10} viable *L. monocytogenes* cells in spleens at 24 h (± SEM)
Poly(I)(C)	0	2.0
L. monocytogenes	0	5.54 (±0.24)
Poly(I)(C)	−2	4.49 (±0.21)
L. monocytogenes	0	

TABLE 6. Influence of *L. monocytogenes* on the primary in vivo anti-vaccinia virus (VACC) cytotoxic T-cell response in C57BL/6 mice

Effector	Day	K/T cell ratio	% specific lysis of MC57 target cells[a]	
			Uninfected	VACC
L. monocytogenes	0	30:1	<1	9
		10:1	<1	7
		3:1	<1	7
		1:1	1	2
VACC	0	30:1	<1	74
		10:1	<1	53
		3:1	<1	24
		1:1	<1	10
L. monocytogenes	−6	30:1	<1	25
VACC	0	10:1	<1	18
		3:1	<1	9
		1:1	<1	1
L. monocytogenes	0	30:1	7	78
VACC	0	10:1	3	64
		3:1	<1	33
		1:1	<1	11

[a] Spontaneous release ranged from 10 to 16%.

TABLE 7. Influence of sheep anti-interferon antibodies on effect of poly(I)(C) on anti-vaccinia virus T-cell responses

Group	Treatment of mice at time:				K/T cell ratio	% Specific lysis from MC57G (H-2^b) target cells[a]	
	−24 h	−18 h	−6 h	0		VACC	Uninfected
A1		Poly(I)(C)		VACC	30	6	3
					10	3	<1
					3	1	<1
A2		Poly(I)(C)		VACC	30	7	1
					10	2	<1
					3	2	<1
B1	Anti-interferon	Poly(I)(C)		VACC	30	40	<1
					10	20	<1
					3	10	<1
B2	Anti-interferon	Poly(I)(C)		VACC	30	33	6
					10	19	<1
					3	6	<1
C1	Normal sheep serum	Poly(I)(C)		VACC	30	12	3
					10	4	<1
					3	1	3
C2	Normal sheep serum	Poly(I)(C)		VACC	30	15	10
					10	6	2
					3	3	<1
D1		Poly(I)(C)	Anti-interferon	VACC	30	22	<1
					10	10	3
					3	3	<1
D2		Poly(I)(C)	Anti-interferon	VACC	30	9	4
					10	2	<1
					3	<1	<1
E1		Poly(I)(C)	Normal sheep serum	VACC	30	3	<1
					10	1	<1
					3	1	<1
E2		Poly(I)(C)	Normal sheep serum	VACC	30	6	<1
					10	3	3
					3	6	2
F1	Anti-interferon			VACC	30	58	4
					10	32	2
					3	11	<1
G1	Normal sheep serum			VACC	30	51	<1
					10	23	<1
					3	12	<1
H1			Anti-interferon	VACC	30	53	2
					10	24	1
					3	6	<1
I1			Normal sheep serum	VACC	30	49	1
					10	27	1
					3	12	<1
K1				VACC	30	53	5
					10	26	<1
					3	11	<1
K2				VACC	30	50	1
					10	20	3
					3	12	5

[a] Spontaneous release was 14% for vaccinia virus-infected cells and 20% for uninfected cells.

(iv) The inhibition of the vaccinia response was only observed when the first virus was injected at least 24 h before allowing sufficient time for interferon to be produced and to act.

(v) Anti-α,β-interferon antiserum neutralized the inhibition of a primary anti-vaccinia virus response caused by poly(I)(C).

These results from a double viral infection are in agreement with previous data of nonspecific protection mediated by interferon or interferon inducers during a single viral infection (7, 14, 16, 20).

The importance of interferon in antiviral resistance has been stressed, but the multiple

origins and effects have complicated analyses. Interferon can act directly by inhibition of viral replication, preventing adequate viral expression, or indirectly on cells involved in immunological defense mechanisms (18). Since vaccinia virus does not replicate in mice, our results probably document indirect regulation involving macrophages and NK cells.

Evidence for macrophage activation in spleen cell populations by interferon or poly(I)(C) was shown by the increased clearance of *L. monocytogenes* colonies (Table 5). Further indirect evidence for the immunoregulatory role of activated macrophages was shown by inhibition of the vaccinia virus response by prior immunization with *L. monocytogenes*—a potent macrophage activator (Table 6). These results correlate with previous reports of increased phagocytic activity of macrophages in vitro by NDV and poly(I)(C) (2, 5, 12). Macrophages activated as the result of the first virus may induce inhibition to the second virus through a reduction in the number of available macrophages, which are necessary for the expression of viral antigens to induce a cytotoxic T-cell response. Activated macrophages may also have a diminished antigen presentation capacity.

The involvement of NK cells in viral inhibition was monitored by lysis of NK cell-sensitive YAC cells. Increased YAC killing correlated with increased interferon titers. Interferon is known to activate NK cells (20, 21), and since activated NK cells are capable of lysing virus-infected cells (1), this may be an important mechanism for reducing virus replication. However, the correlation between high interferon titers and NK cell activity is not absolute for all viruses—the herpes simplex virus-resistant mouse strain SJL/2 produces high interferon titers, but low NK cell activity (6).

In summary the results in this paper show that there is a correlation between the interferon titers produced by different viruses with macrophage and NK cell activity and the inhibition of primary in vivo viral cytotoxic T-cell responses. These results may have important implications for determining resistance in the presence of more than one viral infection.

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