ANTIVIRAL PROTECTION BY VIRUS-IMMUNE CYTOTOXIC T CELLS: INFECTED TARGET CELLS ARE LYSED BEFORE INFECTIOUS VIRUS PROGENY IS ASSEMBLED*

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Cytotoxic, thymus-derived lymphocytes (T cells) which can specifically lyse cultured target cells in vitro also function in vivo in a number of immune reactions of mice; these include: graft versus host reactions, host versus graft reactions (reviewed in 1), the response to virus infections (reviewed in 2, 3), and tumor rejection (reviewed in 1). Cytotoxic T cells appear to be preoccupied with cell surface structures coded for by the *K* and *D* regions of the major murine histocompatibility (*H-2*) complex (1–13). Thus, these T cells seem to monitor cell surfaces for the foreign antigen in functional association with self-markers (probably the serologically defined transplantation antigen).

Although cytotoxic T cells have been studied in vitro with respect to their specificity and the actual mechanism of cytotoxicity (reviewed in 1), our knowledge about their physiological effector role in vivo remains elusive. Circumstantial evidence exists that alloreactive T cells are essential for the elimintion of *H-2*-incompatible tumor cells or tissue grafts (1, 12). There is also strong suggestive evidence that in vitro cytotoxic virus-immune T cells act similarly against virus-infected target cells in vivo (2, 3, 6). This conclusion derives from the fact that the parameters of T-cell-mediated immunity (i.e., kinetics of generation of effector T cells, specificity, and *H-2* restriction), of mice infected with ectromelia virus (mouse pox) or lymphocytic choriomeningitis virus (LCMV)[1] as assayed in vitro parallel the ones in vivo (2, 3, 7, 8). In these two murine virus infections, T cells are necessary for the rapid elimination and/or for restricting growth of virus (reviewed in 2, 3) during the early 3–5 days of a primary infection as shown in adoptive transfer experiments. Antibody-forming cells are, at least in the adoptive transfer models studied, not mandatory; however, they may be important in inhibiting spreading of extracellular virus and in protecting the host against reinfection.

What then is the role in vivo of these virus-specific T cells that are cytotoxic in vitro and, if they are also lytic in vivo, how do they exert their antiviral function? Two possible mechanisms are: (*a*) Cytotoxic T cells can lyse virus-infected cells before infectious virus progeny is assembled and released (Fig. 1*a*); (*b*) T cells do not lyse infected cells, but rather upon recognition of the

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[1] *Abbreviations used in this paper:* LCMV, lymphocytic choriomeningitis virus; MOI, multiplicity of infection; PFU, plaque-forming unit

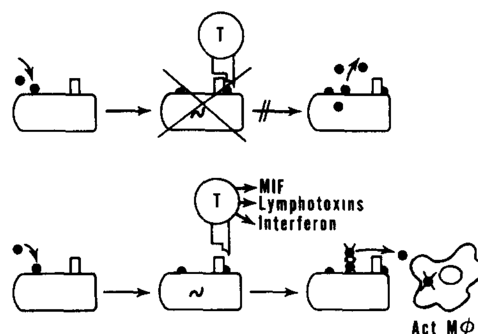

FIG. 1. Two possible mechanisms by which immune T cells could control virus production and/or spreading: (*a*) Cytotoxic T cells lyse acutely infected cells during the eclipse phase before infectious viral progeny are assembled and released (*b*) T cells do not actually lyse acutely infected target cells, but upon recognition of specific virally induced cell surface antigens release immune interferon or other lymphokines such as migration inhibitory factor (MIF), which can cause inactivation of released virus directly or by activation of macrophages (M ϕ). (The T-cell receptor may be formed by one single or two linked, but independent clonally expressed recognition sites, 6)

relevant viral and self-cell surface antigens the T cells release soluble mediators which then indirectly prevent virus spread. The release of immune interferon or lymphokines that activate macrophages to increased virocidal capacity could be involved in this process, and some supporting evidence for this second mechanism exists (reviewed in 2, 14) (Fig. 1*b*). The results of experiments with vaccinia virus described here provide evidence in support of the first mechanism.

Materials and Methods

Mice. 6- to 9-wk-old B10 D2 (*H-2* ^{d}), BALB/c (*H-2* ^{d}), and B10.BR (*H-2* ^{k}) were purchased from The Jackson Laboratory, Bar Harbor, Maine.

Virus and Immunization The WR strain of vaccinia virus was a gift from Dr W. K. Joklik, Duke University, Durham, N. C Mice were infected intravenously (i v.) with 8×10^6 plaque forming units (PFU), and spleen cells were harvested 6 days later (6, 10, 11). The methods employed for the preparation of cells and tissue culture medium have been described (6).

Virus Titrations Virus PFU were determined on Vero cells as described by Early et al. (15). PFU present in individual wells of hemagglutination trays were determined by vigorously pipetting supernate plus all cells out of each well. The suspensions were freeze-thawed and sonified for 10 s before fourfold dilutions were plaqued on Vero cell according to described techniques (15) in 24-hole, 16-mm plates (Linbro Chemical Co., New Haven, Conn.). For the infectious center assay, the infected target cells were pipetted out of individual wells and washed twice in medium Fourfold dilutions were mixed with Vero cells and plated into 24-hole, 16-mm plates.

Antiserum AKR anti-θ C3H was purchased from Bionetics, Kensington, Md. (cat. 8301-01, Lot no 231-61-5). 5×10^7 spleen cells were treated with 1 ml of a 1:10 dilution of the antiserum for 30 min at room temperature Rabbit complement (C) was added at a 1:6 dilution for 30 min at 37°C (6).

^{51}Cr Release Assay. The fibrosarcoma J774 (*H-2* ^{d}) target cell line originating from BALB/c and the fibroblast L929 (*H-2* ^{k}) cell line originating from C3H mice have been described previously (6). The tests were all performed in flat-bottomed hemagglutination trays (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) 1–3 $\times 10^4$ target cells were seeded per well and after adherence labeled with ^{51}Cr, washed, and then infected with vaccinia virus at a multiplicity of infection (MOI) of about three. Spleen cells were then overlaid and percent ^{51}Cr release determined after various intervals, using water release as 100% (6).

Presentation of Data Means ± SEM of triplicates or quadruplicates were compared by using Student's *t* test.

Results

In a preliminary experiment it was determined at what time after acute infection of cultured cells with vaccinia virus, virus progeny was demonstrable in J774 target cells. 1×10^4 J774 were infected with about three PFU per cell and washed three times after 1 h. At intervals, total $\log_{10}$ PFU were determined per 10^4 cells. Between 2.5 ± 0.2 and 2.8 ± 0.3 $\log_{10}$ PFU/10^4 cells were found at 1, 2, and 3 h, 4.0 ± 0.2 at 4 h, 4.8 ± 0.3 at 5 h, 5.2 ± 0.4 at 7 h, 5.4 ± 0.5 at 10 h after infection. Thus, virus titers increase after 3–4 h. After 12 h infected J774 released virtually all of their ^{51}Cr label spontaneously into the supernate indicating cell death. Antiviral and cytotoxic activity of immune spleen cells were therefore assayed from 1–5 h and 1–10 h after acute infection of target cells (Table I).

The vaccinia infected J774 target cells were lysed completely by immune spleen cells from *H-2* compatible mice immunized with vaccinia virus. When assayed 5 h after infection, the virus titers measured as PFU were reduced by 1.65 $\log_{10}$, i.e. about 30-fold, in the wells overlaid with immune syngeneic lymphocytes (Table I). Virus titers in the test wells overlaid with normal syngeneic or normal or immune allogeneic B10.BR spleen cells were not reduced, although the allogeneic immune B10.BR (H-2^k) spleen cells were cytotoxic on syngeneic infected L-cell target cells (H-2^k). During the interval of 5–10 h after initiation of infection of the target cells virus titers increased slightly, about two to threefold in all wells except in those wells where H-2^d immune spleen cells had lysed virtually all targets by 5 h (Table I). This suggests that if soluble factors had been released upon specific T-cell interactions they were not efficient in reducing viral titers measurably.

The kinetics of cytolytic and antiviral activity correlated (Table II). 4-day immune spleen cells were cytolytic and reduced viral titers, but both to a lesser extent than 6 day immune spleen cells. By 8 days both specific activities were low.

To demonstrate more directly that cytolytic activity of T cells was antiviral when the target cells were lysed before assembly of infectious virus progeny we examined the antiviral effect of cytotoxic, virus-immune T cells separately during the period of 1–4 h or during the interval of 4–8 h after initiation of infection of target cells with virus (Table III). Cytotoxic T cells caused lysis of about 90% of the target cells irrespective of the time interval chosen. During the period of 1–4 h after infection of the target cells immune spleen cells lysed 91% of the infected target cells and reduced, when compared with normal spleen cells (Group C), the number of virus-producing target cells (infectious centers) per well by about 1.2 $\log_{10}$ (Group D). In the same wells the total number of PFU per well was also decreased by about 1.3 $\log_{10}$ as compared to control wells. In contrast, although immune spleen cells were highly cytotoxic when tested on infected targets during the period of 4–8 h after infection, they did not reduce the number of infectious centers or of the total number of PFU per well (Group G) when compared with controls (Groups E and F). The total numbers of PFU are

TABLE I
Antiviral and Cytotoxic Activity of Vaccinia Immune T Cells Assayed In Vitro on Acutely Vaccinia Virus-Infected Target Cells

Mouse strain	Spleen cells	Test time-interval after initiation of the infection of J774 (H-2^d) target cells*			
		1-5 h		1-10 h	
		Log_{10} PFU/well	Percent ^{51}Cr release	Log_{10} PFU/well	Percent ^{51}Cr release
B10.D2 (H-2^d)	Normal	4.60 ± 0.35‡	8.7 ± 0.5	5.11 ± 0.30	32.7 ± 1 1
	Immune	2.95 ± 0 17§	80.9 ± 1.1‖	2.97 ± 0.20‖	100.1 ± 2 2‖
B10 BR (H-2^k)	Normal	4.47 ± 0 20	12.3 ± 0.6	5.17 ± 0.18	27.0 ± 0.8
	Immune¶	4.30 ± 0.15	10.9 ± 1.1	4.96 ± 0.22	27.1 ± 1 0

* 3 × 10^4 target cells per well were labeled with ^{51}Cr, washed, and infected with vaccinia virus at a MOI of three for 1 h before. ^{51}Cr release and total virus PFU/well were determined in parallel at the end of the time intervals tested The killer to target cell ratio was 60:1.
‡ Means ± SEM of triplicates.
§ Significantly different from control values, $P < 0\ 01$.
‖ $P < 0.001$
¶ B10.BR immune spleen cells lysed vaccinia infected L cells (H-2^k) to 65 2 ± 1 2% as compared to 20 1 ± 0.6% by immune B10 D2 cells.

TABLE II
Time-Course of the Generation In Vivo of Virus-Specific Cytotoxic Antiviral Activity Assessed In Vitro

B10 D2 spleen cells	Time after infection	J774 (H-2^d) target cells were infected for 1 h and tested during the interval 1-5 h after infection*	
		Log_{10} PFU/well	Percent ^{51}Cr release
	days		
Normal		4 55 ± 0.18‡	7 0 ± 0.5
Vaccinia immune	4	3.80 ± 0.15§	40.7 ± 0.9‖
Vaccinia immune	6	3 09 ± 0.37§	85.3 ± 2.0‖
Vaccinia immune	8	4.38 ± 0.20	18.7 ± 1.1‖

* See Table I.
‡ Means ± SEM of triplicates.
§ Significantly different from control value, $P < 0.05$.
‖ $P < 0.001$.

relatively low when compared to numbers of infectious center. However, the two determinations differ technically, since only the samples that were examined for total PFU per well were freeze-thawed and sonicated for 10 s. Furthermore, vaccinia virus is strongly membrane associated and not readily dissociated. This could explain some considerable loss of detectable PFU. The effector cell responsible for the cytolytic and antiviral effect in vitro is a T cell by several criteria: (*a*) the effector cells are anti-θ + c′ sensitive (Table IV); (*b*) the effector functions are restricted by the *H-2* gene complex; (*c*) the kinetics of the activities are

TABLE III

Comparison of Cytotoxic with Antiviral Activity of Vaccinia Immune Lymphocytes Measured by ^{51}Cr Release and by Infectious Center Assay to Assess Reduction of Virus-Progeny-Producing Target Cells

Group	After acute infection for 1 h, target cells were overlaid with	Total hours elapsing between infection of targets and determination of	Infectious centers per well*	Total PFU/well*	Percent ^{51}Cr release* ‡
A	Medium for 1 h	2	3 62 ± 0 09§	2 81 ± 0 20§	5 1 ± 0 3§
B	Medium for 3 h	4	3 68 ± 0 05	4 61 ± 0 25	9 2 ± 0 5
C	Normal BALB/c for 3 h	4	3 86 ± 0 11	4 50 ± 0 16	9 7 ± 0 7
D	Immune BALB/c for 3 h	4	2 46 ± 0 12‖	3 21 ± 0 10‖	91 1 ± 1 0¶
E	Immune BALB/c for 7 h	8	2 63 ± 0 15‖	3 38 ± 0 13‖	98 4 ± 1 3¶
F	Medium for 3 h + medium for 4 h	8	4 27 ± 0 15	4 87 ± 0 15	22 3 ± 1 0
G	Medium for 3 h + normal BALB/c for 4 h	8	4 25 ± 0 22	4 90 ± 0 10	20 3 ± 0 8
H	Medium for 3 h + immune BALB/c for 4 h	8	4 17 ± 0 20**	4 76 ± 0 12**	88 5 ± 1 2¶

* The numbers of infectious centers or of total PFU per well were determined as described in Materials and Methods

‡ 3×10^4 J774 target cells were overlaid with 2×10^6 spleen cells

§ Means ± SEM of quadruplicate determinations

‖ Significantly smaller than medium or normal cells control, $P < 0\ 001$

¶ Significantly greater than ^{51}Cr release by medium or normal cells, $P < 0\ 001$

** Not significantly different from medium or normal cells control

TABLE IV

*T-Cell Dependence of Antiviral Effect of Cytotoxic Spleen Cells In Vitro**

Spleen cells	Cell treatment	J774 (H-2^d) targets tested 1–5 h after infection with vaccinia virus	
		Log_{10} PFU/well	Percent ^{51}Cr release
B10.D2 (H-2^d)			
Normal	None	4 79 ± 0 02	25 2 ± 1.0
Immune	None	2.78 ± 0 10	97.5 ± 1 9
	Normal AKR + C′	2 73 ± 0.09	101 3 ± 2.2
	AKR anti-θ C3H + C′‡	4 66 ± 0.10§	21.1 ± 0.7§
B10.BR (H-2^k)			
Immune‖		4.60 ± 0 15	21 0 ± 0 8
Normal	None	4.85 ± 0.10	18.7 ± 0 7

* Target cells and ^{51}Cr release test were performed as described in Table I

‡ AKR anti-θ C3H treatment lysed 43% of the spleen cells specifically.

§ Means ± SEM of triplicates. Significantly different ($P < 0.001$) from normal AKR + C′ or no treatment, but not different from normal spleen cells.

‖ B10.BR immune spleen cells lysed vaccinia-infected L929 (H-2^k) target cells to 40.5 ± 0.8% as compared to 10.9 ± 0.3 by B10.D2 immune spleen cells

typical of the T-cell response with a rapid onset after day 4 and a rapid decline 6 days after infection (Table II).

Discussion

These experiments demonstrate that T-cell-mediated, virus-specific cytolysis acts antivirally only during the period after virus penetration and uncoating when the virus has ceased to exist as infectious and biological entity (eclipse

phase) but before virus progeny assembles. If infected target cells are lysed after virus reassembles, virus-specific cytotoxic T cells do not have an obvious antiviral effect.

Since vaccinia immune cytotoxic T cells are generated very early during virus infections, can be detected in vitro by day 4, and peak at 4–6 days when neutralizing antibodies are not yet, or barely, demonstrable (2, 14), the cytolytic T-cell-mediated effector mechanism is probably crucial in rapidly controlling primary virus infections. Although (Fig. 1), this is certainly not the only immune antiviral effector mechanism, all evidence suggests that in the pox and LCMV models it is an early and very potent one.

The results are compatible with those obtained in vivo with mouse pox and LCMV (7, 8) in which adoptively transferred immune spleen cells (that were cytolytic in vitro) reduced up to four $\log_{10}$ of PFU in spleens of preinfected recipients within 24 h. They also confirm the conclusions that cells infected with mouse pox become susceptible to attack by cytotoxic T cells at about 3 h after infection, and that the relevant changes on cell membranes occur probably within the first hour after infection (15). T cells are specific not only for a viral antigen but also for a self-cell surface marker coded in *H-2K* or *H-2D*. Whether this is best explained by the "altered self" or the "dual recognition" hypothesis of T-cell recognition is not relevant for this discussion (6). However, this direct T-cell-mediated cytolytic antiviral effector mechanism could yield a mechanistic explanation for the finding that these T cells recognize and react to viral antigen together with *H-2K* or *H-2D* rather than with the *H-2I* structures.

All viruses as a whole group can, although differentially according to certain host-cell ranges (tropisms), infect virtually any cell of multicellular organisms. Therefore, if this cytolytic effector mechanism is important, the immunologically relevant self-markers recognized by T cells must have two characteristics: first, they have to be ubiquitous, and second, the self-markers or structures closely linked to them have to be structures where the cytolytic signal can be delivered efficiently. Major transplantation antigens satisfy both conditions. They have been demonstrated in various amounts on all cells (17) and T-cell-mediated cytolysis apparently is most efficient when delivered through *H-2K*- or *H-2D*-coded cell surface structures (18). In contrast *H-2I*-coded structures (e.g., Ia) are not ubiquitous and are expressed mainly on macrophages and B cells (17). Also, although under selected experimental conditions of allogeneic stimulation Ia may serve as target for allogeneic cytolysis, in syngeneic virus-specific cytolysis, such a mechanism is not detectable in vitro or in vivo (3, 5–8). The facts that viruses lose their infectious and biological identity for some hours while the host cell is susceptible to immunological attack, and that virus can actively infect virtually any cell distinguishes them (and cells carrying mutations or tumor antigens) from biologically inert (i.e., nonviral) or chemically inert antigens (i.e., chemically nonreactive with cell surface markers [9]) such as toxins.

Considerable experimental evidence indicates that, like viruses, inert antigens have to be presented on cell surfaces in order to be immunogenic for T cells (for review see 19). For this to occur, however, these antigens have to be actively phagocytized since they are inert, i.e., passive towards cells. Obviously the T-

cell-mediated effector mechanism is not wanted to be cytolytic for handling toxins or probably intracellular bacteria.[2] Since these agents do not lose their infectious and biological identities and properties, cytolysis would release them from cells but not destroy their harmful activities. The cell surface structures coded by the *H-2* gene complex may thus be regarded as off-on switches for various partially cell-specific functions. Noncytolytic T cells interacting with distinct *H-2I*-coded structures may differentially trigger release of lymphokines, activation of macrophages, or B cells to produce specific (neutralizing) antibodies. Such mechanisms could lead to destruction or inactivation of inert antigens intracellularly or extracellularly. *H-2K*- or *D*-coded structures probably function as the cells' punch-hole openers which can be specifically operated by cytotoxic T cells.

Summary

Virus-immune cytotoxic T cells can inhibit effectively growth of vaccinia virus in acutely infected target cells in vitro by destroying infected target cells before infectious virus progeny is assembled. Together with the fact that virus-specific T cells are demonstrable after 3 days, very early during infection, and with strong circumstantial evidence from adoptive transfer models in vivo, these data suggest that in some virus infections T cells may in fact act cytolytically in vivo to prevent virus growth and spread and be an important early antiviral effector mechanism.

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