Antivirally Protective Cytotoxic T Cell Memory to Lymphocytic Choriomeningitis Virus Is Governed by Persisting Antigen

By Stephan Oehen, Hanspeter Waldner, Thomas M. Kündig, Hans Hengartner, and Rolf M. Zinkernagel

From the Institute of Experimental Immunology, Department of Pathology, University of Zürich, CH-8091 Zurich, Switzerland

Summary

The basis of antiviral protection by memory cytotoxic T lymphocytes (CTL) was investigated in vivo and in vitro using lymphocytic choriomeningitis virus (LCMV) and recombinant vaccinia viruses expressing the LCMV-glycoprotein (vacc-GP) or -nucleoprotein (vacc-NP). The widely replicating LCMV with a tendency to persist induced solid long-term protective memory. The poorly replicating vaccinia recombinant viruses revealed in the vaccinated host that the antiviral capacity of the secondary immune T cell response and the protection against lethal LCM was dependent upon the immunizing antigen and its dose. Protection against lethal choriomeningitis is less sensitive to assess memory because it depends upon high levels of CTL precursors (p) and/or on an activated state of memory CTL. In contrast, antiviral protection measured as the capacity of the primed host to reduce virus titers after challenge infection correlated with elevated CTLp frequencies after immunization with live LCMV or recombinant vaccinia virus expressing the major LCMV epitope. CTLp frequencies were constantly increased up to 70 d for LCMV immune mice, but rapidly decreased a few weeks after immunization with low dose vaccinia recombinant virus. For example, mice primed with 2×10^6 plaque-forming units (PFU) of vacc-NP, or 2×10^2 PFU, or 2×10^6 PFU of vacc-GP were antivirally protected on day 7 but not after day 30 when CTLp could not be measured any longer in vitro. However, greater priming doses of vacc-NP (10^4 or 2×10^6 PFU) as well as LCMV (2×10^2 PFU) induced elevated levels of CTLp and antiviral protection for 60 d or longer. Adoptive transfer experiments of immune spleen cells into syngeneic recipients without addition of antigen demonstrated that maintenance of the antiviral protective capacity of the transferred cells depended on the presence of viral antigen. Thus, antiviral protection by memory CTL may be rather short-lived since it is based on activated T cells continuously stimulated by persisting antigen. This is best achieved by high immunizing antigen doses yielded either by widely replicating viruses or high doses of poorly replicating recombinant vaccines.

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Immunological memory is one of the key characteristics of the immune system, and evidence for T or B cell memory has been collected for many years (for reviews see references 1–3). Although immunological memory as defined operationally has been studied widely, there is relatively little known about the molecular and cellular basis of this phenomenon. Its nature has been explained in several ways. First, memory may reflect either numerically increased precursor frequencies (4) or second, a qualitatively altered differentiation state of memory T or B cells as compared with naive cells (5–9). Third, memory may simply represent repeated restimulation by crossreactive environmental antigens (3, 10) or antigen persistence that continuously drives T or B cell responses (11–14). These three mechanisms may all apply.

Studies on antigen persistence and its role in maintaining memory have revealed an important role of antigen–antibody complexes on follicular dendritic cells for B cell memory (11, 14). Older studies showing that T cell memory may be short-lived (15–19) have recently been corroborated by transfer studies using the cytotoxic T cell response to the male H-Y antigen or Th cell function specific for a carrier protein. This study implied a role for antigen persistence in the maintenance of T cell memory (20).

Immunological memory is the basis for vaccines against infectious agents or their toxins. The question of whether antigen persistence or other factors maintain memory T cells is therefore not only of great interest in general, but also relevant to the rational design of vaccines. The experiments presented here were aimed at analyzing the basis of antiviral protective cytotoxic T cell memory in mice using lympho-

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cytic choriomeningitis virus (LCMV)[1]. Recovery from and protection against acute infection with LCMV are almost exclusively dependent on virus-specific CTLs (21, 22). Normally, antibodies do not play a major role (23, 24) in this process. Also, protection against immunopathologically mediated lethal LCM induced by intracerebral infection (25, 26) is usually strictly CTL dependent. There exists a clear correlation between immunizing LCMV antigen, mouse H-2 MHC haplotype, and the capacity of mice to generate LCMV-specific CTLs mediating protection from LCM (27, 28). For BALB/c (H-2^d) mice, nucleoprotein (NP) (aa118-132) is the major CTL target, whereas glycoprotein (GP)-specific CTLs are found only at low frequencies (28–30). CTL memory to viruses including LCMV has been analyzed by several groups (31–35), but the potential role of persisting antigen, (24, 36) has not been formally analyzed as yet.

The experiments presented here were aimed at assessing protective memory in the vaccinated host itself and comparing it with memory analyzed in vitro or in adoptive transfer experiments. We had argued before (37) and presented some evidence (17) indicating that virus-specific crossreactive T help may be very short-lived in the vaccinated host, which might explain why primed crossreactive T help may not be able to improve antibody responses to new virus variants.

This study therefore compared in the vaccinated host in vivo the protective capacity and duration of T cell memory with the induction and maintenance of elevated CTLp frequencies after infection of mice with either well-replicating LCMV or the poorly replicating recombinant vaccinia viruses (38–40) expressing the LCMV-glycoprotein (vacc-GP) or the LCMV-nucleoprotein (vacc-NP) (28, 41). Since in contrast to LCMV, vaccinia virus replicates only to a rather limited extent in adult mice (42), the latter virus permitted an approximate titration of the vaccine. In fact, after the intravenous infection of mice with 2×10^6 PFU of the tyrosine kinase negative variant, we have not been able to isolate detectable (>10^3 PFU/g organ) virus from any of the organs tested. The limited antigen reservoir initially provided by the immunizing recombinant vaccinia viruses may therefore be expected to decline over time and drop below levels necessary for maintenance of protective T cell memory. This is not readily seen in LCMV-immune mice since even low doses of LCMV are shown to replicate to very high titers and since despite efficient elimination, LCMV has been shown to often persist in mice (23, 24, 36, 43, 44).

The experiments here show that depending upon the detection assay used, antivirally protective memory T cells assessed in vivo correlated with CTLp detected by limiting dilution assays in vitro. Under conditions where the immunizing antigen seemed limited, protective memory T cells declined rapidly within a few weeks. Thus, long-term antivirally protective T cell memory against LCMV in a vaccinated host itself seems to be driven by persisting antigen. This conclusion was supported by adoptive transfer experiments showing that in the absence of antigenic restimulation, antivirally protective T cell memory declined rapidly within a week.

Materials and Methods

Mice. BALB/c (H-2^d) were purchased from the Institut für Zuchthygiene (Zürich, Switzerland). Mice were immunized intravenously by injecting 200 PFU of the LCMV WE isolate. Immunizations with recombinant vaccinia viruses (vacc-NP, vacc-GP) were given intravenously with the virus doses indicated in the separate experiments.

Viruses. The LCMV WE isolate (24) was used in this study. Recombinant vaccinia virus expressing the LCMV-GP (vacc-GP) was a gift from D. Bishop, Oxford University. The LCMV-NP recombinant vaccinia virus (vacc-NP) has been described in detail earlier (28, 38, 39, 45).

Viral Titers. Four mice per group immunized with the indicated virus doses were challenged intravenously with 200 PFU of LCMV WE after various time intervals. 3 d later, the LCMV titers in the spleens were measured in a plaque assay (46). Control mice were primed with vaccinia virus (2×10^6 PFU) not expressing LCMV antigens.

Protection against Lethal LCM. Four to five mice per experimental group were challenged intracerebrally after different time intervals with a lethal dose (either 10^4 or 10^2 PFU in 30 μl) of LCMV WE. The incidence of lethal LCM was registered over a time period of 3 wk and the mean time to death ± SEM of each group was calculated.

Protective Capacity of Immune Spleen Cells. Mice immunized at various time points with 100 PFU of LCMV WE or the indicated vacc-NP doses served as donors for immune spleen cells. Single spleen cell suspensions were prepared and the indicated numbers of cells were injected in 0.4 ml balanced salt solution (BSS) into syngeneic recipients. Some of the recipients were infected intravenously with 100 PFU of LCMV WE 1 d before adoptive transfer to permit virus to replicate and viral antigen to be expressed. Others were infected at various time points after the transfer of immune lymphocytes. To test the protective capacity of the transferred spleen cells, the virus titers in the recipients' spleens were measured on day 4 after challenge infection, i.e., when the recipients' endogenous LCMV-specific immune response had not yet developed.

Limiting Dilution Assays. Single spleen cell suspensions from two to three mice per group were prepared. 10^5 immune spleen cells per well were placed into 24 wells of a round-bottomed tissue culture microtiter plate and titrated in eight steps of twofold dilutions. Total spleen cell numbers (3×10^5/well) were held constant by adding irradiated syngeneic normal spleen cells. After stimulation with 2×10^4 LCMV-infected peritoneal macrophages for 7 d, each individual culture was tested in a ^{51}Cr-release assay on H-2 matched target cells infected with LCMV or left uninfected as controls. The percentages of 24 cultures in one dilution step that were negative for LCMV-specific cytotoxicity were measured and logarithmically plotted against the number of responder cells (see Fig. 2 *A*). The cutoff to consider a well positive was determined by subjecting vaccinia-immune spleen cells to the same procedure and calculating the mean cpm from 24 cultures plus three times the SD. CTLp were read from the graph at 37% negative wells (47, 48).

Results

T Cell Memory Assessed by Protection Against Choriomeningitis after Intracerebral Challenge Infection. The influence of the immunizing dose, the time between immunization and

[1] *Abbreviations used in this paper:* GP, glycoprotein; LCMV, lymphocytic choriomeningitis virus; NP, nucleoprotein; p, precursor.

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infection and the challenge dose on the protective capacity of vacc-NP and vacc-GP against lethal LCM disease was investigated in BALB/c ($H\text{-}2^d$) mice. These mice generate a dominant LCMV-NP–specific and only a very weak GP-specific CTL response (28). Antiviral and immunopathological responses in both normal mice and mice vaccinated with vacc-GP or vacc-NP are mediated by cytotoxic $CD8^+$ T cells and not antibodies as could be shown by treatment with anti-CD8 mAbs (25, 30). Four to five mice were vaccinated with various doses of vacc-NP or vacc-GP and challenged intracerebrally after different time intervals with two doses of LCMV WE (Table 1). The mortality rate and the mean time to death was then registered.

At a time interval of 10 d between priming and challenge infection, immunization with the two doses (2×10^6 and 2×10^4 PFU) of vacc-NP protected mice against both LCMV challenge doses. Vacc-GP–immune mice were never fully protected against any challenge dose, but 25–40% of the mice survived the LCMV challenge infection (Table 1). Vaccinia virus-immunized control mice all succumbed to lethal LCM. A shift of 1–2 d in the mean times to death was seen in vacc-GP–immune groups, indicating a low level of immunization causing more rapidly developing immunopathology. Similar results were seen 20 d after vaccination. At a time interval of 100 d between priming and challenge infection, immunization with 2×10^6 PFU of vacc-NP could not protect against an LCMV dose of 10^4 PFU, whereas the same immunizing dose resulted in protection of 80% of the animals that were challenged with a lower LCMV dose of 10^2 PFU (Table 1, *bottom*). It is noteworthy that (except for vacc-GP 2×10^6 PFU day 10 primed mice) an acceleration by 2–3 d of the average time to death was observed in high dose challenged mice (i.e., 5.0 + 0.5 d for vacc-NP primed mice day 100 vs. 7–8 d in controls), indicating that vaccination did have an accelerating effect on progression of disease even if it did not protect (Table 1). Thus, immunization with low doses and/or less immunogenic vaccines resulted in diminished protection that lasted for a shorter time. Note, however, that antiviral protection is not an absolute readout parameter but also depends upon the LCMV challenge dose. Accordingly, protection against lower challenge doses tended to be longer-lasting than against high challenge doses. Also it must be stated that protection against lethal LCM depends upon a markedly accelerated immune response when compared with normal mice, as has been shown earlier (23, 49).

Kinetics of Anti-LCMV Protection Assessed by Virus Titer Reduction. The antivirally protective capacity of vaccinated mice was analyzed as follows: BALB/c mice were immunized with various doses of vacc-NP (Fig. 1) and challenged intravenously with 200 PFU of LCMV WE after different time intervals. LCMV-immune mice served as positive controls with optimal protection, and vaccinia-immune mice as negative controls. 3 d after the challenge infection, LCMV titers were measured in spleens. The antivirally protective capacity of vaccination with the two high doses of vacc-NP was extensive, causing about a 1,000-fold reduction of viral titers, and was comparative with LCMV-immune mice when monitored during a time interval of 56 d (Fig. 1 *A*). In contrast, BALB/c mice immunized with a low dose of vacc-NP (2×10^2 PFU)

Table 1. *Effects of Virus Vaccine, of Vaccination Dose, and of Time after Vaccination on Susceptibility to LCM*

Priming infection (PFU)	Challenge infection with 10^4 PFU LCMV		
	day 10	day 20	day 100
		%	
LCMV (10^2)	100	100	100
Vacc-NP (2×10^6)	100	100	0 (5.0 ± 0.5)
Vacc-NP (2×10^4)	100	100	ND
Vacc-GP (2×10^6)	40 (7.0 ± 1.0)	25 (5.7 ± 0.3)	ND
Vacc-GP (2×10^4)	0 (6.0 ± 0.5)	0 (7.0 ± 0.5)	ND
Vaccinia (2×10^6)	0 (7.4 ± 0.4)	0 (7.0 ± 0.5)	0 (8.0 ± 0.5)
	Challenge infection with 10^2 PFU LCMV		
	day 10	day 20	day 100
LCMV 10^2	100	100	100
Vacc-NP 2×10^6	100	100	80 (7.0 ± 0.5)

Groups of four to five BALB/c mice were primed intravenously with the indicated doses of LCMV and vaccinia viruses on day 0 and challenged on the given days thereafter with 10^4 or 10^2 PFU of LCMV-WE intracerebrally. Data represents survival after challenge infection. Time to death was monitored every 12 h and the mean times to death ± SEM are given in parentheses; percent lethality was determined on day 21 after challenge infection. All deaths occurred before day 11.

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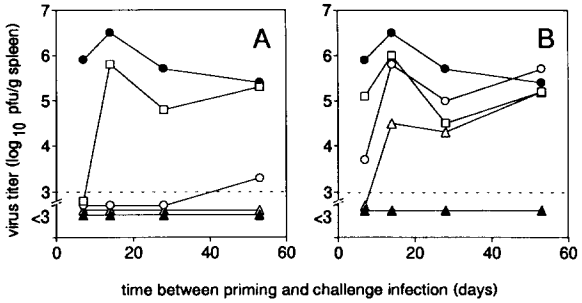

Figure 1. Antiviral protection after immunization with various doses of vacc-NP (*A*) or vacc-GP (*B*). Four BALB/c (H-2^d) mice per group were immunized intravenously with 2 × 10^6 (–△–), 2 × 10^4 (–○–), or 2 × 10^2 PFU (–□–) of vacc-NP and vacc-GP, respectively. Two additional groups of four mice were immunized with 200 PFU of LCMV-WE (–▲–) or 2 × 10^6 PFU of vaccinia (–●–) as controls. After various time intervals, the mice were challenged intravenously with 100 PFU of LCMV. 3 d later, the spleens were removed and the virus titers were determined (46). The $\log_{10}$ values of virus titers per gram of spleen are indicated.

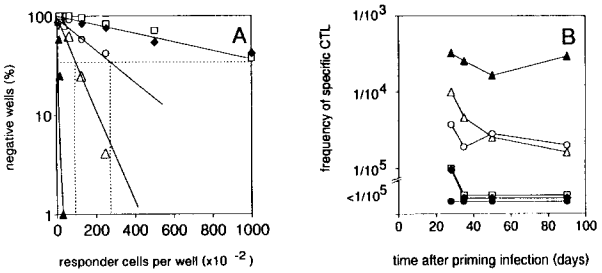

Figure 2. Kinetics of CTLp frequencies of mice primed with various doses of vacc-NP or vacc-GP. BALB/c (H-2^d) mice were primed intravenously with 2 × 10^6 PFU vacc-GP (–◆–), 2 × 10^2 (–□–), 2 × 10^4 (–○–), or 2 × 10^6 (–△–) PFU of vacc-NP; 2 × 10^2 PFU of LCMV (–▲–), or 2 × 10^6 PFU of wild-type vaccinia virus (–●–). After different time intervals, the CTLp frequencies were determined in the spleens by limiting dilution analysis (47, 48) in 7-d cultures in medium conditioned with 10% con A supernatant. (*A*) Representative plot for the determination of CTLp frequencies on day 28 (see also Materials and Methods). (*B*) CTLp frequencies of the individual experimental groups are plotted.

were protected when challenged 7 d after immunization, but not at 14 d or later.

Vaccination with various doses of vacc-GP (Fig. 1 *B*) revealed that immunization with a high dose of vacc-GP induced efficient antiviral protection on day 7. This protection decreased with the vaccination dose and with time. No protection was measured after 56 d as indicated by high virus titers in mice vaccinated with all immunizing doses of vacc-GP. Thus, again the immunizing dose and the quality of the antigen influenced the antiviral protective capacity, depending upon the time after vaccination.

Analysis of the Kinetics of Cytotoxic Anti-LCMV-specific CTLp Frequencies. There is experimental evidence from several model systems that immunological memory correlates with increased CTLp frequencies (1, 4). Therefore, limiting dilution assays were performed with spleen cells of BALB/c mice immunized with vacc-NP, vacc-GP, wild-type vaccinia virus, or LCMV WE (Fig. 2 *A* and see Materials and Methods). Priming with 2 × 10^6 or 2 × 10^4 PFU of vacc-NP induced LCMV-NP–specific CTLp frequencies decreasing eight- or threefold, respectively, between day 28 and days 50 or 90 (Fig. 2 *B*).

Priming with LCMV resulted in 3–10-fold higher overall frequencies that did not decline with time; e.g., CTLp were determined on day 170, yielding a frequency of 1:5 × 10^3 for 2 × 10^2 PFU LCMV primed mice (data not shown). Immunization with a low vacc-NP dose (2 × 10^2 PFU) or a high vacc-GP dose (2 × 10^6 PFU) did not induce any detectable LCMV-specific CTLp above background levels 30 d or later after priming. For technical reasons, frequencies of <1:10^5 could not be measured reliably (4, 50). But note that anti-LCMV protection measurable in vivo after immunization with 2 × 10^2 PFU of vacc-NP on day 7 was specific since vaccination with an irrelevant vaccinia virus failed to protect (Fig. 1 *A*).

Need for Antigen to Maintain Antivirally Protective T Cell Memory Demonstrated by Adoptive Transfer. Enhanced antiviral protection may be explained by long-lived memory T cells and/or by persisting antigen that maintains CTLs activated at low levels. To evaluate the role of persistence of viral antigen in T cell–mediated antivirally protective memory, adoptive transfer experiments were carried out (Table 2). BALB/c mice were immunized as indicated with 2 × 10^6 PFU of vacc-NP, 2 × 10^6 PFU of vaccinia, or 10^2 PFU of LCMV. At different time intervals after immunization, the spleens were removed and 10^8 immune spleen cells were adoptively transferred into syngeneic recipients (Table 2, Expt. *1*). The recipients were infected intravenously with 200 PFU of LCMV on the day before (to enable the virus to replicate and induce immunogenic antigen before cell transfer [27]) or on various days after transfer. 4 d after infection, i.e., before the endogenous antiviral response had developed, LCMV titers were measured in the recipients' spleens. Vacc-NP–immune spleen cells from mice vaccinated 28 d previously reduced virus titers about 200-fold. 77-d immune cells reduced titers only by 10-fold if recipient mice had been infected with LCMV on the day before adoptive transfer (reduction by a factor of about 100 from 6 × 10^6 to 3 × 10^4 PFU/g spleen or by a factor of about 10 from 2 × 10^6 to 2 × 10^5 PFU/g spleen on day 28 or 77, respectively). The protective effect was markedly decreased when recipient mice were challenged only on day 14 after transfer (about a 30-fold reduction from 2 × 10^6 to 9 × 10^4 vs. no reduction from 5 × 10^6 to 6 × 10^6 PFU/g spleen on day 28 or 77, respectively). To test whether spleen cells still contained replicating LCMV, an aliquot containing about 10^7 spleen cells was injected in 30 μl into the footpads of a normal mouse (23, 28). Typically, this detection assay is more sensitive than plaqueing; it detects one replicating unit of LCMV (which is equivalent to about 2–5 PFU in 30 μl of test material). LCMV initiates a local infection and induces a specific CTL response that causes the footpad to dramatically swell after days 7–8 after infection into the footpad (23, 26). No footpad swelling was detected, suggesting that less than 10 replicating LCMV particles per

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Table 2. *Antiviral Protection Mediated by Adoptively-transferred Spleen Cells: Influence of Type of Vaccine and of Time between Adoptive Transfer and Challenge Infection*

Expt.	Vaccination of donor mice* (No. cells transferred)	Time after vaccination (days)	Viral titers (log 10 PFU/g spleen)‡ Challenge infection of recipient mice with respect to cell transfer on day				
			−1	+1	+5	+7	+14
1	Vacc (10^8)	28	6.8 ± 0.1	ND	ND	ND	6.3 ± 0.3
	Vacc-NP (10^8)	28	4.5 ± 0.1	ND	ND	ND	4.8 ± 0.1
	LCMV (10^8)	28	<2.7§	ND	ND	ND	<2.7
	Vacc (10^8)	77	6.2 ± 0.1	ND	ND	ND	6.7 ± 0.2
	Vacc-NP (10^8)	77	5.3 ± 0.1	ND	ND	ND	6.8 ± 0.2
	LCMV (10^8)	77	<2.7	ND	ND	ND	6.7 ± 0.1
2	Vacc (8×10^7)	90	ND	6.9 ± 0.1	7.2 ± 0.2	7.2 ± 0.2	ND
	LCMV (8×10^7)	90	ND	<2.8	5.4 ± 0.2	6.3 ± 0.1	ND
	LCMV (10^7)	90	ND	3.7	6.8 ± 0.3	6.8 ± 0.2	ND

* BALB/c mice were immunized intravenously with 2×10^2 PFU LCMV-WE or 2×10^6 PFU vacc-NP. Immune cells were transferred after the indicated times of vaccination of donors.

‡ Recipient mice were challenged with 2×10^2 PFU of LCMV-WE 1 d before, or the indicated days after transfer of immune cells intravenously. Virus titers were determined 96 h after challenge infection in four to five individual mice per group. Mean ± SEM were determined.

§ Level of infection was below the detection level of 2.7 $\log_{10}$ PFU/g spleen.

10^8 spleen cells could have been transferred. The same test did not reveal LCMV in any of the spleen cell populations.

In contrast to vacc-NP–immune cells, the 28-d LCMV-immune spleen cells conferred maximal antiviral protection irrespective of the time when the challenge infection was given on the day before (day −1) or +14 (Table 2, *Expt. 1*). The protective capacity of 77-d LCMV-immune spleen cells from mice primed with 2×10^2 PFU of LCMV still provided maximal antiviral protection if recipients were challenged with LCMV on the day before, but importantly, not when challenged 14 d later. Thus 77 d after vaccination, LCMV- and vacc-NP–immune spleen cells "parked" for 14 d in a previously uninfected recipient completely failed to control virus spread in the spleen. This strongly suggests that absence of viral antigen in recipient mice resulted in loss of the antiviral protective capacity of the transferred immune spleen cells. The kinetics of loss of antiviral protection adoptively transferred by immune spleen cells was evaluated in greater detail by varying the timing of the challenge infection and by transferring different numbers of cells (Table 2, *Expt. 2*). After adoptive transfer, 8×10^7 immune spleen cells (90 d immune) were antivirally protective in hosts challenged 1 d later (10^4-fold virus titer reduction from 8×10^6 in controls to $<6 \times 10^2$ PFU/g spleen), less so in hosts challenged 5 d later (virus titer reduction by a factor of about 100 from 2×10^7 in controls to 3×10^5 PFU/g spleen in transfused mice), and least active 7 d later (virus titer reduction by a factor of 10 from 2×10^7 in controls to 2×10^6 PFU/g spleen). Eight times fewer cells, i.e., 10^7 LCMV-immune spleen cells transferred excellent protective capacity to recipients challenged on day +1, but not to those challenged on day +5 or day +7 after cell transfer (Table 2, *Expt. 2*).

Discussion

In immunized mice, virus-specific CTL-mediated antiviral protection declined with time after immunization. The type of vaccine strongly influenced the duration of the protection. If replication of the vaccine was limited, the duration of antiviral protection depended upon the vaccine dose. This is revealed in Fig. 1 *B* by decreased antiviral capacity of vacc-GP–immunized mice as a function of immunizing dose and time. The same phenomenon can be seen with the lowest dose of vacc-NP (2×10^2), whereas the two higher doses probably reflected antigen saturating conditions over the time period tested. A similar correlation is found for a short time period in the induction of LCMV-NP–specific CTLp (Fig. 2 *B*). Note, however, that CTLp frequencies of mice immunized with two higher doses of (2×10^4 or 2×10^6 PFU) vacc-GP were below detection level after more than 28 or 40 d. Thus, the antiviral protective potential in vivo correlated with in vitro-measurable CTLp frequencies. The small decline with time in CTLp frequencies of mice primed with 2×10^4 or 2×10^6 vacc-NP (Fig. 2 *B*) did not influence the measurable antiviral protection as assessed in vivo by reduction of challenge virus titers. Maximal antiviral protection was measured despite reduced CTLp frequencies in recombinant vaccinia virus-primed mice when compared with LCMV-(2×10^2 PFU) primed mice that exhibited ten times higher CTLp frequencies. This reflects the fact that

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with a given challenge dose of LCMV, the scale of protection measurable by the virus titer reduction assay is limited and that therefore experimental conditions cannot reveal the existent, more substantial difference. However, the more potent protection is revealed in the protection assay against lethal LCM (compare 2×10^6 vacc-NP in 10^4 vs. 10^2 PFU LCMV-challenged mice, Table 1). The role of stimulating antigen in the maintenance of T cell memory was in addition shown by adoptive transfer of memory CTLs. Efficiency of transferred protection depended upon the presence of stimulating antigen in the recipients. If stimulating antigen was absent or not provided within 7–14 d after cell transfer, protective CTL memory declined rapidly to undetectable levels.

Thus, antigen dependence of antivirally protective immune T cells was shown in the original host without disruption of the structures of lymphoid organs and without forcing memory T cells to recirculate and home under experimental conditions, but was also shown in adoptive transfer experiments. Taken together, our results show a remarkable correlation between the existence of memory T cells as defined by both in vitro (CTLp) and in vivo (antiviral protection) assays. Apparently and understandably, the method of assaying for protection against lethal LCM was less sensitive for detecting T cell memory than for measuring virus titer reduction in spleens. Once a sufficient number of choriomeningeal cells are infected, the immunopathology will be lethal. Therefore, protective memory against LCM needs to be very efficient and quick to be protective. Besides the need for high levels of CTLp, this may require relatively high levels of activation as well. For assaying memory in the spleen, the need for activation may be less than for memory T cells that have to recirculate to mediate effector functions in the choriomeninges for LCM. Collectively, the results thus demonstrate that antiviral protection in vivo reflects primed T cell populations that are continuously driven and activated by persisting antigen.

What are the limitations of the presented experiments and of these interpretations? The presented and many earlier experiments clearly show that the sensitivity of the readout systems chosen obviously determines what generally is called "CTL memory". Accordingly, CTL memory assessed as capacity to prevent LCM is relatively short-lived because the assay is insensitive (see Table 1). This is because LCMV infects choriomeningeal cells rapidly and extensively without CTL having a chance of eliminating virus promptly, except when present and activated during, or briefly after, an acute systemic infection. Paradoxically, and as has been shown in a previous study (30) CTL memory may even be able to accelerate LCMV under certain circumstances. The assessment of antiviral protection by virus titer reduction is usually a more sensitive parameter and obviously in most instances, the most relevant one to validate vaccine-induced immunological memory. This parameter, but also CTLp determinations in vitro, revealed rapid decline of vacc-GP–induced CTL memory when antigen is limiting. Since LCMV-GP codes for a minor T cell epitope in H-2^d mice, this finding is not surprising. The primary cytotoxic T cell response in H-2^d mice against LCMV-NP is at least 30–100 times stronger than against LCMV-GP (28). Accordingly, the antivirally protective T cell response induced in H-2^d mice by vacc-NP vs. vacc-GP (Fig. 2) revealed a substantial difference of detectable CTLp in favor of NP.

One may argue that had the virus titer reduction been measured 1–2 d later even lower levels of CTL memory could have been detected, but the interference of the recipients' own immune response (even after low level irradiation) would complicate the possible benefits of this prolonged protocol. Operationally, any lack of acceleration of virus elimination is biologically meaningful and signals absence of protective T cell memory and/or absence of activated T cells.

The findings obtained by adoptive transfer experiments are limited by the uncertainties of whether transferred cells recirculate and home properly under the chosen experimental conditions (8, 19, 20, 51). However, since 28-d LCMV or vacc-NP–immune spleen cells were able to confer high levels of protection irrespective of whether recipients were infected with LCMV on day −1 or day +14, there was no obvious limitation to recirculation or homing by the recipient. Also, this was not caused by transfer of virus, because we were not able to detect LCMV or vacc-NP in the transferred spleen cells, nor in the uninfected recipients above the stated detection levels of about 50–100 infectious units per spleen (or 5×10^2–10^3 PFU/g of spleen). However, these findings cannot exclude that infectious virus below this detection level was transferred.

Collectively, the presented results suggested that protective memory cytotoxic T cells are T cell populations continuously activated by persisting antigen. Therefore, the previously reported modulations of various surface molecules on naive or memory T cells may reflect physiologically activated T cells, rather than specially defined memory T cell sets. Support for such an assumption may also be deduced from recent work showing that modulation of CD45R is not unidirectional but that so called memory T cells can revert to the phenotype of naive T cells (52). However, and this is important in view of previous studies on memory, simple lack of space (19) for adoptively transferred memory T cells may not be invoked to explain the differing, strictly antigen-dependent kinetics of adoptively transferred protection shown here.

Several earlier studies have analyzed CTL memory against LCMV (31–35). In these studies, as in the present one, CTL-mediated protection has been found to function for more than 1 yr, i.e., for life. The role of antigen persistence was discussed but not evaluated as a limiting factor. This was difficult to achieve in the models used, either because LCMV was not completely cleared or because too much antigen had accumulated to become limiting. Both the poorly replicating vaccinia recombinant viruses and adoptive transfer experiments could overcome this problem in this study. Persistence of virus as a possible source of continuously stimulating antigen has been invoked to explain memory for many viruses including herpes zoster or herpes simplex (53, 54). For other viruses to which CTL memory is very long-lived, such as influenza

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virus, careful search has failed to reveal evidence for persistence of viral genome (55). However, this does not exclude the possibility that viral antigen may be stored on special cells or specialized structures yet to be defined in a class I-presentable form with a long half-life. Such a possibility may be suggested by a recent study showing that class I-associated peptides may have a half-life of >200–600 h (56). The results showing that the in vivo antiviral protection specific for the main T cell epitope is achieved with fewer PFU of vaccinia recombinant virus and lasts for a considerably longer time than found for the minor epitopes, may suggest that the binding affinity of the peptide, together with its quantity, may determine the crucial half-life of memory-promoting antigen. Follicular dendritic cells have been shown to retain antibody–antigen complexes to form antigen reservoirs for the maintenance of B cell memory (11–13). Replicating antigen (including persisting virus) driven into the class I-associated antigen processing pathway may be a prerequisite of antivirally protective activated T cells. It remains to be seen whether other forms of antigen and antigen depot may be processed in a class I-associated form to maintain both CTLp and protective memory, as may be suggested by experiments with OVA (57, 58).

Besides very many examples to the contrary (1, 3, 10, 59–61) there are nevertheless several studies that signal a relatively short half-life for T cell memory, which is again crucially dependent upon the sensitivity of the readout used. The implication is that the more insensitive the test (e.g., as a result of measurements under oversaturating conditions or in parameter ranges where changes have no overt effect) the more long-lasting T cell memory will appear. For example, studies on T cell protection against Listeria (18) or T h cells specific for bovine gamma globulin (15) for sheep RBC (16), for cross-reactive T help against vesicular stomatitis virus (17), or KLH and for H-Y–specific CTLs (20) have revealed short-lived kinetics by adoptive transfer or, and most importantly, even in the host itself (17). Most of the above experimental approaches did not use limiting antigen conditions and therefore failed to reveal the short duration of T cell memory. In fact, the general use of adjuvant is the corollary to viral antigen persistence and usually blurs this conclusion because an antigen depot is formed in granulomas and on follicular dendritic cells. CFA (which includes *Mycobacterium tuberculosis*) in particular causes granuloma formation known to form ideal long-lasting antigen depots. Note that also in our experiments (Table 1; challenge with 10^2 PFU of LCMV), the short duration of antivirally protective T cell memory revealed by vaccination with 2×10^2 PFU of vacc-NP could be masked by immunization with an oversaturating dose of vacc-NP.

What are the consequences of these findings for the design of vaccines to induce and maintain CTL responses? If for some viruses CTL memory is of crucial importance, such as probably against LCMV in mice or HIV in humans (62), then antigen persistence plays a crucial role. This may be achieved either by persistence of the replicating infectious agents or by formation of an appropriate long-lasting depot. The examples studied here with vaccinia recombinant viruses in the mouse, or earlier ones with class I-presentable peptides in incomplete adjuvants revealed a functioning, antivirally protective CTL memory (63). Under antigen-limiting conditions, it is apparently too short for effective long-term vaccination. From this point of view, a virus with similar characteristics with respect to persistence, such as LCMV in the mouse, might be an ideal expression system for CTL vaccine determinants in humans.

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Address correspondence to Rolf M. Zinkernagel, Institute of Experimental Immunology, Department of Pathology, University of Zurich, Sternwartstr. 2, CH-8091 Zurich, Switzerland. S. Oehen is currently at the University of San Diego, Cancer Center, 9500 Gilman Drive 0063, La Jolla, CA 92093.

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