Priming of Virus-Immune Memory T Cells in Newborn Mice

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Neonatal BALB/c mice can be primed at birth by intravenous inoculation of a small dose of A/Puerto Rico/8/34 (H1N1) (PR8) influenza virus, UV-inactivated PR8 virus, or PR8 virus complexed with monoclonal antibody to give a secondary cytotoxic T lymphocyte response when restimulated in vitro as adults. The frequency of responding T cells after secondary stimulation in vitro is approximately 40% of that found for adult mice primed intraperitoneally with a large dose of PR8 virus. The majority of the T cells generated from mice primed at birth or as adults are cross-reactive for H-2-compatible targets infected with the PR8 (H1N1) or A/Hong Kong/X31 (H3N2) viruses. Splenocytes from neonates receiving UV-inactivated vaccinia virus at birth give an augmented secondary cytotoxic T lymphocyte response when restimulated 8 days later in adoptive irradiated adult hosts. We found no indications of specific immunological unresponsiveness in mice exposed to either virus.

We have analyzed previously the development of virus-specific T cell competence in neonatal mice and have reported the presence of vaccinia virus-specific cytotoxic T lymphocyte (CTL) precursors in the thymus of 0- to 3-day-old mice and in the spleens of 4- to 5-day-old mice (19). Furthermore, the antigen-presenting cells capable of stimulating vaccinia virus-specific CTL are present in the spleen by days 5 to 8 of postnatal life (19).

The present report describes experiments in which mice were injected within 24 h of birth with influenza or vaccinia virus. We wished to determine whether tolerance or priming would occur and whether any such effects would persist into adulthood.

We found that spleen cells from adult mice injected at birth with UV-inactivated, antibody-complexed, or low-titer influenza virus generated a strong secondary antiviral CTL response in vitro. Similarly, spleen cells from 8-day-old mice injected at birth with UV-inactivated vaccinia virus gave an augmented CTL response when restimulated in irradiated adoptive hosts. No evidence of specific tolerance was observed in these experiments with influenza or vaccinia virus.

MATERIALS AND METHODS

Mice. The BALB/c (H-2^d) and (B6xC3H)F1, (B6C3)F1 (H-2^{kxb}) mice were bred at The Wistar Institute.

Influenza viruses. The influenza viruses used, obtained originally from W. Gerhard, The Wistar Institute, were A/Puerto Rico/8/34 (H1N1) (PR8) and A/Hong Kong/X31 (H3N2) (HK). Virus stocks were stored frozen in allantoic fluid containing between 1,200 and 3,000 hemagglutinating units (HAU) per ml.

Limiting dilution culture system. Spleen cells from virus-primed mice were cultured in RPMI 1640 medium, 10% fetal calf serum, 4 mM glutamine, 5×10^{-5} M 2-mercaptoethanol, 50 µg of gentamicin per ml, and 10 mg of alpha methyl mannoside per ml, with 25% concanavalin A-stimulated rat spleen culture supernatant. Stimulator cells for the limiting dilution cultures were prepared by exposing irradiated ($1,500$ rad) normal spleen cells to a 1:10 dilution of virus at 1×10^7

cells per ml for 1 h at 37°C. Graded numbers of effector spleen cells and 2×10^5 syngeneic virus-infected, irradiated stimulator cells were distributed in 100-µl portions to the 60 inside wells of 96-well, V-bottom plates. The wells on the periphery of the plate were filled with medium only.

After 8 days, the contents of culture wells were split with a Titertek (Flow Laboratories, McLean, Va.), and samples were brought to a final volume of 100 µl in round-bottomed, Linbro microtiter plates for assay.

Influenza cytotoxic assays. Target cells (P815, H-2^d) were incubated at 37°C for 1 to 2 h at a concentration of 5×10^6 cells per ml with 0.35 mCi of Na₂⁵¹CrO₄ (New England Nuclear Corp., Boston, Mass.). Target cells were infected at 37°C in 7% CO₂ with a 1:6 dilution of stock virus at 5×10^6 cells per ml for 1 h. Then, 5×10^3 infected or uninfected ⁵¹Cr-labeled targets were added to each well in a 100-µl volume and incubated for 7 to 8 h at 37°C in 8% CO₂.

Specific lysis was evaluated as the percentage of ⁵¹Cr release relative to spontaneous release (SR) in medium alone and maximum release (MR) in cetrimide (4 mg/ml; Eastman, Rochester, N.Y.) alone calculated as:

$$\frac{\text{experimental release} - \text{SR}}{\text{MR} - \text{SR}} \times 100$$

Influenza-specific lysis was the difference between the killing of infected and uninfected targets.

Generation and assay of vaccinia-specific CTL. Single-cell suspensions of thymus and spleen (at least 2.0×10^7) were injected intravenously (iv) into lethally irradiated (850 rads, 24 h before injection) mice (4). Three hours later, the recipients were inoculated iv with 5×10^6 PFU of the WR isolate of vaccinia virus, and spleen cells from these mice were assayed for CTL activity 6 days later. The virus-infected and -uninfected target cells used were L cells (K^bD^k), MC57G(K^bD^b), and KD2SV(K^dD^d). Cells were ⁵¹Cr-labeled as described above and infected with vaccinia virus (final dilution; 1:3 of stock virus, 5×10^6 cells per ml) for 2 h at 37°C in 7% CO₂. Target cells were plated at 10^4 cells per well with graded numbers of effector spleen cells and incubated for 7 to 8 h at 37°C in 7% CO₂. Specific lysis was evaluated as described above.

Neonatal inoculation. Newborn mice (<24 h old) were injected via the anterior facial vein with various prepara-

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tions. Details of the different protocols appear in Tables 1 through 3.

RESULTS

At first, we tried to induce either priming or immunological tolerance by injecting newborn BALB/c mice with a relatively large dose (30 HAU) of infectious PR8 (H1N1) virus. However, all mice died within 3 to 6 days of such inoculation. In earlier experiments (11), we found that we could suboptimally prime adult mice to generate CTL memory by injecting them either with much smaller amounts of virus or with virus complexed with monoclonal antibody. These methods, as well as UV inactivation, reduced the pathogenicity of the virus and permitted survival of inoculated newborn BALB/c mice. When spleen cells from the neonates were sampled when they were adults, all three regimens were found to have induced T cell memory (Table 1). The frequency of virus-specific T cells was found to be at least 40% of that observed for memory spleens of adult mice that were previously stimulated with a large dose (300 HAU) of live virus. When adults were injected with UV-inactivated virus, they generally did not show priming as compared with the neonates (data not shown). Results in the literature concerning the ability or inability of UV-inactivated influenza (1, 6, 18) and vaccinia (12) viruses to induce primary or secondary CTL in adult mice are variable.

The specificity patterns of the virus-immune CTL that were generated in neonates were assessed by splitting the contents of individual culture wells and assaying with PR8 (H1N1) and HKX31 (H3N2)-infected P815 cells (Table 2). As is the case for the adult spleen, the majority of the cultures were highly cross-reactive for targets infected with either influenza A virus (1-3, 13, 16), and a small percentage

TABLE 1. Frequency of influenza-specific memory T cells in the spleens of adult BALB/c mice that were primed as neonates with PR8 (H1N1)

Expt. no.	Neonatal mice primed iv with:	Frequency of influenza-immune T cells, in spleen ^a		
		Neonate	% adult frequency	Adult control
1	Infectious PR8 (0.06 HAU)	1/3,800	(47)	1/1,800
		1/4,600	(39)	
2	PR8 + H24B3 ^b	1/8,100	(41)	1/3,300
3	PR8 + H24B3	1/2,100	(67)	1/1,400
4	PR8 + H24B3	1/17,000 ^c	(44)	1/7,500
5	UV-treated virus ^d	1/7,800	(42)	1/3,300

^a Influenza-specific lysis was calculated as the difference between lysis of PR8-infected and -uninfected targets (P815: H-2^d). Wells showing greater than 10% influenza-specific lysis were considered positive. Frequencies were calculated by plotting the percentage of nonresponding wells versus the number of cells per well. The adult controls were primed by a single intraperitoneal injection of 300 HAU of PR8 at least 3 weeks before assay. The percentage is relative to the frequency for the adult controls.

^b Virus was mixed with an over-neutralizing dose of the H24B3 monoclonal antibody (13 mg/ml) so that the neonates were given the equivalent of 30 HAU of virus in the presence of excess antibody (11).

^c Other spleen cells from this pool were treated with anti-Thy1.2 (J1; provided by J. Sprent) plus complement before stimulation in limiting-dilution cultures, and the frequency of positive cells was <1/80,000. Control unprimed spleen cells also showed a frequency of positive cells at <1/80,000.

^d Virus (3,000 HAU/ml) was exposed to UV light for 4 min at a dosage rate of 1,940 μ W/cm². Mice were given 30 HAU of virus after inactivation.

TABLE 2. Specificity of positive cultures for P815 target cells infected with the homologous PR8 (H1N1) or heterologous HK (H3N2) influenza A viruses^a

Protocol for neonatal priming	Spleen population	No. of wells tested ^b	Cultures with specificity pattern (%): ^c		
			PR8 only	HK only	PR8 + HK cross-reactive
Low-titer virus	Neonates, 0.06 HAU	64	9	0	91
	Adult control, 300 HAU PR8	37	16	3	81
Antibody-complexed virus	Neonates, H24B3 + 30 HAU PR8	124	16	4	80
	Adult control, 300 HAU PR8	109	8	1	91
UV-inactivated virus	Neonates, 30 HAU UV PR8	81	15	6	79
	Adult control, 300 HAU PR8	57	3	1	96

^a Data are a compilation of multiple experiments including those described in Table 1. Neonate and adult priming are as described in Table 1.

^b Wells showing $\geq 20\%$ influenza virus-specific lysis were considered for analysis of specificity patterns. These were selected from culture plates in which the majority of wells were negative. The contents of individual positive wells were split and tested on P815-PR8, P815-HK, and P815-normal targets.

^c A culture was regarded as specific for a particular virus if the influenza virus-specific lysis on targets infected with one virus was four times the lysis of targets infected with the heterologous virus. Otherwise, cultures were designated as cross-reactive between PR8 and HK.

of CTL were specific for the homologous priming virus (3, 5, 13, 21).

We also found in previous experiments (19) that thymocytes from 1-day-old and spleen cells from 4-day-old (B6C3)F₁ (H-2^{kxb}) mice could be stimulated on adoptive transfer into irradiated (850 rad) recipients to give a strong, primary vaccinia virus-specific CTL response. Effector function was much more apparent for the H-2^k (L cell) than for the H-2^b (MC57G) target, which is not the case for adult mice (19). A similar phenomenon was found when spleen cells from 8-day-old mice primed at birth with UV-inactivated vaccinia virus were stimulated in irradiated adult recipients (Table 3). A strong CTL response on the H-2^k virus-infected target was seen for spleen populations from the virus-primed, but not the normal, mice. The priming effect of inoculation at birth is much more pronounced for transferred spleen cells than for thymocytes, either because viral antigens are not presented intrathymically or because intrathymically stimulated CTL precursors (CTL-P) subsequently migrate to the spleen. It would also appear that only H-2^k-restricted components of the early neonatal vaccinia virus-specific CTL-P population are present or functionally available for priming. This would parallel our earlier findings for primary CTL (19).

DISCUSSION

Neonatal cytotoxic T cell tolerance to virus has been reported in some experimental systems, particularly the lymphocytic choriomeningitis model (7). However, the mechanism of this nonresponsiveness has not been conclusively established and may be peripheral rather than central

TABLE 3. Priming of newborn (B6C3) mice with UV-inactivated vaccinia virus^a

Lymphocyte population primed on adoptive transfer ^c	E:T ratio	% Specific ⁵¹ Cr release ^b				
		L cells (K ^k D ^k)		MC57G (K ^b D ^b)		KD25V (K ^d D ^d)
		Vacc.	N	Vacc.	N	Vacc.
Normal						
Spleen	30:1	14	2	14	2	2
	100:1	18	3	19	2	3
Thymus	30:1	43	12	28	4	6
Primed						
Spleen	30:1	55	6	19	2	9
	100:1	65	6	22	2	5
Thymus	30:1	54	8	38	6	10
Controls ^d						
B6 (K ^b D ^b)	30:1	5	5	58	4	6
C3H (K ^k D ^k)	30:1	40	3	7	5	7

^a Newborn B6C3F₁ mice (≤ 24 h old) were injected iv with 0.1 ml of a 1:15 dilution of vaccinia stock which had been UV irradiated (1,940 μ W/cm²) for 4 min. The stock contained 10⁸ PFU/ml.

^b Vacc., Virus-infected target cells; N, virus-uninfected target cells.

^c Normal and vaccinia-primed B6C3F₁ mice were killed at 8 days of age, and 3×10^7 spleen or thymus cells were stimulated with infectious virus (3×10^6 PFU) for 6 days in irradiated (850-rad; ~ 24 h) B6C3F₁ recipients.

^d Primary spleen cell response in adult mice given 5×10^6 PFU of vaccinia virus 6 days previously.

(15). Moreover, lymphocytic choriomeningitis virus grows in almost every tissue throughout the body (7) and is presumably seen as "self" in both the bone marrow and thymus where T cell tolerance is likely to be induced (8). The influenza A and vaccinia viruses used here are much less widely distributed. Failure to develop tolerance may simply reflect the absence of viral antigens presented in an appropriate anatomical niche at a particular stage of T-cell ontogeny. Newborn mice received, on a relative weight basis, >10 times the adult dose of influenza. Nevertheless, it is conceivable that even larger amounts or repeated injections of inactivated virus might reveal a degree of tolerance. The use of large doses of nonirradiated virus invariably results in the death of injected baby mice within days. Perhaps these lethally infected newborn mice are also made tolerant to the virus. If enough of their cells could be harvested and adoptively transferred, some degree of tolerance might be detectable.

The fact that T cells in baby mice can be primed to generate a secondary CTL response indicates that early presentation of virus does not cause antigen-induced clonal abortion or the development of suppressors capable of deleting CTL precursors (9). We have demonstrated previously that CTL precursors, at least for vaccinia virus, are present in the thymus from the first day of postnatal life and are present in the spleen from day 4 (19). These T cells were stimulated with virus by adoptive transfer into lethally irradiated, adult recipients, which may have provided the necessary milieu for CTL generation. The baby mouse is thought to have defective antigen-presenting function, at least for *Listeria*-immune T cells (14). Nevertheless, any such defect does not prevent the generation of primary virus-immune CTL in mice infected with vaccinia virus at 1 week of age or older (19), and it does not prevent the generation of

vaccinia-specific T cell memory after exposure at birth. The mice inoculated with influenza A at birth were not tested until adulthood. Probably, stimulation of memory T cells occurred early, but it is possible that the viral antigen persisted until maturation of the necessary physiological environment. Poor clearance of UV-inactivated influenza virus in neonates, as compared with adults, may explain the superior priming for memory in the former. Also, a small amount of residual viable virus in the irradiated inoculum may represent a significant priming dose in the tiny newborn, whereas it would be insufficient in the much larger adult. Once again, poor clearance of this residual active virus in the newborn might enhance priming.

In the virus stimulation experiments discussed so far, the cellular immunity of newborn mice seems remarkably competent. One deficit which we have tentatively identified is at the level of the primary cytotoxic response. We have never succeeded in recovering primary CTL from the spleen or the thymus of 5- to 6-day-old neonates that had been injected at birth with UV-inactivated, antibody-complexed, or low-titer viruses (unpublished data). This could be due simply to low frequencies of CTL not detectable by our assay or anatomical dispersion of the relevant CTL throughout the body. For B cells, there is strong evidence that the ability to proliferate in response to cooperative signals (17) and to generate memory cells in response to antigen (10, 20) ontogenically precedes the ability to secrete antibody. This cannot be the full explanation for T cells since adoptive transfer demonstrates primary CTL-P specific for vaccinia virus in the thymus almost from birth (19). The defect may, instead, reflect a lack of differentiation signals in the neonatal environment. The presence of macrophages (14), T helper cells, or helper factors may be limiting in the 0- to 1-day-old neonate. Passive transfer to neonates of adult macrophages or T helper cells may provide further information regarding this issue.

ACKNOWLEDGMENTS

We thank Pam Warwick and Pauline Angerman for technical assistance.

These experiments were funded by Public Health Service grant AI-14162 from the National Institutes of Health. J.L.H. is a fellow of the Cancer Research Institute.

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