Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 5558–5562, July 1990
Medical Sciences

Vaccination with a live retrovirus: The nature of the protective immune response

(Rauscher murine leukemia virus/postexposure prophylaxis/3'-azido-3'-deoxythymidine and interferon α combination therapy/cellular immunity)

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Communicated by Hilary Koprowski, April 25, 1990

ABSTRACT **We tested 3'-azido-3'-deoxythymidine (zidovudine) combined with interferon α as chemoprophylaxis after exposing mice to Rauscher murine leukemia virus. Therapy started 4 hr after inoculation and administered for 20 days prevented viremia and disease in all 234 mice tested. When the animals were rechallenged with live virus after cessation of therapy, 96% were resistant. The nature of this protective immune response was analyzed: Passive serotherapy of naive mice challenged subsequently with Rauscher murine leukemia virus was only protective at a high dose of immune serum. Immune, but not naive, T cells alone were fully protective against virus challenge. We conclude that vaccination with a live retrovirus that cannot replicate because of pharmacological blockade induces a T-cell response capable of protecting against a lethal retrovirus-induced disease.**

The epidemic of AIDS, caused by human immunodeficiency virus type 1 (HIV-1), calls for the development of effective vaccines. Although results of animal models for vaccination against retroviruses are encouraging (1–6), the development of successful anti-HIV-1 vaccines may have to consider antibody-mediated enhancement of HIV-1 infectivity (7–11); vaccine strategies resulting in such enhancing antibodies may actually accelerate the spread of infection. We decided to elucidate the nature of retroviral immunity in a murine system in which we found, unexpectedly, an effective vaccination protocol involving live virus. Our data point to an important role played by cellular immunity alone.

Initially, our experiments focused on chemoprophylaxis after acute retroviral exposure. Postexposure antiretroviral drug therapy is a current treatment strategy, in contrast to postexposure immunization, which is used to modify some infections (12, 13). We have reported (14) that a 20-day course of high-dose 3'-azido-3'-deoxythymidine (AZT) as well as low-dose AZT combined with recombinant human interferon αA/D (rHuIFN-αA/D) (15), initiated 4 hr after inoculating mice with Rauscher murine leukemia virus (RLV), effectively prevented viremia and disease. The RLV system is a quantitative model for retroviral viremia because the degree of virus-induced splenomegaly measured 3 weeks after inoculation is proportional to the virus titer (16). In this system, we demonstrated that AZT plus rHuIFN-αA/D were highly synergistic without toxicity (17). In our pilot experiment, postexposure prophylaxis with AZT plus rHuIFN-αA/D not only protected all RLV-exposed mice from viremia and disease, but also resulted in protective immunity against live-virus challenge after cessation of therapy (17). Here we report that live virus pharmacologically blocked from replicating was an effective vaccine. Taking advantage of our inbred-mouse system, we investigated the nature of the protective immune response by passive immunization.

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MATERIALS AND METHODS

Mice and Virus. Five- to 10-week-old female BALB/c Antac mice (Taconic Farms) were used. Source and preparation of RLV (strain RVB3) were as described (18). Viral titers were determined by XC plaque assay (19). RLV was administered i.v. at 10^4 plaque-forming units (pfu) per mouse.

Cell Lines. SC-1 (mouse fibroblasts) and XC rat cells (used to test RLV-*env*-induced fusion) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, L-glutamine, penicillin at 100 units/ml, and streptomycin at 100 μg/ml (complete DMEM).

XC Plaque Assay. Viral titers were calculated from an average of three tests carried out as published (19), except that Polybrene at 8 μg/ml (Sigma) was present during infection.

RLV Neutralization Test. To test for RLV neutralization, the XC cell assay was modified (20). All sera were heat-inactivated (56°C, 30 min) before analysis. SC-1 cells were plated at 4.5×10^4 cells per well (6-well plates, Falcon) in 1.3 ml of complete DMEM, and Polybrene (8 μg/ml) was added 1 day later. Virus diluted with ice-cold DMEM and guinea pig complement (Rockland, Gilbertsville, PA, or Sigma) were mixed to give 200 pfu/0.1 ml and a 1:5 dilution, respectively. After incubation (37°C, 5 min), 0.1-ml aliquots were added to 0.1 ml of serum dilutions. Immune mouse sera were tested at 1:20, 1:40, and 1:80 with or without complement. The samples were mixed in 96-well plates, incubated (45 min, CO_2 incubator, 37°C) and added to SC-1 cells. After incubating for 24 hr, the cultures were rinsed with complete DMEM and grown in 2 ml of fresh medium until confluence, after which they were processed as published (19). Neutralizing titers from duplicate samples are expressed as the dilution of immune serum yielding 50% reduction of RLV-induced plaques. No plaques were seen when complement, naive sera, or immune sera, even without heat denaturing, were tested alone. Naive mouse serum (1:40 and 1:80), goat anti-RLV serum (1:160 and 1:1280), and naive goat serum (1:160 and 1:1280), all with virus in the presence or absence of complement, were used as controls.

Abbreviations: RLV, Rauscher murine leukemia virus; AZT, 3'-azido-3'-deoxythymidine; rHuIFN-αA/D, recombinant human interferon αA/D; pfu, plaque-forming units; HIV-1, human immunodeficiency virus type 1; FLV, Friend murine leukemia virus; FLV-N, N-tropic FLV; FLV-B, B-tropic FLV; DMEM, Dulbecco's modified Eagle's medium.

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Postexposure Prophylaxis and Evaluation for Viremia. Groups of mice were inoculated at time 0 with RLV, and 4 hr later (unless noted otherwise), therapy was begun with AZT (given via drinking water at 0.1 mg/ml) and rHuIFN-αA/D, given i.p. at 10,000 units per 20 g of mouse once daily. On day 20, therapy was stopped; blood samples were collected on day 25 and analyzed for RLV antigens by immunoblot analysis (17) using a polyclonal goat anti-RLV antiserum (National Institutes of Health, Bethesda, MD) or for p30 gag antigen (21) as described.

Determination of Viral Immunity. Mice that were nonviremic after cessation of therapy were rechallenged with RLV (days 25–35 after inoculation). After 3 weeks without antiviral therapy, the animals were tested for evidence of viremia and disease as outlined.

Passive Serotherapy. Serum (not heat-inactivated) from immunized or naive mice was pooled, filtered, and stored at −70°C until use. Naive mice were given 1.0 ml of serum at various dilutions i.p. and challenged with RLV 24 hr later. After 3 weeks, they were bled and sacrificed; inhibition of splenomegaly was determined as described (14). Blood samples were analyzed for infectious virus by XC assay and/or for viral antigens by immunoblot analysis.

Adoptive Cell Transfer. Single-cell suspensions were injected i.v. into naive recipients. For T-cell transfers, pooled splenocytes were passed through nylon wool to remove B cells and macrophages, which resulted in >95% T cells as judged by flow cytometry with fluorescinated Thy-1.2 antibody (Becton Dickinson). One hour after adoptive cell transfer, recipients were challenged i.v. with RLV and followed without drug therapy for 3 weeks.

RESULTS

Postexposure Prophylaxis with AZT and rHuIFN-αA/D. To evaluate the regimen of AZT plus rHuIFN-αA/D, 234 mice were inoculated with RLV and started on therapy 4 hr later. After cessation of therapy, all RLV-exposed mice remained clinically well and showed no signs of viremia by immunoblot analysis (Table 1).

Response to Live RLV Rechallenge After Cessation of Therapy and Design of Vaccine Trials. When rechallenged with live virus, 94–100% of the mice treated with AZT plus rHuIFN-αA/D after the initial RLV exposure developed neither viremia nor disease (Table 1). We conclude that our protocol represents an effective vaccine strategy. Sera and spleens from mice resisting live-virus rechallenge were used for adoptive transfer experiments (Fig. 1).

Protection Against RLV Challenge by Passive Serotherapy. Only a high dose of immune serum was protective against RLV challenge in five of eight mice, as demonstrated by negative spleen immunoblots (Table 2), in contrast to nonimmune serum. No infectious virus was transferred by passive serotherapy; control mice given 1:2 dilution of (nonheat-denatured) immune mouse serum but left unchallenged developed no viremia. In parallel, naive recipients were given a goat anti-RLV antibody that provided more effective protection against RLV challenge than homologous immune sera (Table 2).

Table 1. Immunization with live RLV pharmacologically blocked with AZT plus rHuIFN-αA/D

Experiment	*n*	Virus-free after drug treatment*, %	Virus-free after live-virus challenge*, %
1	42†	100	100
2	46	100	96
3	83	100	94
4	63	100	94

*Measured by immunoblot analysis.
†Only 21 mice were re-challenged.

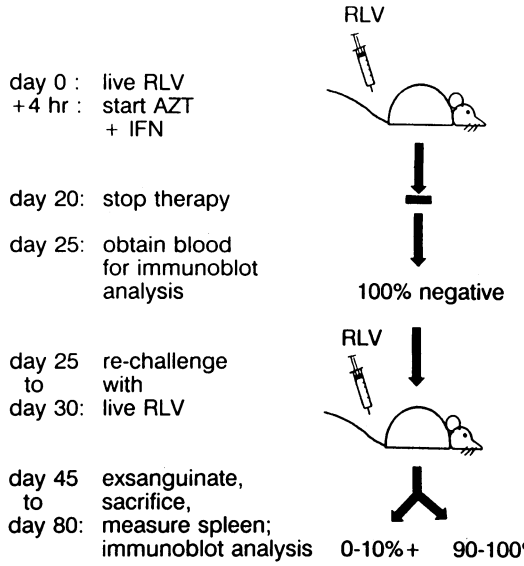

FIG. 1. Vaccination schema. Unless noted otherwise, vaccination was as follows: On day 0, mice were inoculated with RLV. Therapy with AZT plus rHuIFN-αA/D (IFN) was started 4 hr later and continued to day 20. On day 25, blood samples were collected for immunoblot analysis. Animals testing negative were challenged with RLV, sacrificed 3 weeks later, and tested for viremia and disease. Nonviremic mice were used as immune donors for subsequent transfer experiments.

Neutralizing Antibody Titers. Homologous and heterologous sera were analyzed for neutralizing antibodies *in vitro*. In contrast to goat anti-RLV antiserum, little RLV neutralization was seen in sera derived from immunized mice (Fig. 2), even at a 1:20 dilution. The addition of complement did not increase the neutralizing antibody titers significantly (data not shown), in contrast to earlier observations (20).

Protection Against RLV Challenge by Immune Splenocytes. In a pilot experiment, splenocytes from one immune donor were transferred into one naive recipient, followed by RLV challenge. The start of therapy varied from 3 days before (−3) to 4 days after (+4) inoculation in these donors. The time span between the second virus challenge and collection of splenocytes from donors was 72 days. After this follow-up period, donor animals were nonviremic by immunoblot analysis. Splenocytes from RLV-immune mice were protective: All recipients were nonviremic by XC plaque assay of serum and immunoblot analysis of spleens (Table 3 and Fig. 3), in contrast to recipients given splenocytes from naive mice. We conclude that effective protection against RLV can be provided by specific cellular immunity alone; because adoptive transfer of nonimmune splenocytes offered no protection, a significant role of natural killer cells was ruled out. Splenocytes derived from donor mice started on therapy as late as 96 hr after inoculation and harvested 72 days after the second RLV challenge could still protect recipients, indicating the persistence of protective cellular immunity.

Protection Against RLV Challenge by Immune T Cells. To further analyze the nature of protective cellular immunity, B cells and macrophages were removed from immune splenocytes. Various numbers of the remaining T cells were injected into naive recipients, followed by RLV challenge. A dose of 10^8 immune T cells per mouse protected all recipients, in contrast to a 10 times lower dose or 10^8 nonimmune T cells (Table 4). We conclude that exposure to live RLV unable to replicate because of combination antiviral therapy was able

Table 2. Protection against RLV challenge with passive serotherapy

Group	*n*	RLV	Antibody		Spleen weight, mg	Inhibition of splenomegaly, %	Immunoblot
			Source	Dilution			
1	20	+	None		2087 ± 318	–	+
2	10	–	None		82 ± 2	–	–
3	5	+	Naive mouse	1:2	2067 ± 293	–	+
4	4	S	Immune mouse	1:2	109 ± 2	–	–
5	8	+	Immune mouse	1:2	268 ± 106	91.3	+ (3/8)
6	7	+	Immune mouse	1:5	1676 ± 391	23.1	+
7	7	+	Immune mouse	1:10	1457 ± 573	34.8	+
8	5	+	Naive goat	1:5	1991 ± 162	–	+
9	4	S	Goat 294	1:5	134 ± 10	–	–
10	8	+	Goat 294	1:5	176 ± 16	98.0	+ (1/7)
11	7	+	Goat 294	1:15	253 ± 35	93.7	+
12	7	+	Goat 294	1:30	589 ± 366	76.7	+

Naive mice were injected with serum at indicated dilutions and challenged with RLV, except for control groups 4 and 11, which were not challenged. All animals tested positive by immunoblot analysis in groups listed +, except for groups 5 and 12 where the numbers in parentheses indicate the number of mice with positive spleens per number tested. S, mock infection with saline. Goat 294, goat anti-RLV antibody with a neutralizing titer of 1:3200.

to elicit a strong cellular immune response that by itself was sufficient to confer immunity by adoptive T-cell transfer.

DISCUSSION

We have investigated the efficacy of postexposure prophylaxis and the nature of the protective immunity to RLV. In a large series of animals started on therapy 4 hr after virus exposure, treatment with AZT plus rHuIFN-αA/D was 100% effective in preventing viremia and disease, leading to permanent cure. Of considerable interest is that such therapy also led to resistance to reinfection. In a pilot experiment, RLV-exposed mice given the combination regimen were virus-free and able to resist rechallenge, even though the onset of antiviral therapy was delayed to 96 hr after inoculation.

It is not yet clear whether our results are applicable to humans inoculated with HIV-1 because the latter belongs to the subfamily of Lentivirinae, as opposed to RLV, which belongs to the Oncovirinae. Thus, HIV-1 and RLV differ in molecular architecture as well as pathobiology. Nevertheless,

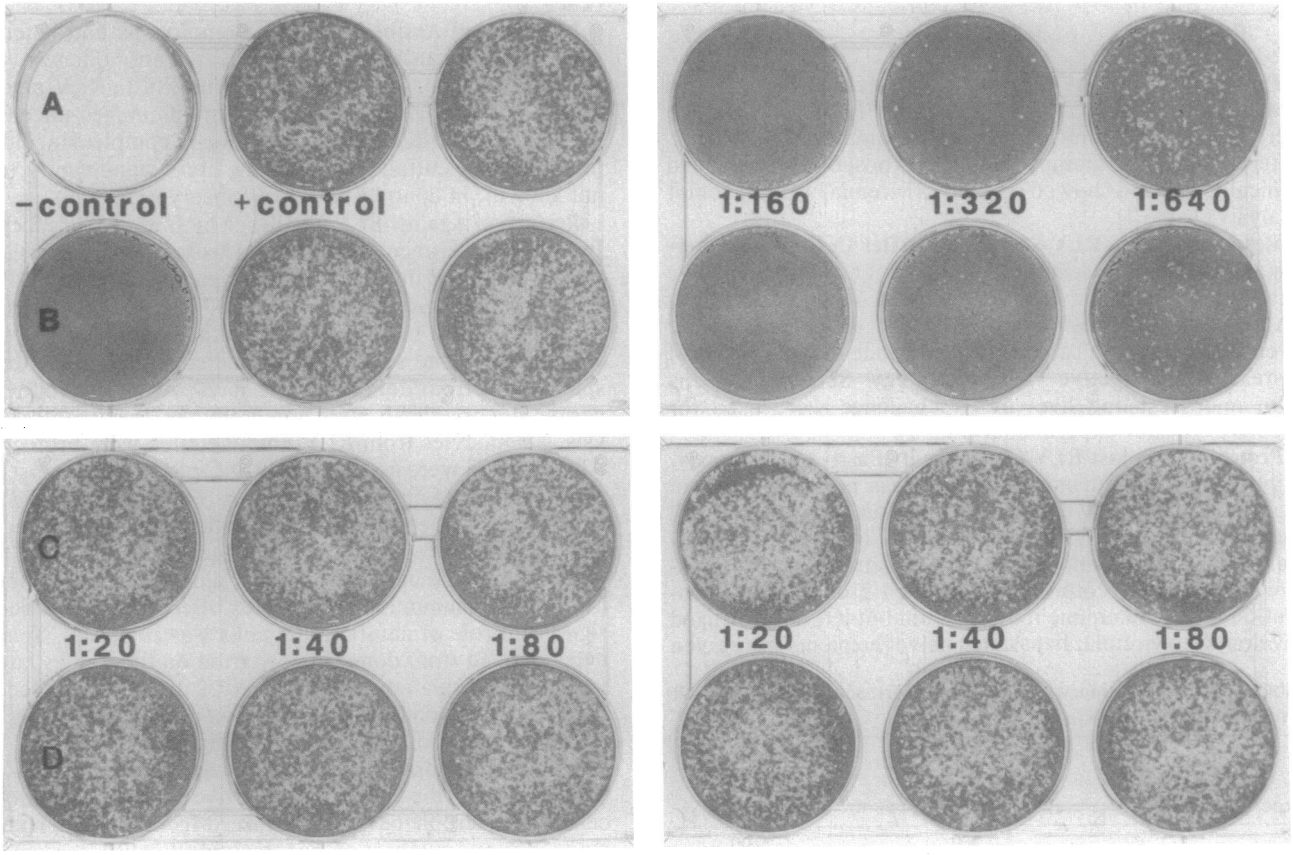

FIG. 2. Titer of neutralizing antibodies by XC plaque assay. Sera were tested for neutralizing antibodies as described. (Top left plate) Well A, SC-1 cells grown to confluence and UV-killed; no XC cells were added. Well B, RLV-negative control. The other four wells show RLV-positive controls at 1:50 dilution. The same RLV inoculum was used for all other plates. (Bottom two plates) Duplicate samples containing RLV were preincubated (37°C, 45 min) with heat-inactivated sera from immune animals at indicated dilutions. (Top right plate) A heterologous goat anti-RLV antiserum with a neutralizing titer of 1:3200 was used as indicated.

Table 3. Immune splenocytes protect against RLV challenge

		Donor			Recipient		
Group	*n*	Spleen cell transfer	AZT + rHuIFN-αA/D treatment	Start	Spleen weight, mg	Immunoblot	Serum titer, pfu/ml
1	3	Naive	None		1828 ± 106	+	(1 ± 0.9) × 10^4
2	2	Immune	+	−3d	267 ± 57	−	0
3	3	Immune	+	+4h	161 ± 43	−	0
4	2	Immune	+	+24h	204 ± 105	−	0
5	3	Immune	+	+96h	175 ± 69	−	0
6*	3	Naive	None		83 ± 4	−	0

Donor mice were inoculated with RLV and at the times (d, day; h, hr) indicated, therapy was started. On day 25, all mice were tested for viremia by RIA (21) or immunoblot analysis. Only RLV-negative mice were used as donors for cell transfer. *, Not challenged.

it should be noted that HIV-1 is susceptible to inhibition with both AZT and recombinant human interferon α, and when these two agents were used in combination against HIV-1 *in vitro*, synergistic inhibition of HIV-1 propagation resulted (22).

The mechanism of induction of viral immunity in our experiments is unknown. In prior *in vitro* studies with other type C retroviruses, proviral DNA was detected 9 hr after exposing cultured cells to virus (23, 24). Theoretically, post-exposure therapy may have prevented synthesis of all proviral DNA and consequently precluded true infection, as defined by permanent insertion of proviral DNA into the host genome, at least in mice started on treatment 4 hr after inoculation. The effective immunity in our RLV system may be due to the fact that live virus stocks contain viral epitopes in their native configuration. Alternatively, our experimental protocols may permit a low level of viral replication—too low to overwhelm host defense mechanisms but sufficient to induce a potent cellular immune response. The latter would destroy any host cells actively synthesizing viral gene products and, thus, force any integrated provirus to remain latent.

Because in our pilot experiment viremia was prevented, even though therapy was not started until 96 hr after exposure, well beyond the time proviral DNA can be detected (23, 24), limited viral replication presumably occurred. Nevertheless, the animals exhibited a protective cellular immune response in adoptive transfer experiments, indicating that transient low-level virus replication may play a role in inducing strong antiviral T-cell responses.

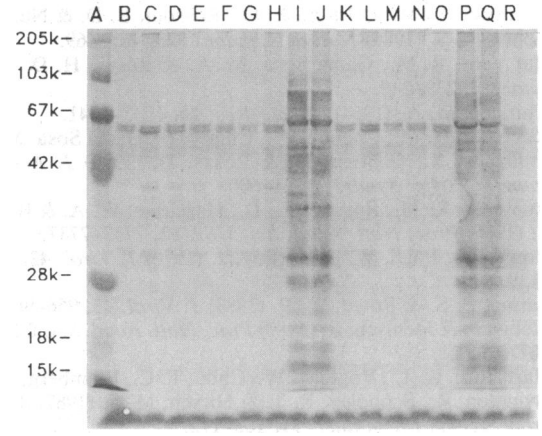

FIG. 3. Immunoblot of spleen homogenates derived from mice given splenocytes before RLV challenge. Naive mice received splenocytes from various donors and were then challenged with RLV. Three weeks later, these mice were sacrificed, and spleen homogenates were analyzed by immunoblot analysis. The donors were either naive (lanes I, J, and Q), or treated with AZT plus rHuIFN-αA/D (10 kilounits i.p. every 12 hr) until day 20 after inoculation. Groups of donor mice were started on therapy either at −72 hr (lanes L, P, and R), at +4 hr (lanes E, H, and K), at +24 hr (lanes B and D), or at +96 hr (lanes C, F, and O). Control mice were left untreated (lanes G, M, and N). Lane A: Molecular mass standards in kDa (k). One donor had a positive p30gag RIA initially that became negative 3 weeks later; this is the only instance in which adoptive cell transfer failed to protect a naive recipient (lane P).

When we examined humoral immunity, mouse immune sera were protective only at high doses, possibly due to the lack of high neutralizing antibody titers. Because the immune sera were collected 3 weeks after live virus rechallenge and neither immunoblot analysis nor passive transfer into secondary recipients revealed ongoing virus replication, it is unlikely that the passive protection was due to lymphokines or other acute-phase reactants rather than to specific antibodies. Possibly, the effect of immune serum on prevention of virus-induced disease is related to its ability to "arm" an effector cell (e.g., a macrophage or natural killer cell).

More dramatic than the antibody effects were the effects of T-cell transfers on the prevention of viremia. As few as 5 × 10^7 T cells (a normal spleen contains 10^8 cells) transferred from immune mice to naive recipients were protective. Administration of AZT plus rHuIFN-αA/D to mock-infected animals did not result in protection against subsequent RLV challenge, indicating a requirement for live virus in generating the specific cellular immunity to subsequent challenge (17).

Earl *et al.* (25) compared various vaccine strategies against Friend murine leukemia virus (FLV) infection, including immunization with (*i*) live recombinant vaccinia virus expressing the FLV glycoprotein envelope, (*ii*) formalin-killed FLV, and (*iii*) live attenuated N-tropic FLV (FLV-N) the replication of which was genetically restricted in the experimental mice used, in contrast to that of FLV-B. Whereas formalin-killed FLV preparations could not protect mice from lethal challenge with pathogenic FLV-B, both recombinant vaccinia virus and live FLV-N stimulated antibody as well as cytotoxic T-lymphocyte responses and prevented leukemia. However, residual FLV-infected cells were seen in mice challenged with pathogenic FLV-B after vaccination with recombinant live vaccinia virus. The immunity induced by recombinant live vaccinia virus was weaker than that induced by live FLV-N. Our studies, in which pathogenic RLV was blocked pharmacologically from replication, demonstrate that live virus is a potent immunogen; after adoptive transfer of immune T cells followed by RLV challenge, neither viral antigens nor infectious virus were seen in recipient mice.

Because antibody titers are easily measured, investigators have used the ability of a vaccine to stimulate specific antibody as the major criterion of vaccine efficacy. However, in certain situations this criterion may be misleading. Although polypeptide and killed-virus vaccines often stimulate excellent antibody responses, they are less capable of inducing virus-specific T cells than live-virus vaccines (26, 27). Furthermore, many retroviral diseases, including HIV-1-induced AIDS in humans, are characterized by the pres-

Table 4. Immune T cells protect against RLV challenge

Mouse	Donor: T cells transferred	Donor: AZT + rHuIFN-αA/D treatment	Donor: Start	Recipient: Spleen weight, mg	Recipient: Immunoblot Spleen	Recipient: Immunoblot Serum	Recipient: Serum titer, pfu/ml
1-1*	None	None		83	−	−	0
2-1	10^8	+	− 3d	377	−	−	0
3-1	10^8	+	+ 4h	131	−	−	0
4-1	10^8	+	+ 24h	237	−	−	0
5-1	10^8	+	+ 4d	218	−	−	0
3-2	5×10^7	+	+ 4h	126	−	−	0
4-2	5×10^7	+	+ 24h	286	−	−	0
5-2	5×10^7	+	+ 4d	251	−	−	0
3-3	10^7	+	+ 4h	2450	+	+	1.9×10^5
4-3	10^7	+	+ 24h	2331	+	+	9.2×10^4
5-3	10^7	+	+ 4d	3532	+	ND	ND
6-1	10^8	None		2623	+	ND	ND
6-2	5×10^7	None		2564	+	ND	ND
6-3	10^7	None		2262	+	ND	ND

Groups of mice were treated as outlined in Table 3. T cells from RLV-immune donors were injected i.v. into naive recipients as indicated. One hour later, recipients were challenged with RLV and tested for virus 20 days later. ND, not done; d, day; h, hr.
*, Not challenged.

ence of large quantities of specific antibodies that do not prevent disease progression (28).

Our studies confirm the efficacy of live-virus preparations in producing protective immunity to retroviruses in >90% of a large number of animals. Additionally, they show that if a lethal retrovirus can be attenuated pharmacologically after inoculation, protective immunity can still be achieved in an otherwise fatal infection. The exact mechanism by which T cells protect against rechallenge is not yet defined, but the present studies have importance in two regards: (*i*) one cannot assume that no immune response exists in an otherwise uniformly fatal disease; and (*ii*) by giving chemotherapeutic agents to block virus replication, one may allow development of an immune response that would otherwise be inhibited or overwhelmed by the disease itself.

The authors thank Dr. S. Nusinoff-Lehrman (Burroughs Wellcome) for the gift of AZT, Dr. I. Sim (Hoffmann–La Roche) for rHuIFN-αA/D, and Drs. R. Jaenisch and A. Sharpe for carrying out RIAs. This work was supported in part by National Institutes of Health/National Institute of Allergy and Infectious Diseases Contract NO1-AI-72664 and National Institutes of Health Grant UO1-AI-74845 to R.M.R. and National Institutes of Health Grant CA34979 and a contract from the U.S. Army, USAMRDC DAMD17-87-C-7151, to R.W.F. R.M.R. is the recipient of a Faculty Research Award from the American Cancer Society.

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