ing buds and to orient the mitotic spindle. These functions are consistent with earlier proposals that intermediate filaments organize cytoplasmic space (*1*) and mediate organelle distribution (*24*).

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13. A unique Nde I site was created at the initiator methionine of the *MDM1* gene (*7*) with the MutaGene M13 in vitro Mutagenesis Kit (Bio-Rad). This site allowed the isolation of a 1519-bp Nde I–Nhe I fragment containing the entire *MDM1* gene, which was cloned into the pET11-a vector (*27*) to create plasmid pET11-a-*MDM1*. We constructed a bacterial expression vector containing the mutant gene (pET11-a-*mdm1*) by replacing the Stu I–Nhe I fragment in plasmid pET11-a-*MDM1* with the corresponding sequence from the cloned *mdm1* gene. Wild-type and mutant forms of MDM1 were expressed in *E. coli* strain BL21(DE3)pLysS and purified from inclusion bodies as described (*14*), except that leupeptin and pepstatin A were added to extraction buffers to a final concentration of 1 mg/liter, and final extracts were clarified by centrifugation at 16,000*g* for 1 min. Protein concentrations were determined with the use of the bicinchoninic acid protein assay reagent (Pierce). The purity of wild-type and mutant MDM1 was analyzed by SDS–polyacrylamide gel electrophoresis followed by Coomassie staining or immunoblotting.
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16. Efficiency of filament formation, as defined previously (*28*), was determined by differential centrifugation and analysis of fractions by electron microscopy. The dialysate was centrifuged at 16,000*g* for 2 min. The pellet contained 50 to 75% of the total MDM1 and appeared as clumps and nonfilamentous aggregrates by electron microscopy. The supernatant (25 to 50% of the total protein) contained numerous filaments. When this latter material was centrifuged at 250,000*g* for 1 hour, at least 80% (20 to 40% of the total protein) was found in the pellet and appeared as filaments and filamentous aggregrates in the electron microscope.
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Rapid Loss of CD4$^+$ T Cells in Human-PBL-SCID Mice by Noncytopathic HIV Isolates

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Human immunodeficiency virus (HIV) isolates differ in cell tropism, replication, pathogenicity, and syncytial induction in vitro. CD4$^+$ T cells were enumerated in severe combined immunodeficient mice transplanted with human peripheral blood leukocytes (hu-PBL-SCID mice) and infected with HIV isolates with different in vitro cytopathicity. Two noncytopathic, macrophage-tropic strains, HIV-1_{SF162} and HIV-2_{UC1}, induced extensive CD4$^+$ T cell depletion, whereas HIV-1_{SF33}, which is highly cytopathic for T cells in vitro, caused little CD4$^+$ T cell depletion at equivalent virus burden. In vitro cytopathicity assays therefore do not predict CD4 depletion in the hu-PBL-SCID model.

The central immunologic defect after infection with HIV-1 is the decline in CD4$^+$ T lymphocytes, which precedes the progression from asymptomatic infection to the acquired immunodeficiency syndrome (AIDS). The duration of this process is usually greater than a decade, and an animal model with accelerated CD4$^+$ T cell depletion would be useful for investigating the mechanism of pathogenesis in vivo (*1*). The simplest model for CD4$^+$ T cell loss would be a direct, cytopathic infection of cells by HIV. However, some isolates of HIV-1 show no cytopathic effect on cultured human T cells and yet have been recovered from patients with CD4$^+$ T cell depletion (*2*). These observations have suggested that HIV-1 infection leads to dysfunction and eventual loss of CD4$^+$ T cells by indirect mechanisms that do not require virus infection of the target cell. Understanding such mechanisms of pathogenesis is also made difficult by the extensive biologic and genetic diversity of HIV-1 or HIV-2 isolates (*2–4*). HIV isolates differ in their cell tropism, cytopathicity, ability to induce syncytia, and rate of replication (*4–11*). Several of these properties are related to changes in the *env* gene encoding the gp120 envelope glycoprotein (*12–20*). It would aid our understanding of AIDS pathogenesis if these changes in the behavior of HIV in vitro could be related to the process of CD4$^+$ T cell depletion in vivo.

To address these issues, we have studied the fate of human CD4$^+$ T cells in severe combined immunodeficient mice reconstituted with adult human peripheral blood mononuclear leukocytes (hu-PBL-SCID mice) and infected with several well-characterized HIV-1 viruses and one HIV-2 strain. The human T cells (including both CD4$^+$ and CD8$^+$ T cells and CD45RO$^+$

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memory T cells) survive for several months after the injection of mononuclear PBL preparations into the peritoneal cavity of SCID mice (*21*), and hu-PBL-SCID mice are highly susceptible to infection with multiple strains of HIV-1 and HIV-2 (*22, 23*). For these experiments, we used the following molecularly cloned viral isolates: HIV-1_{SF2}, HIV-1_{SF13}, HIV-1_{SF33}, HIV-1_{SF162}, and HIV-2_{UC1}. These viruses differ in their in vitro host range, replication rate, and cytopathic effects (Table 1). HIV-1_{SF2} and HIV-1_{SF13} were derived from the same individual when asymptomatic (*24*) and when AIDS symptoms had appeared, respectively, and are ≥97% genetically identical (*6, 17*).

In three replicate experiments, these viruses were used at the same tissue culture infectious dose (as defined by culture with mitogen-activated human mononuclear PBLs) to infect hu-PBL-SCID mice 2 weeks after reconstitution with PBLs (*25*). Five animals per group were examined for numbers of human $CD4^+$ and $CD8^+$ T cells at 2 or 4 weeks after infection (Fig. 1) (*26*). $CD4^+$ T cell depletion was HIV strain-dependent. HIV-1_{SF162} and HIV-2_{UC1} gave the most rapid and extensive depletion, and HIV-1_{SF33}, the most cytopathic strain in tissue culture, produced the slowest and least extensive depletion. After HIV infection, virtually all recovered $CD45^+$ human cells were $CD3^+$, $CD8^+$ T cells, so the decrease in $CD4^+$ T cells was not due to CD4 down-modulation nor to total lymphocyte loss (see Fig. 1 legend). These results on $CD4^+$ T cell loss are surprising because HIV-1_{SF162} and HIV-2_{UC1} are macrophage-tropic isolates with virtually no cytopathic effect on cultured T cells (Table 1) (*7, 11, 12*). Viruses recovered from hu-PBL-SCID mice infected with HIV-1_{SF162} and HIV-1_{SF33} had the same distinct biologic properties as outlined in Table 1 (*27*). Passage in hu-PBL-SCID mice thus had not selected for phenotypic variants.

Although all of the animals shown in Fig. 1 were infected (*25*), as confirmed by isolation of virus by coculture and detection of proviral genomes, the extent of viral replication could have differed among animals infected with different viral strains. To assess this possibility, we did quantitative polymerase chain reaction (PCR) analysis of proviral genome copy number and determined plasma concentrations of the viral core p24 antigen (Fig. 2). These data show that HIV-1_{SF33} replicated to at least as high a copy number in hu-PBL-SCID mice as HIV-1_{SF162} and that HIV-1_{SF2} and HIV-1_{SF13} showed similar and lower proviral copy number. Because $CD4^+$ T cells are the likely target for infection and the numbers of residual $CD4^+$ T cells differed between groups, we also expressed the data as proviral copies per residual $CD4^+$ T cell (Fig. 2D). This recalculation still resulted in no discernible difference in viral burden between hu-PBL-SCID mice infected with HIV-1_{SF33} or HIV-1_{SF162}. In addition, plasma p24 concentrations were higher in hu-PBL-SCID mice infected with HIV-1_{SF33} than in mice infected with other HIV-1 strains (Fig. 2E). The extent of $CD4^+$ T cell depletion thus does not seem to be directly related to the extent of viral replication. HIV-1_{SF33} maintains its high replicative capacity in hu-PBL-SCID mice. Nonetheless, infection with this virus does not lead to as rapid $CD4^+$ T cell depletion as infection with the macrophage-tropic strains.

These results, although examining only five molecularly cloned viruses, establish several points that could prove useful in understanding the pathogenesis of AIDS in humans: (i) reproducible $CD4^+$ T cell depletion can be demonstrated in a small animal model for HIV infection; (ii) the rate and extent of $CD4^+$ T cell depletion is dependent on the virus strain, implying that the virus itself (as opposed to the immune response to HIV) plays a dominant role in the mechanism of $CD4^+$ T cell loss; (iii) $CD4^+$ T cell depletion is not necessarily correlated with extent of virus replication; and (iv) the rate and extent of $CD4^+$ T cell depletion in hu-PBL-SCID mice was not predicted by in vitro studies of viral replication rate or cytopathic effect. Moreover, the lack of correlation between proviral copy number and rate of $CD4^+$ T cell depletion observed here advises caution about the use of this measure of viral burden as a surrogate clinical marker for predicting AIDS progression. Finally, both isolates that caused rapid $CD4^+$ T cell depletion, HIV-1_{SF162} and HIV-2_{UC1}, are macrophage-tropic as well as noncytopathic for $CD4^+$ T cells in vitro. Although this correlation might be coincidental given the

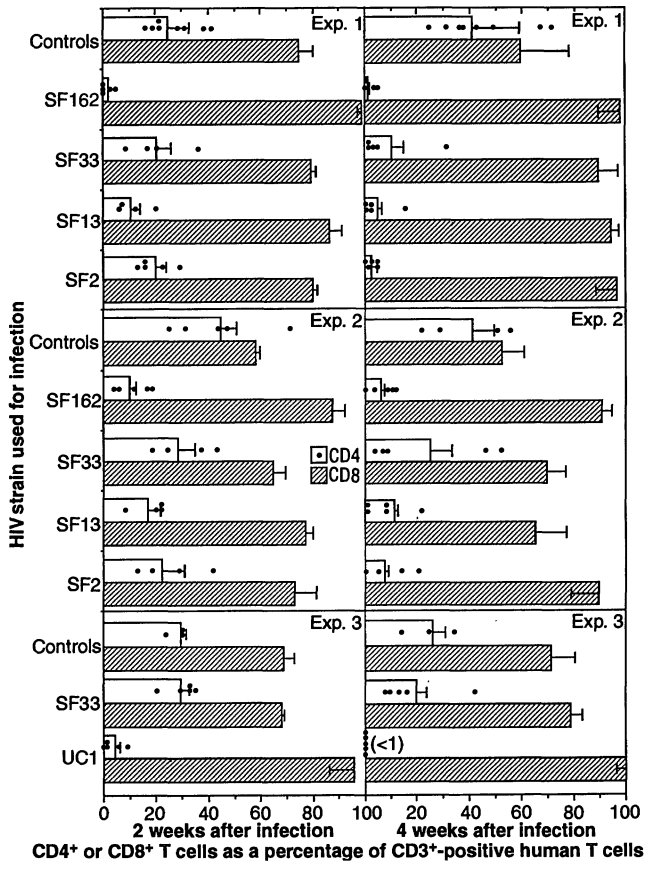

Fig. 1. Infection of hu-PBL-SCID mice with HIV-1_{SF162}, HIV-1_{SF33}, HIV-1_{SF13}, HIV-1_{SF2}, or HIV-2_{UC1} leads to a strain-dependent decline in $CD4^+$ T cells. Data are from fluorescence-activated cell sorter analysis of human cells recovered from the peritoneal cavity of five hu-PBL-SCID mice per group infected with 10^2 tissue culture infectious doses of virus 2 or 4 weeks previously. Controls are uninfected hu-PBL-SCID mice. Error bars represent the standard error of the mean, and $CD4^+$ T cell percentages for individual mice are shown by the filled circles. Mice infected with HIV-1_{SF162} or HIV-2_{UC1} had significantly fewer $CD4^+$ T cells than either control groups or mice infected with HIV-1_{SF33} ($P < 0.001$ at 2 weeks, $P < 0.05$ at 4 weeks; Kruskal-Wallis nonparametric analysis of variance). Three experiments using different PBL donors are shown. In Exp. 1 the control data were derived from a contemporaneous experiment using the same PBL donor ($n = 8$ for controls). Internal controls were used in Exp. 2 and 3. Data are presented as a percentage of $CD3^+$ human T cells, which accounted for 90 to 98% of recovered $CD45^+$ human cells. The mean percentage of $CD45^+$ cells was 72.7 ± 4.4% and 37.3 ± 5.3% of recovered peritoneal lavage cells at 2 or 4 weeks after HIV infection, respectively. Although there was no statistically significant difference in human cell recovery between control mice and mice infected with any HIV strain at 2 weeks after infection or infected with HIV-1_{SF33} or HIV-1_{SF162} at 4 weeks after infection, somewhat lower cell recovery was seen at 4 weeks after infection of mice with HIV-1_{SF2}, HIV-1_{SF13}, and HIV-2_{UC1}. The percentage of $CD4^+$ and $CD8^+$ T cells thus parallels their absolute numbers, except for hu-PBL-SCID mice infected with HIV-1_{SF2}, HIV-1_{SF13}, and HIV-2_{UC1} at 4 weeks after infection, when absolute numbers were lower.

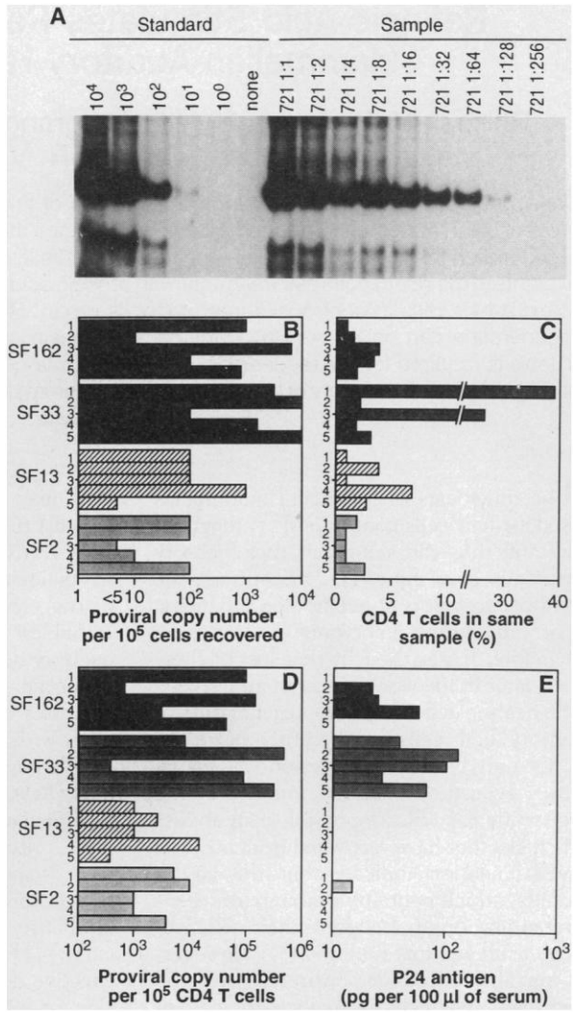

Fig. 2. Quantitation of HIV infection in hu-PBL-SCID mice infected with different HIV-1 or HIV-2 strains. (**A**) Sensitivity of the quantitative PCR assay used to detect proviral copy number of HIV-1 *gag* genes. DNA was prepared from the indicated number of 8E5 T cells, a subclone of the A3.01 HIV-1–infected CEM T cell line containing a single integrated provirus (*30*), or from dilutions of DNA from 10^5 peritoneal cavity cells of HIV-infected hu-PBL-SCID mice. DNA was subjected to 35 cycles of amplification by PCR as described previously (*22, 23*). The limit of detection of amplified *gag*-specific sequences was approximately ten proviral copies. The last dilution of DNA giving a visible band was accordingly assumed to contain ten copies of the HIV-1 provirus, and the proviral copy number per input 10^5 total cells was calculated. These data are presented in (**B**) for peritoneal cells recovered from each animal at 4 weeks after HIV-1 infection in Exp. 2. (**C**) The percentage of residual CD4+ T cells present in the peritoneal cells recovered from each mouse (from Fig. 2 and Exp. 2) is shown to allow comparison of the proviral copy number and the extent of CD4+ T cell depletion. (**D**) The proviral copy number shown in (B) is recalculated to show the copy number per recovered CD4+ T cell [from (C)], to correct for different amounts of CD4+ T cells among different hu-PBL-SCID mice. (**E**) Plasma p24 antigen levels in individual hu-PBL-SCID mice infected with different strains of HIV-1 4 weeks previously. Data are from Exp. 1, so p24 antigenemia and proviral copy number shown in (B) and (D) cannot be directly compared. The sensitivity of the antigen-capture enzyme-linked immunosorbent assay (Coulter Diagnostics, Hialeah, Florida) is ~10 pg of p24 core antigen.

Table 1. Biologic properties of viruses derived from molecular clones of HIV-1_{SF2}, HIV-1_{SF13}, HIV-1_{SF33}, HIV-1_{SF162}, and HIV-2_{UC1} (*31*).

Strain	Replication in PBLs*		Cytopathic effect in CD4 cells†	MT4 plaque formation in vitro‡	CD4 down-modulation in vitro§	CD4 T cell depletion in hu-PBL-SCID mice
	CD4 T cells	MØ				
SF2	++	±	+	−	+	Intermediate
SF13	++	+	++	+	+	Intermediate
SF33	++	±	+++	++	+	Slow
SF162	++	++	−	−	−	Fast
UC1	++	++	−	−	−	Fast

*Plus signs indicate relative levels of the indicated property of each virus strain. Replication rates were measured both in primary cultures of CD4+ T lymphocytes or monocyte-macrophages (MØ) derived from human PBLs and in a variety of cell lines, as described (*6, 7, 11, 12, 14, 17–19, 28, 31*). For virus replication, (++) indicates >500,000 cpm/ml maximum reverse transcriptase activity in culture fluid, (+) >100,000 cpm/ml, and (±) replication only detected after coculture with mitogen-activated PBLs. Background was <2000 cpm/ml. †Cytopathic effect was measured by syncytial formation or balloon cell degeneration of CD4+ T lymphocytes and SupT-1 cells. Control cultures showed no cytopathic effects. ‡Conducted as described (*28*). Plus signs indicate size of plaques when formed. Plaque formation correlated with cytopathic effects in vitro (*28*). §CD4 expression on cultured CD4+ T cells or the SupT-1 cell line as determined by flow cytometry (*31*).

small number of HIV isolates examined, it is also possible that the sequence variation in the HIV gp160 envelope protein that conveys macrophage-tropism is also associated with enhanced pathogenicity in vivo.

These experiments were conducted to determine whether the in vitro properties of HIV strains would be reflected in studies in vivo. Our results in hu-PBL-SCID mice suggest that viral variation contributes to the rate and extent of CD4+ T cell depletion in infected individuals and moreover that macrophage-tropic, noncytopathic strains may be of particular importance. The findings are surprising because previous observations on HIV isolates have associated the recovery of synctia-inducing, cytopathic viruses with progression to AIDS (*6, 8*). This apparent discrepancy could mean that the hu-PBL-SCID model is not appropriate for demonstrating the full range of pathogenic potential of HIV infection in humans. Alternatively, the emergence of more cytopathic HIV variants after long-term infection of humans could be the result of immune deficiency rather than the cause of it. The ability to disassociate the in vitro cytopathic effects of HIV isolates from their capacity to cause CD4+ T cell depletion in hu-PBL-SCID mice should provide insight into the mechanisms by which HIV infection leads to immune deficiency.

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25. Human PBLs were collected from Epstein-Barr virus seronegative donors, and mononuclear cells were prepared by Ficoll-Hypaque density centrifugation. Mononuclear PBLs (2×10^7) were injected intraperitoneally (i.p.) into C.B-17 SCID mice that were "non-leaky" (mouse immunoglobulin levels <5 μg/ml). HIV was injected i.p. into hu-PBL-SCID mice 2 weeks later. Human immunoglobulin (hIg) was determined at this time and when mice were killed 2 or 4 weeks later. Of the 106 mice used in these experiments, three were excluded from further data collection because of low hIg levels before HIV infection and two because of lack of HIV isolation.
26. Cells were recovered from the peritoneal cavity by lavage with 5 to 10 ml of warm RPMI 1640 medium. Single-cell suspensions were prepared from spleens. Viable cell counts were performed with trypan blue exclusion, and samples of cells were stained with monoclonal antibodies directly conjugated with fluorescein or phycoerythrin and specific for human CD3, CD4, CD8, or CD45 or mouse H-2K^d [Becton-Dickinson (B-D), Mountain View, CA, or Pharmingen, San Diego, CA]. Nonspecific staining of murine cells was blocked by addition of 10% mouse serum and antibody to the mouse Fcγ2 receptor to the staining buffer. Cells were evaluated for staining with a FACScan instrument (B-D) after gating on lymphocytes by forward and side light scatter. A minimum of 5×10^3 cells were evaluated, and more commonly 1×10^4 to 2×10^4. We observed no evidence of CD4 down-modulation by the intensity of CD4 staining in these experiments.
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31. The results summarized in Table 1 represent more than 20 separate experiments with the virus strains listed, and the relative expression of the biologic properties was consistent in all experiments. The amounts of virus replication shown in CD4$^+$ T cells and macrophages were reached in all experiments and were exceeded by up to 40% when mononuclear PBLs were derived from donors with high sensitivity to HIV (*2, 11*). Cytopathic effects (CPE) were estimated by the number of syncytia and balloon cell degeneration induced in either CD4$^+$ T cells from peripheral blood or the SupT-1 cell line. HIV-1_{SF162} and HIV-2_{UC1} infection never caused CPE. Infection of T cells with HIV-1_{SF33} gave >90% CPE within 7 to 10 days of culture, infection with HIV-1_{SF2} gave <5% CPE, and infection with HIV-1_{SF13} gave 25 to 50% CPE in both PBL CD4$^+$ T cells and SupT-1 cells. MT4 plaque formation correlated with the CPE of the virus strains (*28*). HIV-1_{SF162} and HIV-2_{UC1} do not replicate in MT4 cells, HIV-1_{SF2} replicates but does not cause plaques, HIV-1_{SF13} produces small plaques, and HIV-1_{SF33} rapidly induces large plaques. CD4 down-modulation was measured both in CD4$^+$ T cells from PBLs and the SupT-1 cell line. Down-modulation was considered positive if CD4 staining was reduced from 100% positive cells before infection to <25% positive cells after infection (as observed with HIV-1_{SF2}, HIV-1_{SF13}, and HIV-1_{SF33}) and negative if infected cells showed >90% of control values (as observed with HIV-1_{SF162} and HIV-2_{UC1}).
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Retinoic Acid Stimulates Regeneration of Mammalian Auditory Hair Cells

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Sensorineural hearing loss resulting from the loss of auditory hair cells is thought to be irreversible in mammals. This study provides evidence that retinoic acid can stimulate the regeneration in vitro of mammalian auditory hair cells in ototoxic-poisoned organ of Corti explants in the rat. In contrast, treatment with retinoic acid does not stimulate the formation of extra hair cells in control cultures of Corti's organ. Retinoic acid–stimulated hair cell regeneration can be blocked by cytosine arabinoside, which suggests that a period of mitosis is required for the regeneration of auditory hair cells in this system. These results provide hope for a recovery of hearing function in mammals after auditory hair cell damage.

The inner ears of fishes and amphibians produce hair cells continuously throughout life and thus can self-repair these sensory structures after injury (*1*). In contrast, the auditory receptors of avians stop producing hair cells during embryonic development; therefore, it was thought that loss of these cells later in life would result in an irreversible hearing deficit (*2*). The regeneration of sensory hair cells has recently been described in birds after a lesion caused by either acoustic overstimulation or ototoxic poisoning (*3*). Electrophysiological studies of chicks that have recovered from acoustic overstimulation indicate that the basilar papilla, which contains a mixture of regenerated and original auditory hair cells, recovers full auditory function (*4*). However, it remains to be demonstrated that the regenerated hair cells actually participate in the observed recovery of function.

Deafness resulting from a loss of hair cells in mammals has also been assumed to be permanent because the production of sensory cells is normally completed at the end of the first half of the gestation period (*5*). However, the repair of stereocilia bundles and cuticular plates after mechanical injury to Corti's organ has been described in cochlear duct explants from newborn mice; this result suggests that replacement of hair cells may be possible in the mammalian cochlea (*6*). This finding implies that some regenerative capabilities must exist in the mammalian inner ear. Such a response may be triggered by a stimulus in addition to the initial lesion, as demonstrated in cultures of auditory neurons in which a limited ability to regenerate severed or damaged neuronal processes (*7*) could be stimulated by treatment with neurotrophic growth factors (*8*).

We have tested the potential of retinoic acid (RA) to stimulate the regeneration of mammalian auditory hair cells in organotypic cultures (*9*) of Corti's organ excised from 3-day-old rat pups (*10*) after exposure to an ototoxic dose of the aminoglycoside antibiotic neomycin. A dose-response curve (*11*) determined that 10^{-3} M was an effective dose of neomycin for the destruction of 99% of the auditory hair cells in these organotypic cultures (Fig. 1A). After treatment with a dose of 10^{-3} M neomycin, the explants were compared with control (untreated) cultures and to neomycin-treated explants that were also treated after exposure with 10^{-8} M RA for 4 or 7 days (*12, 13*) (Fig. 1B; Table 1). The neomycin-treated cultures did not stain for the presence of hair cell stereocilia bundles either after 2 days of exposure to neomycin (post-exposure day 0) or after the removal of neomycin from these cultures and an additional 4 or 7 days of culture in normal growth medium. The lack of hair cells in the ototoxin-exposed cultures contrasts with the results observed in stereocilia bundles in the control (untreated) cultures (Figs. 1B and 2A and Table 1).

When ototoxin-exposed cultures were treated after neomycin exposure with 10^{-8} M RA for a period of either 4 or 7 days, they contained increasing numbers of new hair cells, as we determined by counting cells with fluorescein isothiocyanate (FITC)–phalloidin–stained stereocilia bundles. After 4 and 7 days of treatment with RA, a 16% and a 78% replacement, respectively, of the original auditory hair cell

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