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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}).
32. Supported by NIH grants AI29182 and AI30238 (D.E.M.) and AI24499 (J.A.L.). We thank K. Von-Sederholm and R. MacDowell for animal care, H. Struble for technical assistance, and P. Luciw, C. Cheng-Mayer, T. Shioda, M. Quiroga, and D. York-Higgins for their contributions in the initial cloning and characterization of the HIV strains. All work with HIV-infected hu-PBL-SCID mice was performed under biosafety level 3 containment (*23, 29*). This is publication 7723-Imm from The Scripps Research Institute.

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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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population was observed (Figs. 1B and 2B; Table 1). In contrast to the observation that 10^{-8} M RA can evoke the formation of supernumerary hair cells in vitro (*12*), we observed that treatment with the same concentration of RA in 3-day-old rat organ of Corti cultures for 11 days did not initiate new hair cell formation as determined by FITC-phalloidin staining (Table 1). A photomicrograph of a control culture of Corti's organ labeled with FITC-phalloidin at 11 days shows that hair cell integrity was maintained in these untreated explants (Fig. 2A). Ototoxin- and RA-treated explants showed, after 4 days of RA exposure, a band of epithelial cells stained densely with FITC-phalloidin. Approximately 13% of the labeled cells possessed the beginning of a stereocilia bundle (Table 1). At 7 days after exposure to RA, the presence of hair bundles on the apical surfaces of the cells labeled with FITC-phalloidin was nearly 100% (Fig. 2B and Table 1).

To confirm that these labeled cells were immature regenerating hair cells, we undertook an ultrastructural study (*14*). Electron micrographs of a control Corti's organ after 11 days in vitro showed inner and outer hair cells with stereocilia as well as cuticular plates (Fig. 3A). Normal-appearing synapses of afferent auditory neurites were also

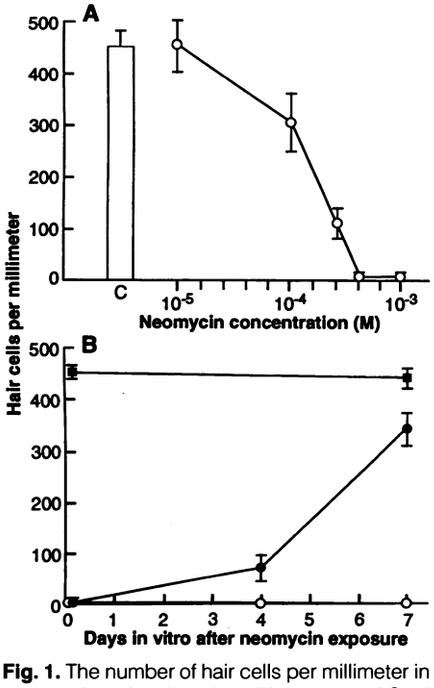

Fig. 1. The number of hair cells per millimeter in the cochlear duct in 3-day-old rat organ of Corti explants. (**A**) A dose-response curve of the ototoxic effect of neomycin on auditory hair cell survival; C, control. (**B**) A comparison of hair cell counts in controls (■), in neomycin-treated (10^{-3} M) explants (○), and in neomycin-treated (10^{-3} M) explants subsequently treated with RA (10^{-8} M) (●) (n = 12, bars = ±SEM).

Table 1. Auditory hair cell counts from 3-day-old rat organ of Corti explants; U, untreated cultures; RA, 10^{-8} M retinoic acid, continuous or postexposure-treated cultures; and RA + Ara C, 10^{-5} M Ara C +10^{-8} M RA, postexposure-treated cultures. Hair cells per millimeter of cochlear duct were determined by counts of cells with FITC-phalloidin–stained stereocilia bundles. Each count represents the mean value of 12 specimens from four separate experiments. Standard errors of the means (SEM) are expressed as ± values; ND, hair cell counts were not performed.

Time in vitro (days)	Time after exposure (days)	Control		Neomycin (10^{-3} M)		
		U	RA	U	RA	RA + Ara C
4	0	454 ± 13		4 ± 2		
8	4	ND	ND	0	71 ± 26	0
11	7	440 ± 28	442 ± 20	0	344 ± 32	0

observed at the base of these sensory hair cells (*15*). When organ of Corti explants that had been treated with 10^{-3} M neomycin for 48 hours were examined, severely damaged auditory hair cells were present. These dying cells were vacuolized, and many were in the process of being extruded from the apical surface of the sensory area of Corti's organ (Fig. 3B). In the cultures treated with neomycin and then 10^{-8} M RA, regenerating hair cells were identified by their long cell-surface microvilli (between 4 and 6 μm) that contained an arrangement of actin-like filaments that extended short rootlets into the apical surfaces of these immature hair cells (Fig. 3, C and D). We often observed that the immature regenerating hair cells contained a single rudimentary kinocilium (Fig. 3C) resembling a normally developing auditory hair cell (*16*).

The stimulatory effect of RA on hair cell regeneration in the neomycin-treated explants could be completely blocked if a mitosis-blocking dose of 10^{-5} M cytosine arabinoside (Ara C) was added at the same time as the 10^{-8} M RA. After exposure to RA plus Ara C for 4 or 7 days, no new hair cells were detected by FITC-phalloidin staining for stereocilia bundles (Table 1). To control for a possible toxic effect of 10^{-5} M Ara C on these organotypic cultures, we exposed unlesioned organ of Corti explants for the same period of time to this antimitotic drug. No significant changes in hair cells were observed in the explants stained with FITC-phalloidin.

Retinoic acid is a potent morphogen for patterning of the cephalic area (*17*). In combination with fetal calf serum, RA can induce precocious differentiation of inner ear sensory epithelium in cultures of early chick embryo otocysts (*12*). In mammals, the gene that encodes the cellular RA binding protein is uniquely expressed in that portion of developing inner ear that will form Corti's organ (*18*). Moreover, RA can affect the pattern of hair cell differentiation if applied before the onset of overt cytodifferentiation in cultures of embryonic mouse cochlear duct by initiating the formation of supernumerary hair cells (*12*). Conversely, our cultures of postnatal rat organ of Corti that already contain differentiated but not yet fully functional auditory hair cells did not develop supernumerary hair cells in response to the addition of exogenous RA. However, the addition of exogenous RA to these cultures did stimulate hair cell regeneration in Corti's organ after an initial period of ototoxic poisoning

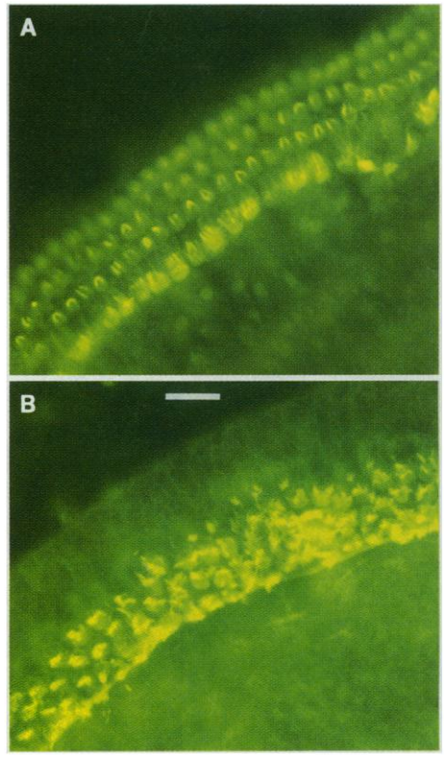

Fig. 2. Photomicrographs of FITC-phalloidin–stained 3-day-old rat organ of Corti explants, after 11 days in vitro. (**A**) An untreated culture with auditory hair cells (11 days in normal medium); explants grown in 10^{-8} M RA had the same pattern of FITC-phalloidin staining. (**B**) An ototoxin-damaged, RA-treated culture with regenerated auditory hair cells (2 days in normal medium, 2 days + 10^{-3} M neomycin, 7 days + 10^{-8} M RA; in ototoxin-treated cultures there were no FITC-phalloidin–stained stereocilia bundles. Bar = 25 μm.

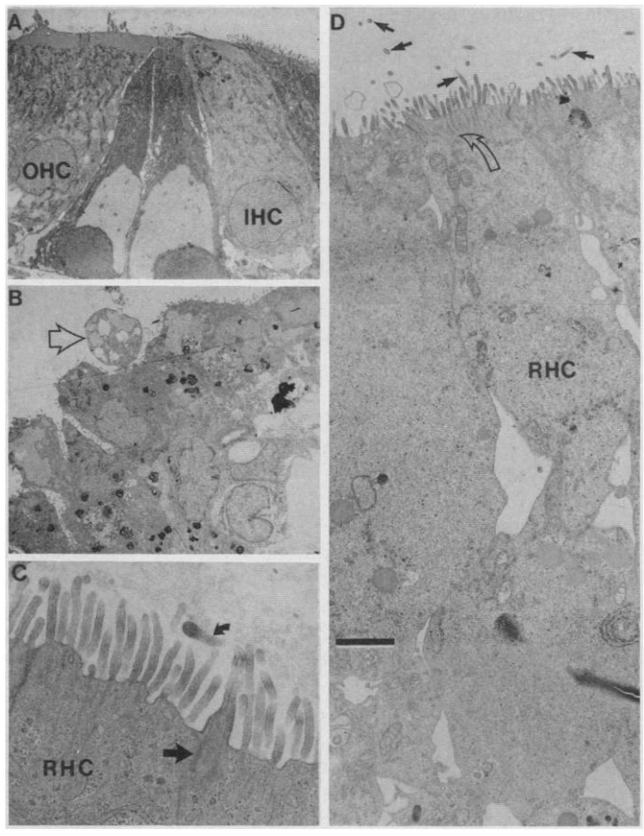

Fig. 3. Electron micrographs of the auditory hair cell area of Corti's organ from 3-day-old rat organ of Corti explants. (**A**) A control culture after 11 days in vitro (11 days in normal medium). Both inner (IHC) and (OHC) outer hair cells are present, separated by a pair of pillar cells. (**B**) An ototoxin-damaged culture, after 4 days in vitro (2 days in normal medium, 2 days + 10^{-3} M neomycin); numerous myelin figures are present in the damaged cells of Corti's organ, with a dying cell (open arrow) being extruded from the apical surface of this sensory area. (**C** and **D**) Areas of regenerated auditory hair cells in ototoxin-damaged, RA-treated cultures, after 11 days in vitro (2 days in normal medium, 2 days + 10^{-3} M neomycin, and 7 days + 10^{-8} M RA); numerous immature stereocilia (small solid arrows) are present on the apical surfaces of the regenerated hair cells (RHC), with rootlets (open arrow) extending into the apical cytoplasm of these sensory cells. A single rudimentary kinocilium (heavy solid arrow) is present at the apical surface of a regenerating hair cell and was a frequent finding in these immature hair cells. Bar = 7.9 μm (A and B); 0.7 μm (C); 2 μm (D).

that destroyed 99% of the original hair cell population. This stimulatory action of RA on hair cell regeneration in vitro was blocked by an antimitotic drug (Ara C), suggesting that mitosis is required for regeneration of mammalian auditory hair cells. This result agrees with similar findings of mitotic activity during the repair and hair cell regeneration period in avian labyrinths (*3*) and in the lateral line system of amphibians (*19*).

Two hypotheses can be formulated in relation to hair cell regeneration in mammals. (i) Healthy auditory hair cells can suppress proliferation in the other cells that compose Corti's organ through the release of a diffusible molecule or molecules. Destruction of hair cells by an ototoxin or other trauma may remove the inhibition to cell proliferation and thus allow the suppressed cell population to enter the mitotic cycle and become capable of responding to a RA stimulus to differentiate into auditory hair cells. (ii) Injured hair cells can release a diffusible factor or factors that initiate cell division in a quiescent population of cells of Corti's organ, which can then be stimulated by RA to differentiate into immature auditory hair cells. The identification of the actual mechanism and the factor or factors responsible for the stimulation of cell proliferation in the damaged organ of Corti is a key issue in the restoration of hearing after a lesion to the auditory receptor. Consequently, administration of a mitosis-stimulating factor together with a differentiation factor like RA might be used as a therapeutic technique for repopulating damaged inner ears in mammals.

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14. The organotypic cultures were fixed overnight at 4°C in freshly prepared solution of 4% paraformaldehyde and 4% glutaraldehyde in PBS (pH 7.2) and then exposed to 1% OsO_4 in PBS for 1 hour at 4°C, dehydrated in an ethanol series and then in a propylene oxide series, and infiltrated and imbedded in Imbed 812. Thick sections (1 μm) were sampled to identify areas of interest. Thin sections (silver gray) were cut and mounted on 200-mesh grids, stained with lead citrate and uranyl acetate, carbon-coated, and examined on a Hitachi model

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Linkage on Chromosome 3 of Autoimmune Diabetes and Defective Fc Receptor for IgG in NOD Mice

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A congenic, non-obese diabetic (NOD) mouse strain that contains a segment of chromosome 3 from the diabetes-resistant mouse strain B6.PL–*Thy-1*^{a} was less susceptible to diabetes than NOD mice. A fully penetrant immunological defect also mapped to this segment, which encodes the high-affinity Fc receptor for immunoglobulin G (IgG), FcγRI. The NOD *Fcgr1* allele, which results in a deletion of the cytoplasmic tail, caused a 73 percent reduction in the turnover of cell surface receptor–antibody complexes. The development of congenic strains and the characterization of Mendelian traits that are specific to the disease phenotype demonstrate the feasibility of dissecting the pathophysiology of complex, non-Mendelian diseases.

Complex diseases such as autoimmune, insulin-dependent diabetes mellitus (IDDM) (*1–4*), hypertension (*5*), and epilepsy (*6*) are largely determined by a number of gene effects. The NOD mouse strain spontaneously develops IDDM (*7*), which is determined by at least nine unlinked loci [*Idd-1* through *Idd-9* (*1, 2, 8*)]. With the exception of *Idd-1*, which is encoded by genes within the major histocompatibility complex (MHC) on chromosome 17, no individual locus appears to be absolutely essential for disease onset. Heterozygosity even at the MHC has a low, but significant penetrance that makes fine mapping by standard linkage analysis impractical (in the absence of any obvious candidate genes) (*1–4*). Proof that any of the non-MHC loci encode significant effects and progress in fine mapping depend on the demonstration that NOD strains that are congenic for chromosomal regions encoding diabetes-resistant alleles are actually less susceptible to disease.

Idd-3, which is linked to both diabetes and insulitis, was mapped to a 48–centimorgan (cM) interval on chromosome 3, approximately between *D3Nds6* [interleukin-2 (*Il-2*)] and *D3Nds9* (*Adh-1*) (*1*). In this 48-cM interval, the marker loci *D3Nds7* [*Cacy* (*9*)], *D3Nds11* [*Fcgr1* (*10*)], and *D3Nds8* (*Tshb*), which are in a 7-cM region, show strong linkage to diabetes and insulitis (Table 1). These backcross data do not, however, permit assignment of the disease locus within this 7-cM interval or even to an adjacent interval. In order to fine map the disease locus and assess the role of the chromosomal region encompassing these three marker loci in diabetes, we measured the frequency of disease in the NOD.B6.PL–*Thy-1*^{a}–*D3Nds7 D3Nds8* (NOD.*D3Nds7 D3Nds8*) congenic strain, which contains a segment of chromosome 3 from B6.PL–*Thy-1*^{a} in the homozygous state. The cumulative frequency of diabetes, as compared with cohorts of male and female NOD mice, was reduced significantly in both male ($P < 0.0001$) and female ($P < 0.0001$) congenic mice (Fig. 1, A and B). We obtained further evidence that this segment, and not a region on a different chromosome, is the cause of the reduction in diabetes frequency by demonstrating linkage between chromosome 3 and diabetes in a backcross analysis (*11*). Statistical analyses of backcross data (*3*) and the frequency of diabetes in other congenic strains encompassing different regions of chromosome 3 (*4*) indicate that *Idd-3* is encoded by two or more distinct chromosome 3 loci, one of which is in the *D3Nds7-D3Nds8* region. The occurrence of diabetes in the NOD.*D3Nds7 D3Nds8* congenic strain shows that the NOD alleles of genes in this region are not essential for disease development but do contribute significantly.

Another approach to the dissection of a complex disease is to identify Mendelian phenotypes that are caused by single, fully penetrant genes that can be mapped easily and precisely and that might indicate candidate genes (*12*). We have identified such a trait in NOD mice. After injection of complete Freund's adjuvant (CFA), the number of peripheral blood cells expressing Mac-1, an integrin molecule present on macrophages, monocytes, and neutrophils (*13*), increased in both NOD (Fig. 2A) and nondiabetic strains such as C57BL/10SnJ (B10) (Fig. 2B). A portion of Mac-1$^+$ cells in NOD (Fig. 2, C and E) but not in B10 (Fig. 2D) mice bound a monoclonal antibody (MAb) to immunoglobulin G2a (IgG2a). The expression of this IgG2a$^+$ phenotype was unrelated to *Idd-1* because NOD.B10–*H-2*^{b} mice (NOD.*H-2*^{b}) (*14*) also developed this trait (Fig. 3).

The IgG2a$^+$ phenotype was inherited in backcross and F_2 progeny as a single gene dominant trait: 70 out of 129 or 54% of the progeny from the backcrosses (B6.PL–*Thy-1*^{a}

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Table 1. Linkage of *D3Nds11* (*Fcgr1*) and flanking marker loci to insulitis and diabetes (*40*). He, heterozygous; Ho, NOD homozygous.

Marker locus	Recombination fraction (SE)	Insulitis He:Ho	χ^2	Diabetes He:Ho	χ^2
D3Nds7 (*Cacy*)		31:58	8.2*	22:84	36.3***
D3Nds11 (*Fcgr1*)	0.031 (0.015)	27:61	13.1**	24:82	31.7***
D3Nds8 (*Tshb*)	0.039 (0.017)	32:57	7.0*	25:81	29.6***

*$P < 10^{-2}$. **$P < 10^{-3}$. ***$P < 10^{-4}$.