Comparison of High– and Low–Diabetes-Incidence NOD Mouse Strains

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The nonobese diabetic (NOD) mouse is a model of insulin-dependent diabetes mellitus. These mice develop insulinopenia and hyperglycemia secondary to β-cell destruction, which is associated with insulitis and autoantibody production. We have two strains of NOD mice: a low-incidence strain (NOD/Wehi), in which <10% females and <1% males develop diabetes by 150 days despite intense insulitis, and a high-incidence strain (NOD/Lt), in which most females and many males develop diabetes by 150 days. This phenotypic difference has been maintained for 24 mo despite identical housing in our specific pathogen-free unit. Reciprocal skin grafting and allozyme electrophoresis have not identified a difference between the strains. Mixed-lymphocyte cultures were performed with splenic T-lymphocytes cultured with equal numbers of irradiated stimulator splenocytes for 3–6 days. NOD/Wehi mice demonstrated a heightened syngeneic mixed-lymphocyte response (SMLR), averaging 19% of the allogeneic response to CBA/CaHWehi cells. The response to NOD/Lt stimulator cells was not significantly different from the syngeneic response. In contrast, NOD/Lt mice had an SMLR similar to that of BALB/cAnBradleyWehi control mice, averaging 5% of the allogeneic response. NOD/Lt cells also responded similarly to NOD/Wehi stimulator cells and briskly to allogeneic cells. The heightened SMLR in NOD/Wehi mice may reflect active generation of suppressor function, and this may account for the low incidence of diabetes. *Diabetes* 38:1296–1300, 1989

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Most autoimmune diseases are genetically determined, but usually only a subset of susceptible individuals develop overt disease. Insulin-dependent diabetes mellitus (IDDM) shows this pattern. Nonobese diabetic (NOD) mice, a rodent model of IDDM, develop diabetes abruptly between 100 and 150 days of age associated with rapid loss of weight, polyuria, polydipsia, and abdominal distention. Makino et al. (1) described mononuclear cell infiltrates in the islets of Langerhans (insulitis), a progressive loss in number and size of islets, and a total decrease in pancreatic weight.

The Walter & Eliza Hall Institute (WEHI) is unique in having two strains of NOD mice. The first, NOD/Wehi, inherit the genetic susceptibility to diabetes in that they exhibit intense insulitis, and overt diabetes may be precipitated by administration of cyclophosphamide (2). Nonetheless they have an exceptionally low incidence of spontaneous disease, with <10% of females and <1% males becoming diabetic by 150 days of age. The second strain, NOD/Lt, shows the pattern of disease found in most NOD colonies, with most females and many males becoming diabetic by 150 days. This phenotypic difference has been maintained for 24 mo despite identical housing in our specific pathogen-free unit.

This study was undertaken to compare the two strains for evidence of major heterogeneity by skin grafting, allozyme analysis, and mixed-lymphocyte culture (MLC). Serreze and Leiter (3) have recently demonstrated that NOD/Lt responder cells proliferate poorly and produce little interleukin 2 (IL-2) when stimulated by syngeneic non–T-lymphocytes. They speculated that this represents a defect in suppressor T-lymphocyte activation that could be responsible for unmasking autoreactive helper T-lymphocytes, resulting in islet destruction and autoantibody production. We have therefore compared the level of the syngeneic response in MLC of the high- and low-incidence NOD mouse strains.

RESEARCH DESIGN AND METHODS

NOD/Wehi mice aged 70–188 days (av 120 days) were obtained from the colony maintained at WEHI for 17 generations. The colony was originally provided by H. Asamoto (Kyoto, Japan) in 1984. NOD/Lt mice aged 40–161 days (av 110 days) were obtained from the colony maintained at WEHI for 6 generations. This colony was originally obtained from E.H. Leiter (Jackson, Bar Harbor, ME) in January 1987. Experimental stocks of both NOD lines were maintained on separate racks in the same room, with a 12-h light-dark cycle and a constant ambient temperature of 21°C. BALB/cAnBradleyWehi, CBA/CaHWehi, BALB/c.Ig-I^{b}nu/Wehi, B6.Ig.I^e.nu/Wehi, CBA/CaH.bg, and NZB/Wehi mice were obtained from the animal breeding facilities of WEHI. All mice were housed under specific pathogen-free conditions and were allowed free access to food (Barastoc mouse pellets, Bendigo, Victoria, Australia) and acidified water. Mice were excluded if polydipsic, polyuric, or obviously ill.

Preparation of MLC assays. Mice were killed by cervical dislocation, and the spleen was removed under aseptic conditions and placed in cold, sterile phosphate-buffered balanced salt solution with 10% fetal calf serum (BSS-FCS). One mouse of each strain was killed to provide stimulator cells, and two mice of one strain were killed for the responder cells. A cell suspension was made, and the erythocytes were lysed in 5 ml 0.168 M NH_4Cl at 37°C for 5 min and washed and resuspended in BSS-FCS.

Responder cells were enriched for T-lymphocytes by nylon-wool incubation. The nylon-wool columns were prepared as follows: 1.4 g of nylon wool from LP-1 Leuko-Pak leukocyte filters (discontinued product, Fenwal, Morton Grove, IL) was weighed and carded on fleece cards (Ashford Handicrafts, Ashburton, New Zealand), packed into 10-ml syringes, and compacted, and the barrel containing nylon wool was sterilized in a preevacuation moist-heat autoclave (Harts, Perth, Western Australia) at 121°C and 106 kPa for 20 min. Before loading the cells, the nylon wool was saturated with BSS-FCS, capped, and incubated at 37°C for 60 min. The column was drained immediately before loading.

Cell concentration and viability were assessed in an aliquot of each sample of responder cells by eosin exclusion, and the remaining cells were decanted into a column and incubated for 30 min. The column was then washed with warmed (37°C) BSS-FCS, and the first 18 ml of effluent was collected and pelleted. The responder cells were pooled, and an aliquot was counted and appropriately diluted to concentrations of 1, 2, 3, 4, 5, and 6 × 10^6 cells/ml with mouse-tonicity Dulbecco's modified Eagle's medium and 10% FCS (DMEM-FCS).

Stimulator cells were suspended in 2 ml BSS-FCS and irradiated with 1500 rad by means of a Cobalt 60 γ-irradiator (Eldorado 6, Atomic Energy of Canada), an aliquot was counted, and the cells were diluted. Responder and stimulator cells were titrated as 100-μl aliquots into 96-well, round-bottom microtiter plates (Flow, Hamden, CT) with quadruplicate cultures for each cell concentration. Responder cells were titrated alone as a negative control, against irradiated syngeneic cells for autologous response, and against cells completely mismatched at all *H-2* loci as a positive control. A responder-to-stimulator cell ratio of 1:1 was used for all cultures. The plates were incubated for 3–6 days at 37°C with 10% CO_2 in a Kevatron (Vic) model 102 water-jacketed incubator. Six hours before harvesting, 10 μl of 100 μCi/ml tritiated thymidine (1 μCi) was added to each well. The cells were harvested with a Skatron AS cell harvester (Lierbyen, Norway). The glass filter paper (Enzo, New York) was allowed to dry, and the cells were counted for 1 min in a Prias PL Tricarb liquid-scintillation counter (Packard, Downers Grove, IL).

Skin grafting. Donor mice were killed by cervical dislocation, and the tail was removed and bathed in 70% ethanol. The skin was stripped and cut into 0.5-cm^2 patches on filter paper soaked with phosphate-buffered saline. Recipient mice were anesthetized by methoxyflurane (Abbott, North Chicago, IL) inhalation, and the left flank was prepared by shaving with small animal clippers and irrigated with 70% ethanol. An area of left flank skin was removed with curved dissection scissors, leaving the vascular bed of the subdermis. The graft was placed over the defect, covered with sterile petroleum jelly–impregnated gauze, and held in place with Micropore adhesive tape (3M). A protective layer of Transpore adhesive tape (3M) was then applied. Grafted mice were then observed at 30, 60, and 90 days. After 100 days, recipient mice were killed, and the graft was examined histologically.

Allozymes. Allozyme electrophoresis of kidney homogenates was performed according to the procedures described by Richardson et al. (4). The following enzymes and proteins were used as genetic markers: aldehyde dehydrogenase (EC 1.2.1.5), alkaline phosphatase (EC 3.1.3.1), carboxylesterase (EC 3.1.1.1), aspartate aminotransferase (EC 2.6.1.1), glucose-6-phosphate isomerase (EC 5.3.1.9), alanine aminotransferase (EC 2.6.1.2), glutathione reductase (EC 1.6.4.2), β-glucuronidase (EC 3.2.1.31), hemoglobin, isocitrate dehydrogenase (EC 1.1.1.42), malate dehydrogenase (EC 1.1.1.40), mannose-6-phosphate isomerase (EC 5.3.1.8), dipeptidase (EC 3.4.13.11), and phosphoglucomutase (EC 5.4.2.2).

Statistical analysis. For each experiment, the cell concentration that provided submaximal allogeneic response was selected for comparison. Because both 3- and 4-day cultures produced maximal stimulation at the same cell concentration (5 × 10^5 responder cells/well), the data from these two time points were pooled for statistical analysis.

A comparison of the syngeneic responses was made. The means of allogeneic and syngeneic responses at 4 × 10^5 cells/well were calculated. The mean syngeneic response was then expressed as a proportion of the mean allogeneic response obtained at the same cell concentration in the same experiment. It was assumed that each experiment was independent and each estimated value maintained the same distribution. Wilcoxon's rank-sum test was applied.

A comparison of the response to syngeneic cells and cross-strain cells was made. Each of the quadruplicate measurements of the syngeneic and cross-strain responses was expressed as a proportion of the mean allogeneic response obtained at the same cell concentration in the same experiment. Two-way analysis of variance was then used to test for column effect.

TABLE 1
Reciprocal skin grafts between NOD mouse strains

Donor	Recipient	Graft survival (days)
NOD/Wehi	NOD/Lt	60* (n = 1), 90† (n = 1), 94‡ (n = 1), 100 (n = 5)
NOD/Lt	NOD/Wehi	100 (n = 8)
NOD/Wehi	NOD/Wehi	30 (n = 1), 100 (n = 21)

*Became diabetic, suffered rectal prolapse, and died with graft intact.
†Became diabetic and died with graft intact.
‡Became diabetic, suffered facial abscess, and died with graft intact.

RESULTS

Reciprocal skin grafts were not rejected after 100 days (Table 1). Histological examination revealed no evidence of infiltration or scar formation. During the 100-day observation period, 3 of 8 NOD/Lt mice developed diabetes, whereas none of the 30 NOD/Wehi mice were affected. The allozyme profiles of the strains were also identical (Table 2).

Both NOD/Lt and NOD/Wehi were able to mount a brisk proliferative response to CBA/CaHWehi stimulator cells (Figs. 1 and 2). These responses were maximal with 5 × 10^5 responder cells/well at 3 or 4 days, 4 × 10^5 cells/well at 5 days, and 3 × 10^5 cells/well at 6 days, because high cell numbers cultured for extended periods resulted in medium depletion. Because the syngeneic response was measured as a proportion of the allogeneic response, cell concentrations resulting in submaximal allogeneic reactivity were selected for further analysis, i.e., 4 × 10^5 responder cells/well for 3 or 4 days, 3 × 10^5 cells/well at 5 days, and 2 × 10^5 cells/well at 6 days. There was no significant difference between the responses of responder cells from either strain to stimulator cells derived from either NOD/Wehi or NOD/Lt mice (Table 3). The NOD/Wehi responder cells exhibited a greater syngeneic proliferative response than either NOD/Lt ($P < .005$) or BALB/cAnBradleyWehi ($P < .005$) controls (Fig. 3). This difference was maintained at 5 and 6 days (data not shown). There was no significant difference between the syngeneic responses of NOD/Lt and BALB/cAnBradleyWehi mice.

DISCUSSION

Skin grafts, allozymes, MLCs, and the presence of insulitis in most mice of both sexes and both strains demonstrated their near-genetic identity. The acceptance of tail skin grafts between strains is highly significant because lines segregated within the same strain of NOD mice rejected reciprocal grafts by 40 days (E.H. Leiter, unpublished observations). The allozyme profiles are also identical, including the rare *b* allele at *Gpt-1*, which is found in only a few inbred strains, including the NOD colony bred at The Jackson Laboratory. MLCs have also failed to demonstrate reactivity between the strains. Therefore, it is unlikely that strain contamination resulted in differential susceptibility to diabetes, and the two strains seem virtually identical. However, we estimate that NOD/Wehi was segregated from the parental strain after 19 generations of brother-sister mating from the observation by Makino et al. (1) of a single diabetic female cataract-prone mouse. Thus, there is potential for genetic heterogeneity (5). Although we have examined many loci in these studies, we have not excluded genetic divergence.

The two NOD strains have been maintained in near-identical conditions for 24 mo. Their difference in incidence has been maintained in three different animal houses and is unaffected by housing the strains in the same room. Although the restricted passage of a pathogen responsible for susceptibility or resistance to disease may be occurring within one strain, it seems unlikely.

The autologous mixed-lymphocyte response (AMLR) in

TABLE 2
Allelic profiles of NOD mouse strains

Enzyme	Chromosome location	NOD/Wehi	NOD/Lt	BALB/c.Ig-I^{b}nu/Wehi	B6.Ig.I^e.nu/Wehi	CBA/CaH.bg	NZB/Wehi
Ahd-1	4	*a*	*a*	*b*	*a*	*b*	*a*
Akp-1	1	*b*	*b*	*b*	*a*	*b*	*b*
Es-1	8	*b*	*b*	*b*	*a*	*b*	*b*
Es-3	11	*c*	*c*	*a*	*a*	*c*	*c*
Got-2	8	*b*	*b*	*b*	*b*	*b*	*b*
Gpi-1	7	*b*	*b*	*b*	*b*	*b*	*b*
Gpt-1	15	*b**	*b**	*a*	*a*	*a*	*b**
Gr-1	8	*a*	*a*	*a*	*b*	*b*	*a*
Gus-s	5	*b*	*b*	*a*	*b*	*b*	*b*
Hbb	7	*s*	*s*	*d*	*s*	*d*	*d*
Idh-1	1	*a*	*a*	*a*	*a*	*b*	*a*
Itp	2	*b*	*b*	*a*	*b*	*b*	*b*
Mod-1	9	*b*	*b*	*a*	*b*	*b*	*b*
Mpi-1	9	*b*	*b*	*b*	*b*	*b*	*b*
Pep-3	1	*b*	*b*	*a*	*a*	*b*	*c*
Pgm-1	5	*a*	*a*	*a*	*a*	*b*	*b*

Standard nomenclature used for allelic profiles (16,17). Allelic profile for NOD mice identical with that found by The Jackson Laboratory in the Leiter colony of NOD mice (18). Profiles of 4 other inbred strains are included for comparison.
*Rare *Gpt-1*^{b} allele found in only a few inbred strains.

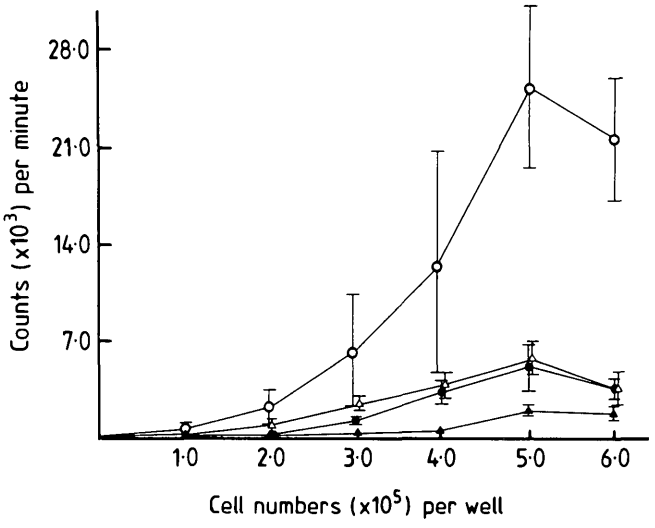

FIG. 1. Mixed-lymphocyte cultures in NOD/Wehi mice. NOD/Wehi responder cells were cultured for 3 days with CBA/CaHWehi (○), NOD/Lt (△), or NOD/Wehi (●) stimulator cells in 1:1 ratio. Proliferation was assayed by thymidine incorporation and results expressed as mean ± SD of quadruplicate cultures. ▲, Background.

humans is an in vitro T-lymphocyte response to class II antigens that demonstrates the characteristics of an immune reaction, i.e., memory and specificity (6). The AMLR produces immune effector cells, described as mediating help (7) or suppression (8) and acting as suppressor-inducers (9). T-lymphocytes generated in culture with autologous non–T-lymphocytes suppress both the proliferative and cytotoxic effects of fresh unstimulated T-lymphocytes to allogeneic cells in MLC (10) and inhibit pokeweed mitogen–stimulated immunoglobulin synthesis by autologous T-lymphocytes (11). The association between a defective AMLR and systemic lupus erythematosus (SLE) was first described by Sakane et al. (12) in 1978. Since then, the same defect has been found in patients with many other autoimmune diseases, including IDDM (13).

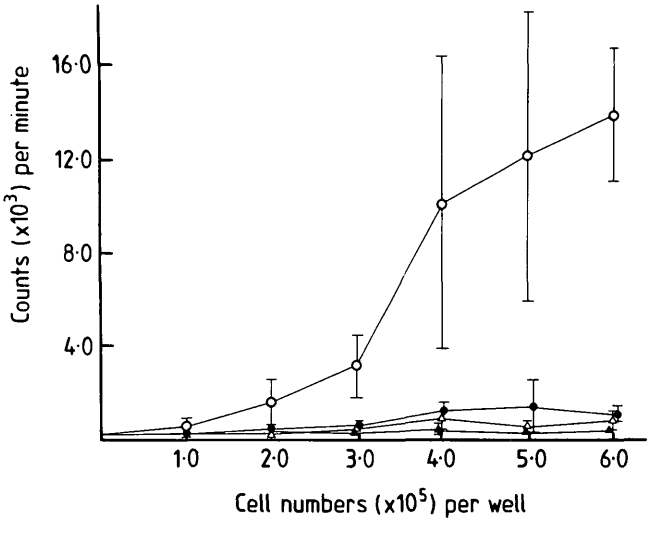

FIG. 2. Mixed-lymphocyte cultures in NOD/Lt mice. NOD/Lt responder cells were cultured for 3 days with CBA/CaHWehi (○), NOD/Lt (△), or NOD/Wehi (●) stimulator cells in a ratio of 1:1. Proliferation was assayed by thymidine incorporation, and results were expressed as mean ± SD of quadruplicate cultures. ▲, Background.

TABLE 3
Mixed-lymphocyte response in NOD mice in 6 experiments

Responder	Stimulator	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6
NOD/Wehi	NOD/Wehi	2064 ± 331 (13)	2774 ± 801 (23)	2199 ± 278 (17)	2691 ± 1258 (25)	2426 ± 950 (18)	2551 ± 564 (16)
	NOD/Lt	2406 ± 950 (15)	3239 ± 868 (27)	1220 ± 621 (9)	18 ± 480 (0)	1322 ± 724 (10)	5177 ± 929 (32)
	CBA/CaHWehi	16,222 ± 2376	12,140 ± 7894	13,001 ± 2353	10,885 ± 2228	13,207 ± 1563	16,213 ± 3777
NOD/Lt	NOD/Lt	465 ± 423 (5)	790 ± 191 (6)	809 ± 402 (2)	633 ± 1138 (3)	1013 ± 298 (9)	2319 ± 416 (7)
	NOD/Wehi	756 ± 366 (8)	1305 ± 548 (11)	1061 ± 435 (3)	1253 ± 1781 (6)		
	CBA/CaHWehi	9724 ± 6270	12,226 ± 1384	41,014 ± 6022	19,932 ± 895	11,311 ± 3037	35,160 ± 1539
BALB/cAnBradleyWehi	BALB/cAnBradleyWehi	411 ± 196 (4)	884 ± 438 (9)	1263 ± 403 (12)	382 ± 432 (3)		
	CBA/CaHWehi	9230 ± 2049	9791 ± 2285	10,566 ± 864	13,585 ± 1378		

Results of culturing each responder sample with syngeneic and allogeneic stimulator cells. For NOD/Lt and NOD/Wehi responder cells, reactivity to the other NOD strain is also shown. Responder and stimulator cells were each cultured at an initial concentration of 4×10^5 cells/well for 3 or 4 days, and proliferation was assayed by thymidine incorporation. The δ-counts/min ± SD of quadruplicate cultures is given. Numbers in parentheses are responses as a percentage of the allogeneic response.

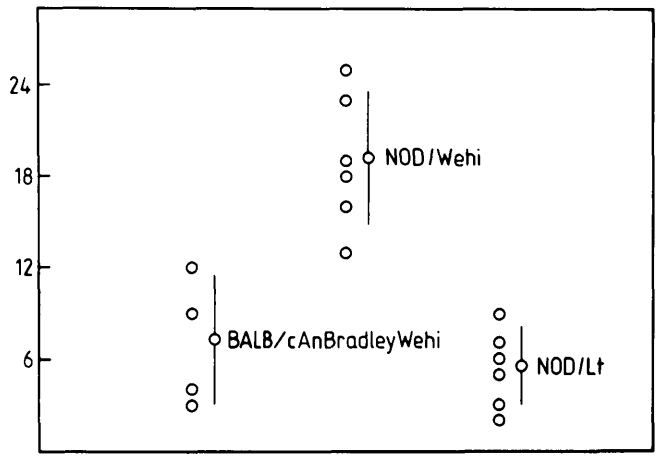

FIG. 3. Syngeneic responses of NOD/Lt, NOD/Wehi, and BALB/cAnBradleyWehi mice are shown as percentage of allogeneic response. Responder cells (4×10^5) were cultured with equal number of either allogeneic or syngeneic stimulator cells for 3 or 4 days. Proliferation was assayed by thymidine incorporation, and mean of quadruplicate cultures was calculated. For each responder sample, mean incorporation of syngeneic cultures was expressed as percentage of mean incorporation of allogeneic cultures. *Error bars* indicate mean ± SD of each group.

Smith and Pasternak (14) demonstrated a deficiency in the murine homologue of the AMLR (syngeneic mixed-lymphocyte response [SMLR]) of New Zealand Black (NZB) mice, a model of SLE, and Hom and Talal (15) found that autoimmune disease–prone NZB/W and MRL/Mp (*lpr/lpr*) mice also exhibit a poor SMLR. This defect precedes the onset of clinical disease and further decreases with the progression of disease. SMLR in the NOD/Lt mouse was examined by Serreze and Leiter (3), who found it to be severely depressed compared with that in SWR mice. They also found that NOD/Lt T-lymphocytes harvested from an SMLR were functionally defective when tested for ability to induce suppression of an allogeneic MLR. A low response was also observed when NOD stimulator cells were cultured with T-lymphocytes from F_1 mice from an SWR × NOD cross. This was interpreted as a defective stimulator function in the NOD mouse, which is not supported by the data presented herein because NOD/Lt cells are capable of stimulating the rapid proliferation of NOD/Wehi responder cells. Indeed, the finding that NOD/Wehi responder cells are also incapable of stimulating NOD/Lt responder cells suggests a defect in the NOD/Lt response rather than stimulation.

The high- and low-incidence strains of NOD mice may be useful models of human identical twins discordant for IDDM. Our NOD lines are virtually genetically identical and housed in very similar conditions. They are both susceptible to diabetes, as evidenced by the spontaneous occurrence of disease in a few NOD/Wehi mice and the precipitation of diabetes in most of the mice with cyclophosphamide (2), although one strain is able to resist spontaneous disease. Thus, these mouse strains may offer the opportunity to investigate and modulate both genetic and environmental factors to identify the mechanism of disease resistance. One possible mechanism may be through the heightened generation of suppressor function in a process reflected by SMLR. This hypothesis is consistent with the finding of raised SMLR in the NOD/Wehi mouse and the abrogation of resistance to disease by cyclophosphamide.

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