The NOD Mouse: A Model For Insulin-Dependent Diabetes Mellitus

Nonobese diabetic (NOD) designates an inbred, genetically well characterized mouse strain. Like the DP-BB/Wor rat (*UNIT 15.3*), NOD mice spontaneously develop autoimmune T cell–mediated insulin-dependent diabetes mellitus (IDDM). However, the two models are sharply contrasted in that NOD mice, unlike BB rats, do not exhibit T lymphocytopenia, but rather the inverse (Serreze and Leiter, 1995). NOD mice are very easy to breed and are available from suppliers in the United States, Europe, and Japan. In addition to the extensive genetic characterization of the NOD genome, the availability of monoclonal antibodies to murine leukocyte antigens as well as the availability of a variety of congenic and transgenic stocks of NOD mice makes these mice especially useful for the immunologic dissection of autoimmune IDDM. Diabetogenesis in NOD mice is the consequence of heritable immunodeficiencies under complex polygenic control. The penetrance of these susceptibility-contributing polygenes is strongly influenced by the physical environment, particularly the diet and exposure to microbial pathogens. One of the intriguing features of NOD mice is that stimulation of the NOD immune system by environmental pathogens actually leads to development of a more normal immune system and IDDM resistance. Thus, NOD mice must be raised under stringent specific-pathogen-free (SPF) conditions for expression of the diabetes phenotype. This unit will define a protocol required for maintaining NOD mice under conditions permissive to full expression of their autoimmune potential (see Basic Protocol 1). Methods are also described for diagnosing IDDM on the basis of glycosuria and glycosemia (see Basic Protocol 2) as well as for the semiquantitation of insulitis, a valuable subphenotype diagnostic of prediabetes in these mice (see Alternate Protocol), including a procedure for aldehyde fuchsin staining to identify β granules in β islet cells for diagnostic purposes. An adoptive-transfer method is also included (see Basic Protocol 3) in which leukocytes, purified T cells, or T cell infiltrates obtained from the insulitic pancreas tissue of NOD mice are injected into prediabetic NOD or diabetes-resistant F1 mice, which then develop disease in an accelerated fashion. Basic Protocol 3 also includes alternative steps in which bone-marrow cells from NOD mice are transferred to syngeneic, irradiated NOD mice, allowing for reconsistution with a diabetogenic immune system. This method can also be used to reconstitute NOD/LtSz-*scid/scid* mice with NOD bone-marrow cells. Steps for isolating pancreatic islet cells, which can then be used for a variety of purposes (e.g., as a source of islet antigens to establish and maintain autoreactive T cell lines) are included (see Support Protocol). Finally, steps are outlined that can be used to introduce transgenes into NOD mice (see Basic Protocol 4). Basic Protocol 4 also discusses important considerations for introduction of targeted mutations produced in embryonic stem cells derived from other inbred strains, or introduction of other genes from non-diabetes-prone strains.

MAINTENANCE OF SPECIFIC-PATHOGEN-FREE (SPF) NOD MICE

BASIC PROTOCOL 1

Prior to receiving NOD mice from an specific-pathogen-free (SPF) colony, each investigator should check with institutional veterinary staff as to available resources for maintaining the SPF status of the imported mice. This requires provision of space, autoclavable diet, autoclavable bedding, acidified or chlorinated drinking water, and health monitoring. See *UNIT 1.2* for additional information on managing immunocompromised animals. In North America, there are two major distributors supplying NOD mice (see *APPENDIX 5* for contact information). The Jackson Laboratory provides NOD/Lt mice and related strains, including NOD/LtSz-*scid*/*scid*, NOD/Lt-RIP-Tag and NOD/LtSz-*scid*/*scid*-RIP-Tag transgenic mice. In addition, The Jackson Laboratory distributes related control strains

Table 15.9.1 Potential Diabetes-Free Control Strains for NOD Mice

Strain	Advantages	Disadvantages	Best uses	References
NOR/Lt	NOD-derived recombinant congenic stock; same MHC, differs at relatively few non-MHC *Idd* loci	Exhibits some but not all of the immune dysfunctions of NOD	Good source of islets; MHC ($H2^{g7}$)-non-MHC interactions different than in NOD	Prochazka et al., 1992a; Serreze et al., 1994a
NON/Lt	Closely related to NOD; diabetes-resistant MHC	Develops obesity, impaired glucose tolerance, and immunodeficiencies; difficult to breed	Genetic analysis of *Idd* genes; potential model for type II diabetes	Leiter et al., 1986
ICR (available as CD-1)	Progenitor stock for NOD and related strains	Randomly bred	Analysis of population frequency of rare genetic polymorphisms present in NOD	Ikegami et al., 1990
SWR/J	Swiss-derived like ICR and NOD, but inbred and without immunodeficiencies; readily available (The Jackson Laboratory)	Genetically very different from NOD, including MHC	Control for certain immune functions that are aberrant in NOD	Serreze and Leiter, 1988
NOD-Ea^d transgenics	Diabetes-free control stock for comparison to NOD mice; expresses I-E molecules on antigen-presenting cells; available from The Jackson Laboratory	Exhibits some but not all immune dysfunctions of NOD	Analysis of T cell repertoire development and presentation of β cell antigens	Uehira et al., 1989; Lund et al., 1990; Hanson et al., 1996
NOD.NON-$H2^{nb1}$	Diabetes-resistant MHC from NON/Lt; available from The Jackson Laboratory	Exhibits some but not all immune dysfunctions of NOD	Useful in dissecting the role of MHC versus non-MHC genes in producing aberrant immunophenotypes	Leiter and Serreze, 1991; Serreze and Leiter, 1991
NOD.SWR-$H2^{q}$	Diabetes-resistant MHC from SWRIJ; available from The Jackson Laboratory	Exhibits some but not all immune dysfunctions of NOD	Useful in dissecting the role of MHC versus non-MHC genes in producing aberrant immunophenotypes	Serreze et al., 1996
NOD.B10-$H2^{b}$	Diabetes-resistant MHC from C57BL/10J; available from The Jackson Laboratory	Exhibits some but not all immune dysfunctions of NOD	Useful in dissecting the role of MHC versus non-MHC genes in producing aberrant immunophenotypes	Wicker et al., 1992; Todd et al., 1991
NON.NOD-$H2^{g7}$	Diabetogenic MHC from NOD/Lt; available from The Jackson Laboratory	Exhibits some but not all immune dysfunctions of NOD	Useful in dissecting the role of MHC versus non-MHC genes in producing aberrant immunophenotypes	Leiter and Serreze, 1991; Serreze and Leiter, 1991
NOD-*scid*	No endogenous T or B lymphocyte functions; available from The Jackson Laboratory	Develops high incidence of thymoma with age	Delineation of the role of T cell subsets; source for insulitis-free islets	Prochazka, 1992b; Christianson et al., 1993; Shultz et al., 1995
NOD.$B2m^{null}$	MHC class I–negative, CD8-deficient diabetes resistant-stock congenic for targeted mutation of the β2-microglobulin gene	CD4 T cells are not tolerant to MHC class I–positive syngeneic cells	Excellent source for insulitis-free, MHC class I–bare NOD islets for transplantation studies	Serreze et al., 1994b, 1997; Wicker et al., 1994

listed in Table 15.9.1 (including NON/Lt, NOR/Lt, and NOD.NON-*Thy1*^{a}). Choice of an appropriate control strain will depend upon the nature of the experiments. Pedigreed breeding pairs are also available upon request from The Jackson Laboratory. NOD/MrkBrTac mice are available from Taconic Farms. In Japan, NOD/Shi mice and breeders are available from CLEA. In Europe, NOD mice are distributed by Bomholtgård in Denmark.

Materials

NOD and control mice (see Table 15.9.1 for control strains)
Appropriate SPF vivarium (see Critical Parameters; also see *UNIT 1.2*)
Autoclaved white pine shavings or cellulose paper bedding (Alpha-Dri, Shepard Specialty Papers)
Autoclaved mouse food (Purina 5001, Purina NIH-31, or equivalent)
Acidified water (pH 2.8 to 3.2, adjusted using HCl) or hyperchlorinated water containing 10 ppm sodium hypochlorite

Additional reagents and equipment for diagnosis of IDDM (see Basic Protocol 2 and Alternate Protocol; also see *UNIT 15.3*)

1. Verify in advance the specific-pathogen-free (SPF) status of the mice received from an outside distributor.

   The presence of known murine pathogens (see Critical Parameters for discussion of screening) would require that the mice be held in microisolator cages in a quarantine facility and rederived into the institution's SPF facility either by Caesarean derivation or by embryo transfer. This level of caution is required not only to insure a high frequency of insulin-dependent diabetes mellitus (IDDM) in the NOD mice being imported, but also to protect other SPF mouse colonies on site from the introduction of new pathogens.

   Additional special requirements should be followed for maintenance of NOD/LtSz-scid/scid mice (see Critical Parameters).

2. After imported NOD mice have been passed into the SPF facility and cleared by the veterinary staff for introduction into the vivarium space assigned to the investigator, implement animal health status monitoring for pathogens. Maintain mice on autoclaved chow and acidified or hyperchlorinated water ad libitum, and keep in a cage with autoclaved shavings.

   All experimental agents derived from murine sources that may potentially harbor pathogens, such as monoclonal antibodies derived from ascites fluid that may be used for studies in vivo, should be prescreened for the presence of murine pathogenic agents (murine antibody production or MAP test), or, in the case of animal protein preparations, sterilized by γ irradiation or other suitable means.

   A partial list of pathogens specifically demonstrated to decrease IDDM incidence in NOD colonies includes murine hepatitis virus (MHV), lymphocytic choriomeningitis virus (LCMV), lactate dehydrogenase virus (LDHV), and encephalomyocarditis virus (EMCV). Colonies should be kept free of Sendai virus and Mycoplasma pulmonis (see Critical Parameters).

3. Establish a diabetes-incidence curve. Begin to monitor mice for signs of IDDM (glycosuria, glycosemia, and insulitis) at 8 to 10 weeks of age (see Basic Protocol 2 and Alternate Protocol).

   Figure 15.9.1 shows the frequency of IDDM in virgin mice of both sexes produced in a SPF colony of NOD/Lt mice maintained by the author at The Jackson Laboratory. The mice were reared in a research colony (non-barrier protected). The male mice serve as useful "barometers" of the presence of extrinsic environmental influences that suppress autoimmune IDDM. As indicated by Figure 15.9.1, an incidence in excess of ~50% should be achieved by the time the males have aged to 30 weeks. If the incidence in males is <20%,

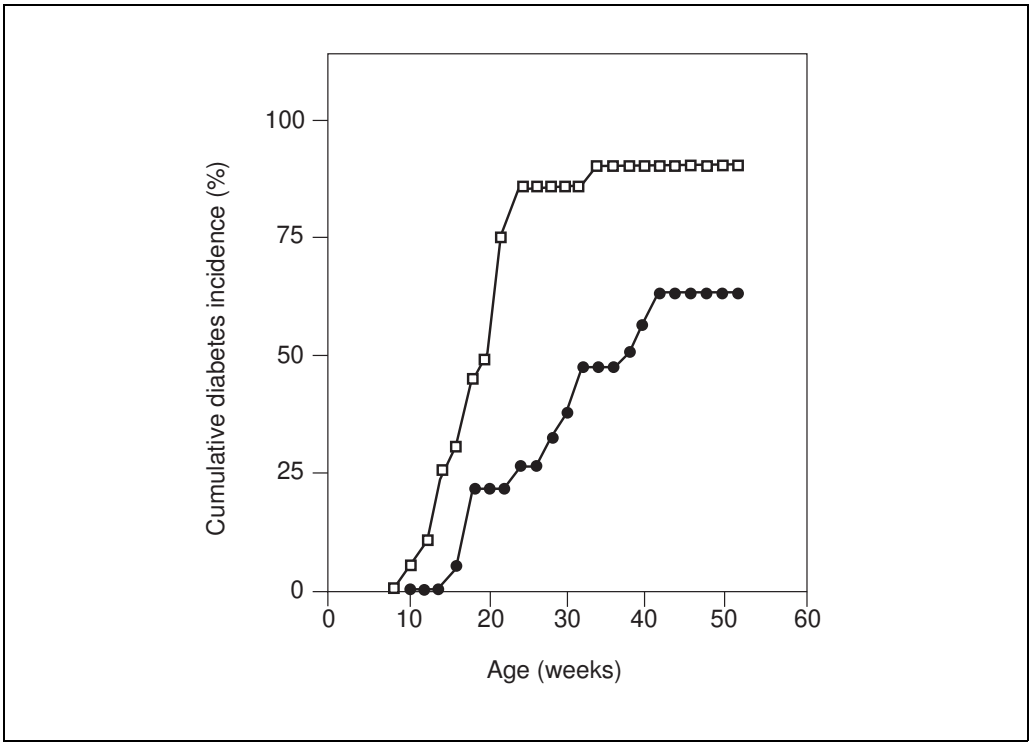

Figure 15.9.1 Diabetes incidence curve for NOD mice. Circles represent males ($N = 20$); squares represent females ($N = 16$), where N is the number per group in the incidence study.

and if the female incidence is <60% by 30 weeks, the presence of a pathogen or other immunomodulatory agent in the colony is indicated.

BASIC PROTOCOL 2

DIAGNOSIS OF INSULIN-DEPENDENT DIABETES MELLITUS (IDDM)

Monitoring of glycemic status should begin when NOD mice reach 10 weeks of age. Generally, this is done at weekly intervals by using Diastix (Bayer Diagnostics; *APPENDIX 5*) or similar reagent strips to measure urine glucose. Picking a mouse up leads to immediate urination, allowing a drop to be collected on the test area (tip) of the reagent strip. High levels of glucose in the urine (glycosuria) appear when plasma glucose is ≥300 mg/dl. A nonfasting plasma glucose of ≥300 mg/dl for 2 consecutive weeks indicates IDDM. The nonfasting plasma glucose levels of young, prediabetic NOD mice ranges between 130 and 180 mg/dl. Plasma glucose can be measured directly in small samples of venous blood using glucose oxidase methods (either commercially available glucose analyzers or small portable analyzers and glucose oxidase–coated test strips; see *UNIT 15.3* for details). Onset of IDDM can also be accelerated in young prediabetic NOD mice by intraperitoneal administration (*UNIT 1.6*) of cyclophosphamide (Sigma; 200 to 300 mg per kg body weight). It is quite difficult to maintain hyperglycemic NOD mice by insulin treatment; mice are usually euthanized after the diagnosis is certain. Transition from mild to severe hyperglycemia occurs over a period of 3 to 4 weeks. During this time, the mouse will survive without insulin therapy. If insulin therapy is part of the investigator's experimental protocol, doses of 1 to 2 U of a 1:1 mixture of regular and slower-acting porcine insulin (both available from Novo Nordisk; *APPENDIX 5*) are injected intraperitoneally as required by empirical determination of hyperglycemic status immediately before injection in the morning and evening (also see *UNIT 15.3*).

SEMIQUANTITATION OF INSULITIS AS A SUBCLINICAL PHENOTYPE OF PROGRESSION TO INSULIN-DEPENDENT DIABETES MELLITUS

ALTERNATE PROTOCOL

Leukocytic infiltration of the pancreatic islets (insulitis) is the major histopathologic feature of IDDM development. This complex phenotype is often used as a measure of the extent of autoimmune pathogenesis by the calculation of an insulitis index. The pancreas is fixed in Bouin's fixative, paraffin-embedded, and sectioned by standard histological methods. Sections mounted on microscope slides are dehydrated and stained with hematoxylin and eosin to permit microscopic visualization of islets. The granulated β cell component of the islets (~70% to 80% of total islet cells) can be inexpensively visualized by use of the aldehyde fuchsin stain as described below. It is useful to have the β cell mass delineated, since the insulitic process selectively eliminates this cell type. The consequence of this is that, in older NOD mice, some islets visualized as "insulitis-free" may in fact represent post-insulitic "pseudoislets" containing only non–β endocrine cells.

Materials

NOD mice (see Basic Protocol 1)
Modified Bouin's fixative (see recipe)
50%, 70%, 80%, 95%, and 100% ethanol
1:1 (v/v) 100% xylene/100% ethanol
Xylene
Aldehyde fuchsin stain (see recipe)
Mayer's hematoxylin (e.g., Sigma)
1% (w/v) eosin Y in 80% ethanol
Mounting medium (e.g., Permount, HSR, or Coverbond)

Dissecting instruments
Histology or pathology laboratory equipped for paraffin embedding and sectioning
Glass microscope slides
Staining dishes
Coverslips

Additional reagents and equipment for euthanasia of mice by carbon dioxide asphyxiation (*UNIT 1.8*)

Remove, fix, and mount pancreas

1. Euthanize mouse by CO_2 asphyxiation (*UNIT 1.8*).
2. Open the abdomen. Grasp the spleen with forceps and withdraw the spleen and attached pancreas from the peritoneal cavity. Use scissors to free the pancreas from the duodenum. Remove the pancreas and attached spleen to a flat surface and use fine scissors to separate the pancreas from the spleen.
3. Place pancreas in a histology cassette and fix by immersing for 24 hr in modified Bouin's fixative.
4. Rinse pancreas for 8 to 24 hr in a gentle stream of running tap water.

   Complete removal of the acetic/picric acid from the Bouin's fixative is essential since the presence of acid in the subsequent ethanolic solutions will extract insulin from the tissues and result in a weaker stain.

5. Dehydrate pancreas by passing through the following series:

50% ethanol	quickly
95% ethanol	quickly
100% ethanol	2 min
100% ethanol (fresh)	3 min

100% ethanol (fresh)	5 min
1:1 ethanol/xylene	1 to 1.5 min
Xylene, several changes	5 min each.

The tissue should be dehydrated and embedded on the same day if possible. Leave in 70% ethanol for as short a time as possible, as long periods of storage in 70% ethanol may extract some insulin from the β granules and result in a weaker stain. The exocrine portion of the mouse pancreas may section with some difficulty if stored in Bouin's fixative for extended periods.

6. Embed tissue in paraffin, prepare 5-µm sections, and mount on glass microscope slides.

   This step is usually performed in a dedicated histology facility. Zeller (1989) describes the details of paraffin embedding and mounting of fixed tissues.

7. Rehydrate paraffin sections on slides by passing through the following series:

Xylene	5 min (twice)
1:1 ethanol/xylene	1 min
100% ethanol	5 min
100% ethanol	3 min
100% ethanol	2 min
95% ethanol	quickly
70% ethanol	quickly
Distilled H_2O	rinse.

8. Place slides in fresh 70% ethanol again for 2 min.

Stain β granules with aldehyde fuchsin

9. Stain slides by immersing 10 to 13 min in filtered aldehyde fuchsin stain.

10. Wash stained slides in 2 to 3 changes of 95% ethanol as follows:

Wash 1	30 sec to 1 min
Wash 2	2 to 3 min
Wash 3	3 to 5 min (optional; if there are many slides).

11. Wash slides briefly in 70% ethanol, then briefly in distilled water.

Stain sections with hematoxylin/eosin and dehydrate

12. Immerse slides in Mayer's hematoxylin for 15 sec (or longer as determined empirically with multiple sections, depending on strength of hematoxylin).

13. Wash slides in gently running tap water until they turn a navy-bluish color, then rinse in distilled water.

14. Dehydrate and stain slides with eosin by passing through the following series:

50% ethanol	quickly
1% eosin Y in 80% ethanol	usually 15 to 30 sec, empirical
95% ethanol	quickly
100% ethanol	2 min
100% ethanol	3 min
100% ethanol	5 min
1:1 ethanol/xylene	1 to 1.5 min
Xylene, several changes	5 min each.

Examine slides microscopically, score for insulitis infiltration, and calculate insulitis index

15. Mount coverslips on sections with mounting medium (e.g., Permount, HSR, or Coverbond). Examine microscopically.

    A mouse islet with a normal complement of darkly staining (navy-blue color) β cells is shown in Figure 15.9.2A.

    β cells will stain a deep purple color if well granulated or a weaker blue if partially degranulated. Any mast cells in the pancreas will also be stained purple, as will the elastin lining of capillaries and arteries.

    The advantage of using the aldehyde fuchsin histochemical stain for insulin is that immunocytochemistry (UNIT 21.4) using standard immunocytochemical protocols (to detect other islet cell peptide hormones or any antigen stable after Bouin's fixation/paraffin embedding) can be applied on the same slide. This is done at the distilled water step of the rehydration process (step 7) before staining with aldehyde fuchsin.

16. Score all pancreatic islets observed in at least three nonoverlapping sections per pancreas (include at least 30 to 40 islets) for the extent of insulitic infiltration as follows:

No infiltration	grade 0
Perivascular/periductular infiltrates with leukocytes touching islet perimeters, but not penetrating	grade 1
Leukocytic penetration of up to 25% of islet mass	grade 2
Leukocytic penetration of up to 75% of islet mass	grade 3
End-stage insulitis, <20% of islet mass remaining	grade 4

    Several grades of insulitis in the NOD pancreas using the aldehyde fuchsin stain to identify granulated β cell mass are illustrated in Figure 15.9.2.

17. Calculate insulitis index (I) according to the following formula:

$$I = \frac{(0 \times n_0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4)}{4 \times (n_0 + n_1 + n_2 + n_3 + n_4)}$$

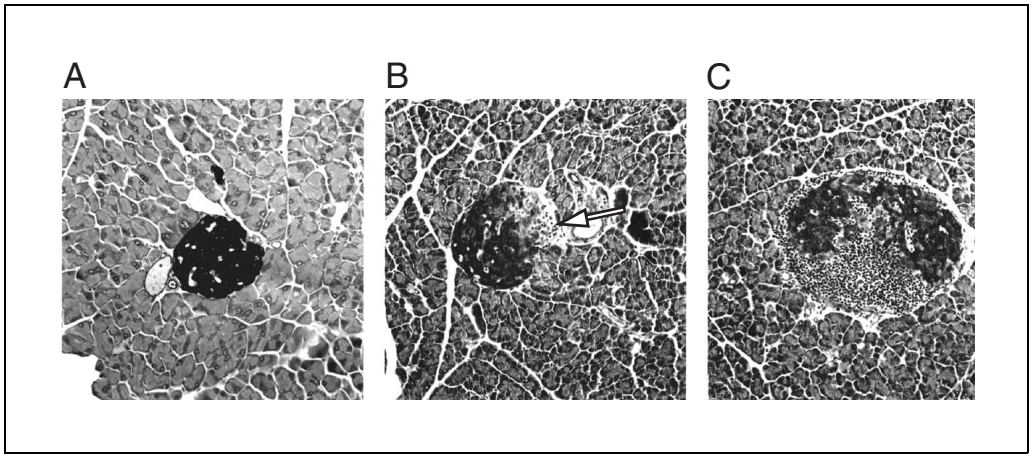

Figure 15.9.2 Photomicrographs (156×) of aldehyde fuchsin–stained pancreatic sections in NOD mice. (**A**) Insulitis-free islet in pancreas of NOD-*scid/scid* mouse stained with aldehyde fuchsin to identify granulated β cells (dark color). (**B**) Islet in pancreas of 8-week-old NOD/Lt-+/+ female showing stage 2 insulitis at one pole of islet (arrow). It is scored as "2" because leukocytes are clearly penetrating into the islet. If there were no leukocytic penetration, the score would be "1" ("perinsulitis"). (**C**) Islet in same pancreas as (B), but showing stage 3 insulitis. The original boundaries of this large islet are now filled in with infiltrating leukocytes, producing erosion of the β cell (dark staining) mass. An "end-stage" islet (4.0 score) would contain even fewer, if any, granulated β cells.

where n_0, n_1, n_2, n_3, and n_4 are the number of islets scored in grades 0, 1, 2, 3, and 4, respectively.

For example, if 40 islets all are scored as 0 (no lesions), the index would be (40 × 0)/(40 × 4) = 0. If 10 islets scored 0, 10 islets scored 1, 20 islets scored 2, 15 islets scored 3, and 6 islets scored 4, the index would be: [(10 × 0) + (10 × 1) + (20 × 2) + (15 × 3) + (6 × 4)]/(61 × 4) = 119/244 = 0.49.

CAUTION: *An insulitis index of 1.0 means that all islets have essentially been destroyed. Insulitis can be quite extensive in the pancreases of NOD mice necropsied prior to onset of IDDM. However, it does not necessarily follow that histologic documentation of a high insulitis index (>0.5) necessarily equates to an incipient onset of IDDM that would have occurred had the mouse not been necropsied. In many colonies, high insulitis index in NOD males is accompanied by a <30% incidence of clinical disease. An Australian substrain, NOD/Wehi, exhibits high insulitis indices in mice of both sexes, but low diabetes incidences. Pancreatic insulin content must decline to <10% of normal levels for hyperglycemia to be manifest.*

Extent of pancreatic β cell loss should be estimated physiologically by performing a glucose-tolerance test on mice that have been deprived of food overnight (i.e., for 16 hr). Determine fasting plasma or blood glucose from venous blood (obtained from the retroorbital sinus of the eye or from the tail vein; UNIT 1.7) prior to injecting the bolus of glucose. Inject 2 g glucose/kg body weight intraperitoneally (UNIT 1.6) from a 20% (w/v) solution. Sample venous blood 60 min post-injection. Plasma or blood glucose levels should be <300 mg/dl if sufficient pancreatic β cell mass is present. If values do not decline below this threshold, the mouse is at high risk for development of hyperglycemia. Splenic leukocytes from NOD/Lt mice that do not pass the glucose-tolerance test efficiently transfer IDDM into NOD/LtSz-scid/scid mice (see Basic Protocol 3).

BASIC PROTOCOL 3

ADOPTIVE TRANSFER OF IDDM INTO NOD AND NOD/LtSz-*scid/scid* MICE

Adoptive transfer of IDDM can be achieved by intravenous or intraperitoneal injection of populations of leukocytes or purified subsets of T cells obtained from lymphoid organs from donor NOD mice into young, prediabetic recipients ("accelerated transfer" model). Leukocytes or purified T cells from isolated pancreatic islet cells (see Support Protocol) may also be used. Cells may be transferred directly into neonates; adolescent and older recipients require whole-body irradiation. Alternatively, bone-marrow cells from NOD mice can be injected into lethally irradiated IDDM-resistant F1 mice, allowing for reconstitution with a diabetogenic immune system. Unirradiated NOD/LtSz-*scid/scid* mice can also be used as recipients of donor leukocytes or purified T cells from diabetic or prediabetic NOD donors.

NOTE: All solutions and equipment coming into contact with cells should be sterile, and proper sterile technique should be used accordingly. Injections should be performed in a laminar flow hood in the animal room, with as much adherence to sterile technique as possible.

Materials

Donor strain: diabetic or prediabetic NOD or NOD/LtSz-*scid*/*scid* mice (see Basic Protocol 1)

Recipient strain: NOD, NOD/LtSz-*scid*/*scid*, or IDDM-resistant F1 mice (choice of strain is at the discretion of the investigator)

Phosphate-buffered saline (PBS; APPENDIX 2)

^{137}Cs γ irradiator (e.g., Gammacell 1000, Nordion)

Additional reagents and equipment for preparing spleen or lymph node cells (UNIT 3.1), T cell enrichment (UNIT 3.2 or 3.3), T cell fractionation (UNIT 3.5A), intravenous injection (UNIT 1.6), preparation of bone-marrow cells (UNIT 6.4), and diagnosis of IDDM (see Basic Protocol 2 and Alternate Protocol)

Irradiate recipient mice

1a. *For prediabetic, postpubertal NOD recipients ("accelerated transfer"):* Irradiate recipient mice with 600 to 750 rad on the day of adoptive transfer.

Neonatal recipients do not need to be irradiated.

1b. *For IDDM-resistant F1 hybrid recipients:* Irradiate with 1200 rad on the day of adoptive transfer.

This lethal dose may be subdivided into two 600-rad doses administered in the morning and afternoon of the same day.

F1 hybrids are quite radiation resistant; thus a higher dose of γ irradiation must be used to insure complete destruction of the hematopoietic system when donor marrow from various congenic stocks are tested for diabetogenic potency.

1c. *For NOD/LtSz-scid/scid recipients:* Do not irradiate. Proceed directly to adoptive transfer.

Prepare donor cells and perform adoptive transfer

2a. *For transfer of leukocytes from overtly diabetic NOD mice:* Prepare spleen-cell (*UNIT 3.1*) or purified and/or fractionated splenic/lymphoid T cell suspensions (*UNITS 3.2, 3.3 & 3.5A*) or T cells from pancreatic islet cells (see Support Protocol) from donor mice and adjust to 2×10^8 cells/ml in PBS. Inject each recipient intravenously (*UNIT 1.6*) with 0.1 ml of cell suspension (2×10^7 T cells or splenic leukocytes).

NOD mice respond to male sex-limited (H-Y) antigen; therefore cells from female donors should not be transferred into male recipients.

If purified T cells are to be transferred, spleens or insulitic pancreas tissue (see Support Protocol) should be used as a source of cells. Inject 5×10^6 to 1×10^7 cells. It should be noted that purified $CD4^+$ splenic T cells will transfer IDDM in the absence of $CD8^+$ cells only when using cells from overtly diabetic donors. Donor cells prepared from other peripheral lymphoid tissue are not an optimal choice for transferring IDDM (Christianson et al., 1993; Leiter and Atkinson, 1998).

2b. *For transfer of leukocytes from prediabetic mice:* Prepare spleen-cell (*UNIT 3.1*) or purified and/or fractionated T cell suspensions (*UNITS 3.2, 3.3 & 3.5A*) from donor mice and adjust to 2×10^8 cells/ml in PBS. Inject each recipient intravenously (*UNIT 1.6*) with 0.1 ml of cell suspension (5×10^6 to 1×10^7 purified T cells or 2×10^7 splenic leukocytes).

NOD mice respond to male sex-limited (H-Y) antigen, therefore, cells from female donors should not be transferred into male recipients.

If T cells are to be transferred using prediabetic donors, both $CD4^+$ and $CD8^+$ subsets are necessary to transfer IDDM.

2c. *For transfer of bone marrow cells into NOD mice:* Prepare bone-marrow cell suspension using femurs of donor mice (*UNIT 6.4*) and adjust to 5×10^7 cells/ml in PBS. Inject each recipient intravenously (*UNIT 1.6*) with 0.1 ml of cell suspension (5×10^6 bone-marrow cells).

If desired, bone-marrow cells can be depleted of T cells (UNIT 3.4) prior to injection, so as to eliminate mature T cells in the inoculum.

NOD/LtSz-scid/scid recipients of adoptively transferred leukocytes should be used at 5 to 6 weeks of age since the stock develops a high incidence of thymic lymphoma with age. By 25 weeks of age, histologically identifiable thymic lymphomas have been observed in 76.2% of females and 38% of males. A NOD/LtSz-scid-Emv30^{null} stock lacking an endogenous NOD-specific ecotropic provirus previously associated with lymphomagenesis has recently

been produced at The Jackson Laboratory (Serreze et al., 1995). This stock exhibits a retarded rate of histologically identifiable thymic lymphomas (20.8% in females and 35% in males at 25 weeks) and hence is recommended for studies entailing long-term engraftment with marrow or other tissues.

3. Observe mice for signs of diabetes (see Basic Protocol 2 and Alternate Protocol).

   If T cells from diabetic donors or diabetogenic T cell clones are used, monitoring should be done weekly; if prediabetic T cells are used, monitoring can be initiated 2 to 4 weeks after transfer.

   Depending upon the donor/recipient combination used, extensive insulitis will be manifest within 2 weeks of transfer; overt hyperglycemia/glycosuria will be detectable within 3 to 5 weeks in 80% to 100% of recipients.

SUPPORT PROTOCOL

ISOLATION OF PANCREATIC ISLET CELLS

Studies involving autoimmune IDDM in NOD mice often require the availability of isolated syngeneic islets to be used: (1) as a source of leukocytes from insulitic pancreas tissue for use in adoptive-transfer studies (see Basic Protocol 3); (2) in tissue transplantation as a therapy for diabetes; (3) for isolating T cells used for establishing lines of β cell–autoreactive T cells; or, (4) to serve as a source of islet antigens to maintain T cell lines and clones in vitro. The NOD/LtSz-*scid*/*scid* mouse serves as an excellent islet donor in transplantation studies, since the islets can be isolated from donors of any age and free of insulitic infiltrates (Christianson et al., 1993). If NOD islets must be obtained, the best yields are obtained from young, prediabetic males aged 5 to 7 weeks. NOD/Lt-RIP-Tag (Hamaguchi et al., 1991) and NOD/LtSz-*scid*/*scid*-RIP-Tag transgenic mice (Serreze et al., 1997) develop large pancreatic adenomas by 14 weeks of age and hence are especially useful as a source for islet material when large quantities are required. It should be noted that an alternative method for isolating islet cells has recently been published (Liu and Shapiro, 1995). The modified method is more rapid since it employs density-gradient separation to purify islets instead of manual washes and transfers by micropipet. Higher islet yields per pancreas have also been reported using this modified approach.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique must be used accordingly.

Materials

NOD mice or controls (see Basic Protocol 1)
70% ethanol
DNase/collagenase P working solution (see recipe), freshly prepared
Supplemented HBSS (see recipe)

Dissecting microscope with reflected lighting
Artery clamp
30-G, 0.5-in. hypodermic needle
10-ml syringe
60-mm glass petri dish, sterile
Silanized glass micropipet: hand-pulled from silanized soft-glass tubing (PROSIL28 from PCR) according to manufacturer's instructions
Tabletop centrifuge
Amber rubber tubing connected to gentle vacuum, preferably applied by turning a micrometer-operated suction device

Additional reagents and equipment for euthanasia of mice (*UNIT 1.8*), T cell enrichment (*UNIT 3.2* or *UNIT 3.3*), and T cell fractionation (*UNIT 3.5A*)

1. Euthanize the mouse by asphyxiation with CO_2 (*UNIT 1.8*) and swab the abdomen with 70% ethanol. Make a ventral incision, reflect the skin, and open the peritoneal cavity.

   If intact islets from NOD mice are desired, islets should be harvested from young prediabetic donors not older than 7 weeks, since insulitic destruction of islets becomes progressively more severe beyond that time. This problem can be eliminated by use of NOD/LtSz-scid/scid mice or MHC-matched NOR/Lt mice (Prochazka et al., 1992a,b) as islet donors.

2. Place the mouse beneath a good-quality dissecting microscope with reflected lighting and affix a small artery clamp to occlude the common bile duct at its junction with the duodenum.

   Figure 15.9.3 illustrates this and subsequent steps of the dissection.

3. Cannulate artery with a 30-G, 0.5-in. hypodermic needle attached with flexible tubing to a 10-ml syringe. Manually infuse 3 ml of room temperature DNase/collagenase working solution into the pancreas through the common bile duct.

   This is the method described by Gotoh et al, 1985. A successful ligation/cannulation is evidenced by visible inflation of the pancreas.

4. Aseptically excise the inflated pancreas and transfer to a sterile, empty 60-mm glass petri dish. Carefully place the dish in a 37°C water bath (making sure no contaminating water gets in) and incubate 20 min to allow digestion of the pancreas.

   NOD/LtSz-scid/scid pancreases require 20 min; other strains may require different lengths of time that must be established empirically.

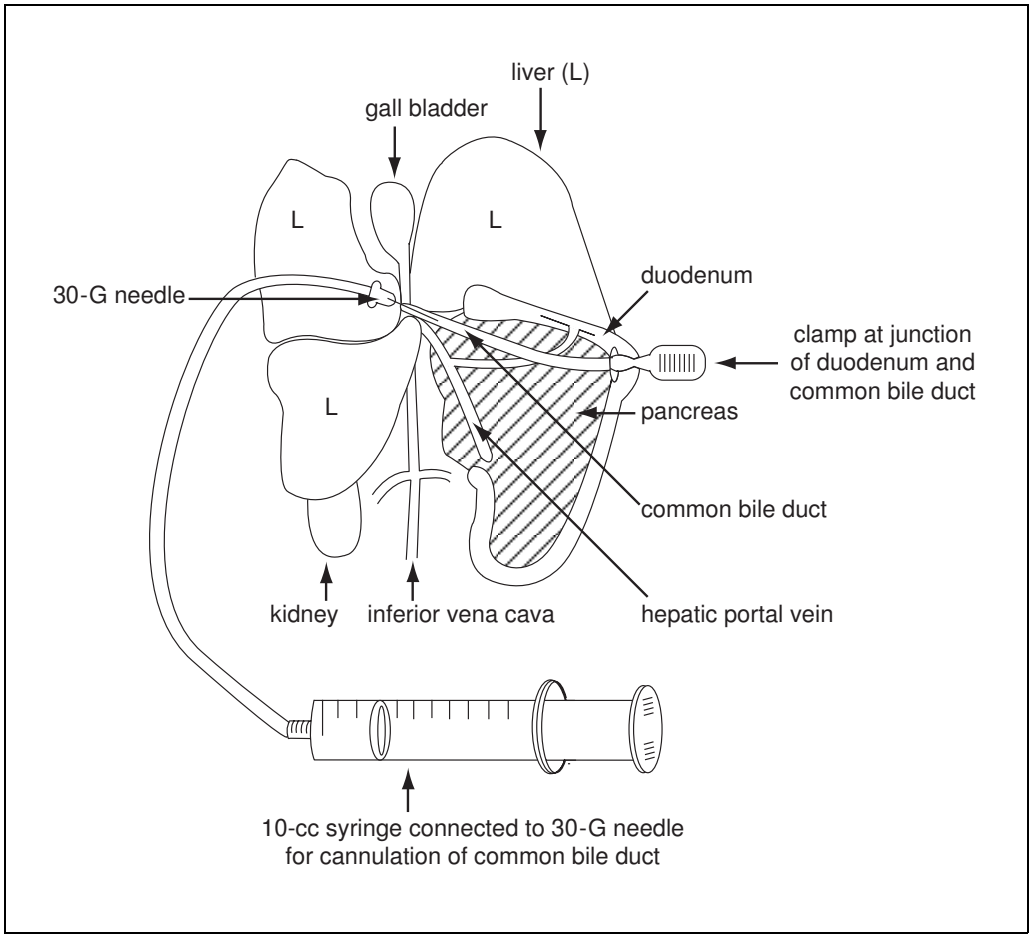

Figure 15.9.3 Setup for collagenase digestion of pancreas to obtain pancreatic islets.

5. Following incubation, disperse the digested pancreas by adding 10 ml supplemented HBSS and triturating with a silanized glass pipet. Centrifuge 1 min at 200 × *g*, room temperature.

6. Carefully decant supernatant and discard. Resuspend pellet of islet cells in 10 ml supplemented HBSS, triturate again, and centrifuge as in step 5. Repeat this washing/dispersion procedure four times.

7. Plate the pellet from a single pancreatic digest (containing dispersed islets and single exocrine cells) into two sterile 60-mm glass petri dishes and examine under a dissecting microscope with reflected lighting and a dark base.

   Islets are recognized as ovoid, translucent shapes on a "lawn" of disperse exocrine cells. See Figure 15.9.4A.

8. Using a silanized micropipet (preferably controlled by a micrometer-operated suction device), withdraw islets selectively from the dish into another dish containing supplemented HBSS, excluding as much exocrine tissue as possible. Repeat this picking procedure through two successive rounds.

   After the third pick, a nearly pure population of islets will be obtained (see Figure 15.9.4B). If the pancreas has been properly inflated with the collagenase solution, a yield of ~100 islets/NOD donor may be expected.

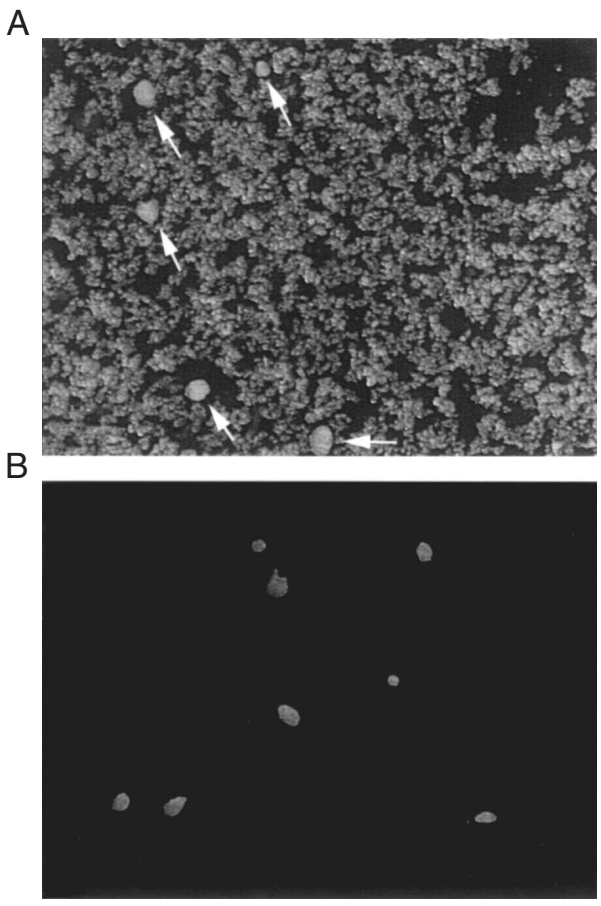

Figure 15.9.4 (**A**) Plated pellet from a pancreatic digest containing islet cells (ovoid shapes) and dispersed exocrine cells (21× magnification). (**B**) Nearly pure population of islets obtained by picking (13× magnification).

9. Isolate T cells or T cell subsets (optional; see *UNITS 3.2, 3.3 & 3.5A*) following Ficoll gradient (or Lymphocyte M) separation from islet cells after dissociation of the islet into a single-cell suspension.

Pancreatic islet–infiltrating T cells can be used in adoptive transfer experiments (see Basic Protocol 3) or to prepare T cell clones (see UNIT 3.13). See Varlamov et al. (1997) for an isolation method.

PRODUCTION OF TRANSGENIC NOD STOCKS

BASIC PROTOCOL 4

Immunologists studying the NOD mouse often wish to study a transgenic stock of NOD mice. Because IDDM is under complex multigenic control, the transfer of transgenes of interest from other inbred strain backgrounds onto the NOD inbred strain background by outcross/backcross is labor-intensive, since multiple NOD-derived susceptibility (*Idd*) loci must first be fixed to homozygosity before any conclusions regarding the effect of the transgene on IDDM pathogenesis can be reached. This goal can be accomplished within six backcross cycles ("speed congenics"). To produce a "speed congenic," breeders at each backcross generation must be typed for homozygosity for as many polymorphic simple sequence-repeat markers as possible that are closely linked to key *Idd* susceptibility loci. However, to avoid residual genetic contamination from the donor strain at other loci, the easiest approach with regard to transgenesis is to introduce the transgene construct(s) directly into NOD zygotes. The skills necessary to make such mice require special training. A laboratory manual outlining the procedures in detail is available (Hogan et al., 1994a). The purpose of including this protocol is not to provide a step-by-step methodology, but to acquaint the immunologist with the NOD-strain-specific peculiarities that affect production of transgenic mice. The information in this section should be brought to the attention of the specialist who is charged with the task of making NOD transgenic mice, since this knowledge will greatly improve the chances for successful insertion of transgenes of interest directly into NOD zygotes. Further, there are genetic issues regarding the development of NOD stocks congenic for gene "knockouts" that must be considered in studies designed to probe the effect of the disrupted gene function on IDDM pathogenesis (see Critical Parameters).

Tips for Successful Recovery of NOD Transgenic Mice

NOD mice produce litters of 11 to 14 pups. This means that NOD females are good natural ovulators. Although the specialist trained in producing transgenic mice normally would not use natural matings to obtain preimplantation embryos, but instead would "superovulate" young females by injection of pregnant mare's serum, NOD mice do not superovulate well, and the embryos obtained following treatment with gonadotropins are fragile and do not survive microinjection/reimplantation. Therefore, it is recommended that only fertilized eggs recovered from natural matings of NOD mice be used. For this purpose, females between 8 and 12 weeks of age are either selected at estrus and mated with 12-week "stud" males, or else pairs of unselected females are placed in a cage with a "stud" male. In the latter case, mice are checked for copulation plugs over the next 3 days and microinjections are done on each of the days that plugged mice are identified. An injection of complete Freund's adjuvant into a hind footpad of these mice shortly after weaning (see Critical Parameters) will reduce the possibility that mice used in these experiments will develop diabetes.

Another procedure likely to be performed by specialists making transgenic mice is overnight culture of the microinjected zygotes. This permits exclusion of nonviable embryos from the embryos to be transferred back into pseudopregnant females. This procedure will not work for NOD zygotes, as they typically arrest at the 2-cell stage in

Whitten's medium, which is the medium commonly used for maintaining preimplantation-stage embryos. Dr. John Eppig of The Jackson Laboratory has observed that NOD preimplantation embryos can transit this 2-cell block if a more advanced KSOM medium is used (Ho et al., 1995; Lawitts and Biggers, 1993). The important differences between this medium and Whitten's medium are reduced sodium, increased potassium, decreased glucose and phoshate, and addition of EDTA (J. Eppig, pers. comm.). This medium is not commercially available but is not difficult to prepare from the published formulations. However, the simplest course is to transfer the microinjected embryos directly into the oviduct of pseudopregnant nonalbino F1 females without overnight culture. If culturing is attempted, specific details are provided in Section I of the laboratory manual by Hogan et al. (1994). The experience at The Jackson Laboratory has been that recovery of transgene-positive progeny is low, but that transgene-positive mice generally breed well and express the transgenes.

Materials

DNA construct of interest
TE buffer, pH 7.5 (*APPENDIX 2*)
NOD mice (females, 10- to 12-weeks old, and "stud" males, usually 10- to 12-weeks old)
Supplemented MEM (see recipe)
Nonalbino pseudopregnant females: e.g., (C57BL/6J × BALB/cByJ)F1 or (C57BL/6J × SJL/J)F1 females
Kit for purifying DNA construct: e.g., QiaQuick (Qiagen) or GeneClean (Bio 101)
Equipment for microinjection (see Hogan et al., 1994b)

NOTE: It is critical to use the mostly highly purified water available (18 MΩ obtained using Mill-Q still or equivalent) for all reagents. The specialist operating the transgenic facility will normally supply this, or it can be purchased from Life Technologies).

1. Purify DNA construct.

   If the transgene is ≤10 Kb in size, the QiaQuick kit from Qiagen can be used; for larger constructs, the GeneClean kit from Bio 101 can be used. The technician who does the microinjection will require highly purified constructs. Details regarding purification are found in Section E of the laboratory manual by Hogan et al. (1994b) and in Ausubel et al. (1997), which contains protocols for purification of DNA constructs as well as for the linearization described in step 2.

2. Linearize the construct and dissolve at a concentration of 10 to 20 µg/ml in TE buffer, pH 7.5. Store at −20°C until ready for microinjection.

3. Set up breeding cages for mating between male and nonsuperovulated female NOD mice. Check females for uterine plugs on a daily basis.

   Plugs may be evident the day after setting up the breeding cage.

   The use of zygotes from superovulated females is not recommended since only a low yield is obtained and those embryos do not produce a high recovery of liveborn offspring in comparison to embryos from superovulated females.

4. On day that plug is evident, collect zygotes from female NOD mice. Place in supplemented MEM.

   The procedure for collecting zygotes is detailed in section C of Hogan et al. (1994a).

5. Microinject the purified DNA construct (usually at a final concentration inside the cell of 2.0 ng/µl) into the best visible pronucleus of the zygote (although protocols

usually call for injection into the male pronucleus). While observing the injection, watch until the pronucleus swells, then carefully withdraw micropipet.

Hogan et al. (1994) contains full details regarding necessary instrumentation and skills required for this procedure.

6. Immediately transfer embryos with the DNA construct into pseudopregnant recipient females.

At The Jackson Laboratory, (C57BL/6J×BALB/cByJ)F1 or (C57BL/6J×SJL/J) F1 females that have been selected in estrus and made pseudopregnant by pairing with a vasectomized C57BL/6J male are used as recipients. Usually, 30 to 40 one-cell embryos are injected into a single pseudopregnant recipient within 12 hr after identification of a copulation plug from the vasectomized mating. All vasectomized males should be test-mated to insure sterility. FVB/NJ, BALB/cByJ, or hybrid males are often used as vasectomized "studs" in preference to C57BL/6J because of the mediocre breeding performance of the latter strain.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 5****.*

Aldehyde fuchsin stain

100 ml 60% (v/v) ethanol
0.5 g pararosaniline hydrochloride (e.g., Sigma)
1 ml paraldehyde
1.5 ml concentrated HCl
Allow to ripen 2 days at room temperature
Filter through Whatman no. 1 paper before use
Stable for 7 to 10 days at room temperature

It is important to use pararosaniline and not basic fuchsin as called for in older formulations.

DNase/collagenase P working solution

To prepare 10 ml, combine:
1 ml 0.1 mg/ml DNase (from bovine pancreas; Sigma) in supplemented HBSS (store in 1-ml aliquots at –20°C; see recipe for supplemented HBSS)
1 ml 40 mg/ml collagenase P (Boehringer Mannheim) in supplemented HBSS (store in 1-ml aliquots at –20°C; see recipe for supplemented HBSS)
8 ml supplemented HBSS (see recipe)
Prepare fresh and keep on ice

The 10 ml of working solution can be used to isolate islet cells from ~3 mice.

HBSS, supplemented

Hanks' balanced salt solution (HBSS; APPENDIX 2) containing:
9 mM HEPES
100 U/ml penicillin
100 µg/ml streptomycin
Adjust pH to 6.9
Store up to 2 months at 4°C

MEM, supplemented

Rehydrate powdered Eagle's Minimal Essential Medium containing Earle's salts and L-glutamine (Life Technologies), using water of the highest purity (18 MΩ obtained using Milli-Q still or similar equipment, or purchased from Life Technologies). Add $NaHCO_3$ to a final concentration of 26.2 mM, sodium pyruvate to a final concentration of 0.23 mM, and EDTA to a final concentration of 10 µM. Gas for 10 min with 5% O_2/5% CO_2/90% N_2. Add 3 mg/ml bovine serum albumin (BSA). Filter sterilize and store ≤2 weeks at 4°C.

Modified Bouin's fixative

85 ml saturated aqueous picric acid
5 ml glacial acetic acid
10 ml 38% to 40% formalin
Prepare fresh

COMMENTARY

Background Information

A number of reviews describe the immunology of IDDM in NOD mice (Kikutani and Makino, 1992; Leiter, 1993; Wicker et al., 1995). Furthermore, a book devoted to NOD and its related strains is available (Leiter and Atkinson, 1998). These should be consulted for the relative contributions of T lymphocytes, B lymphocytes, and macrophages to the immune pathogenesis. Susceptibility to IDDM is inherited as a polygenic threshold liability (McAleer et al., 1995) with diabetogenesis requiring a complex interaction with numerous other MHC-unlinked genes. Chromosomal regions shown to segregate with IDDM susceptibility are putatively termed *Idd* (insulin-dependent diabetes) loci until their molecular basis is discovered. Many chromosomal regions reported to contain an *Idd* locus in the literature probably contain multiple genes important for diabetogenesis. For example, the major *Idd* locus contributing to susceptibility (at least 40% of the relative risk) is the unique $H2^{g7}$ MHC haplotype of NOD (designated *Idd1*). Susceptibility is clearly associated with the uncommon I-A^{g7} MHC class II molecule, coupled with the absence of cell-surface I-E molecules (Serreze and Leiter, 1994). However, susceptibility is not exclusively engendered by MHC class II–regulated events. T lymphocytes from prediabetic NOD/Lt mice cannot adoptively transfer IDDM into NOD/LtSz-*scid*/*scid* mice rendered MHC class I "bare" by congenic introgression of the $B2m^{null}$ targeted mutation (Serreze et al., 1997). These results indicate that the initiation of IDDM pathogenesis at the β-cell level requires antigen presentation in the context of the common MHC class I products. In outcross/backcross or outcross/intercross genetic-segregation analyses, homozygosity of the MHC class II region of the unique $H2^{g7}$ haplotype of this strain is usually necessary, but not sufficient, for overt IDDM (Leiter and Serreze, 1992). Similarly, homozygosity for numerous other non-MHC *Idd* loci compound risk of IDDM development, but individually no single non-MHC locus is absolutely required for diabetogenesis in the presence of $H2^{g7}$ homozygosity.

Many, but not all *Idd* genes have been shown to express in the progeny of bone-marrow derived antigen-presenting cells (APCs). These genetic defects have been associated with a functional immaturity of marrow-derived macrophages (see below). One of the features differentiating the NOD mouse from the BB rat (*UNIT 15.3*), another well-characterized rodent model of IDDM, is the phenomenon of T lymphoaccumulation in the NOD mouse, whereas T lymphopenia characterizes the BB rat (Serreze and Leiter, 1995). Defects in apoptotic events in NOD thymus and in the periphery have been reported, perhaps accounting for anomalies in positive and negative selective mechanisms in the NOD mouse.

It should be noted that immune infiltrates in NOD mice are not limited to the pancreatic islets, but are widely distributed through multiple organ systems (Leiter, 1993). Further, if NOD mice do not develop IDDM, they are susceptible to development of a broad spectrum of neoplasias, of which lymphomas are the most common.

Penetrance of diabetes-susceptibility genes in the NOD mouse is strongly influenced by agents in the extrinsic environment, particularly microbial pathogens (Bowman et al., 1994) and dietary components (Hoorfar et al., 1993). The protective effects of exposure to microbial pathogens are of great interest for several reasons. Rather than exacerbating IDDM pathogenesis, the immunostimulation produced by microbial challenge suppresses it (Leiter, 1993). A recent review of NOD colonies worldwide (Pozzilli et al., 1993) showed cumulative (30-week) disease frequencies to be much more variable (and lower) in males than in females. Although some of the colony differences may be explained by genetic divergence as substrains of NOD mice remain separated from the original source colony, most of the differences probably are caused by environmental effects. As noted previously, NOD males are particularly susceptible to environmental modulation of diabetes development.

Both developmental and functional defects

have been reported in APC of NOD mice (Langmuir et al., 1993; Serreze et al., 1993). These defects appear to selectively perturb presentation of "self" antigens in the course of tolerance induction. Some of these defects in antigen presentation and tolerance induction are associated with defective secretion of endogenous cytokines, including IL-1, IL-2, and IL-4 (Rapoport et al., 1993; Serreze et al., 1990). Exposure to microbial pathogens would counterbalance these defects via elevation of inflammatory cytokine levels. Indeed, exposure of NOD mice to viral pathogens (EMCV, LCMV, LDHV, or MHV), or to bacteria (*Mycobacterium* or *Streptococcus*) or their components (complete Freund's adjuvant or OK432) circumvents IDDM (Bowman et al., 1994). This presumably results from up-regulation of APC function produced by inflammatory cytokine production, since the IDDM-protective effects produced by virologic challenge to the immune system can be mimicked by treating young NOD mice with polyinosinic-polycytidylic acid (poly[I:C]), a potent inducer of immune interferons.

NOD/LtSz-scid/scid mice

Lymphocyte precursors in *scid/scid* mice exhibit defects in repair of double-stranded DNA breaks and cannot productively rearrange antigen receptors on T and B lymphocytes. This mutation has been transferred from the C.B-17 stock on which it arose by multiple backcrosses to the NOD/Lt substrain (NOD/LtSz-*scid*/*scid*) at The Jackson Laboratory. Like standard NOD/Lt mice, large litter sizes characterize this congenic stock and females are excellent mothers. In the absence of functional T and B lymphocytes, NOD/LtSz-*scid*/*scid* mice are completely insulitis- and IDDM-free. Unlike other stocks of *scid* mice, the NOD/LtSz-*scid*/*scid* stock exhibits very little leakiness of immunocompetent effectors as the mice age (as assessed by absence of serum immunoglobulins from B lymphocytes or T cell–mediated allograft rejections; Shultz et al., 1995). The combination of the *scid* mutation with the NOD strain–specific deficiencies in innate and adaptive immunity render the NOD/LtSz-*scid* stock exceptionally useful for the growth of allogeneic and xenogeneic cells and tissues that grow poorly in other available *scid* stocks.

Among the NOD/Lt strain-specific immunodeficiencies are low NK-cell activity, absence of the C5 component of hemolytic complement, low levels of IL-1 and IL-4 secretion, a depressed syngeneic mixed-lymphocyte reaction, and defects in macrophage maturation.

Because NOD/LtSz-*scid*/*scid* mice lack functional lymphocytes, they are heavily utilized in adoptive-transfer studies designed to assess the diabetogenic potency of NOD T lymphocytes (either purified or in bulk from lymphoid organs) or antigen-specific T cell lines (cloned and uncloned). The obvious advantage of transfers into NOD/LtSz-*scid*/*scid* mice as compared to transfer into neonates (Bendelac et al., 1987), young prediabetic immunocompetent NOD males ("accelerated transfer model"; Bach et al., 1990), or heavily irradiated (700- to 1000-rad) recipients (Miller et al., 1988) is that fully mature mice can be used without prior irradiation, since there are no functional endogenous lymphocytes to complicate interpretation of the transfer results. As a further aid in identification of NOD donor T cells transferred into NOD/LtSz-*scid*/*scid* mice, a stock of NOD/Lt mice congenic for the $Thy1^a$ allotypic marker is available (standard NOD mice are $Thy1^b$). A double-congenic stock of NOD/LtSz-*scid*/*scid* mice has recently been produced in which the β2-microglobulin (*B2m*) null mutation generated by homologous recombination has been introduced into the *scid* stock by intercross with a preexisting NOD/Lt.$B2m^{null}$ congenic stock (Serreze et al., 1994b; Wicker et al., 1994). This NOD/LtSz-*scid*/*scid* $B2m^{null}$ stock does not express MHC class I molecules and hence is proving to be an even better recipient for growth of human lymphoid tissues. The NOD-*scid* stock also is useful for carrying certain transgenes that accelerate IDDM immunopathogenesis by activating T lymphocytes. For example, the B7-1 (CD80) transgene ligated to an insulin promoter (to target expression to pancreatic β cells) activates islet-infiltrating $CD8^+$ CTL, effecting not only an accelerated IDDM onset, but also an efficient means of establishing lines and clones of islet-reactive $CD8^+$ T cells (Wong et al., 1995). However, this NOD transgenic line is difficult to maintain because of the early onset of IDDM. Introgression of the transgene into the NOD-*scid* stock would eliminate this problem, while still providing a source of readily isolatable pancreatic islets expressing the B7-1 (CD80) costimulatory molecule useful for propagation of $CD8^+$ islet-reactive T cells.

Critical Parameters

Maintaining NOD mice

The immunologist interested in studying autoimmune processes in the NOD mouse has

to chose between two options: either to maintain a breeding colony on site or to order the requisite numbers of mice necessary for specific studies from an outside breeder. Whichever option is selected, the investigator must still obtain space in an SPF vivarium that is maintained to high standards of cleanliness and good animal-care practice. When NOD mice are purchased from a extramural breeding facility, they usually cannot be obtained before 4 weeks of age, thereby precluding the use of neonates or pre-weaning mice. Moreover, NOD mice are not distributed commercially at ages beyond 8 to 10 weeks. Although IDDM may initiate as early as 10 to 12 weeks, peak incidence is attained only between 16 to 20 weeks in females, and later in males. This relatively late onset of IDDM necessitates that the investigator maintain the mice for extended periods after receiving them, if the clinical onset of IDDM is an endpoint of the study. The advantage of maintaining a breeding colony is that NOD mice are excellent breeders, producing large litters (average size ~11 pups per litter). Since NOD mice are costly, and shipment of the mice produces stresses and chance exposure to murine pathogenic agents that may influence the proposed experiments, maintenance of an institutional colony has advantages if the per diem costs of maintaining mice is not prohibitive. This requires a commitment on the part of the colony manager to maintain strict sibling matings and to keep pedigree records. Given the current heavy demand for NOD/LtSz mice homozygous for the severe combined deficiency (*scid*) mutation, the advantages to investigators of maintaining an institutional pedigreed breeding colony of this congenic stock are numerous. It should be noted that whenever inbred colonies are maintained separate from the source colony for >10 generations, the satellite colony is considered to be a substrain, requiring a holder or institutional designation. These are assigned upon request by the National Research Council Institute of Laboratory Animal Resources, Washington, D.C. To avoid substrain divergence resulting from accumulation of recessive mutations distinct from those being fixed in the source colony, new breeders from the source colony can be imported every 10 to 20 generations to maintain uniformity with the mice being studied by other investigators.

Note that the annual December issue of the journal *Lab Animal* contains a "Buyer's Guide," which provides consumer information regarding materials for mouse-colony maintenance.

Mouse room

Access to the mouse room should be limited to the personnel involved in care of the mice. Procedures for entry will depend upon the barrier level. A complete barrier level is not necessary for maintaining a high-IDDM-incidence NOD colony. Covering of the body by donning a clean lab gown is a minimum requirement; donning of clean booties over shoes and a hair net are recommended. The room and associated clean supply area should be positively pressurized by HEPA-filtered, humidified air to remove particles ≥0.3 microns. Temperature should be maintained between 22° and 24°C, and humidity should be maintained between 35% and 45%. Light/dark cycles of 12:12 or 14:10 are commonly employed. Mouse cages may be kept on open shelves if covered by sterilizable filter bonnets, or they may be held in pressurized individually ventilated (PIV) caging systems or in microisolator cages. Autoclaved or otherwise processed clean materials should be kept in a clean supply area and dirty materials removed from the mouse room into a separate dirty-materials corridor. HEPA-filtered cage-changing stations are recommended.

Diet

A chow diet must be fed in order to achieve high incidence of IDDM in NOD mice of both sexes. In North America, the Purina 5001, and NIH-31 autoclavable formulations have been found suitable for this purpose (Pozzilli et al., 1993). In Europe, SSniff MR diet (SSniff, Soest, Germany) supports a high IDDM incidence in SPF mice of both sexes, as does NFM diet from Oriental Yeast Company in Tokyo, Japan (Pozzilli et al., 1993). A semidefined diet lacking in complex vegetable matter, such as AIN-76, generally will not support a high IDDM incidence (Coleman et al., 1990). Diabetogenic factors have been reported in both wheat- and soybean-derived components of the chow diets. Thus, experiments entailing supplements of non-chow diets must be carefully controlled, and IDDM incidence may not be compared to the incidence expected if chow diet is fed.

Drinking water should be acidified (with hydrochloric acid to attain a pH of 2.8 to 3.2) to prevent growth of *Pseudomonas* species. Alternatively, hyperchlorinated water (10 ppm sodium hypochlorite) may be used.

Bedding

In the United States, autoclaved white pine shavings are commonly used for bedding. Cellulose paper bedding is also used; this material is quite hygroscopic and thus is useful for maintaining adult diabetic mice, which urinate excessively. However, such material may be excessively dry for litters in breeding cages. Cages should be changed at least once per week; two or more changes per week are necessary if diabetic NOD mice are being held, since they are polyuric. Caretakers should handle mice using smooth-tipped dressing forceps sterilized by dipping in an iodine solution.

Reproduction

NOD mice are excellent breeders; brother-sister mating pairs can be established at the time mice are weaned. Little preweaning mortality is experienced as long as the dam remains IDDM-free. Pregnancy appears to accelerate IDDM onset in females. To avoid constant turnover in the breeding pens resulting from early loss of females to IDDM, permission should be obtained from the Institutional Animal Care and Use Committee to inject a single dose of 50 µl of complete Freund's adjuvant (e.g., Sigma) into one hind footpad of each breeder female and male. This treatment prevents IDDM onset in NOD/Lt females until after at least two litters have been born and weaned (Leiter and Atkinson, 1998). During the period of footpad swelling, pellets of food should be placed in the bottom of the cage so that injected mice do not have to stand on their hind legs to feed.

Screening mice for pathogens

A variety of pathogens have been shown to specifically decrease IDDM incidence in NOD colonies, including murine hepatitis virus (MHV), lymphocytic choriomeningitis virus (LCMV), lactate dehydrogenase virus (LDHV), encephalomyocarditis virus (EMCV), and Sendi virus. Mice should be screened for the presence of antibodies to these viruses using appropriate ELISAs (*UNIT 2.1*). If institutional laboratory animal medicine services cannot provide this screening, tests may be commercially contracted from Charles River Laboratories (see *APPENDIX 5*). Ideally, testing should be performed at quarterly intervals using randomly selected mice from the mouse room. When NOD/LtSz-*scid*/*scid* mice are used, antibody screening should be performed using immunocompetent sentinel mice housed in the same mouseroom.

High levels of exposure to certain bacteria (e.g., *Mycoplasma pulmonis*) and intestinal protozoa may also compromise the autoimmune pathogenesis in NOD mice. A listing of some of these may be found in Ohsugi and Kurosawa (1994).

Special requirements for maintenance of NOD/LtSz-scid/scid mice

Immunodeficient NOD-*scid*/*scid* mice are especially vulnerable to pulmonary infections by the opportunistic pathogen, *Pneumocystis carinii*. Hence, if these mice are to be used in environments where *P. carinii* is present, mice must be maintained in microisolator caging or in other protected caging systems such as pressurized individually ventilated (PIV) racks. The drinking water must be supplemented with thiomethoprim-sulfamethoxazole (Goldline Laboratories). This product contains 40 mg thiomethoprim and 200 mg sulfamethoxazole per 5 ml of suspension. A 31.25-ml aliquot of this suspension is mixed per liter of water and administered for 3 consecutive days per week. In SPF barrier colonies maintained to the highest standards, the treatment appears to be unnecessary. *Pneumocystis* is monitored either by PCR assay or by staining of lung sections using the Gomori metheamine-silver stain.

NOD congenic stocks produced by outcross/backcross to other stocks carrying transgenes or functionally disrupted ("knock-out") genes of interest

When NOD mice are outcrossed to other strains of mice, the complex multigenic interactions between the diabetogenic MHC ($H2^{g7}$) and other required non-MHC genes are disrupted so that IDDM does not develop. Hence, if an investigator wishes to establish the role of a particular gene expression or absence of expression on diabetogenesis, it must first be ensured that the background genome contains sufficient numbers of NOD susceptibility genes to permit IDDM development in non-transgenic or non-gene-targeted segregants at the same backcross generation. PCR primer sets based upon 6600 polymorphic simple sequence repeats are available interspersed throughout the mouse genome (Dietrich et al., 1996; Miller et al., 1996). Expected NOD product sizes for most of these markers have been established by the supplier (Research Genetics; see *APPENDIX 5*). A list of some of these genetic markers that can be typed to produce a speed congenic is given in Table 15.9.2. This list is based on susceptibility-modifying genes iden-

tified by outcross of NOD with C57BL strains and the related NON/Lt strains. Other susceptibility-modifying loci not yet identified would very likely be introduced by outcrossing NOD mice to strains commonly used to produce transgenics, such as the FVB strain. In the case of targeted mutations generated by homologous recombination within an embryonal stem cell line, these mutations are usually available on segregating B6-129 chimeric backgrounds. The speed congenic procedure is also useful for rapid transfer from the background of origin onto the NOD inbred strain background. It is essential to establish on which chromosome the targeted gene resides before initiation of congenic stock development. If the targeted gene is located very close to a locus exerting strong effects on susceptibility (e.g., *Idd1, Idd3*, *Idd10*, or *Idd13*), congenic introduction of the targeted gene will very likely be accompanied by cointroduction of resistance alleles that are difficult to eliminate by recombination in successive backcross cycles. For example, congenic transfer of a targeted mutation of an MHC class II gene within the MHC complex on chromosome 17 will bring the entire MHC haplotype carried by the ES cell line used (usually a strong diabetes-resistance-conferring $H\ 2^b$ haplotype from strain 129 or B6). Similarly, cointroduction of strain 129–derived IDDM-resistance genes on chromosome 14 (*Idd8?*) in linkage disequilibrium with a functionally disrupted TCR-α locus apparently led to IDDM resistance independent of the level of TCR-α expression (Elliott and Altmann, 1996).

Information regarding polymorphic alleles containing simple sequence repeats (SSRs) whose lengths distinguish NOD from other strains can be obtained from Web sites maintained by The Jackson Laboratory (*http://www.informatics.jax.org*), The Whitehead Institute (*http://www.genome.wi.mit.edu/ftp/*), or Research Genetics (*http://www.resgen.org*). The latter company is the vendor of SSR-based primer pairs for NOD.

Table 15.9.2 Markers Used to Make NOD Speed Congenics[a]

Idd locus	Chromosome	Marker locus[b]	Comments regarding *Idd* gene(s)
Idd 1	17	*D17Nds5 (H2-Qb1)*	MHC; major susceptibility locus that should be fixed at first backcross
Idd 2	9	*D9Nds2 (Thy1)*	Gene controls rate of T cell activation
Idd 3	3	*D3Nds2 (Il2)*	The *Il2* gene may be *Idd3*
Idd 4	11	*D11Nds16 (Acrb)*	Numerous candidate genes in the area proximal and distal to this marker
Idd 5	1	*D1Mit3 (Il1r1)* *D1Mit24 (Vil)* *D1mit26 (Bcl2)*	Probably multiple *Idd* loci on this chromosome so that multiple markers should be used
Idd 6	6	*D6Mit52* *D6Mit15*	C57BL allele protective; NON allele more diabetogenic than NOD allele
Idd 7	7	*D7Nds6 (Ckmm)*	NOD allele not as diabetogenic as C57BL allele
Idd 8	14	*D14Mit11 (Plau)*	NOD allele not as diabetogenic as C57BL allele
Idd 9	4	*D4Mit59*	Apparently not the *Tnf* receptor gene in this area
Idd 10	3	*D3Nds8 (Tshb)*	Distal to *Idd3*: advisable to fix for NOD alleles within the entire region between *Il2* and D3Nds8 since multiple Idd loci have been described around Idd10
Idd 11	4	*D4Mit16*	Proximal to *Idd9*; may not be different
Idd 12	14	*D14Nds3*	Unlike *Idd8*, the NOD allele is diabetogenic
Idd 13	2	*D2Mit257 (Pcna)*	Identified in outcross with NOR/Lt
Idd 14	13	*D13Mit61*	Identified in outcross with NON/Lt
Idd 15	5	*D5Mit48*	Identified in outcross with NON/Lt

[a] Data based primarily on outcross to strains containing C57BL genome.

[b] Reference for DMit primers is Dietrich et al., 1996; reference for Nds primers is Miller et al., 1996.

Anticipated Results

An SPF colony of NOD mice should yield a high frequency of diabetes (at least 50% in females) by 18 to 20 weeks of age. When transgenes or genetically targeted mutations are sufficiently backcrossed into the NOD-inbred background, transgene-negative female segregants (or segregating females that are wild type at the targeted gene locus) should exhibit the same high incidence as standard NOD females. A diabetes frequency of 40% to 70% should be obtained in older NOD males, 30 to 40 weeks of age.

Literature Cited

Ausubel, F.A., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (eds.) 1997. Current Protocols in Molecular Biology. John Wiley & Sons, New York.

Bach, J.-F., Boitard, C., Yasunami, R., and Dardenne, M. 1990. Control of diabetes in NOD mice by suppressor cells. *J. Autoimmun.* 3:97-100.

Bendelac, A., Carnaud, C., Boitard, C., and Bach, J.F. 1987. Syngeneic transfer of autoimmune diabetes from diabetic NOD mice to healthy neonates: Requirement for both L3T4$^+$ and Lyt-2$^+$ T cells. *J. Exp. Med.* 166:823-832.

Bowman, M.A., Leiter, E.H., and Atkinson, M.A. 1994. Autoimmune diabetes in NOD mice: A genetic programme interruptible by environmental manipulation. *Immunol. Today* 15:115-120.

Christianson, S.W., Shultz, L.D., and Leiter, E.H. 1993. Adoptive transfer of diabetes into immunodeficient NOD-*scid/scid* mice: Relative contributions of CD4$^+$ and CD8$^+$ T lymphocytes from diabetic versus prediabetic NOD.NON-Thy-1^a donors. *Diabetes* 42:44-55.

Coleman, D.L., Kuzava, J.E., and Leiter, E.H. 1990. Effect of diet on the incidence of diabetes in non-obese diabetic (NOD) mice. *Diabetes* 39:432-436.

Dietrich, W.F., Miller, J., Steen, R., Merchant, M.A., Damronboles, D., Husain, Z., Dredge, R., Daly, M.J., Ingalls, K.A., O'Connor, T.J., Evans, C.A., Deangelis, M.M., Levinson, D.M., Kruglyak, L., Goodman, N., Copeland, N.G., Jenkins, N.A., Hawkins, T.L., Stein, L., Page, D.C., and Lander, E.S. 1996. A comprehensive genetic map of the mouse genome. *Nature* 380:149-152.

Elliott, J.I. and Altmann, D.M. 1996. Non-obese diabetic mice hemizygous at the T cell receptor alpha locus are susceptible to diabetes and sialitis. *Eur. J. Immunol.* 26:953-956.

Gotoh, M., Maki, T., Kiyoizumi, T., Satomi, S., and Monaco, A.P. 1985. An improved method for the isolation of mouse pancreatic islets. *Transplantation* 40:437-438.

Hamaguchi, K., Gaskins, H.R., and Leiter, E.H. 1991. NIT-1, a pancreatic β cell line established from a transgenic NOD/Lt mouse. *Diabetes* 40:842-849.

Hanson, M.S., Cetkovic-Cvrlje, M., Ramiya, V.K., Atkinson, M.A., Maclaren, N.K., Singh, B., Elliot, J.F., Serreze, D.V., and Leiter, E.H. 1996. Quantitative thresholds of MHC class II I-E expressed on hemopoietically derived antigen-presenting cells in transgenic NOD/Lt mice determine level of diabetes resistance and indicate mechanism of protection. *J. Immunol.* 157:1279-1287.

Ho, Y., Wigglesworth, K., Eppig, J., and Schultz, R. 1995. Preimplantation development of mouse embryos in KSOM: Augmentation by amino acids and analysis of gene expression. *Mol. Reprod. Dev.* 41:232-238.

Hogan, B., Beddington, R., Costantini, F., and Lacy, E. 1994a. Recovery, culture, and transfer of embryos and germ cells. *In* Manipulating the Mouse Embryo: A Laboratory Manual (Section C). pp. 127-188. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Hogan, B., Beddington, R., Costantini, F., and Lacy, E. 1994b. Production of transgenic mice. *In* Manipulating the Mouse Embryo: A Laboratory Manual, (Section E). pp. 217-254. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Hoorfar, J., Buschard, K., and Dagnaes-Hansen, F. 1993. Prophylactic nutritional modification of the incidence of diabetes in autoimmune nonobese diabetic (NOD) mice. *Br. J. Nutr.* 69:597-607.

Ikegami, H., Eisenbarth, G.S., and Hattori, M. 1990. Major histocompatibility complex-linked diabetogenic gene of the nonobese diabetic mouse. Analysis of genomic DNA amplified by the polymerase chain reaction. *J. Clin. Invest.* 85:18-24.

Kikutani, H. and Makino, S. 1992. The murine autoimmune diabetes model: NOD and related strains. *In* Adv. Immunol., (F. J. Dixon, ed.) pp. 285-322. Academic Press, New York.

Langmuir, P., Bridgett, M., Bothwell, A., and Crispe, I. 1993. Bone marrow abnormalities in the nonobese diabetic mouse. *Int. Immunol.* 5:169-177.

Lawitts, J. and Biggers, J. 1993. Culture of preimplantation embryos. *In* Methods in Enzymology: Guide to Techniques in Mouse Development, (P. Wassarman and M. DePamphilis, eds.) pp. 153-155. Academic Press, San Diego.

Leiter, E.H. 1993. The nonobese diabetic mouse: A model for analyzing the interplay between heredity and environment in development of autoimmune disease. *ILAR News* 35:4-14.

Leiter, E.H. and Atkinson, M.A. (eds.) 1998. NOD mice and related strains: Origin, husbandry, and biology. *In* NOD Mice and Related Strains: Research Applications in Diabetes, AIDS, Cancer, and Other Diseases. R.G. Landes, Austin, Tex.

Leiter, E.H. and Serreze, D.V. 1991. Autoimmune diabetes in the nonobese diabetic mouse: Suppression of immune defects by bone marrow transplantation and implications for therapy. *Clin. Immunol. Immunopathol.* 59:323-334.

Leiter, E.H. and Serreze, D.V. 1992. Antigen presenting cells and the immunogenetics of autoimmune diabetes in NOD mice. *Reg. Immunol.* 4:263-273.

Leiter, E.H., Prochazka, M., Coleman, D.L., Serreze, D.V., and Shultz, L.D. 1986. Genetic factors predisposing to diabetes susceptibility in mice. *In* The Immunology of Diabetes Mellitus, (M.A. Jaworskil, G.D. Molnar, R.V. Rajotte, and B. Singh, eds.) pp. 29-36. Elsevier/North-Holland, Amsterdam.

Liu, M. and Shapiro, M.E. 1995. New method for isolation of murine islets with markedly improved yields. *Transplant Proc.* 27:3208-3210.

Lund, T., O'Reilly, L., Hutchings, P., Kanagawa, O., Simpson, E.R.G., Chandler, P., Dyson, J., Picard, J.K., Edwards, A., Kioussis, D., and Cooke, A. 1990. Prevention of insulin-dependent diabetes mellitus in non-obese diabetic mice by transgenes encoding modified I-A β-chain or normal I-E α-chain. *Nature* 345:727-729.

McAleer, M.A., Reifsnyder, P., Palmer, S.M., Prochazka, M., Love, J.M., Copeman, J.B., Powell, E.E., Rodrigues, N.R., Prins, J.-B., Serreze, D.V., DeLarto, N.H., Wicker, L.S., Peterson, L.B., Todd, J.A., and Leiter, E.H. 1995. Crosses of NOD mice with the related NON strain: A polygenic model for type I diabetes. *Diabetes* 44:1186-1195.

Miller, B.J., Appel, M.C., O'Neil, J.J., and Wicker, L.S. 1988. Both the Lyt-2^+ and L3T4^+ T cell subsets are required for the transfer of diabetes in nonobese diabetic mice. *J. Immunol.* 140:52-58.

Miller, J.C., Dietrich, W.F., Steen, R.G., Joyce, D.C., Merchant, M.A., Wessel, M.T., Damron, D.M., Nahf, R.W., Stein, L.D., Dredge, R.D., Marquis, A.L., Daly, M.J., Reeve, M.P., Goodman, N., Lord, C.J., Montague, C.T., Prins, J.B., Todd, J.A., and Lander, E.S. 1996. SSLP/microsatellite genetic linkage map of the mouse. *In* Genetic Variants and Strains of the Laboratory Mouse, 3rd ed., (M. Lyons, S. Rastan, and S. Brown, eds.) pp. 1671-1755. Oxford University Press, Oxford.

Ohsugi, T. and Kurosawa, T. 1994. Increased incidence of diabetes mellitus in specific pathogen–eliminated offspring produced by embryo transfer in NOD mice with low incidence of the disease. *Lab. Anim. Sci.* 44:386-388.

Pozzilli, P., Signore, A., Williams, A., and Beales, P. 1993. NOD mouse colonies around the world: Recent facts and figures. *Immunol. Today* 14:193-196.

Prochazka, M., Serreze, D.V., Frankel, W.N., and Leiter, E.H. 1992a. NOR/Lt; MHC-matched diabetes-resistant control strain for NOD mice. *Diabetes* 41:98-106.

Prochazka, M., Gaskins, H.R., Shultz, L.D., and Leiter, E.H. 1992b. The NOD-*scid* mouse: A model for spontaneous thymomagenesis associated with immunodeficiency. *Proc. Natl. Acad. Sci., U.S.A.* 89:3290-3294.

Rapoport, M., Zipris, D., Lazarus, A., Jaramillo, A., Serreze, D., Leiter, E., Cyopick, P., and Delovitch, T. 1993. IL-4 reverses thymic T cell anergy and prevents the onset of diabetes in NOD mice. *J. Exp. Med.* 178:87-99.

Serreze, D.V. and Leiter, E.H. 1988. Defective activation of T suppressor cell function in nonobese diabetic mice. Potential relation to cytokine deficiencies. *J. Immunol.* 140:3801-3807.

Serreze, D.V. and Leiter, E.H. 1991. Development of diabetogenic T cells from NOD/Lt marrow is blocked when an allo-*H2* haplotype is expressed on cells of hematopoietic origin but not on thymic epithelium. *J. Immunol.* 147:1222-1229.

Serreze, D.V. and Leiter, E.H. 1994. Genetic and pathogenic basis for autoimmune diabetes in NOD mice. *Curr. Opin. Immunol.* 6:900-906.

Serreze, D.V. and Leiter, E. H. 1995. Insulin dependent diabetes mellitus (IDDM) in NOD mice and BB rats: Origins in hematopoietic stem cell defects and implications for therapy. *In* Lessons from Animal Diabetes. V (E. Shafrir, ed.) pp. 59-73. Smith-Gordon, London.

Serreze, D.V., Hamaguchi, K., and Leiter, E.H. 1990. Immunostimulation circumvents diabetes in NOD/Lt mice. *J. Autoimmun.* 2:759-776.

Serreze, D.V., Gaskins, H.R., and Leiter, E.H. 1993. Defects in the differentiation and function of antigen presenting cells in NOD/Lt mice. *J. Immunol.* 150:2534-2543.

Serreze, D.V., Prochazka, M., Reifsnyder, P.C., Bridgett, M., and Leiter, E. 1994a. Use of recombinant congenic and congenic strains of NOD mice to identify a new insulin dependent diabetes resistance gene. *J. Exp. Med.* 180:1553-1558.

Serreze, D.V., Leiter, E.H., Christianson, G.J., Greiner, D., and Roopenian, D.C. 1994b. MHC class I deficient NOD-$B2m^{null}$ mice are diabetes and insulitis resistant. *Diabetes* 43:505-509.

Serreze, D.V., Leiter, E.H., Hanson, M.S., Christianson, S.W., Shultz, L.D., Hesselton, R.A.M., and Greiner, D.L. 1995. *Emv30*^{null} NOD-*scid* mice: An improved host for adoptive transfer of autoimmune diabetes and growth of human lymphohematopoietic cells. *Diabetes* 44:1392-1398.

Serreze, D.V., Gallichin, W.S., Snider, D.P., Croiturn, K., Rosenthal, K.L., Leiter, E.H., Christianson, G.J., Dudley, M.E., and Roopenian, D.C. 1996. MHC class I—mediated antigen presentation and induction of $CD8^+$ cytotoxic T lymphocyte responses in autoimmune diabetes—prone NOD mice. *Diabetes* 45:902-908.

Serreze, D.V., Chapman, H.C., Varnum D.S., Gerling, I., Leiter, E.H., and Shultz, L.D. 1997. Initiation of autoimmune diabetes in NOD/Lt mice is MHC class 1-dependent. *J. Immunol.* 158:3958-3986.

Shultz, L.D., Schweitzer, P.A., Christianson, S.W., Gott, B., Birdsall-Maller, I., Tennent, B., McKenna, S., Mobraaten, L., Rajan, T.V., Greiner, D.L., and Leiter, E.H. 1995. Multiple defects in innate and adaptive immunological function in NOD/LtSZ-*scid* mice. *J. Immunol.* 154:180-191.

Todd, J.A., Aitman, T.J., Cornall, R.J., Ghosh, S., Hall, J.R.S., Hearne, C.M., Knight, A.M., Love, J.M., McAleer, M.A., Prins, J.-B., Rodrigues, N., Lathrop, M., Pressey, A., DeLarato, N.H., Peterson, L.B., and Wicker, L.S. 1991. Genetic analysis of autoimmune type 1 diabetes mellitus in mice. *Nature* 351:542-547.

Uehira, M., Uno, M., Kurner, T., Kikutani, H., Mori, K., Inomoto, K., Uede, T., Miyazaki, J., Nishimoto, H., Kishimoto, T., and Yamamura, K. 1989. Development of autoimmune insulitis is prevented in $E\alpha^d$ but not in $A\beta^k$ NOD transgenic mice. *Int. Immunol.* 1:209-213.

Varlamov, O., Fricker, L., Furukawa, H., Steiner, D. Langley, S., and Leiter, E.H. 1997. β cell lines derived from transgenic Cpefat/Cpefat mice are defective in carboxypeptidase E and proinsulin processing. *Endocrinology* 148:4883-4888.

Wicker, L.S., Appel, M.C., Dotta, F., Pressey, A., Miller, B.J., DeLarato, N.H., Fischer, P.A., Boltz, R.C., and Peterson, L.B. 1992. Autoimmune syndromes in major histocompatibility complex (MHC) congenic strains of nonobese diabetic (NOD) mice. The NOD MHC is dominant for insulitis and cyclophosphamide-induced diabetes. *J. Exp. Med.* 176:67-77.

Wicker, L.S., Leiter, E.H., Todd, J.A., Renjilian, R.J., Peterson, E., Fischer, P.A., Podolin, P.L., Zijlstra, M., Jaenisch, R., and Peterson, L.B. 1994. β2 microglobulin-deficient NOD mice do not develop insulitis or diabetes. *Diabetes* 43:500-504.

Wicker, L.S., Todd, J.A., and Peterson, L.B. 1995. Genetic control of autoimmune diabetes in the NOD mouse. *Annu. Rev. Immunol.* 13:179-200.

Wong, S., Guerder, S., Visintin, I., Reich, E.P., Swenson, K.E., Flavell, R.A., and Janeway, C.A. 1995. Expression of the co-stimulator molecule B7-1 in pancreatic beta-cells accelerates diabetes in the NOD mouse. *Diabetes* 44:326-329.

Zeller, R. 1989. Fixation, embedding, and sectioning of tissues, embryos, and single cells. *In* Current Protocols in Molecular Biology (Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., eds.) pp. 14.1.1-14.1.8. John Wiley & Sons, New York.

Key References

Kikutani and Makino, 1992. See above.

Summarizes immunology and immunopathology of NOD mice.

Wicker et al., 1995. See above.

Summarizes immunogenetics of NOD mice.

Leiter and Atkinson, 1998. See above.

Covers husbandry, related strains, immunogenetics, T cell biology, antigen-presenting cell defects, immunopathology, and multiple uses of NOD-scid/scid mice.

Contributed by Edward H. Leiter
The Jackson Laboratory
Bar Harbor, Maine

Figure 15.9.1 Diabetes incidence curve for NOD mice. Circles represent males ($N = 20$); squares represent females ($N = 16$), where N is the number per group in the incidence study.

Figure 15.9.2 Photomicrographs (156×) of aldehyde fuchsin–stained pancreatic sections in NOD mice. (**A**) Insulitis-free islet in pancreas of NOD-*scid/scid* mouse stained with aldehyde fuchsin to identify granulated β cells (dark color). (**B**) Islet in pancreas of 8-week-old NOD/Lt-+/+ female showing stage 2 insulitis at one pole of islet (arrow). It is scored as "2" because leukocytes are clearly penetrating into the islet. If there were no leukocytic penetration, the score would be "1" ("perinsulitis"). (**C**) Islet in same pancreas as (B), but showing stage 3 insulitis. The original boundaries of this large islet are now filled in with infiltrating leukocytes, producing erosion of the β cell (dark staining) mass. An "end-stage" islet (4.0 score) would contain even fewer, if any, granulated β cells.

Figure 15.9.3 Setup for collagenase digestion of pancreas to obtain pancreatic islets.

Figure 15.9.4 (**A**) Plated pellet from a pancreatic digest containing islet cells (ovoid shapes) and dispersed exocrine cells (21× magnification). (**B**) Nearly pure population of islets obtained by picking (13× magnification).