# Prevention of Type I Diabetes in NOD Mice by Adjuvant Immunotherapy

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**The nonobese diabetic (NOD) mouse is an excellent model of insulin-dependent (type I) human diabetes mellitus. We report that a single injection of complete Freund's adjuvant (CFA) given at an early age (5 wk) prevented the appearance of diabetes and greatly increased the life span of NOD mice without additional therapy. No treated mouse developed hyperglycemia by the age of 12 mo ($n$ = 13), whereas all untreated mice died of diabetes before 8 mo of age ($n$ = 38). All CFA-treated mice were alive and healthy at 12 mo of age. Some CFA-treated NOD mice that were monitored for long-term survival are still alive with no sign of disease at 18 mo of age ($n$ = 5). Administration of CFA resulted in decreased in vitro splenic lymphocyte proliferative responses to alloantigen and mitogen. Cell-mixing experiments indicated that antigen-nonspecific inhibitory cells were elicited in the spleen and increased in the bone marrow. These regulatory cells were Thy-1$^-$ and nonadherent to nylon wool, as has been described for natural suppressor (NS) cells. These data lend support to a relationship between the boosting of endogenous NS activity and the establishment of tolerance to self in the context of autoimmunity. Our results suggest that early nonspecific immunotherapy of genetically predisposed individuals could prevent the development of autoimmune diabetes.**
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The nonobese diabetic (NOD) mouse spontaneously develops an insulin-dependent diabetes mellitus that has many immunological and pathological similarities to human insulin-dependent diabetes (1,2). The autoimmune nature of the disease is suggested by the lymphocytic infiltration of the islets of Langerhans, preceding the destruction of insulin-producing β-cells (3). By 3–5 wk of age, mononuclear cells infiltrate periductal and perivascular spaces, leading to insulitis and diabetes within 3–6 mo of age, and by 8 mo, most mice are affected. As in humans, diabetes in NOD mice is multigenic and possibly controlled by three recessive loci, including one that is linked to the major histocompatibility complex (MHC; 4–6). The genetically determined diabetes and insulitis in NOD mice are dependent on the presence of NOD hematopoietic stem cells, based on data from radiation chimeras (7,8). The disease can be transferred with splenocytes from NOD mice, and both CD4$^+$ and CD8$^+$ T-lymphocyte subsets are necessary for this transfer (9–11). Because T lymphocytes may determine the development of diabetes, we investigated the effect of treatments that affect the immune response on the course of disease. In particular, we used adjuvant immunotherapy and found that a single injection of complete Freund's adjuvant (CFA) given at an early age prevents the appearance of diabetes and confers normal life spans to NOD mice without any additional treatment. Adjuvants, in addition to being immunopotentiators, also act on the regulatory arm of the immune system by inducing nonspecific natural effector cells, which in turn may influence the effector T lymphocytes (12–15). Examination of in vitro lymphocyte proliferative responses to alloantigen and mitogen revealed profound modifications in the response of adjuvant-treated mice. Both splenic T- and B-lymphocyte responses were significantly reduced. This reduction was associated with the detection of a radioresistant, Thy-1$^-$, nylon-wool–nonadherent cell population in the spleen and an increase in natural suppressor (NS) activity in the bone marrow.

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## RESEARCH DESIGN AND METHODS

**Mice.** NOD/Alt, BALB/cJ, C57BL/6J, and BALB/cJ × C57BL/6J $F_1$ mice were bred in our facility at the University of Alberta. NOD mice were kindly provided by E. Leiter of Jackson (Bar Harbor, ME) and bred by brother-sister mating. The incidence of diabetes in female mice in our NOD/Alt colony was >95% by 250 days of age (50% of the mice

were hyperglycemic by 150 days of age). All mice were kept under standard housing conditions with filter tops. Mice were randomized and litter matched for this study, and only females were used. Glycemia was monitored weekly with a Glucoscan 2000 glucometer (Lifescan, Mountain View, CA). CFA was purchased from Difco (Detroit, MI).

**Mixed-lymphocyte reaction (MLR).** The primary in vitro allogeneic MLR was studied by culturing 2 × $10^5$ responder splenocytes with 8 × $10^5$ stimulator splenocytes irradiated with 30 Gy from C3H/HeJ or C57BL/10J mice. After 96 h in 8% fetal bovine serum–containing RPMI-1640 medium (Flow, McLean, VA), each well was pulsed with [*methyl*-$^3$H]thymidine (Du Pont-NEN, Boston, MA) for 24 h before harvesting. All cultures were arranged in quadruplicate wells in flat-bottom 96-well Linbro plates (Flow). In cell-mixing experiments, 2 × $10^5$ spleen or bone marrow cells were added at the onset of culture (1:1 cell-mixing ratio).

**Mitogen response.** Two-hundred thousand splenocytes per well were cultured for 72 h in the presence of 2.5 μg/ml concanavalin A (ConA; Calbiochem, La Jolla, CA), 10 μg/ml *Escherichia coli*, phenol-extracted lipopolysaccharide (LPS; Sigma, St. Louis, MO), or medium alone to measure background, followed by a 12-h pulse with [*methyl*-$^3$H]thymidine. Cultures were carried out in medium containing 5% fetal bovine serum in flat-bottom 96-well Linbro plates, each culture performed in quadruplicate. In cell-mixing experiments, 2 × $10^5$ spleen or bone marrow cells were added at the onset of culture (1:1 mixing ratio) and eventually titrated by adding $10^5$ or 5 × $10^4$ bone marrow cells to 2 × $10^5$ responder spleen cells, defining 1:2 and 1:4 bone marrow–spleen mixing ratios, respectively.

**Cellular phenotyping.** Adherence to nylon wool has been described (12). Nonadherent cells were washed once after filtration through the column and counted. Thy-1$^+$ cells, which accounted for 80% of the nonadherent fraction of normal splenocytes by fluorescence-activated cell sorter analysis (data not shown), were depleted by antibody (Thy-1.2, Du Pont-NEN) and complement (Low-Tox M rabbit complement, Cedarlane, Hornby, Canada) as previously described (12). This treatment removed 80% of the nonadherent cells by trypan blue exclusion and abrogated response to ConA in vitro. In some experiments, the cocultured cells were added after in vitro exposure to 15 Gy γ-irradiation, establishing that the regulatory function is partly radioresistant. The splenic regulatory cells were characterized in CFA-treated mice 8 days after intraperitoneal administration of 100 μl of adjuvant and concomitantly prepared from normal spleen. All cellular fractions were added to 2 × $10^5$ responder cells at the onset of culture at various regulator-responder ratios (2:1, 1:1, 1:2, 1:4).

## RESULTS

**Prevention of development of diabetes with single CFA injection.** To investigate the effect of adjuvant on the development of T-lymphocyte–dependent diabetes in NOD mice, 5-wk-old prediabetic female NOD mice were administered 50 μl CFA as an emulsion in an equal volume of saline in the hind foot pad. A single injection of CFA prevented the development of hyperglycemia (Fig. 1). Serum glucose levels in CFA-treated mice remained <10 mM up to 11 mo after administration of the adjuvant, whereas in control saline-injected mice, hyperglycemia developed as early as 3 mo after birth, usually rising to ≥27.8 mM within 2–4 wk of onset. All 38 control mice monitored weekly for blood glucose died by 5 mo of age, which is generally the case in our colony, whereas all CFA-treated mice ($n$ = 13) were still alive and healthy 1 yr after birth (Fig. 2). Five CFA-treated NOD mice that were monitored for long-term survival are healthy and of normal weight at 18 mo of age with no sign of diabetes. Similar results were obtained when NOD mice were treated with CFA anytime between 4 and 10 wk after birth (data not shown). Histological examination of the pancreas revealed intact islets and insulin-staining β-cells in NOD mice 8 mo after CFA administration, whereas virtually all islets showed varying degrees of mononuclear cell infiltration (insulitis) and islet destruction in untreated NOD mice after 4 mo of age (data not shown). Islets exhibiting insulitis but not destruction were also observed. Studies are in progress to assess whether earlier administration of CFA can prevent both insulitis and diabetes in NOD mice and BB rats. Because of the autoimmune nature of diabetes in NOD mice and the apparent limitation of the destructive potential of the

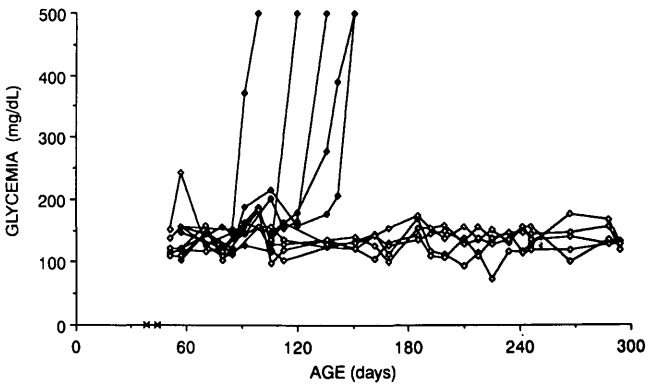


**FIG. 1. Early adjuvant administration prevents development of hyperglycemia in nonobese diabetic (NOD) mice. NOD mice were given 50 μl complete Freund's adjuvant (◇, $n$ = 5) or an equal volume of saline (◆, $n$ = 5) once only in foot pad at age 35 days, and serum glucose levels were monitored weekly for 10 mo. Mice were killed when glycemia was >500 mg/dl.**

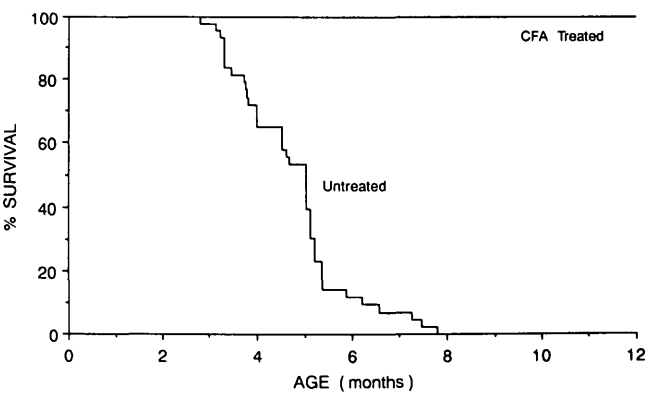


**FIG. 2. Early adjuvant administration extends life span of nonobese diabetic mice. Five-week-old mice were given 50 μl of complete Freund's adjuvant (CFA; $n$ = 13) in foot pad or no treatment ($n$ = 38). Percentage survival is calculated as 100 × (number of surviving mice) × (initial number of experimental mice)$^{-1}$. All mice that died had confirmed hyperglycemia.**

insulitis resulting from CFA administration, we next investigated the effect of adjuvant administration on the proliferative responses of T lymphocytes.

**Decreased splenic lymphocyte proliferative responses with CFA administration.** T-lymphocyte–mediated responses were examined in vitro in the primary allogeneic MLR and mitogen-induced lymphoproliferative responses. The responses of splenocytes from adjuvant-treated mice were compared to the responses of control (saline-injected) age- and sex-matched mice. The primary allogeneic MLR, studied 8 days after CFA administration, was reduced in both NOD mice and age- and sex-matched nondiabetic BALB/cJ × C57BL/6J $F_1$ mice (Fig. 3*A*). This reduction was maximal 8–10 days after CFA treatment (data not shown). In one of three representative experiments, including three separately treated mice per group, the lymphocyte proliferative response dropped from 63,520 ± 4638 to 48,568 ± 2650 counts/min (cpm) in NOD mice and from 62,991 ± 1116 to 26,958 ± 2867 cpm in nondiabetic mice. Comparable results were obtained in the mitogen-induced proliferation assays with either ConA or LPS to stimulate the lymphocytes (Fig. 3*B*). To investigate the mechanism of this reduction, cell-mixing experiments were performed to determine whether regulatory cells elicited by adjuvant administration may account for this effect.

**Antigen-nonspecific inhibitory cells in spleen and bone marrow after CFA administration.** To investigate the presence of regulatory cells, spleen cells from CFA-treated or control mice were mixed with syngeneic responder spleen cells from 2-mo-old untreated donors (Fig. 4). Thus, the addition of splenocytes from CFA-treated but not saline-injected mice reduced the primary allogeneic MLR and the mitogen-induced proliferative responses. Cell-mixing experiments are shown at one responder-spleen–test-spleen cellular ratio (1:1). At this mixing ratio, a comparable reduction of the proliferative response in the MLR and the response to LPS and a lesser reduction in the ConA response of NOD spleen cells than that of BALB/cJ × C57BL/6J $F_1$ cells was achieved. Taken together, these data establish that an inhibitory cell population is present in the spleen after CFA administration. Because nonspecific suppressor cells have been suggested to originate in the bone marrow and migrate to the spleen after adjuvant administration (13–15), we also examined the regulatory potential of bone marrow cells. A naturally occurring suppressor activity in the bone marrow inhibits both the MLR and the proliferative response to LPS and is augmented by CFA administration (Fig. 5). Although this increase is apparent in both diabetic and nondiabetic strains, this activity at the basal level is less in NOD mice than in the BALB/cJ × C57BL/6J $F_1$ strain. Approxi-

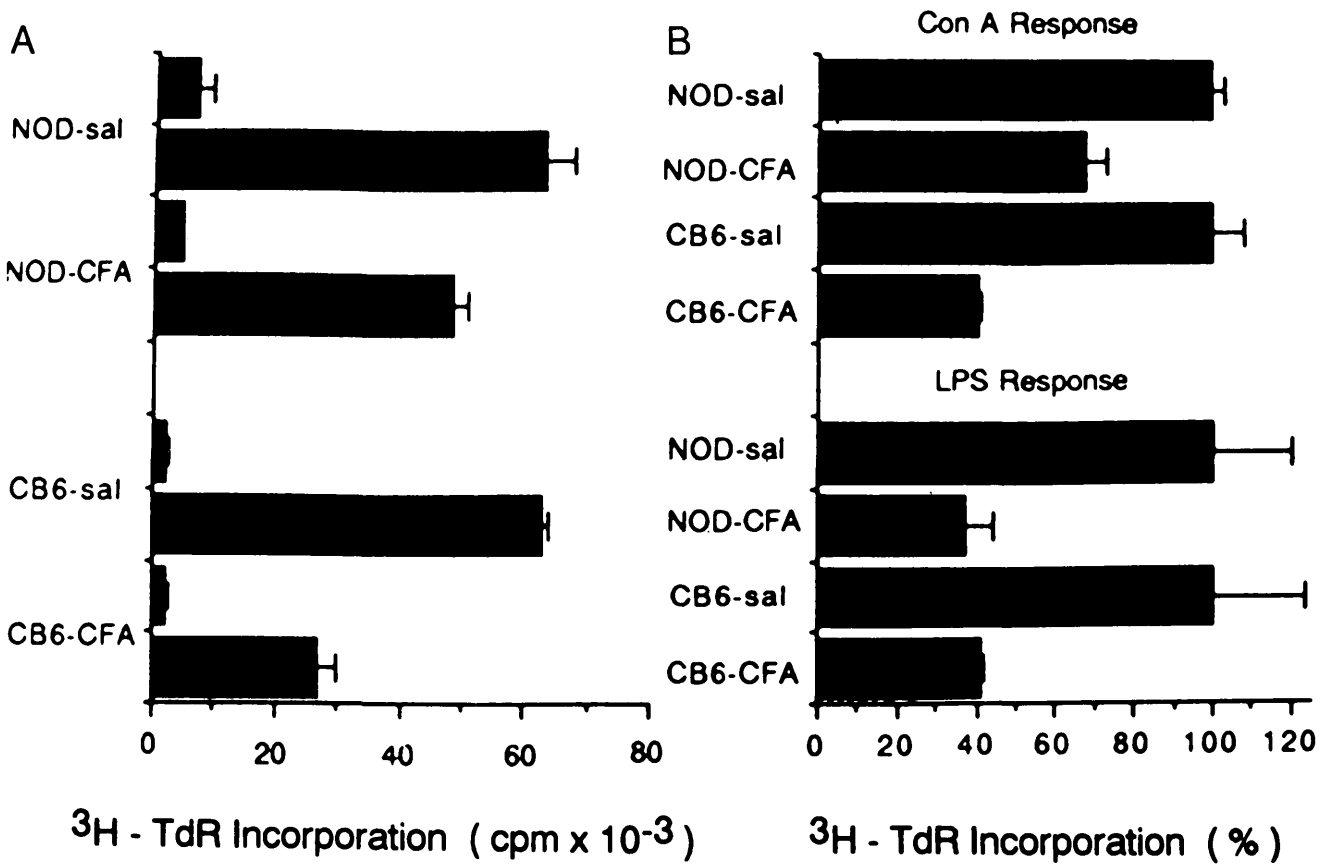


**FIG. 3. Adjuvant administration results in reduced in vitro splenic proliferative responses to alloantigen (*A*) and mitogen (*B*). sal, Control saline-injected mice; CFA, complete Freund's adjuvant–treated mice. For each group of mice in the mixed-lymphocyte reaction, syngeneic proliferative responses are plotted above anti-C3H/HeJ allogeneic response (*left*). Mitogen responses are normalized to facilitate comparisons between treatments and mouse strains (nonobese diabetic [NOD] and BALB/cJ × C57BL/6J $F_1$ [CB6] mice). Control responses (100%) are 130,280 ± 4140 counts/min (cpm) and 68,267 ± 5769 cpm in concanavalin A (ConA) response and 11,629 ± 2209 and 35,694 ± 8923 cpm in lipopolysaccharide (LPS) response in NOD and CB6 mice, respectively. $^3$H-TdR, [*methyl*-$^3$H]thymidine. Data are means ± SD (3 mice/group).**

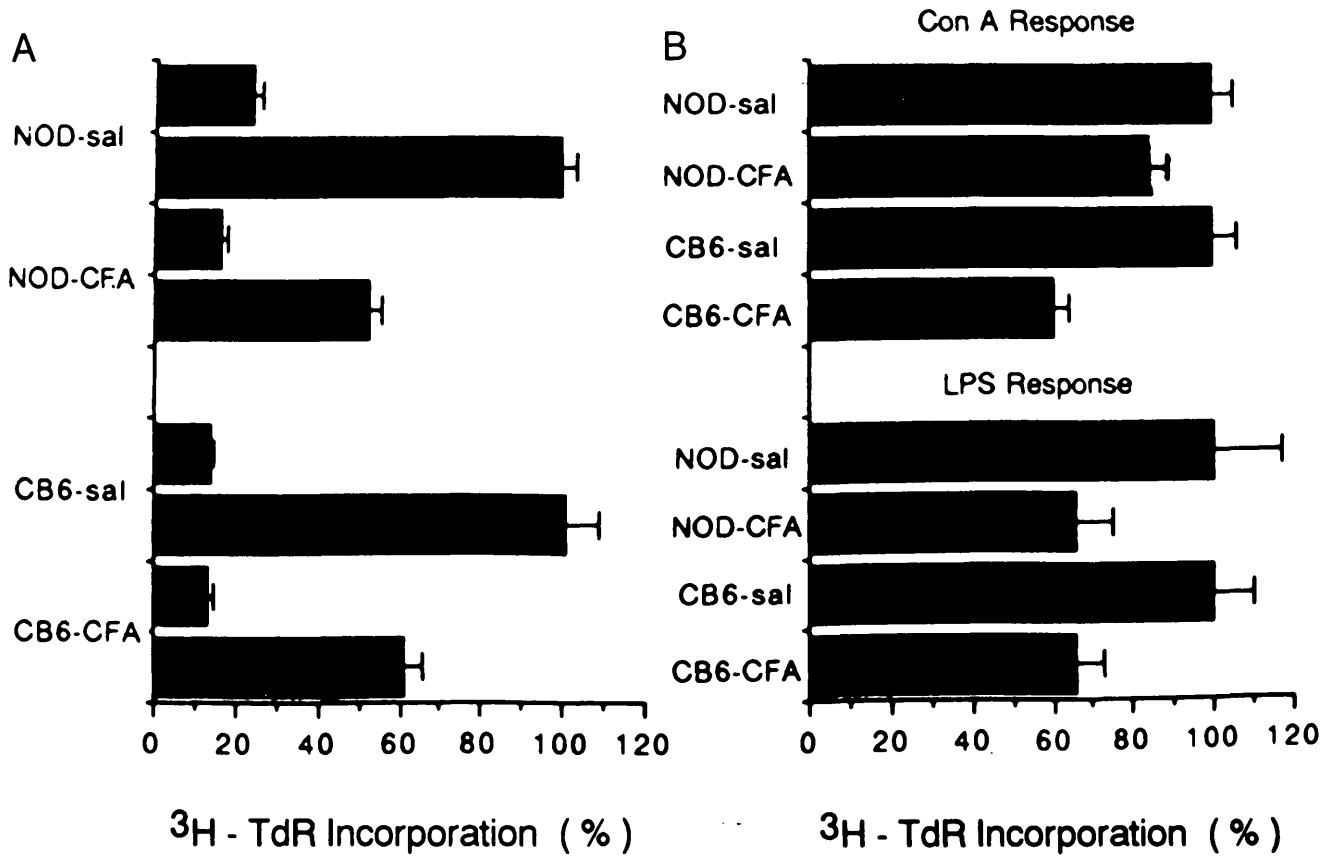


**FIG. 4. Cells that downregulate mixed-lymphocyte reaction (MLR; *A*) and proliferative response to mitogen (*B*) are present in spleen after adjuvant administration. In these cell-mixing experiments, 2 × $10^5$ responder spleen cells were mixed with 2 × $10^5$ syngeneic spleen cells from complete Freund's adjuvant (CFA)–treated or control mice in each culture well (1:1 cell-mixing ratio). Addition of spleen cells from CFA-treated mice resulted in reduction of proliferative response, indicating that natural suppressor activity was present in spleen 8 days after adjuvant administration. Data are normalized to facilitate comparisons. Control responses (100%) are 46,409 ± 1213 and 32,681 ± 2419 counts/min (cpm) in MLR in nonobese diabetic (NOD) and BALB/cJ × C57BL/6J $F_1$ (CB6) mice, respectively, which is not significantly different from response of responder spleen cells alone (data not shown). Responses to concanavalin A (conA) are 113,860 ± 4966 and 84,651 ± 5079 cpm, and responses to lipopolysaccharide (LPS) are 14,979 ± 1178 and 28,664 ± 1251 cpm in NOD and CB6 mice, respectively. $^3$H-TdR, [*methyl*-$^3$H]thymidine. Data are means ± SD.**

mately 20 × $10^4$ NOD bone marrow cells are necessary to reduce the control lymphoproliferative response by 50%, whereas only 9 × $10^4$ cells are required 8 days after CFA administration (Fig. 5*B*). In nondiabetic BALB/cJ × C57BL/6J $F_1$ mice, 8 × $10^4$ marrow cells from untreated mice and only 4 × $10^4$ cells after CFA administration are necessary to reduce the response by half. Thus, we conclude from these mixing experiments that NS activity is increased in bone marrow and elicited in spleen after adjuvant administration.

**Mediation of NS activity by nylon-wool–nonadherent Thy-1$^-$ radioresistant effector cells.** To characterize the effector cells mediating the antigen-nonspecific inhibitory activity that is induced in the spleen after CFA administration, we evaluated the adherence to nylon wool, the presence of surface Thy-1 molecules, and the radioresistance profile of the inhibitory spleen cells. By cell-mixing experiments in the primary allogeneic MLR and the LPS proliferation assays, the regulatory activity resided within the nylon-wool–nonadherent Thy-1$^-$ fraction (Fig. 6). The addition of nylon-wool–nonadherent Thy-1$^-$ cells from CFA-treated mice reduced the mean ± SD proliferative responses by 45.9 ± 9.2% in MLR and 45.8 ± 8.7% in LPS stimulation at the 1:1 regulator-responder cell ratio, whereas the addition of identically prepared spleen cells from untreated age- and sex-matched NOD mice resulted in 11.8 ± 7.8% reduction in MLR ($P < 0.001$) and 20.9 ± 8.2% reduction in LPS stimulation ($P < 0.001$). In our experiments, spleen cells were irradiated (15 Gy) before being added to the culture (Fig. 6), which reduced the inhibitory activity by ≤50% (unpublished observations). The relative suppression measured in both assays was of comparable magnitude at each cell-mixing ratio. We therefore conclude that the NS activity present in the spleen after adjuvant administration is mediated by Thy-1$^-$ cells (unlike splenic T lymphocytes) and nylon-wool–nonadherent cells (unlike B lymphocytes and macrophages, which tend to stick to the columns).

## DISCUSSION

We found that a single injection of CFA given at an early prediabetic stage in NOD mice can prevent the development of hyperglycemia and death. Although this was associated with conservation of insulin-producing β-cells, infiltration of the islets with mononuclear cells was not completely eradicated, suggesting that adjuvant therapy acted by reducing β-cell destruction by the mononuclear infiltrate. Prevention of diabetes in these mice was accompanied by the transient global reduction of lymphocyte proliferative responses, the

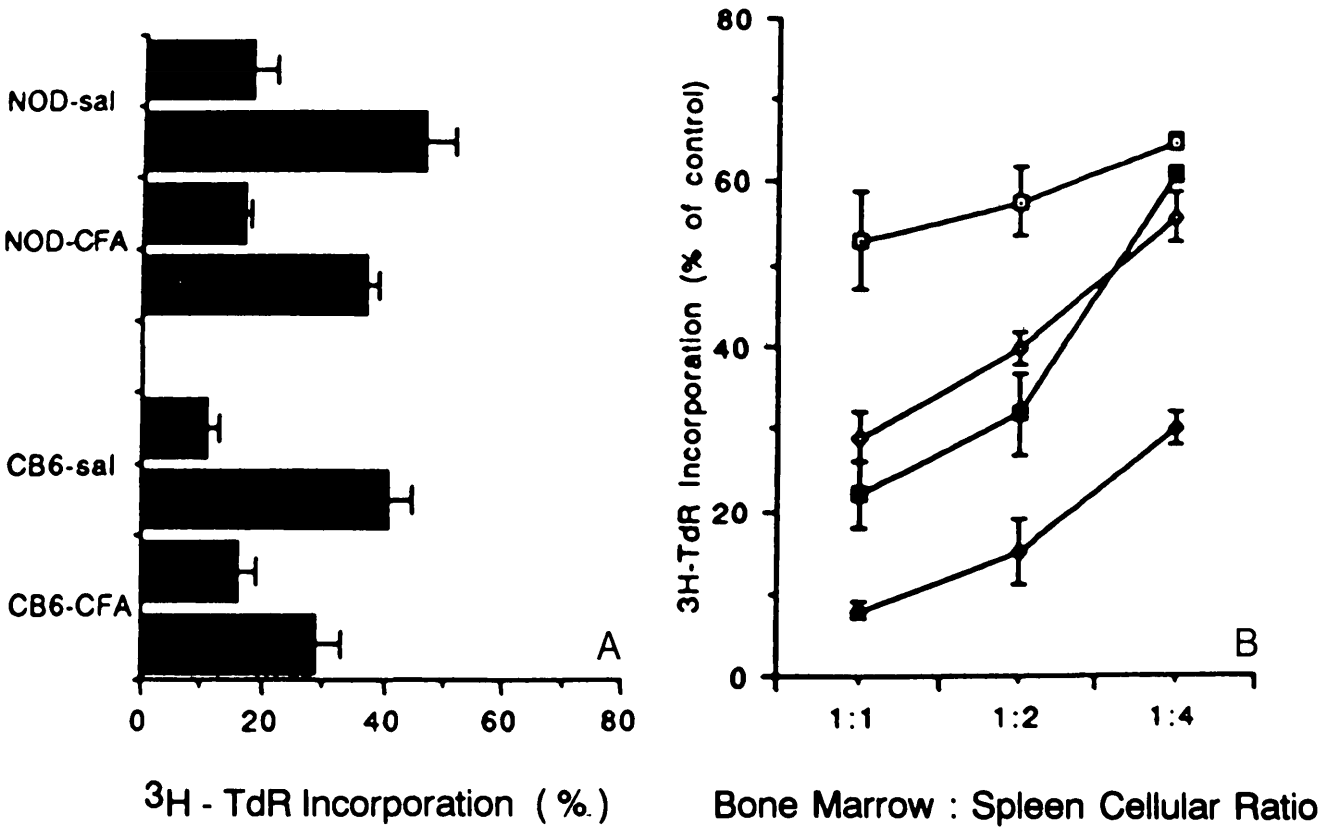


**FIG. 5. Constitutive bone marrow natural suppressor (NS) activity is increased after adjuvant administration. In mixed-lymphocyte reaction (*A*) at 1:1 bone marrow–spleen cellular ratio, proliferative response is reduced from 47 ± 5 to 37 ± 2% when mixed with bone marrow from saline (sal)-treated and complete Freund's adjuvant (CFA)–treated nonobese diabetic (NOD) donors, respectively, and from 41 ± 4 to 29 ± 4% in BALB/cJ × C57BL/6J $F_1$ (CB6) counterparts. In proliferative response to lipopolysaccharide (*B*), bone marrow dilutions are shown to permit semiquantitative appreciation of increase in NS activity. □, NOD mice; ■, CFA-treated NOD mice; ◇, CB6 mice; ♦, CFA-treated CB6 mice. $^3$H-TdR, [*methyl*-$^3$H]thymidine. Data are means ± SD (*n* = 3 for each group).**

relevance of which is suggested by the ability of T lymphocytes to transfer the disease (9–11). Cell-mixing experiments indicate that this reduction may be attributed at least in part to the induction of regulatory cells in the spleen after adjuvant administration. Several protocols that inhibit T-lymphocyte function have been shown to block the progression of disease in most cases with prolonged therapy (16–23). However, our results differ in one major respect: disease prevention was achieved by a single injection without further therapy, thus establishing that early immunotherapy can affect pathogenic events over a prolonged period. We propose that this effect is conferred by adjuvant alone through the activation of endogenous regulatory cells that can prevent the initiation, amplification, and/or effect of the autoimmune response. This may be particularly so because the mice were treated at an early age, probably at the initial stages of insulitis, and at the time of first exposure to an islet antigen.

Our data indicate that the reduction of lymphocytic proliferative responses in adjuvant-treated NOD mice is associated with the presence in the spleen of antigen-nonspecific inhibitory cells. Several reports have established that adjuvant administration activates NS activity, ascribed either to suppressor macrophages or nonadherent Thy-1$^-$ NS cells (for review, see refs. 24–26). NS activity is antigen nonspecific, non–*H-2* restricted, and physiologically present in neonatal spleen (27–31) and adult bone marrow (12–15,32–35). It has been suggested that cells mediating NS activity migrate from bone marrow to spleen after administration of adjuvants alone without antigen (13–15). We report here that the inhibitory activity is mediated by nylon-wool–nonadherent Thy-1$^-$ effector cells in the primary allogeneic MLR and the LPS-induced proliferative response. This inhibition operates across MHC barriers (unpublished observations). Therefore, the data suggest that antigen-nonspecific non–*H-2*-restricted Thy-1$^-$ suppressor (NS) cells are induced in the spleen after adjuvant administration. We have shown elsewhere that this activity is increased in bone marrow and elicited in the spleen after administration of CFA to C57BL/6J and BALB/cJ × C57BL/6J $F_1$ mice, reaching a maximum ~10 days after adjuvant administration, and mediated by Thy-1$^-$ plastic-nonadherent cells in the bone marrow (12). The data shown here do not exclude the possibility that adjuvant administration may also act independently through other mechanisms involving cytokine or growth factor production by lymphocytes or other cells, intrathymic selection (36), clonal anergy (37), or antigenic expression on target cells. It is also possible that regulatory T lymphocytes are induced after adjuvant administration, which suppresses or prevents the destruction of islet β-cells.

The lesser reduction of the proliferative responses in the CFA-treated NOD mice than in the CFA-treated nondiabetic mice we studied in parallel may reflect either a less CFA-inducible NS cell reservoir in the NOD mice of our colony or that some NOD lymphocytes are refractory to NS inhibitory

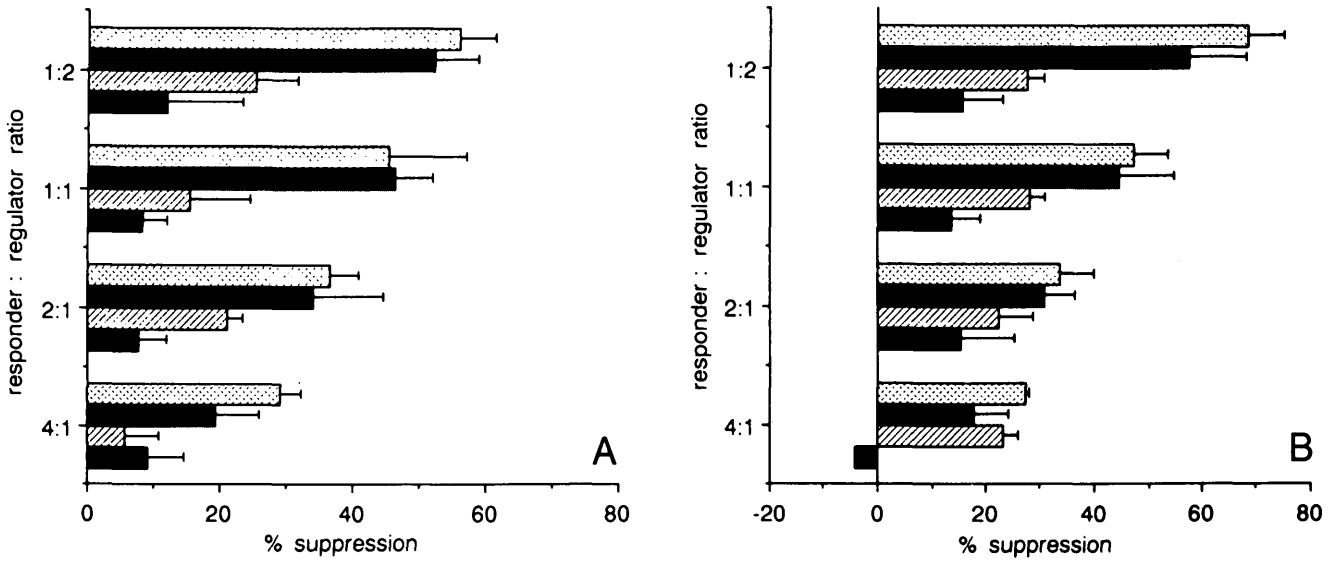


**FIG. 6. Phenotypic characterization of nonobese diabetic (NOD) splenic complete Freund's adjuvant (CFA)–induced natural suppressor effector cell in primary allogeneic mixed-lymphocyte reaction (MLR; *A*) and proliferative response to lipopolysaccharide (LPS; *B*). Spleen cells from CFA-treated NOD mice were filtered through nylon-wool column (*stippled bars*) and depleted of Thy-1$^+$ cells (*gray bars*). Spleen cells from saline-treated NOD mice were also filtered through nylon-wool column (*hatched bars*) and depleted of Thy-1$^+$ cells (*black bars*). Cells were irradiated (15 Gy) before coculture. Regulatory cells were added at different ratios to fixed number (2 × 10$^5$) of syngeneic (NOD) responder spleen cells. Results are means ± SD of suppression of response achieved in absence of added regulatory cells (17,922 ± 412 counts/min [cpm] in MLR and 129,851 ± 14,919 cpm in LPS assay). Each data point represents 3 mice.**

signals. A spontaneous permanent reduction of endogenous NS activity would strongly support a relationship between this immunoregulatory activity and the control of the diabetic phenotype. In the NZB autoimmune mouse strain, a profound deficiency in bone marrow NS activity in vitro has been reported and suggested to play a role in the autoimmune response (38).

NS cells have been suggested to play an important role in the induction of self-tolerance in neonatal mice, the regulation of immune responses, and the success of allogeneic bone marrow transplantation after total lymphoid irradiation (for review, see ref. 25). The administration of adjuvant early in life (or the eventual influence of environmental factors that increase NS activity) may enhance extrathymic mechanisms of tolerance induction to self-antigens by extending and/or amplifying the spontaneously elevated NS activity that characterizes the postnatal period (12,27–31).

Whatever the mechanism of the prevention of diabetes in NOD mice after administration of CFA, this approach provides a simple method for maintaining NOD mice in a disease-free state. Our results establish that early nonspecific immunotherapy in a murine model can prevent the development of diabetes in genetically predisposed individuals. We hope that this approach may also serve as a basis for strategies of preventive therapy in human insulin-dependent diabetes mellitus and perhaps other autoimmune diseases.

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