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Immunology

Autologous CD4 T-cell responses to ectopic class II major histocompatibility complex antigen-expressing single-cell islet cells: An *in vitro* insight into the pathogenesis of lymphocytic insulitis in nonobese diabetic mice

(diabetes/beta cells)

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ABSTRACT **We investigated by flow cytometric analysis the expression of class II major histocompatibility complex (MHC) molecules by viable single-cell islet cells (SCICs) prepared from male and female 4- and 10-week-old nonobese diabetic (NOD) mouse islets. With anti-I-A^k monoclonal antibody (specific for I-A$_\beta^{k,f,r,s}$ and produced by clone 11-5-2), and fluorescein isothiocyanate-conjugated goat anti-mouse IgG as second-step antibody, we found that SCICs from both sexes aberrantly expressed class II MHC molecules, which was not altered after SCICs were cultured for 24 hr or 120 hr in the presence of 10 ng of recombinant murine interferon γ per ml. Double-indirect immunofluorescence of male SCICs indicated that the expression of class II MHC molecules was a property of beta cells. Control experiments documented that macrophages and mononuclear cells did not contaminate the SCIC preparations. Coculture experiments with responder splenic CD4 T cells isolated from diabetic NOD mice and stimulator male SCICs indicated a recognition event evidenced by a 12-fold increase in proliferative response. Monoclonal antibodies to class II MHC and CD4 antigens blocked the proliferative response. Results from control autologous and allogeneic mixed lymphocyte reactions suggest that the responder CD4 T cells are autoreactive self-class II MHC restricted. We tentatively conclude that the ability of SCICs from both sexes of NOD mice to express class II MHC molecules as early as 4 weeks of age may represent a mechanism for targeting immune reactions to beta cells and initiate lymphocytic insulitis.**

The nonobese diabetic (NOD) mouse, an animal model for human type I diabetes, spontaneously develops ketotic-prone, insulin-dependent glucose intolerance mediated through immunological destruction of pancreatic beta cells. Lymphocytic insulitis presumably is the pathogenic lesion responsible, and the events that trigger the onset of the disease seem to be associated with particular major histocompatibility complex (MHC) alleles (1, 2). Although still much in debate, aberrant expression of class II MHC molecules by pancreatic beta cells has been suggested to be the initial event in the immunological sequence of events that culminate in lymphocytic insulitis and onset of diabetes in the human (3–7) and the two animal models of the disease, the NOD mouse (8, 9) and the BioBreeding (BB) rat (10). A prerequisite for these events to occur is an initial situation of immunological intolerance to self-class II MHC molecules expressed by target pancreatic beta cells with acquired capacity to present a specific "nontolerated-self" antigen. A specific interaction of class II MHC–antigen complex with responder class II-restricted CD4 T cells contributes to signals leading to CD4 T-cell activation. In additional support for this hypothesis, aberrant expression of class II MHC molecules has been documented in Graves and Hashimoto diseases (11, 12), in pemphigus vulgaris (13), in juvenile rheumatoid arthritis (14), and in primary biliary cirrhosis (15).

In the present study we investigated by flow cytometric analysis and monoclonal antibodies (mAbs) to I-A antigens the ectopic expression of class II MHC molecules by viable single-cell islet cells (SCICs) prepared from both sexes of 4- and 10-week-old NOD mouse islets. Also we report data from experiments with an *in vitro* model for lymphocytic insulitis in which autologous splenic CD4 T cells isolated from overtly diabetic NOD mice were cocultured with SCICs prepared from 10-week-old male NOD mouse islets.

MATERIALS AND METHODS

Mice. NOD/Sansum mice were bred in our facilities. The spontaneous incidence of diabetes in our colony reaches 60% in females and 10% in males by postnatal week 35. No diabetes was observed in our colony in females or males by postnatal week 12. Diabetes was diagnosed when permanent glycemia >350 mg/dl occurred. C57BL/6J/Sansum mice were used as diabetes-nonsusceptible controls.

mAbs. Anti-L3T4, anti-Lyt-2, anti-Thy-1.2, anti-I-A^k [clone 11-5-2, specific for I-A$_\beta^{k,f,r,s}$ and precipitates the same molecules as the NOD I-A-specific clones 10-2-16 and 10-3-16 (16–18)], and anti-I-A^d (clone MKD6, specific for I-A$_\beta^d$) were purchased from Becton Dickinson.

Preparation of Responder CD4 T Cells. Spleens were removed from newly diagnosed diabetic female NOD or control C57BL/6J mice and disrupted with frosted glass slides. The homogenate was washed in phosphate-buffered saline (PBS), resuspended in erythrocyte lysing agent (Coulter), centrifuged at 400 × *g* for 8 min, and washed in PBS containing 5% fetal calf serum. The washing procedure was repeated twice and the cells were suspended either in PBS for flow cytometric analysis or in complete medium (CM) for coculture [CM: RPMI 1640 medium supplemented with 25 mM Hepes, 10% HyClone fetal calf serum (HyClone), 1% antibiotic mixture (GIBCO), 1% L-glutamine, 0.1% gluthathione reduced, and 0.4% 2-mercaptoethanol]. IgG panning removed B lymphocytes and macrophages expressing receptors for Fc regions of IgG by using Petri dishes coated with goat anti-mouse IgG (Sigma). Nonadherent T cells were washed twice in PBS and resuspended in 2 ml of PBS (2–3 × 10^7 cells per ml) containing anti-Lyt-2 mAb (5 μg/ml, Becton Dickinson).

Abbreviations: MHC, major histocompatibility complex; SCIC, single-cell islet cell; NOD, nonobese diabetic; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; rMuIFN-γ, recombinant murine interferon γ; MLR, mixed lymphocyte reaction; CM, complete medium.

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After incubation at room temperature for 20 min, the cells were washed and added to goat anti-rat IgG-coated plates. After 1 hr at 4°C a CD4 T-cell-enriched fraction was collected. The efficiency of enrichment, assessed by flow cytometric analysis (FACScan, Becton Dickinson), was 80–85% for L3T4$^+$, Thy-1.2$^+$ T cells.

Preparation of Stimulator SCICs. Pancreatic islet donors were newly weaned insulitis-free 4-week-old NOD mice (sex not determined), 10-week-old male or female NOD mice, or diabetes-nonsusceptible male or female C57BL/6J mice. Islets were isolated by a collagenase technique as described (19). To prepare SCICs, islets were washed in PBS containing neither Ca^{2+} nor Mg^{2+} but with the addition of 0.54 mM EDTA and resuspended (≈150) in 1 ml of the same buffer with the addition of 0.5 mg of trypsin per ml. The islets were incubated at 37°C with occasional hand shaking for 10 min followed by centrifugation at 400 × g for 6 min. The SCIC pellet was resuspended and washed three times in CM. Viability determined by trypan blue exclusion on average was >85%.

Flow Cytometric Analysis of SCICs. Suspensions of 10,000–15,000 SCICs per 50 μl were incubated with 50 μl of anti-I-A^k mAb in PBS solutions. As a control for nonspecific binding, anti-I-A^d mAb of the same class (IgG2a), but specific for a different haplotype, was employed. After incubation for 1 hr at 4°C, cells were washed in cold PBS containing 2% fetal calf serum followed by incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (dilution, 1:2000; Bethesda Research Laboratories) as the second-step antibody for 1 hr. Positive controls for I-A^k mAb were similarly performed with (*i*) NOD mouse unfractionated splenic lymphoid cells containing class II MHC antigen-bearing macrophages and B lymphocytes (20) and (*ii*) NOD mouse peritoneal macrophages cultured for 24 hr in medium alone or medium containing 10 ng of recombinant murine interferon γ (rMuINF-γ) per ml (Genzyme; specific activity, 4.5 units/ng) to induce expression of class II MHC molecules (21). The absence of contaminating B lymphocytes and macrophages expressing Fc receptors in the SCIC preparation was controlled by flow cytometric analysis of SCICs incubated with FITC-conjugated goat anti-mouse IgG alone. The absence in the SCIC preparation of cells bearing L3T4, Lyt-2, and Thy-1.2 antigens was controlled by flow cytometric analysis of SCICs incubated with phycoerythrin-conjugated anti-L3T4, FITC-conjugated anti-Lyt-2 mAbs, or anti-Thy-1.2 mAb plus FITC-conjugated goat anti-mouse IgG as the second-step antibody. Fluorescence intensity in arbitrary units on a logarithmic scale was proportional to the level of I-A^k antigen and was analyzed in a fluorescence-activated cell sorter (FACScan, Becton Dickinson).

Cocultures of CD4 T Cells and SCICs. Cocultures were performed in CM in 96-well microtiter plates for 96 hr with ≈4 × 10^4 SCICs as stimulators and 2 × 10^5 CD4 T cells as responders per well in a volume of 0.2 ml at 37°C in a 5% CO_2/95% air humidified atmosphere. Control wells contained medium or SCICs or CD4 T cells alone. To assess proliferative responses, cocultures were incubated in the presence of 0.1 μCi of [^{3}H]thymidine (1 Ci = 37 GBq; New England Nuclear) for an additional 16 hr of culture, and the cultures were harvested onto glass fiber disks by a cell harvester. The disks were assayed for radioactivity in 3 ml of Aquasol-2 in a β-counter. Data are presented as the mean cpm (±SD) of triplicate cocultures minus the mean cpm (±SD) of triplicates of culture medium alone from three independent experiments.

Blocking Experiments with Anti-L3T4 or Anti-I-A^k mAbs. Suspensions of ≈2 × 10^7 CD4 T cells per ml were incubated with 10 μg of anti-L3T4 mAb per ml for 1 hr at 4°C; this was followed by washing thrice in PBS containing 2% fetal bovine serum and then suspensions were used in the proliferation assay described above with SCICs as stimulators. Suspensions of ≈5 × 10^4 SCICs per ml were incubated with 50 μg of anti-I-A^k mAb per ml for 1 hr at 4°C; this was followed by washing thrice in PBS containing 2% fetal bovine serum and then suspensions were used in the proliferation assay described above with CD4 T cells as responders.

Effect of rMuINF-γ on the Expression of I-A^k Antigen on SCICs. SCICs were prepared from overnight cultured islets isolated from 10-week-old male NOD mice and cultured with control or rMuINF-γ medium (10 ng/ml) for 24 hr. Expression of I-A^k antigen was analyzed by flow cytometry as described above. In a second set of experiments islets were isolated from insulitis-free 4-week-old NOD mice and cultured with control or rMuINF-γ medium (10 ng/ml) for 5 days. Then SCICs were prepared and analyzed by flow cytometry for expression of I-A^k antigen.

Double-Indirect Immunofluorescence of SCICs. A double-labeling technique incorporating anti-insulin antibodies was used to investigate whether expression of I-A^k antigen was a property of beta cells. Ten to fifteen thousand SCICs per 50 μl were incubated with 4 μl of anti-I-A^k mAb (1:8 dilution) for 1 hr at 4°C. After washing in PBS containing 0.1% sodium azide, rhodamine-conjugated rabbit anti-mouse IgG (1:200 dilution, Dako, Santa Barbara, CA) was used as the second-step antibody. Surface-stained cells were then fixed in 4%

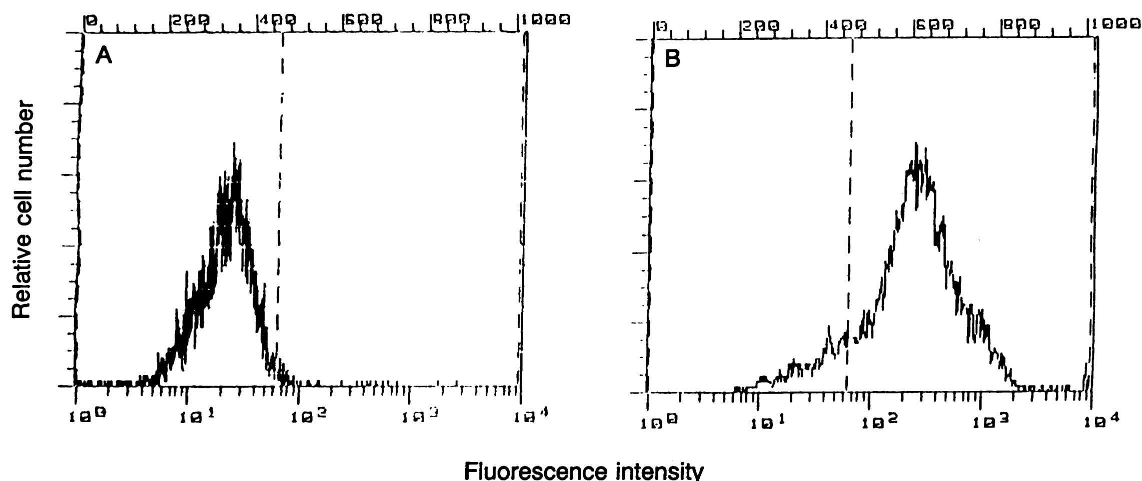

FIG. 1. Representative flow cytometric data showing ectopic expression of I-A^k antigens on SCICs prepared from 10-week-old male NOD mice. (*A*) Control staining of SCICs with FITC-conjugated goat anti-mouse IgG alone. (*B*) Incubation of SCICs with anti-I-A^k mAb followed by staining with FITC-conjugated goat anti-mouse IgG as the second-step antibody.

paraformaldehyde in 0.9% NaCl containing 20 mM Hepes and 1 mg of bovine serum albumin per ml (pH 7.4) for 30 min. After washing in PBS, aliquots of cell suspensions ($\approx 3 \times 10^3$) were cytocentrifuged onto glass slides and air-dried. Cell smears were treated with 50% ethanol for 15 min at −20°C and washed three times in PBS. Cells were then incubated with guinea-pig anti-human insulin antiserum (1:350 dilution, Dako) followed by FITC-conjugated rabbit anti-guinea-pig IgG. Staining was visualized through a fluorescence microscope using appropriate filters for fluorescein and rhodamine excitation and emission.

Autologous and Allogeneic Control Mixed Lymphocyte Reactions (MLRs). Autologous MLRs were performed in 96-well microtiter plates for 96 hr with 1.5×10^5 CD4 T cells as responders and 1.5×10^5 irradiated (2000 rads; 1 rad = 0.01 Gy) unfractionated splenic lymphoid cells as stimulators per well. Proliferative responses were assayed as described above. Allogeneic MLRs were performed exactly as described for autologous MLRs, but with irradiated (2000 rads) unfractionated splenic lymphoid cells as stimulators isolated from the spleens of C57BL/6J mice.

RESULTS

Flow Cytometric Analysis of Ectopic Expression of Class II MHC Molecules of SCICs. Fig. 1*A* shows representative data from flow cytometric analysis of 10-week-old male NOD mouse control SCICs after reaction with FITC-conjugated goat anti-mouse IgG alone. The data clearly demonstrate that nonspecific binding of control FITC antibody was acceptably low, indicating that macrophages and B lymphocytes were not present in the stimulator SCIC preparation. Identical control experiments (n = 11) performed with male and female SCICs confirmed this observation. Fig. 1*B* shows a representative flow cytometric analysis of SCICs from 10-week-old male NOD mice with anti-I-A^k mAb. From additional experiments the mean expression of I-A^k antigens by SCICs prepared from 10-week-old male and female NOD mouse islets was calculated to 84% ± 9% (n = 5) of total SCICs. It is worth noting that no significant difference in the expression of I-A^k antigens by SCICs between male and female NOD mice was found (data not shown). The expression of I-A^k antigens by SCICs prepared from 4-week-old NOD mice was 69% ± 11% (n = 3). Control anti-I-A^d mAb did not recognize an epitope expressed by SCICs. Unfractionated splenic lymphoid cells from NOD mouse spleens containing B lymphocytes and some macrophages with surface expression of class II MHC molecules were analyzed as positive controls for anti-I-A^k and anti-I-A^d mAbs. It was found that 33% (n = 2) of total lymphoid cells reacted with FITC-conjugated goat anti-mouse IgG alone. Ten percent (n = 2) of total lymphoid cells expressed surface I-A^k antigen. Surface expression of I-A^d antigen was not detectable when analyzed with control I-A^d mAb. In the additional positive control experiments it was found that 2.9% ± 0.5% (n = 3)

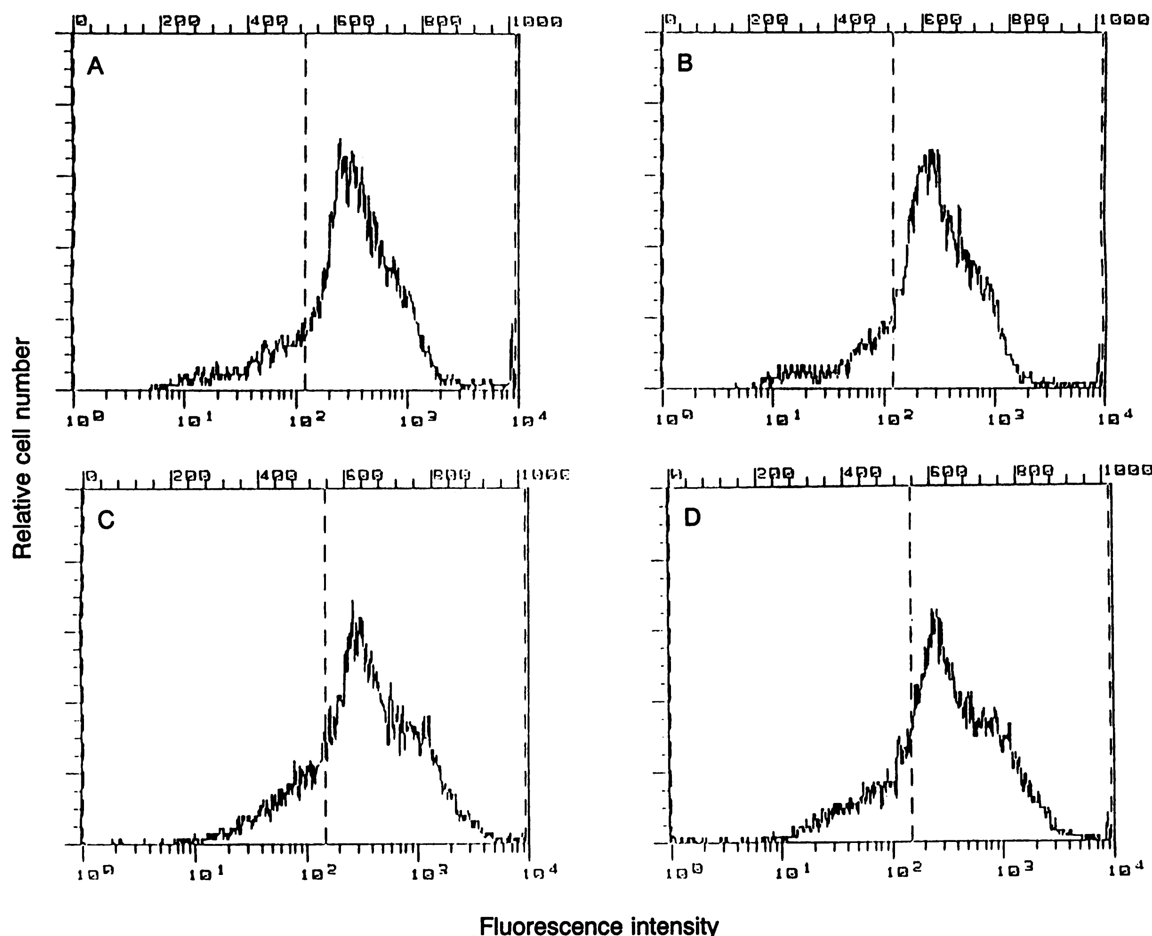

FIG. 2. Effect of rMuINF-γ on expression of I-A^k antigens by SCICs. (*A*) Representative fluorescence histogram showing expression of I-A^k antigens on SCICs prepared from 10-week-old male NOD mouse islets. SCICs were cultured in CM for 24 hr and then incubated with anti-I-A^k mAb followed by staining with FITC-conjugated goat anti-mouse IgG. (*B*) Identical experimental conditions as described in *A*, but SCICs were cultured in CM containing 10 ng of rMuINF-γ per ml. (*C*) Representative fluorescence histogram showing expression of I-A^k on SCICs prepared from 4-week-old NOD mice. Islets were cultured in CM for 5 days. SCICs were prepared and incubated with anti-I-A^k mAb followed by staining with FITC-conjugated goat anti-mouse IgG. (*D*) Identical experimental conditions as described in *C*, but islets were cultured in CM containing 10 ng of rMuINF-γ per ml.

of peritoneal macrophages after culture for 24 hr in medium alone expressed I-A^k antigens. However, after 24 hr of culture in the presence of rMuINF-γ (10 ng/ml), 14.6% ± 1.0% (n = 3, P = 0.005) of the peritoneal macrophages expressed I-A^k antigens. Control stainings with anti-I-A^d mAb were negative. There was no detectable fluorescence in control SCIC preparations for the antigen L3T4, Lyt-2, or Thy-1.2, indicating that T cells of these phenotypes were not contaminating the SCIC preparations.

Effect of rMuINF-γ on Expression of I-A^k Antigens by SCICs. SCICs prepared from islets isolated from 10-week-old male NOD mice were cultured in control or rMuINF-γ medium (10 ng/ml) for 24 hr. Representative fluorescence histograms shown in Fig. 2 *A* and *B* clearly demonstrate that rMuINF-γ did not enhance the expression of I-A^k antigens. Seventy-four percent ± 13% (n = 3) of total SCICs cultured with rMuINF-γ expressed I-A^k antigens versus 85% ± 3% (n = 3) of control SCICs. Fig. 2 *C* and *D* show representative fluorescence histograms of SCICs prepared from islets isolated from 4-week-old NOD mice after culture in control or rMuINF-γ medium (10 ng/ml) for 5 days. rMuINF-γ had no enhancing effect on the expression of I-A^k antigen. The percentage of total SCICs expressing I-A^k antigen after culture with rMuINF-γ was 78% (n = 2) versus 76% (n = 2) in control SCICs. Experiments with higher concentrations of rMuINF-γ were not performed. Of significant interest is the observation that SCICs prepared from 4-week-old NOD mouse islets aberrantly expressed I-A^k antigens. By histological examination we and others (7) have observed that young 4-week-old NOD mice do not have detectable lymphocytic infiltrate of the islets, suggesting that the expression of I-A^k antigen by SCICs is not a secondary phenomenon induced by infiltrating lymphocytes but precedes the invasion of them.

Double-Indirect Immunofluorescence of SCICs. A double-labeling technique incorporating anti-insulin antibodies was used to investigate whether the expression of I-A^k antigens was a property of beta cells. The double immunofluorescence study was performed with SCICs prepared from 10-week-old male NOD mice and revealed that I-A^k antigen-positive SCICs corresponded almost completely with cells whose cytoplasm stained with insulin antibodies (Fig. 3).

Cocultures of CD4 T Cells and SCICs. Fig. 4 summarizes data from coculture experiments. An ≈12-fold increase in proliferative response of CD4 T cells to stimulator SCICs prepared from male NOD mouse islets was found in comparison with that obtained with CD4 T cells alone. The proliferative response was blocked 43% after pretreatment of responder CD4 T cells with anti-L3T4 mAb. A 60% blocking of proliferative response was obtained after pretreatment of stimulator SCICs with anti-I-A^k mAb. Basal SCIC uptake of [^{3}H]thymidine was insignificant. No proliferative responses were observed in control cocultures with CD4 T cells and SCICs isolated from diabetes-nonsusceptible C57BL/6J mice. In autologous MLRs a significant but much lower proliferative response of CD4 T cells to irradiated splenic lymphoid cells was found in comparison with the response obtained with SCICs as stimulators. In allogeneic MLRs CD4 T cells failed to respond since the increase in proliferative response of CD4 T cells (NOD) to irradiated splenic lymphoid cells (C57) as stimulators was not statistically significant, suggesting that the CD4 T cells isolated from diabetic spleens are autoreactive self-class II MHC restricted.

DISCUSSION

The data reported here provide evidence that I-A^k antigens ectopically expressed by 10-week-old male NOD mouse

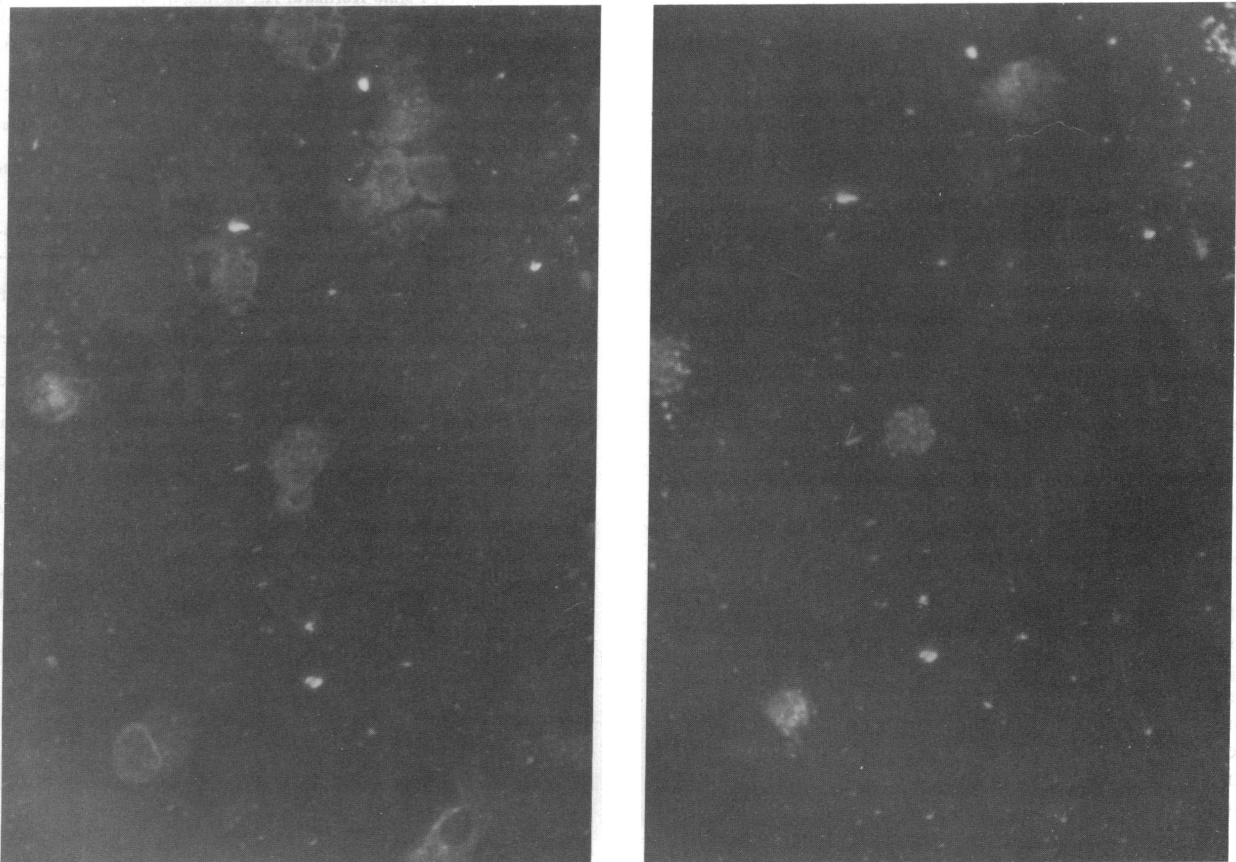

FIG. 3. Double-indirect immunofluorescence staining of SCICs prepared from 10-week-old male NOD mice for insulin (*Left*) or I-A^k antigen (*Right*).

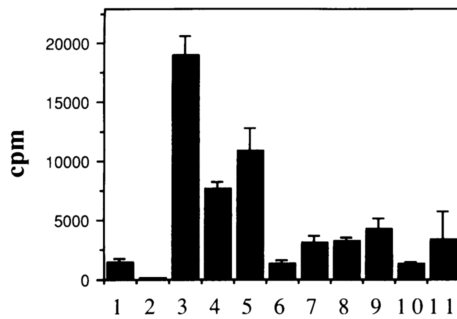

FIG. 4. Proliferative responses of CD4 T cells isolated from the spleens of diabetic NOD mice to either SCICs prepared from 10-week-old male NOD mouse islets or irradiated unfractionated splenic lymphoid cells isolated from autologous NOD mice or allogeneic C57BL/6J mice. Proliferative responses were quantified after 96 hr of coculture by [^{3}H]thymidine uptake for an additional 16 hr. Each bar represents the mean ± SD of three independent experiments. Bars: 1, CD4 T cells alone; 2, SCICs alone; 3, CD4 T cells plus SCICs; 4, CD4 T cells plus SCICs pretreated with anti-I-A^k mAb; 5, CD4 T cells pretreated with anti-L3T4 mAb plus SCICs; 6, CD4 T cells plus intact pancreatic islets; 7, CD4 T cells from control C57BL/6J mice; 8, CD4 T cells and SCICs from control C57BL/6J mice; 9, CD4 T cells from NOD mice plus irradiated splenic lymphoid cells from NOD mouse spleens; 10, same as bar 1; 11, CD4 T cells from diabetic NOD mouse spleens plus irradiated splenic lymphoid cells from allogeneic C57BL/6J mice.

SCICs interact *in vitro* with class II MHC-restricted CD4 T cells isolated from overtly diabetic female NOD mouse spleens. The interaction contributes signals leading to CD4 T-cell proliferation. Results from flow cytometric analyses of SCICs prepared from islets isolated from 10-week-old male or female NOD mice or from NOD mice immediately after weaning (i.e., 4 weeks of age) demonstrated aberrant expression of class II MHC antigens, which suggests the existence of a potential pathogenic triggering mechanism to activate autoimmune T cells. This was demonstrated in cocultures of primed CD4 T cells isolated from overtly diabetic spleens and SCICs prepared from 10-week-old male NOD islets, where CD4 T cells displayed a 12-fold increase in proliferative response. Blocking experiments with anti-L3T4 or anti-I-A^k mAbs indicated that CD4 and class II MHC molecules functionally contribute to signals leading to CD4 T-cell activation. We did not observe any immune responses in control cocultures with target and responder cells isolated from diabetes-nonsusceptible C57BL/6J mice.

In questioning the specificity of CD4 T-cell stimulation by SCICs, data from control autologous and allogeneic MLRs taken together with data from coculture experiments suggest that CD4 T cells isolated from diabetic spleens and presumably islet-derived are autoreactive self-class II MHC restricted. Additional evidence for the important role of CD4 T cells in the pathogenesis of insulitis in NOD mice is (*i*) insulitis induced by adoptive transfer of CD4 T cells in T-cell-depleted NOD mice (22) and (*ii*) the ability to completely prevent onset diabetes in NOD mice by early prophylactic treatment with anti-CD4 mAbs (23, 24).

A problem with SCICs prepared from pancreatic islets is the heterogeneity of the cell population even though ≈65% of the single cells in an islet are beta cells (25). Our double-indirect immunofluorescence demonstrated that beta cells identified by cytoplasm whose staining with anti-insulin antibodies exhibited a marked staining for I-A^k antigens. Data from control flow cytometric analyses made it possible to conclude that macrophages, B lymphocytes, and T cells did not contaminate the SCIC preparations.

Of particular interest is our observation that class II MHC molecules were equally expressed by SCICs from both sexes. Successful adoptive transfer of overt diabetes by injection of spleen cells from diabetic NOD mice has been reported in young female and male preirradiated NOD recipients (26–28). The work presented here suggests that such disease transfer is possible because SCICs in male and female recipients *a priori* aberrantly express class II MHC molecules, hence engendering an autoimmune attack by the adoptive transferred autologous class II MHC-restricted T cells.

Note. After this manuscript was submitted, we obtained data identical to those shown in Fig. 1 by using two alloantisera—i.e., anti-I-A^{NOD} (AS-3) demonstrated by immunoprecipitation to be specific for NOD I-A (29), kindly provided by D. Serreze (The Jackson Laboratory) and A.TH anti-A.TL (I-A^s anti-I-A^k, broadly crossreactive with most standard class II haplotypes except s), purchased from Accurate Chemicals (Westbury, NY).

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