Clinical Modeling of T Cell Vaccination against Autoimmune Disease in Rats

Selection of Antigen-specific T Cells Using a Mitogen

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Abstract

Effective T cell vaccination against experimental autoimmune diseases involves treatment with activated, autoimmune T lymphocytes. The present study was undertaken to learn whether antigen-specific T cells present in low frequency could be selected in vitro without using the specific antigen. The rat models of adjuvant arthritis and experimental autoimmune encephalomyelitis were investigated using proliferation assays and limiting dilution techniques to quantify the changes in reactivity of a heterogenous population of lymphocytes to the relevant antigen. Stimulation with concanavalin A for 2 d and then culture in IL-2–containing medium led to a substantial increase in the activity and frequency of the specific autoimmune T cells.

Enrichment of antigen-specific T cells could be demonstrated using lymph node, spleen, or peripheral blood lymphocytes, from rats late in the course of disease. The effect was not evident in lymphocytes from the thymus.

These results are relevant to the clinical application of T cell vaccination and to investigation of self-antigens in autoimmune disease. (*J. Clin. Invest.* 1990. 85:1594–1598.) autoimmune disease • T cell activation • concanavalin A • interleukin 2 • T cell vaccination

Introduction

Resistance to experimental autoimmune diseases can be induced by treating rats or mice with lines or clones of activated, autoimmune T lymphocytes that have been rendered avirulent, a procedure termed T cell vaccination (1–5). Such vaccination has been found to induce remission of established disease (4), as well as to protect naive animals from a subsequent attempt to induce the disease (1–4). T cell vaccination generates specific antiidiotypic T-helper and T-suppressor cells recognizing the autoimmune clones (6), along with T cells that appear to respond to activated, syngeneic T cells, which we have termed antiergotypic T cells (7). However, the antiidiotypic response seems to be more effective than the antiergotypic response in regulating the disease process (7).

To facilitate the induction of an efficacious antiidiotypic response, it would be desirable for vaccine preparation to enrich a heterogeneous population of T cells with the relevant anti-self idiotypes involved in the disease. The problem posed by many human autoimmune diseases is how to select the autoimmune effector cells from other T cells when the specific autoantigen is unknown and thus cannot be used to stimulate the specific T cells.

It is generally believed that polyclonal T cell mitogens such as Con A activate helper T cells irrespective of their antigen specificity (8). Nevertheless, specific T cell vaccines could be made using Con A to activate populations of lymph node cells from rats suffering from experimental autoimmune encephalomyelitis (EAE) or adjuvant arthritis (AA)[1] (4). Such lymph node cell populations were likely to contain a minority of antigen-specific autoimmune T cells. Could Con A have a bias for the specific autoimmune T cells responsible for the autoimmune disease and required for specific vaccination?

Our strategy here was to immunize rats with myelin basic protein (BP) to induce EAE or *Mycobacterium tuberculosis* (MT) antigen to induce AA, and then to study the effect of Con A and growth factor stimulation in vitro on the relatively low numbers of antigen-specific T cells present in the spleen or lymph nodes late in disease. We now report that incubation with Con A does indeed augment markedly the activity and the frequency of antigen-specific T cells. From a fundamental point of view this finding indicates that the response to Con A can discriminate previously activated T cells from naive T cells and hence can be used to amplify the proportion of antigen-specific T cells. From a practical point of view, Con A can be used to prepare effective T cell vaccines in situations where the specific antigen is unknown or unavailable for selective activation.

Methods

Rats. Inbred Lewis rats were supplied by the Animal Breeding Center of this Institute and were used at 2–3 mo of age. Rats were matched for age and sex in each experiment.

Antigens. MT H37Ra was purchased from Difco Laboratories, Inc., Detroit, MI. BP was prepared as described (9) from the spinal cords of guinea pigs, without the step of purification by column chromatography.

Immunization. AA was induced by intradermal injection at the base of the tail with 0.1 ml of incomplete Freund's adjuvant (Difco Laboratories, Inc.) to which was added 1 mg of MT. The disease appeared on days 13–15 and lasted for several months. EAE was induced by injecting both hind foot pads with 0.05 ml containing 25 μg of BP and 200 μg MT emulsified in equal volumes of incomplete Freund's adjuvant and phosphate-buffered saline.

Antigen-specific T cell proliferation assay. When the peak of T cell responsiveness had passed late after primary immunization, rats were

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1. *Abbreviations used in this paper:* AA, adjuvant arthritis; BP, basic protein; EAE, experimental autoimmune encephalomyelitis; MLR, mixed lymphocyte reaction; MT, *Mycobacterium tuberculosis.*

killed and suspensions of lymphocytes were prepared by pressing lymph nodes, spleens, or thymuses through a fine wire mesh. Peripheral blood lymphocytes were collected using a Ficoll Hypaque gradient. Red blood cells from the spleen were lysed using 2 ml of a solution of 9 vol 0.83% NH_4Cl to 1 vol 0.17% M Tris buffer (pH 7.2) for 5 min at room temperature. To detect enhancement of T cell responses, 10^5 cells per well, less than an optimal number, were seeded in round-bottom microtiter wells (Greiner, Nürtingen, FRG). T cells to be tested for responses to antigens after stimulation with Con A, were plated at 10^5 per well with 5×10^5 irradiated (2,500 R) thymocytes as accessory cells. All proliferation assays were performed in Dulbecco's modified Eagle's medium supplemented with 2 mercaptoethanol (5×10^{-5} M) L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), nonessential amino acids (1 ml/100 ml; BioLab, Jerusalem, Israel) and 1% autologous Lewis rat serum. The antigens, MT or BP, were added to the proliferation assays at 10 μg/ml. Mixed lymphocyte reaction (MLR) assays were performed using 10^5 irradiated (2,500 R) allogeneic Brown Norway (BN) rat splenocytes and 10^5 responding Lewis rat lymphocytes. The cultures were incubated in triplicate for 72 h at 37°C in humidified air containing 5% CO_2. Each well was pulsed with 1 μCi of [^{3}H]-thymidine (10 Ci/mmol sp act; Nuclear Research, Negev, Israel) for the final 18 h. The cultures were then harvested on fiberglass filters and the proliferative response expressed as counts per minute (cpm), delta cpm (mean cpm of test cultures minus mean cpm of control cultures without antigen)±SD or as stimulation index (mean cpm test cultures divided by mean cpm control cultures).

Con A cultures. Lymphoid cells were cultured in proliferation medium (PBL at 5×10^6/ml, lymph node or spleen at 7.5×10^6/ml) in 10-ml plates with Con A at a final concentration of 1.2 μg/ml (Miles-Yeda, Rehovot, Israel). After 2 d, the cells were collected, washed, and cultured for a resting phase in resting medium (identical to the proliferation medium, but without autologous serum) to which was added 10% horse serum (Gibco Laboratories, Grand Island, NY) and 15% supernatant of Con A–stimulated splenocytes as a source of T cell growth factors prepared as described (1). After 5 d of culture in the resting medium, the cells were collected and either tested for antigen-specific proliferation or cultured for additional cycles of Con A (2 d) followed by resting medium (5 d). These cultures were done at cell concentrations of 10^6/ml T cells and 1.2×10^7/ml irradiated (2,500 R) thymocytes as accessory cells. All cultures were kept at 37°C with humidified air and 5% CO_2. Each experiment consisted of two to five such cycles of Con A stimulation and rest, from which samples were investigated for antigen-specific proliferation at the end of the rest phase.

Limiting dilution analysis. Lymph node cells were seeded in 96-well, round-bottom microtiter plates at decreasing concentrations from 4×10^4 to 1 cell per well, in 200 μl resting medium. Irradiated thymocytes were added as feeder cells (10^5 per well). Cultures were performed in the absence or presence of MT (10 μg/ml). 24 wells were seeded at each cell concentration. Plates were incubated for 7 d in 5% CO_2 at 37°C and [^{3}H]thymidine uptake was measured as in proliferation assays. In addition to measuring thymidine incorporation, each well was observed on day 7 for cell growth. A well was considered to be positive if there was a visible cellular aggregation and thymidine uptake exceeded the mean background cpm of cells cultured without antigens by 2 SD. Percent-negative wells was plotted semilogarythmically against the number of cells seeded in the wells. The number of cells per well producing 37% negative wells was read from the plot and this number was considered to represent the frequency of the T cells responding to the antigen (10).

Results

Culture with IL-2 does not select for antigen-specific T cells. As pathogenic effector T cells in the act of causing autoimmune disease were likely to be activated and express IL-2 receptors, we attempted to select MT-responsive T cells from rats with AA by culturing their lymphoid cells with IL-2. However, this strategy failed to generate specific anti-MT T cells, and it is possible that the relevant cells did not express IL-2 receptors (11).

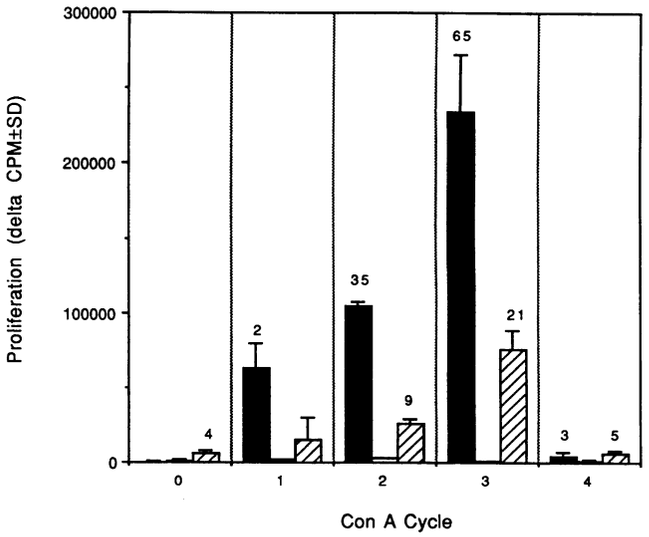

Figure 1. Con A culture cycles amplify the anti-MT proliferation of spleen cells from AA rats. Spleen cells were obtained from five rats 25 d after induction of AA. The proliferation responses were measured as delta cpm to MT (*closed bars*), BP (*open bars*), or to allogeneic BN stimulator cells (*hatched bars*) before or after cycles of culture with Con A followed by resting medium. The numbers above the bars designate stimulation indices of 2 or greater.

Antigen-specific T cells are selected by culturing with Con A. Stimulation of T cells by mitogens or antigens was found to induce the expression of IL-2 receptors by those cells (12). Therefore, we examined the possibility that in vitro mitogen stimulation and then culture in IL-2 might lead to the selection of antigen-specific T cells. Figs. 1 and 2 show the results of the proliferation assays to MT, BP, or allogenic cells of lympho-

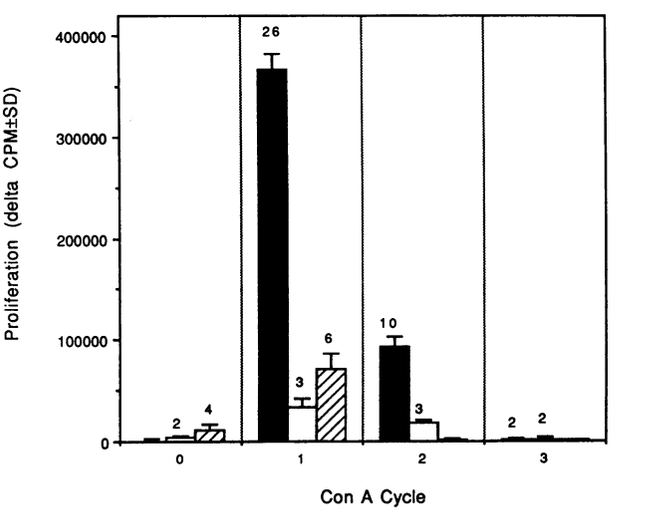

Figure 2. Con A culture cycle amplifies the lymph node response to MT. The experiment was done as described in the legend to Fig. 1. The stimulators were MT (*closed bars*), BP (*open bars*) or BN allogeneic cells (*hatched bars*).

cytes that had been so treated. In Fig. 1, the cells were from the spleen of a rat suffering from AA 25 d after induction of disease with MT. At this period, the rats were beyond their peak proliferative response to MT (around day 10) and as expected there was no detectable response culturing a relatively low number of cells (10^5) for only 72 h. However, it can be seen that the reactivity to MT increased progressively during three cycles of Con A stimulation to reach a peak of 234,000 cpm or a stimulation index of 65 after the third cycle. The reactivity to MT was markedly reduced after subsequent cycles. MLR alloreactivity was also tested in the same experiment. The initial reactivity was low (stimulation index of 4) as expected for 10^5 cells cultured for only 72 h. However, as a consequence of cycles of stimulation with Con A, MLR reactivity increased and decreased in parallel to reactivity to MT. Fig. 2 shows the results of the proliferations from lymph node cells. In contrast to spleen cells that peaked after three cycles, lymph node cells manifested their maximum reactivity to MT after the first Con A stimulation (stimulation index 26, delta cpm 366,000). Decreased reactivity to MT was observed after subsequent cycles of Con A stimulation. Cycles of Con A stimulation induced no increased reactivity to a control antigen, BP, to which the rats had not been immunized.

Increase in specific reactivity is accompanied by an increase in the frequency of antigen-specific T cells. To analyze the increase in antigen reactivity after Con A stimulation, we used a limiting dilution analysis to quantitate the frequency of the antigen-reactive cells at different stages during the procedure. Fig. 3 shows the results of studying popliteal lymph node cells obtained from rats 42 d after induction of AA. The calculated frequency of the MT-reactive cells upon removal from the animals was 1:1,025. This low frequency explains the initial low response to 72 h of culture: only 100 MT-specific T cells would be expected to be present in the population of 10^5 cells. After 2 d of Con A stimulation, the frequency of the cells reactive to the MT increased to 1:125. After 5 d in resting medium, the frequency of anti-MT cells increased further to 1:25. At this frequency, 4,000 antigen-specific T cells would be present in 10^5 cells. Thus, the increase in reactivity to MT demonstrated in the bulk cultures could be attributed to an increase in the frequency of T cells responsive to the antigen. Experiments mixing initial populations of cells with T cells selected in Con A culture failed to detect active suppression (not shown).

Con A selects for BP-reactive T cells after EAE. Fig. 4 shows the results of proliferations taken from rats that had recovered spontaneously from EAE after induction of disease by immunization to BP in adjuvant containing MT. We assayed lymph node, spleen, and thymus cells obtained 44 d

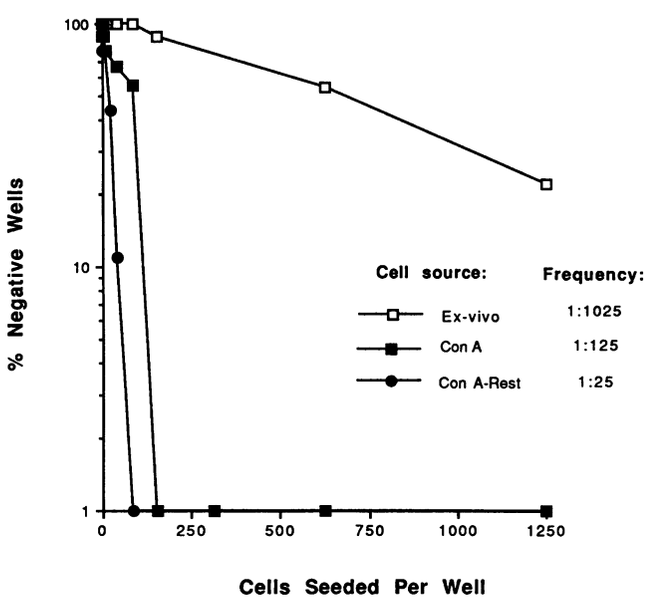

Figure 3. Limiting dilution analysis of frequency of anti-MT T cells. Lymph node cells were studied from a rat in whom AA had been induced by administration of MT, 42 d later. The percent negative wells was plotted as a function of cell number before culture with Con A (*open squares*), after 48 h of culture with Con A (*closed squares*), or after 48 h of culture with Con A followed by 5 d in resting medium (*closed circles*). The frequency of anti-MT T cells was computed to be ~ 1:1,025 before culture, ~ 1:125 after culture with Con A, and ~ 1:25 after five additional days in resting medium.

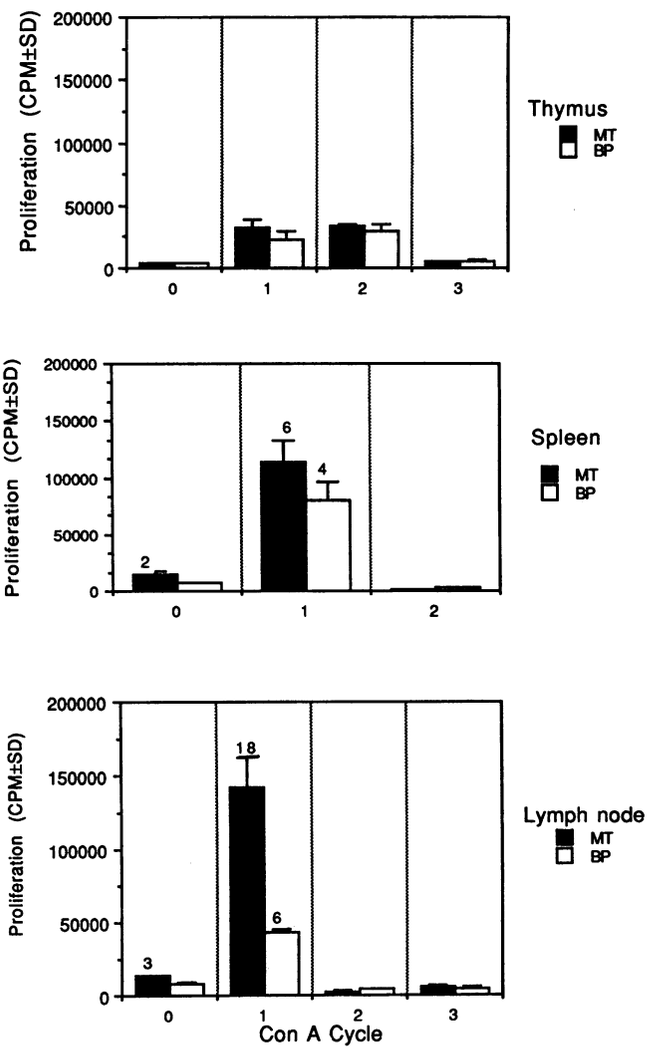

Figure 4. A Con A cycle amplifies reactivity to BP after EAE. 44 d after induction of EAE in three rats with BP in complete Freund's adjuvant, lymph node, spleen, and thymus cells were tested for reactivity (delta cpm) to BP (*open bars*) or MT (*closed bars*) before or after Con A cycles. The numbers above the bars indicate stimulation indices of 2 or greater.

after induction of EAE, a period long after the peak of anti-BP activity associated with EAE. The largest increase in reactivity to both MT and BP was seen in lymph node cells; spleen cells showed a parallel, albeit smaller increase in reactivity. The thymocytes taken from the same rats did not show any change in reactivity after three cycles of Con A stimulation.

Because circulating peripheral blood lymphocytes are a readily accessible source of cells in human patients, we stimulated with Con A peripheral blood lymphocytes from rats with AA and found a significant enrichment of MT-reactive T cells similar in magnitude to that obtained from lymph node populations (data not shown).

Fig. 5 shows the results of subjecting splenocytes from naive rats to the Con A stimulation protocol. Although treatment with Con A induced an increase in the background proliferations for two cycles, there was no increase in reactivity to either MT or BP above the background. Thus, selection of T cells reactive to antigen required that the rat be immunized to that antigen.

Discussion

The experiments presented here indicate that all T cells in a mixed population do not respond equally well to the T cell mitogen Con A; T cells previously activated by antigen in vivo seem to enjoy a selective advantage. Beyond the realm of basic information, this observation could be useful in dealing with T cells of clinical interest which are at a low frequency and for which the specific antigens are lacking.

In AA, using cells from spleens or lymph nodes of rats well after their peak responses to antigen, we were able to increase considerably the reactivity to the MT antigen. Analysis of the frequency of the MT-responding T cells using a limiting dilution technique showed that after 2 d of Con A stimulation, the anti-MT cell frequency was considerably increased (about eightfold) and 5 d of culture in resting medium increased the frequency of these T cells even further about (~ 40-fold). Application of the same protocol to lymph node cells from rats convalescent from EAE showed a marked increase in anti-BP reactivity.

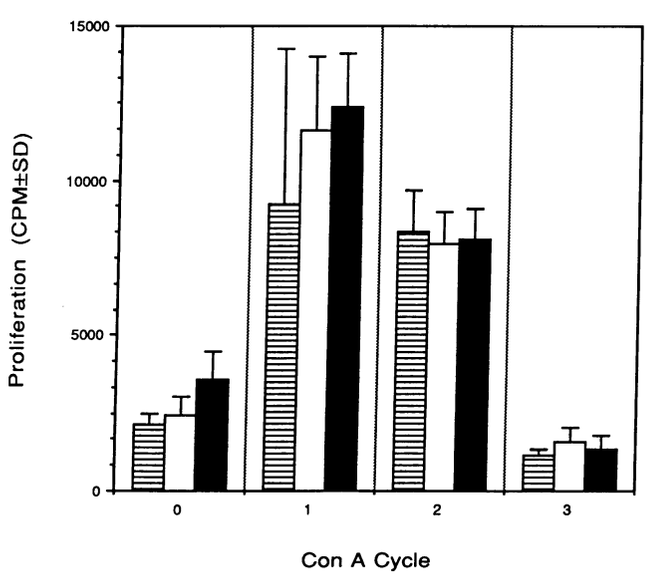

Figure 5. Con A cycles do not enhance antigen reactivity of spleen cells from naive rats. Spleen cells from three naive rats were tested before or after Con A cycles for their reactivity (cpm) without added antigen (*horizontally lined bars*), or with MT (*closed bars*) or BP (*open bars*). All the stimulation indices were ~ 1.

There was no augmented response to antigens to which the cell donors had not been primed in vivo. However, the increase in responsiveness to the specific priming antigens was accompanied by an increase in responsiveness to allogeneic stimulator cells detected by a mixed lymphocyte reaction. The mixed lymphocyte reaction in vitro is attributed to a naturally high frequency of reactive T cells and does not require additional specific priming in vivo. Thus, mixed lymphocyte-reactive T cells may be viewed as primed without experimental intervention.

What is the mechanism of T cell enrichment? Cantrell and Smith detected a decay in the amount of IL-2 receptor on T cells upon removal of the cells from activating lectins or antigens, followed by a rapid reappearance of IL-2 receptor upon restimulation (12). The accelerated kinetics of the expression of IL-2 receptor were also observed when in vivo-activated T cells were secondarily activated in vitro. Accelerated reexpression of the IL-2 receptors of primed T cells upon Con A stimulation in vitro could lead to earlier cell division of the antigen-specific cells.

This hypothetical mechanism is plausible since it could also explain the fact that the enrichment is transient. It is conceivable that the relative advantage of the primed cells seen in the early stimulations is subsequently lost because in later stimulations the other cells are no longer naive and reexpress IL-2 receptors as rapidly as the antigen-primed cells.

The hypothesis could account for the observation that shorter Con A stimulations are more effective in selection. The optimal period of culture in Con A to obtain an increase in antigen reactivity was found to be 1–2 d, whereas the optimal culture period for cell multiplication of unprimed cells is 3–4 d (data not shown). It is conceivable that the decrease in stimulation indices upon prolonged stimulation is caused by diluting the antigen-specific population as more cells enter the division phase.

Hedlund and associates examined the kinetics of the response to Con A of CD45R-positive and CD45R-negative CD4 rat T lymphocytes (13). They found that memory T lymphocytes (CD45R-negative) responded more rapidly to Con A than did naive T lymphocytes as reflected by phenotypic conversion, IL-2 production, and proliferation. In a similar study, Byrne and co-workers compared the activation requirements of naive and memory T cells in humans and found that only the primed population (CD45R-negative, UCHL1-positive) responded to anti-CD3 antibody (14).

The population of activated T cells present in an individual suffering from a severe autoimmune disease is likely to include the disease-causing autoimmune T cells. The results presented here indicate that the relative frequency of these cells can be markedly increased by culturing the individual's lymphoid cells with a T cell mitogen for a brief period of time. This strategy was shown to be useful in developing T cell vaccines for rats suffering from active AA, effective in treating other arthritic rats (15). Recently we have succeeded in treating the spontaneous autoimmune diabetes of 5–6-wk-old nonobese diabetic mice with T cell vaccines prepared from the spleens of 3-mo-old prediabetic mice using Con A–activated T cells (16).

As in human type-1 diabetes, the specific antigen in nonobese diabetic mouse diabetes has yet to be identified. Thus, effective T cell vaccination does not require the use of a specific antigen for vaccine preparation.

Irrespective of mechanism, the observation that in vitro enrichment of antigen-primed T cells can be achieved without use of specific antigen is of importance in planning attempts to identify relevant antigens in human autoimmune diseases and as an aid to developing protocols for T cell vaccination therapy of autoimmune diseases such as rheumatoid arthritis or multiple sclerosis (17).

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