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Brazilin Augments Cellular Immunity in Multiple Low Dose Streptozotocin (MLD-STZ) Induced Type I Diabetic Mice

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Brazilin, an active principle of *Caesalprenia sappan*, was examined for its immunopotentiating effects in multiple low dose streptozotocin (MLD-STZ) induced type diabetic mice. Brazilin was intraperitoneally administered for 5 consecutive days to MLD-STZ induced type I diabetic mice. Delayed type hypersensitivity, Con A-induced proliferation of splenocytes and mixed lymphocyte reaction, which had been decreased in diabetic mice, were significantly recovered by the administration of brazilin. Brazilin increased IL-2 production without affecting suppressor cell activity. Con A-induced and IL-2-induced expression of high affinity IL-2 receptors were also enhanced by brazilin. These results indicate that brazilin augments cellular immune responses, which are suppressed in the MLD-STZ induced type I diabetic mice, by increasing IL-2 production and responsiveness of immune cells to IL-2.

Key words: Brazilin, Immunomodulating agents, Type I diabetes, Delayed type hypersensitivity, Mixed lymphocyte reaction, Proliferation, Splenocytes, IL-2

INTRODUCTION

Brazilin, an active principle of *Caesalpenia sappan*, has been found to have immunomodulating activities; brazilin increased delayed type hypersensitivity in normal mice and also improved the impaired DTH and mitogen-induced lymphocytes proliferation by augmenting IL-2 production in high dose SRBC-induced immune tolerant mice (Mock *et al.*, 1998). In addition, brazilin was found to modulate the altered immune functions in early phase of halothane intoxication (Choi *et al.*, 1997).

Diabetic subjects are highly susceptible to microbial and fungal infections. The susceptibility of diabetic subjects to the infections seems to be concerned with defects in cellular immunity. Mencacci *et al.* reported that a perturbation in the anticandidal T helper responses resulting in the induction of a biased Th2-like antifungal response renders diabetic mice highly susceptible to systemic C. albicans infection (Mencacci *et al.*, 1993). The development of fatal disseminated candidiasis is correlated with the detection of a strong Th2 response, while protective antifungal immunity is associated with a predominant Th1 response (Jones-Carson *et al.*, 2000). Thus, control of balance between Th1 immune response and Th2 immune response may recover the host resistance to infections in diabetic subject (Mencacci *et al.*, 1993). Likewise, recovery of suppressed immune responses as well as control of blood glucose levels is important for healthy life in diabetic subjects. The facts mentioned above motivated us to investigate whether brazilin could improve the impaired immune functions in type I diabetes induced by the administration of multiple low dose streptozotocin (MLD-STZ).

Streptozotocin (STZ) is a toxin derived from Streptomyces acromogenes consisting of a glucose molecule with an alkylating nitrosurea moiety. Administration MLD-STZ to susceptible strains of mice can induce diabetes that resembles in many aspects the spontaneous murine model of diabetes as well as the human type I diabetes mellitus. Development of STZ-induced diabetes is dependent upon the sex and strain of mouse and also the dose and frequency of administration. Since a bolus dose of STZ (~250 mg/kg) is extremely toxic to pancreatic beta cells and induces fulminant diabetes, a multiple dosing regimen has been developed which is characterized by minimal beta cell toxicity and an essential autoimmune T cell component of disease (Steven *et al.*, 1999).

The present study was designed to determine the effects of brazilin on cellular immunity in MLD-STZ induced type I diabetes. We investigated effects of brazilin on delayed type hypersensitivity in mice, Con A induced prolife-

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ration of splenocytes and mixed lymphocyte reaction. And then we also investigated the following parameters to elucidate the immunomodulation-mechanisms; Con A induced IL-2 production from splenocytes, Con A induced expression of high affinity IL-2 receptors on splenocytes, IL-2 induced proliferation of splenocytes and suppressor cell activity which is known to reduce IL-2 production in splenocytes.

MATERIALS AND METHODS

Experimental animals

C57BL/6 mice (Korea Animal Center Co., Korea), 6-7 weeks of age (21-25 g body weight), were used for all studies. Animals were housed in suspended cages and provided rodent chow and water *ad labium*. The environment was maintained at 22°C ± 2 with a 12 hr light/dark cycle.

Induction of type I diabetes

C57BL/6 male mice were given intraperitoneally 50 mg/kg streptozotocin (STZ, Sigma, S-0130) once a day for 5 consecutive days. STZ was dissolved in sodium citrate buffer (pH 4.5) just before use. Two weeks after the first injection of STZ, all of the mice were subjected to determination of blood glucose level. Blood samples were obtained from the retro-orbital venous plexus of non-fasted mice using heparinized hematocrit capillary tubes between AM 09:00 and 11:00. After centrifugation, serum glucose concentrations were assayed by the glucose-oxidase method.

Adminstration of brazilin and cyclosporin A

Fifty milligram brazilin monohydrate (Aldrich, 27687-1) was suspended in 10 ml saline and sonicated. Brazilin (50 mg/kgbw/day) was administered intraperitoneally for 7 consecutive days. As positive control, 40 mg/kg cyclosporin A (Sandoz) was intraperitoneally administered to the mice for 4 consecutive days.

Immunization for delayed type hypersensitivity (DTH) assay

At day 2 after administration of brazilin, 10^6 SRBC were intravenously injected into the tail vein of a mouse. At day 4 after the immunization, 10^8 SRBC were injected into one hind footpad of a mouse. Twenty four hrs later, DTH was assayed by measuring the increase in thickness of the footpad using micrometer (Mitsutoyo, Japan). The specific swelling of the footpad was calculated by subtracting the thickness of a saline-injected footpad from that of the antigen-injected footpad. All the measurements were conducted by the same individual.

Preparation of splenocytes suspension

Spleens were obtained from each treatment group. Spleens were dissociated into a single-cell suspension in culture medium. The culture medium was composed of RPMI 1640 medium (Sigma) supplemented with 100 μU/ml penicillin, 100 mg/ml streptomycin (Gibco), 0.2 mM sodium pyruvate (Sigma), 2 mM glutamine (Sigma), 10 mM HEPES (Sigma), 2 g/l sodium bicarbonate (Sigma), 1 mM non-essential amino acids (Gibco), and 50 μM 2-mercaptoethanol (Sigma) (This medium is hereafter referred to as K-0 medium). Cells were washed with K-0 medium 3 times and cell viability was then determined by tryphan blue exclusion test.

Assay of ConA induced proliferation of splenocytes

The proliferation of splenocytes responding to mitogenic stimuli was determined using the methodology described by Lombardi *et al.*, (1991) with slight modifications. Briefly, 50ml splenocyte suspension (8×10^6 cells/ml) and 100 μl K-10 medium (K medium supplemented with 10% FCS) were added in triplicate into a 96 well flat-bottomed plate (Falcon) and incubated with 50 μl Con A (40 μg/ml). Cells were incubated at 37°C in a humidified 5% CO_2 incubator for 24 hrs followed by the addition of ^{3}H-thymidine (0.5Ci per well, DuPon NEN) to each well. Cells were incubated for a further 24 hrs and then harvested with an automatic cell harvester (Skatron). ^{3}H-thymidine incorporation was determined by Liquid Scintillation Spectroscopy (LKB). Results were expressed as mean counts/minute SD of triplicate cultures

Assay of one way mixed lymphocyte reaction

The proliferation of splenocytes responding to alloantigenic stimuli was determined by a one-way mixed lymphocyte reaction (Lombadi *et al.*, 1991). Briefly, spleens were removed from 3 mice of each group. Splenocyte suspensions (8×10^6cells/ml) were prepared as described above. Splenocytes (8×10^6cells/ml), obtained from Bal b/c mice, were treated with 50 μg/ml MMC (Mitomycin C, Sigma) for 30 min at 37°C and used as stimulator cells. Splenocytes that were obtained from C57BL/6 mice of each group were used as responder cells. Fifty microliter of stimulator cells, 50 μl responder cells and 100 μl K-10 medium were added into a 96 well round-bottomed plate, followed by incubation for 96 hrs at 37°C. ^{3}H-thymidine (0.5 μCi per well, DuPon NEN) was added into each well and the cells were incubated for a further 24 hrs. Cells were then harvested and subjected to liquid scintillation counting. Results were expressed as mean counts/minute ± SD of triplicate cultures.

Assay of ConA induced IL-2 production from splenocytes

Splenocytes from mice of each treatment group were incubated with 10mg/ml Con A in K-0 medium for 20 hrs at 37°C in 24 well plates. The contents of the plates were

then centrifuged, and the cell-free supernatants (Con A sups) were removed, and stored at -20°C until use. Levels of IL-2 in Con A sups from each group were analyzed quantitatively based on the ability to maintain the proliferation of CTLL-2 cells which proliferate via responding to IL-2. CTLL-2 cells, cloned cytolytic T cells derived from the C57BL/6 mouse, were purchased form Korean Cell Line Bank (Seoul, Korea). Cells were cultured in a growth medium (K-10) supplemented with supernatant containing IL-2. The cells were subcultured at 3 to 4 day intervals. Cells in the log phase of growth (day 3) were harvested, washed 3 times with K-0 medium, and suspended to 5×10^5 cells/ml in K-10 medium. A 50 μl cell suspension, 50 μl standard IL-2 or Con A sups from each group and 100 μl K-10 medium were plated in a 96 well plate. Cells were incubated at 37°C in a humidified 5% CO_2 incubator for 24 hrs and ^{3}H-thymidine (0.5 μCi per well, DuPon NEN) was added to the medium in each well. Cells were incubated for a further 24 hrs and then harvested and subjected to liquid scintillation counting. Results were expressed as mean counts/minute ± SD of triplicate cultures.

Assay of IL-2 induced proliferation of resting splenocytes

Fifty-microliter splenocyte suspension (8×10^6 cells/ml) and 100ml K-10 were plated in triplicate in a 96 well flat-bottomed plate (Falcon) and incubated with 50 μl r-IL-2 (4 ng/ml). Cells were incubated at 37°C in a humidified 5% CO_2 incubator for 96 hrs and ^{3}H-thymidine (0.5 μCi per well, DuPon NEN) was added to the medium in each well, after which, the incubation was continued for a further 24 hrs. Cells were harvested and subjected to liquid scintillation counting. Results were expressed as mean counts/minute ± SD of triplicate cultures

Assay of IL-2 induced proliferation of ConA-activated splenocytes

Two hundred-microliter splenocyte suspension (8×10^6 cells/ml), 50 μl Con A (600 μg/ml) and 750ml K-10 medium were plated in duplicate in 24 well plate (Falcon). Cells were incubated at 37 in a humidified 5% CO_2 incubator for 72 hrs. Cells were washed with K-10M (K-10 medium supplemented with 100 mM α-methylmannoside (Sigma)) and adjusted to 1×10^6 cells/ml. Fifty ml splenocyte suspension and 100 μl of K-10 media were plated in triplicate in a 96 well flat-bottomed plate (Falcon) and incubated with 50 μl r-IL-2 (4 ng/ml). Cells were incubated at 37°C in a humidified 5% CO_2 incubator for 24 hrs and ^{3}H-thymidine (0.5 μCi per well, DuPon NEN) was added to the medium in each well. Cells were incubated for further 24 hrs. Cells were then harvested and subjected to liquid scintillation counting. Results were expressed as mean counts/minute ± SD of triplicate cultures.

Assay of non-specific suppressor cell activity

Spleens were removed from 3 mice of each group and pooled. Splenocytes suspensions (8×10^6cells/ml) were prepared as described above and were incubated with 50 μg/ml MMC (Mitomycin C, Sigma) for 30 min at 37°C to use as suppressor cells. Splenocytes (8×10^6cells/ml) obtained from Balb/c mice were incubated with 50 μg/ml MMC (Mitomycin C, Sigma) for 30 min at 37 and used as stimulator cells. Splenocytes obtained from normal C57BL/6 mice were used as responder cells. Fifty l stimulator cells, 50 μl responder cells, 50 μl suppressor cells and 50 μl K-10 medium were placed in 96 well round-bottomed plate and incubated for 96 hours at 37°C. ^{3}H-thymidine (0.5 μCi per well, DuPon NEN) were added to the medium of each well and the cultures were incubated for further 24 hrs. Cells were harvested and subjected to liquid scintillation counting. Results were expressed as mean counts/minute ± SD of triplicate cultures

Statistical analysis

The significance of the differences between the means was evaluated by Student's t-test. Values which differ from control over $P<0.05$ were considered as significant.

RESULTS AND DISCUSSION

There have been many studies examining the functional phenotype of T cells in human diabetic patients. Peripheral blood lymphocytes (PBL) from type I diabetic patients showed a reduced proliferative response to mitogen. Significantly, CD3-mediated signaling is impaired in PBL of Type I diabetic patients and, in addition to poor proliferative responses to mitogen, this is manifested by low IL-2 secretion. Exposure of human patient T cells to either IL-2 or phorbol esters only partially reversed this defect, a finding similar to the NOD mouse model. The impaired ability to respond to mitogen activation in vitro is pronounced in human patient T cells in that PKC translocation and CD69 expression are also reduced (Steven *et al.*, 1999). These functional abnormalities of cellular immunity may lead the diabetic subjects to be susceptible to various infections. In MLD-STZ type I diabetic mice, DTH was found to be suppressed. Con A induced proliferation and mixed lymphocyte reaction of splenocytes obtained from these diabetic mice were also suppressed. These results indicate that cellular immunity was suppressed in MLD-STZ type I diabetes. The suppression of cellular immunity might be caused by the suppression of IL-2 production from splenocytes and their responsiveness to IL-2 in MLD-STZ type I diabetic mice.

The present study aimed to investigate if brazilin recovers the defected cellular immune responses in type I diabetes. Our first trial was to determine the DTH after

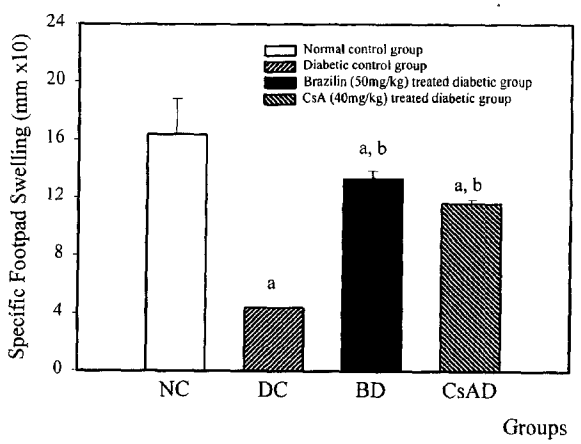

Fig. 1. Effect of brazilin on the delayed type hypersensitivity in MLD-STZ type I diabetic mice. At day 2 after administration of brazilin, 10^6 SRBC were intravenously injected into the tail vein of a mouse. At day 4 after the immunization, 10^8 SRBC were injected into one hind footpad of a mouse. 24 hrs later, DTH was assayed by measuring the increase in thickness of the footpad using micrometer. The specific swelling of the footpad was calculated by subtracting the thickness of a saline-injected footpad from that of the antigen-injected footpad. NC; normal control, DC; diabetic control, BD; brazilin treated diabetic mice and CsAD; CsA treated diabetic mice. Each bar represents the mean ± SEM for 6 mice. a significantly different from normal control mice ($p<0.05$). b significantly different from diabetic control mice ($p<0.05$). The data shown are representative of three independent experiments.

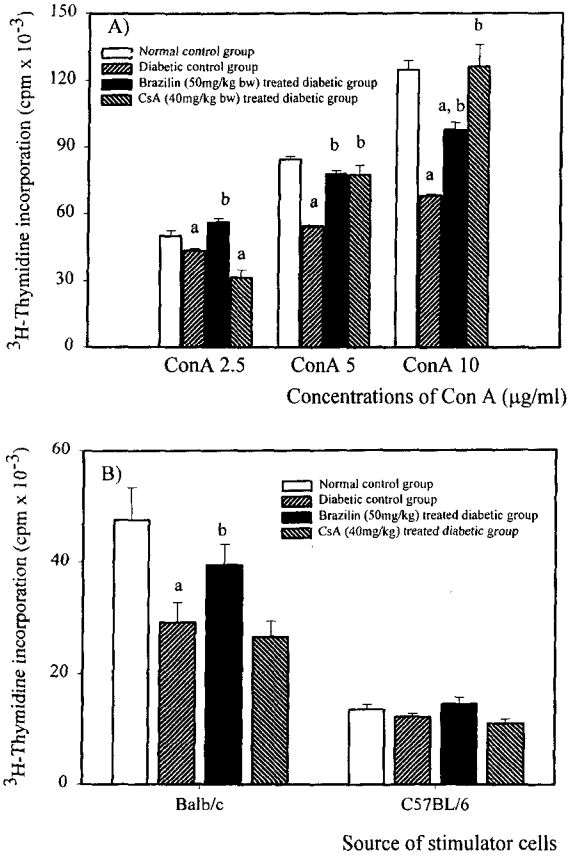

Fig. 2. Con A induced proliferation and mixed lymphocyte reaction of splenocytes obtained from the brazilin treated MLD-STZ type I diabetic mice. Mice were intraperitoneally injected with brazilin. Two days later, splenocytes were obtained from 4 mice of each group. Splenocytes were incubated in the presence of ConA (2.5, 5, 10 μg/ml) for 48 hrs (A) and MMC treated splenocytes from C57BL/6 or Balb/c mice for 120 hrs (B). ^{3}H-Thymidine was pulsed for the last 24 hrs. Each bar represents the mean ± SEM of triplicated cultures. a significantly different from normal control mice ($p<0.05$). b significantly different from diabetic control mice ($p<0.05$). The data shown are representative of three independent experiments.

the administration of brazilin to the MLD-STZ diabetic mice. DTH is one of the most important immune parameters with which we can evaluate cellular immune responses in vivo (Titus and Chiller, 1981; Crowle, 1975). We selected the mice whose blood glucose levels were more than 400 mg/dl at day 14 after the first treatment of streptozotocin. Brazilin was intraperitoneally administered to the mice and then we measured DTH responding to SRBC. Brazilin (50 mg/kg b.w.) significantly augmented DTH lowered in diabetic state (Fig. 1). Similar effects were found in the dose range of 1-200 mg/kg and in the period of 5-14 days after the treatment of brazilin (data not shown). This result indicated that brazilin could improve the impaired cellular immune responses in type I diabetes.

Based on the report that control of blood glucose levels with insulin therapy increased the cellular immunity in type I diabetic subjects (Ishibashi *et al.*, 1980), we first confirmed if brazilin exhibited the hypoglycemic effect of brazilin in MLD-STZ diabetic mice. Brazilin did not show any significant hypoglycemic effect in type I diabetes (data not shown). This fact suggested that brazilin might directly stimulate cellular immunity in MLD-STZ type I diabetic mice.

In a trial to confirm if brazilin increases cellular immune responses by enhancing T cell-proliferation responding to antigenic stimulations, mitogen induced proliferation and MLR of splenocytes were determined. Splenocytes were obtained from each group and incubated with Con A (A) or MMC-treated splenocytes from Balb/c (B) mice. Splenocytes from the brazilin treated MLD-STZ type I diabetic mice significantly proliferated more than those from diabetic mice ($p<0.05$) (Fig. 2).

The findings that brazilin increased the DTH and the proliferation of splenocytes by antigenic stimulations, which were decreased in type I diabetes, motivated us to determine the IL-2 production in splenocytes. Cytokines production and their proliferation are the characteristic responses of splenocytes to antigenic stimulation. IL-2 is one of the most important cytokines which play key roles in cellular immune responses. It enhances the proliferation and differentiation of T cells and activates macrophages or NK

cells (Smith, 1984; Waldmann *et al.*, 1984). Abnormality in the production of IL-2 might cause dysfunction of T cell mediated immunity. Enhanced IL-2 production in splenocytes was observed in the early phase of halothane intoxication (Choi *et al.*, 1997) and the suppressed of IL-2 production was observed in SRBC induced immunotolerance in mice (Mock *et al.*, 1998). Fig. 3 shows that IL-2 production was decreased in the splenocytes from diabetic mice and that brazilin significantly increased the lowered IL-2 production in type I diabetic mice ($p< 0.05$).

This fact suggested that IL-2 production might be related to the immunopotentiating mechanism of brazilin in type I diabetes. Suppressor cell activity is reported to be able to affect the production of IL-2 in splenocytes. In the study of diseases associated with autoimmune reactions, suppressor cells have recently attracted great interest (Reinherz and Schlossman, 1980). Suppressor cell activity has been found to be reduced in systemic lupus erythematosus (SLE) (Morimoto *et al.*, 1979), juvenile rheumatoid arthritis (Strelkiauskas *et al.*, 1978), myasthenia gravis (Zilko *et al.*, 1979) and multiple sclerosis (Bach *et al.*, 1980). In many studies the reduction of suppressor cell activity has been correlated to the induction of the disease. Based on the facts that brazilin augments cellular immune responses via IL-2 production and that IL-2 production is affected by suppressor activity, we investigated the effects of brazilin on non-specific suppressor cell activity using MLR. MLR of normal splenocytes was suppressed in the presence of splenocytes from diabetic mice ($P<0.05$), indicating the enhanced non-specific suppressor cell activity might be related to the suppression of IL-2 production in MLD-STZ type I diabetic mice (Fig. 4). Brazilin did not affect the non-suppressor cell activity in MLD-STZ type I diabetic mice. This result suggests that brazilin might increase IL-2 production by enhancing the intrinsic activity of T cells without affecting suppressor activity.

IL-2 stimulates the proliferation of T cells via its specific receptors (Taniguch *et al.*, 1983). Signals from T cell receptor complex or IL-2 receptor itself can induce the expression of high affinity IL-2 receptors on T cells (Smith, 1984). Binding of IL-2 to its receptors stimulates T cells to proliferate and differentiate (Greene and Leonard, 1986). Thus, the responsiveness of T cells to IL-2 may be an important factor to affect T cell proliferation after antigenic stimulation. To investigate if brazilin could augment the responsiveness of splenocytes to IL-2, we measured proliferation of Con A blast, on which high affinity IL-2 receptors were expressed, and the proliferation of resting splenocytes responding to recombinant IL-2. Proliferation of Con A blast responding to standard IL-2 was increased by the administration of brazilin to diabetic mice (Fig. 5A). IL-2 induced proliferation of splenocytes from diabetic mice was comparable to that of splenocytes from non-diabetic control mice, but this proliferation was significantly increased by brazilin (Fig. 5B). Brazilin significantly increased the sensitivity of splenocytes to IL-2 in diabetic mice in the dose range of

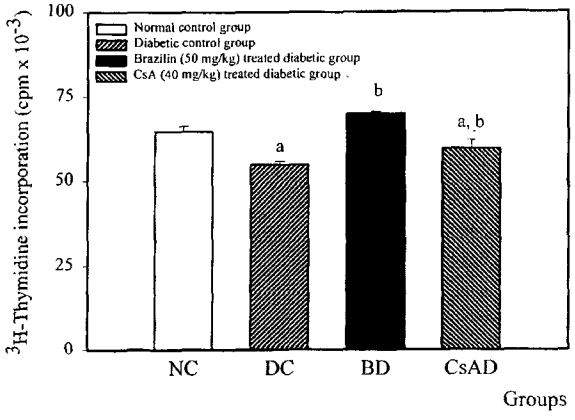

Fig. 3. Con A induced IL-2 production in splenocytes obtained from the brazilin treated MLD-STZ type I diabetic mice. Mice were intraperitoneally injected with brazilin (50 mg/kg b.w.). Two days later, splenocytes were obtained from mice of each group. Splenocytes were incubated with ConA (10 μg/ml) for 20 hrs and the supernatants were collected. IL-2 was quantified by its ability to maintain the proliferation of CTLL-2 cells. NC; normal control, DC; diabetic control, BD; brazilin treated diabetic mice and CsAD; CsA treated diabetic mice. Each bar represents the mean ± SEM of triplicated cultures. a significantly different from normal control mice ($p<0.05$). b significantly different from diabetic control mice ($p<0.05$). The data shown are representative of three independent experiments.

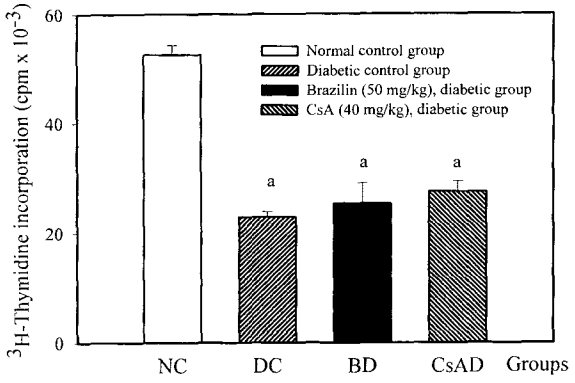

Fig. 4. Effect of brazilin on the non-specific suppressor cell activity of splenocytes obtained from the brazilin treated MLD-STZ type I diabetic mice. Mice were intraperitoneally injected with brazilin (50 mg/kg b.w.). Two days later, splenocytes were obtained from mice of each group. Splenocytes were treated with mitomycin-C (50 μg/ml) for 30 min and used as suppressor cells. Splenocytes from normal Bal b/c mice were treated with MMC and used as stimulator cell. The suppressor cells, stimulator cells, and splenocytes from normal C57BL/6 mice (responder cells) were incubated for 120 hrs at 37°C. ^{3}H-Thymidine was pulsed for the last 24 hrs. NC; normal control, DC; diabetic control, BD; brazilin treated diabetic mice and CsAD; CsA treated diabetic mice. Each bar represents the mean ± SEM of triplicated cultures. a significantly different from normal control mice ($p<0.05$). The data shown are representative of three independent experiments.

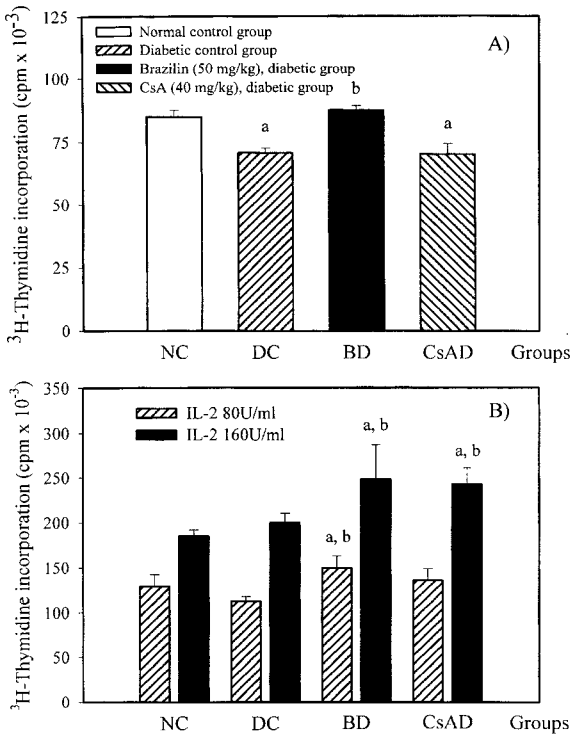

Fig. 5. IL-2 induced proliferation of resting or Con A-activated splenocytes obtained from the brazilin treated MLD-STZ type I diabetic mice. At day 2 after administation of brazilin, splenocytes were prepared from the spleens obtained from mice of each group. To assess the proliferation of ConA-activated splenocytes, they were incubated with Con A (20 μg/ml) for 72 hrs. After washing, the cells were incubated for a further 48 hrs in the presence of r-IL-2 (40U/ml) (A). Splenocytes were incubated for 120 hrs in the presence of r-IL-2 (80, 160U/ml) to assess the proliferation of resting splenocytes (B). ^{3}H-Thymidine was pulsed for the last 24 hrs. NC; normal control, DC; diabetic control, BD; brazilin treated diabetic mice and CsAD; CsA treated diabetic mice. Each bar represents the mean ± SEM of triplicated cultures. a significantly different from normal control mice ($p<0.05$). b significantly different from diabetic control mice ($p<0.05$). The data shown are representative of three independent experiments.

1-200 mg/kg b.w. (data not shown).

In conclusion, brazilin increased cellular immune responses by direct stimulation of IL-2 production in splenocytes and by increasing splenocytes-sensitivity to IL-2 in MLD-STZ type I diabetic mice. Such immunopotentiating effects of brazilin in type I diabetic animals might be utilized to the prevention of diabetic subjects from bacterial or fungal infections.

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