LING ZHI-8: STUDIES OF A NEW IMMUNOMODULATING AGENT

LIEUWE G. VAN DER HEM,1,2 J. ADAM VAN DER VLIEJ,1 C. FRANS M. BOCKEN,1 KOHSUKE KINO,3 ANDRIES J. HOITSMAN,4 AND WIL J.M. TAX4

Department of Surgery and Department of Medicine, Division of Nephrology, University Hospital Nijmegen, Nijmegen, The Netherlands, and Biochemical Genetics Division, Meiji Institute of Health Science, Odawara, Japan

Ling Zhi-8 (LZ-8) is a protein derived from the fungus *Ganoderma lucidum* and has immunomodulatory capacities. It was shown to be mitogenic toward mouse splenocytes in vitro and immunosuppressive in vivo by reducing antigen-induced antibody formation and by preventing completely the incidence of autoimmune diabetes in nonobese diabetic mice. In this study, the mitogenic effects of LZ-8 on human mononuclear cells are reported. In accordance to its mitogenic effect on mouse splenocytes, LZ-8 proved to be mitogenic for human PBMC. This mitogenic effect of LZ-8 apparently required the presence of monocytes. We also demonstrated it to be immunosuppressive in vitro in a human MLC performed in the absence of monocytes, using purified T cells and EBV-transformed allogeneic B cells. Furthermore, we tested LZ-8 for its possible suppressive effects in 2 different models of allogeneic tissue transplantation. LZ-8 proved to have a significant effect on cellular immunity, since its administration in an allografted mouse skin model resulted in an increased survival time. In a model of transplanted allogeneic pancreatic rat islets, LZ-8 was effective in delays the rejection process of allografted islets. More frequent or continuous administration resulted in a further prolongation of survival time. No serious side effects of LZ-8 could be discerned in these experiments.

The first report on a new immunosuppressive agent, Ling-Zhi-8 (LZ-8*), was published by Kino and associates (1) in 1989. It concerned a purified protein with a molecular mass of 13–17 kDa that had been isolated from the fungus *Ganoderma lucidum*. The complete amino acid sequence of LZ-8 was determined subsequently. The LZ-8 molecule consists of a homodimer of 2 polyepitides of 110 amino acids, which bear primary and secondary structural similarities with the immunoglobulin heavy chain. This led to the hypothesis that LZ-8 might be an ancestral protein of the immunoglobulins (2).

In vitro, LZ-8 was mitogenic for mouse splenocytes (1). The immunomodulatory activity of LZ-8 in vivo was explored by determination of its effect on systemic anaphylaxis and on the Arthus reaction in CFW mice. A complete prevention of systemic anaphylactic reactions occurred and a 45% decrease in the incidence of Arthus reactions was observed (1). The ability of LZ-8 to suppress the production of specific antibody was demonstrated in C57Bl10 mice. A 80–95% decrease of antibody production to HBsAg was observed in the LZ-8-treated animals (4). LZ-8 injections in young female nonobese diabetic (NOD) mice completely prevented the occurrence of autoimmune diabetes mellitus in these mice, whereas in a control group of female NOD mice, the incidence of diabetes mellitus was 70% after 40 weeks of age (5). Histological examination of pancreatic islets in both groups revealed only slight insulitis in LZ-8-treated mice, whereas control islets showed massive lymphocytic infiltration. LZ-8 was immunosuppressive without having severe concomitant toxic effects on pancreatic islets. This might be in contrast to other immunosuppressive agents, since prednisone (6, 7), CsA (8, 9), and more recently developed agents, such as FK506 (9, 10), all show toxic side effects on pancreatic islets.

In the present report, we evaluated the effects of LZ-8 administration on survival of allogeneically transplanted pancreatic islets in rats. The immunomodulating effects of LZ-8 were also studied in a mouse skin allograft model. In addition, the mitogenic and immunosuppressive effects of LZ-8 on human mononuclear cells in vitro were investigated and we demonstrate that LZ-8 can suppress the allogeneic response of human T lymphocytes in vitro.

MATERIALS AND METHODS

Drug. Purified LZ-8 was obtained from the Meiji Institute of Health Science, Odawara, Japan. The agent was purified from *G lucidum* mycelia by gel filtration, followed by ion exchange chromatography, as described previously (1). The purified protein is dissolved in PBS in a concentration of 1 mg/ml. It was partly further concentrated for the transplantation experiments in rats using an Amicon YM10 filter to reach a final concentration of 5 mg/ml. The LZ-8 solution was kept frozen at −20°C until use.

Cells. Samples of human peripheral mononuclear cells (MNC), T lymphocytes, and EBV-transformed B lymphocytes were obtained from healthy volunteers. Cells were procured and stored in a previously described manner (11).

T lymphocytes. Lymphocytes were purified from peripheral MNC by counterflow centrifugation. T lymphocytes were isolated from this suspension by rosetting with sheep erythrocytes treated with aminothiophosphorone bromide. The purified T lymphocytes were cryopreserved.

EBV-transformed B lymphocytes. Cryopreserved MNC from some individuals were used for EBV transformation of the B cells. Immunization and stabilization of the cell lines was performed by Dr. F. Uytdehaag and co-workers at the Department for Public Health (RIVM) at Bilthoven, The Netherlands. EBV-transformed B cell lines were cultured in RPMI 1640 medium containing HEPES and sodium bicarbonate, supplemented with 10% heat-inactivated PCS, 2 mM glutamine, 1 mM sodium pyruvate, and gentamycin (40 μg/ml). Only cells negative for *Mycoplasma* were used in the present study.
Mitogenic activity of LZ-8. Mitogenic activity of LZ-8 was determined with 3 different final concentrations of LZ-8 (0.1, 1, and 10 \( \mu \)g/ml). LZ-8 was incubated with either MNC or purified T cells. Each well contained 2\( \times 10^5 \) cells in RPMI 1640 Dutch modification medium (ICN Biomedicals, Costa Mesa, CA) containing 10% heat-inactivated FCS (Gibco, Life Technologies, Breda, The Netherlands), 2 mM glutamine, 1 mM sodium pyruvate, and gentamicin (40 \( \mu \)g/ml) in a final volume of 100 \( \mu \)l. All incubations were performed in triplicate in U-bottom plates (Costar, Cambridge, MA) in a humidified environment at 37\(^\circ\)C and 5% CO\(_2\). Sixteen hours before measurement of mitogenic activity, 50 \( \mu \)l of [\( ^3 \)H]thymidine (0.5 \( \mu \)Ci/well; Amersham International, Amersham, UK) were added. Thymidine incorporation was measured using a cell harvester and a Betaplate liquid scintillation counter (Pharmacia, Uppsala, Sweden). The mitogenic activity of LZ-8 was determined after 3, 4, 5, 6, and 7 days of incubation. In additional experiments, the mitogenic effect of LZ-8 after 3 days of incubation was measured in the complete absence of FCS. In these experiments, 10% autologous serum was present instead.

Immunosuppressive activity of LZ-8 in vitro. MLC were performed by incubation of human peripheral MNC obtained from 2 unrelated donors (10\(^5 \) responder cells plus 10\(^5 \) stimulator cells). Cells were obtained from the same donors as were used for the study on mitogenic activity of LZ-8. Stimulator cells were irradiated with 60 Gy.

In a second series of experiments, 2\( \times 10^5 \) T cells were incubated with 4\( \times 10^4 \) irradiated (60 Gy) allogeneic EBV-B cells. T lymphocytes from 2 different donors were used. LZ-8 solution was added in 3 different final concentrations: 0.1, 1, and 10 \( \mu \)g/ml. After 6 days, 50 \( \mu \)l of [\( ^3 \)H]thymidine were added to each well and 16 hr later, thymidine incorporation was measured with a cell harvester and a Betaplate liquid scintillation counter.

Mouse skin transplantation. B10.D2 mice (H\(_2\)b, Olac, Bicester, Great Britain) weighing 20–25 g served as skin donors and C57B1\( \times \)10 mice (H\(_2\)a, University Animal Facilities, Nijmegen, The Netherlands) served as recipients. These 2 mice strains have the same non-MHC background, but are completely mismatched with respect to their MHC alleles.

Full-thickness skin flaps were procured from tails of donor mice. A patch of donor skin was attached by adhesive wound spray to the flank of recipients after removal of a corresponding skin area. All transplanted sites were checked for signs of rejection on a daily basis. This was considered to have occurred on the day that complete necrosis of the transplanted skin was recorded. Skin transplants were performed in 3 different groups and 2 different regimens of LZ-8 administration were applied after transplantation. Group 1 (controls, n=12) received an intraperitoneal saline injection of 0.3 ml twice a week after transplantation. Group 2 (n=11) had an intraperitoneal injection of 0.3 ml of a 1-mg/ml LZ-8 solution (15 mg/kg) twice a week after transplantation. An intraperitoneal injection of 0.3 ml of a 0.5-mg/ml LZ-8 solution (7.5 mg/kg) was administered 4 times a week to group 3 (n=12) after skin transplantation.

Rat pancreatic islet transplantation. Male Lewis rats (RT1\(^s\)) weighing 250–300 g were obtained from the University Animal Facilities, Nijmegen, The Netherlands, and served as islet donors. Male F344 rats (RT1\(^1\)) with a weight of 250–300 g were obtained from Charles River Wiga, Sulzfeld, Germany, and were the recipients of islet transplants. Recipients were rendered diabetic with an injection of 30 mg/kg streptozotocin (Sigma Chemicals, St. Louis, MO) into the peritoneal cavity with 1 week before transplantation. They were considered to be diabetic with nonfasting blood glucose levels above 20 mmol/L, as measured with a Reflolux glucose analyzer (Boehringer Mannheim, Germany).

Pancreatic islets were obtained according to a previously described method, with some modifications (12). Donor pancreases were dissociated by an intraductal injection of 15 ml of a cold HBSS (Gibco, Life Technologies) containing 1 mg/ml collagenase (Collagenase P, Boehringer Mannheim, intrinsic activity 1.58 Wünsch U/mg). After a pancreatectomy, the gland was incubated in a stationary water bath, followed by a mechanical dissection with filtering through a nylon filter (pore size of 800 \( \mu \)m) and centrifugation on a Dextran gradient (Sigma, m.w. 70,000, industrial grade, density stock solution: 1.084 g/ml). The discontinuous gradient was completed by adding layers of 1.081 g/ml, and 1.041 g/ml on top. Gradients were centrifuged at 40 \( \times \)g for 4 min and 500 \( \times \)g for 12 min. Islets were harvested at the topmost interface. After 2 more washes with HBSS, the islets of 2 donors were suspended in 2.5 ml of HBSS.

Diabetic recipients were anesthetized with ether and underwent a laparotomy. A 23-gauge butterfly needle was used to inject the islets in a mesenteric vein. Hemostasis was assured by hemostatic clips (LS100, Ethicon, Norderstedt, Germany).

After surgery, nonfasting blood glucose levels were determined on a daily basis with the Reflolux glucose analyzer. Rejection of transplanted islets was considered to have occurred on the first of 3 consecutive days with a blood glucose \( \geq \)11 mmol/L.

Pancreatic islet transplants were performed in 4 different groups. Group A (n=9) received an allogeneic islet transplantation on day 0 without concomitant immunosuppression. Group B (n=6) had an intraperitoneal injection of 15 mg/kg LZ-8 twice every week after transplantation until the day of rejection. A daily intraperitoneal injection of 5 mg/kg LZ-8 was given to group C (n=6) after islet transplantation until rejection occurred. The effects of continuous intraperitoneal immunosuppression were studied in 8 other animals (group D). In this group, an osmotic minipump (2 ML1; Alzet, Palo Alto, CA) with a reservoir volume of 2 ml and an operational period of 7 days was filled with LZ-8 (5 mg/ml) during transplantation and inserted in the abdominal cavity. This dosage is an equivalent of nearly 6 mg/kg/day LZ-8. These pumps drained freely in the abdomen. Osmotic minipumps were removed 3 weeks after implantation in persistent normoglycemic animals. Cumulative week doses of LZ-8 were 30 mg/kg body weight in group B, 35 mg/kg body weight in group C, and 40 mg/kg in group D. Body weights were measured on a daily basis in all animals. Nutritional status and possible hepatotoxic or nephrotoxic effects were evaluated by measuring serum total protein, albumin, liver enzymes, and creatinine.

Animals were killed either after a return to the hyperglycemic state, or after 28 days, which was considered to be a long-term function in this experiment. The liver and native pancreas were excised for histological examination. Specimens of the liver were checked for the presence of pancreatic islets and lymphocytic infiltration by immunoperoxidase and hemotoxylin-eosin staining.

Statistics. For statistical analysis of data on mitogenic and suppressive activity of LZ-8 and of survival data, a Wilcoxon rank sum test was used throughout this study. For analysis of weight loss in LZ-8-treated groups, a one-tailed Student's \( t \) test was used. \( P \)-values less than 0.05 were considered to be significant.

RESULTS

Mitogenic activity of LZ-8. When human MNC (obtained from 6 unrelated individuals) were incubated with LZ-8, a strong mitogenic response was measured in all cases. Peak activity of LZ-8-stimulated mitogenesis was observed after 3 days of incubation of human MNC with LZ-8 at all concentrations tested (Fig. 1, \( P<0.01 \) for LZ-8-treated groups vs. controls). The highest mitogenic activity was observed with 1 \( \mu \)g/ml LZ-8 after 3 and 4 days of incubation (\( P<0.05 \) for 1 \( \mu \)g/ml LZ-8 vs. other LZ-8-treated groups). The stimulatory effect of LZ-8 decreased rapidly over time. After an incubation time exceeding 4 days, LZ-8 no longer induced a significant increase of [\( ^3 \)H]thymidine incorporation. In contrast to the results of MNC, the incubation of purified T lymphocytes with LZ-8 hardly resulted in any thymidine incorporation (Fig. 2, note the different scale on the y axis). Only incubation with 10 \( \mu \)g/ml LZ-8 resulted in some T cell activation (\( P<0.05 \)
3H-thymidine incorporation (cpm)

Controls
LZ-8 (0.1 µg/ml)
LZ-8 (1 µg/ml)
LZ-8 (10 µg/ml)

Figure 1. Proliferation of human MNC with different LZ-8 concentrations (mean value ± SD) after increasing incubation times (3-7 days) (*P<0.01 vs. controls).

3H-thymidine incorporation (cpm)

Controls
LZ-8 (0.1 µg/ml)
LZ-8 (1 µg/ml)
LZ-8 (10 µg/ml)

Figure 2. Proliferation of human T cells with different LZ-8 concentrations (mean value ± SD) after increasing incubation times (3-7 days) (*P<0.05 vs. controls).

3H-thymidine incorporation (cpm)

Controls
LZ-8 (0.1 µg/ml)
LZ-8 (1 µg/ml)
LZ-8 (10 µg/ml)

Figure 3. Suppressive activity of different LZ-8 concentrations in an MLC of human T cells and irradiated allogeneic EBV-B cells. Data from a representative experiment are shown; similar results were obtained in 5 additional experiments.
TABLE 1. MST ± SD of allogeneic skin transplants from B10D2 mice (H2b) to C57Bl10 mice (H2b) in LZ-8-treated groups

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MST (days)</th>
<th>P</th>
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<tbody>
<tr>
<td>Group 1: 0.3 ml i.p. saline (n=12)</td>
<td>10.2±1.1</td>
<td></td>
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<tr>
<td>Group 2: 15 mg/kg LZ-8 i.p. (n=12)</td>
<td>11.5±1.8</td>
<td>1 vs. 2: 0.06</td>
</tr>
<tr>
<td>Group 3: 7.5 mg/kg LZ-8 i.p. (n=11)</td>
<td>13.3±2.9</td>
<td>1 vs. 3: &lt;0.01</td>
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<td>Group 4: 4X/week Continuous —-o—</td>
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FIGURE 4. Mean nonfasting blood glucose levels (mM) after pancreatic islet transplantation from Lewis to F344 rats in control animals and in groups treated with different LZ-8 regimens (15 mg/kg twice a week, 5 mg/kg daily, and 5 mg/kg continuously). Rejection of islet grafts is supposed to have occurred in animals with blood glucose >11 mM.

TABLE 2. MST ± SD of allogeneic pancreatic islets from Lewis rats (RT11) to diabetic F344 rats (RT11v) in LZ-8-treated groups

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MST (days)</th>
<th>P</th>
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<tbody>
<tr>
<td>Group A: Control (n=9)</td>
<td>4.7±0.2</td>
<td></td>
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<tr>
<td>Group B: 15 mg/kg LZ-8 i.p. (n=6)</td>
<td>9.7±0.8</td>
<td>A vs. B: &lt;0.01</td>
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<tr>
<td>Group C: 5 mg/kg LZ-8 i.p. (n=6)</td>
<td>11.0±0.7</td>
<td>A vs. C: &lt;0.01</td>
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<tr>
<td>Group D: 5 mg/kg LZ-8 continuous (n=6)</td>
<td>12.5±1.2</td>
<td>A vs. D: &lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B vs. D: &lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C vs. D: NS</td>
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</tbody>
</table>

Figure 5. Mean relative weights of different LZ-8-treated groups after rat allogeneic islet transplantation, compared with mean pre-operative weights of each group (*P<0.05 vs. controls).

Histological examination of samples of liver tissue in normoglycemic LZ-8-treated animals revealed the presence of well-preserved islets, surrounded by normal liver parenchyma. In normoglycemic animals, only small cellular infiltrates could be detected. Immunoperoxidase staining for insulin was evidently positive in the intrahepatically transplanted islets.

DISCUSSION

This study addresses the immunomodulatory properties of LZ-8 in in vitro and in vivo conditions. LZ-8 proves to be a potent mitogen for human MNC. This mitogenic effect on human MNC is in accordance with the results obtained with murine splenocytes (7). The maximal [3H]thymidine incorporation in NOD murine lymphocytes occurred at an LZ-8 concentration of 1.56 μg/ml. Also in our experiment, [3H]thymidine incorporation at 1 μg/ml LZ-8 was significantly higher than at 0.1 or 10 μg/ml. This mitogenic effect turned out to be limited in time, as after 4 days of incubation only background stimulation was recorded, irrespective of the concentration of LZ-8. The mitogenic effect of LZ-8 requires monocytes as accessory cells, since purified T lymphocytes were not activated by LZ-8. When autologous serum was used instead of FCS, a similar mitogenic response to LZ-8 was measured. This finding indicates that FCS is not involved in the observed mitogenic effect of LZ-8, and furthermore suggests that Fc receptors (which will be blocked by the immunoglobulin present in autologous serum) are not required for this mitogenic effect.

The observed mitogenic response of LZ-8 on human MNC overruled possible immunosuppressive effects of LZ-8 in an MLC, since no suppressive effect by LZ-8 could be measured after 6 days in an MLC of human MNC and results obtained were actually higher with LZ-8. Therefore, we modified the MLC by using EBV-B cells as stimulator cells and tested the proliferative response of purified human T cells in the absence of monocytes. In this experiment, an evident immunosuppressive effect of LZ-8 was demonstrated. [3H]Thymidine

during their normoglycemic period. In both groups C and D, significant weight loss was noticed during the first postoperative days, compared with controls and group B (Fig. 5) (P<0.05 vs. controls).

The nutritional status of animals was not jeopardized by LZ-8, since serum albumin and total serum protein ranged between 22–31 g/L and 50–65 g/L, respectively, with no differences among groups. Also liver enzyme and serum creatinine levels were all in the normal range.
incorporation was inhibited around 40% by 1 \( \mu g/ml \) LZ-8 and even stronger inhibition (60%) of T cell activation was noticed with a concentration of 10 \( \mu g/ml \) LZ-8. This is the first demonstration of in vitro inhibition of an allogeneic response by LZ-8. Pretreatment of DBA/2 mice with LZ-8 in vivo before the isolation of splenocytes used for MLC also resulted in a demonstration of in vitro inhibition of an allogeneic response by LZ-8. This is the first demonstration of a potent immunosuppressive effect in vivo, which was reported previously (4, 5) and confirmed by the present data. The mitogenic effect of LZ-8 may seem paradoxical in this respect, but similar in vitro results (inhibition of T cell function as well as mitogenic activity) have been obtained with anti-CD3 antibodies that are immunosuppressive in vivo.

The relatively small increase in graft survival seen in our allogeneic mouse skin model may seem slightly disappointing when compared with the results reported by Kino. His group was able to successfully prevent the incidence of autoimmune diabetes mellitus in NOD mice with the same dosage of LZ-8. This difference may be accounted for by the stronger immunogenic stimulus, evoked by an allogeneic skin graft, as compared with an autoimmunogen process. In the mouse skin allograft model, previous studies have revealed that daily administration of CsA at 25 mg/kg caused only a modest increase in graft survival, with values comparable to the ones obtained with LZ-8. A daily high dosage of 75 mg/kg CsA was required for a substantial prolongation of graft survival (13).

LZ-8 provided a potent immunosuppressive effect in rat allogeneic islet transplantation. All rejection episodes in LZ-8-treated animals were delayed significantly. The most pronounced effect on survival of allogeneic islets was observed in the group that received continuous LZ-8 infusion for over 1 week. Normoglycemia was maintained in all animals after the osmotic pumps had delivered their total content.

The only observed side effect during administration of LZ-8 was a small, albeit statistically significant, weight loss in the early posttransplantation days in animals that received LZ-8 intraperitoneally on a daily or continuous basis. Kidney and liver functions were all in the normal range during the course of the experiment. The nutritional status of the animals was also not disturbed, as albumin and total serum protein levels were comparable to normal values. To be able to relate the immunosuppressive action of LZ-8 in this experimental setting to more well-known immunosuppressive drugs, we also tested the efficacy of continuous CsA administration on prolongation of survival time in the same model. When CsA was administered by an osmotic minipump in the same dosage of 6 mg/kg/day (n=6), an MST of 16.3±4.6 days was recorded, which is comparable to survival times obtained with LZ-8. This is also in accordance with the results of continuous intravenous administration of low dose CsA (2 mg/kg/day) in a rat cardiac allograft model, in which only a modest prolongation of survival time was achieved (14). A higher dosage of 30 mg/kg of CsA, however, was effective in inducing long-term survival of allografted pancreatic islets in a large percentage of cases (15).

The immunological mechanisms, by which LZ-8 exerts its immunosuppressive effect, need further clarification. In an analysis of T lymphocyte subsets in spleen and lymph nodes, Kino et al. (5) found an increased L3T4+ to Lyt-2+ ratio in NOD mice after 11 weeks of treatment with LZ-8 injections twice a week. CsA, also a fungal polypeptide, has immunosuppressive properties in common with LZ-8. CsA is equally effective in the prevention of the incidence of diabetes in NOD mice (16, 17). The modes of action of CsA and LZ-8 are, however, quite different. LZ-8 promotes IL-1 and IL-2 production in vitro in the presence of human peripheral MNC, containing 10% macrophages (5). CsA is a potent inhibitor of IL-2 production at the transcription level (18). LZ-8 is strongly mitogenic for human peripheral MNC and mouse spleen cells. CsA is not mitogenic and can inhibit the mitogenic effect of Con A (19). LZ-8 may exert its immunomodulatory effect by inducing quantitative and qualitative modulation of adhesion molecules on immunocompetent cells (20).

With the widespread use of CsA during the last decade, severe side effects of this immunosuppressive drug have been elucidated. The major drawbacks of CsA therapy are its nephrotoxicity and its toxicity toward pancreatic islets, which causes a diabetogenic effect (8, 9). CsA inhibits glucose-induced insulin synthesis in the pancreas (21) and increases peripheral insulin resistance (22). FK506 is another potent immunosuppressive drug, developed in 1987 from a Streptomyces tsukubakensis species (23). FK506 is equally effective as LZ-8 or CsA in the prevention of insulinitis and diabetes in NOD mice (24). In analysis of lymphocyte subsets, only the population of L3T4+ lymphocytes was significantly decreased by FK506 in comparison with control NOD mice. In contrast, the administration of LZ-8 resulted in overall decreased frequencies of lymphocyte subsets and an increased L3T4+ to Lyt-2+ ratio (5). Controversy exists about a potential toxic effect on pancreatic islets by FK506. Kai et al. (24) found no harmful effects of FK506 on histological examination of grafted pancreatic islets in NOD mice. However, toxic effects by FK506 on pancreatic islets were described in other studies (9, 10). Histological examination of FK506-treated rats revealed vacuolization and degranulation of pancreatic islets (25). Also, intravenously performed glucose tolerance tests in an early phase after pancreas transplantation were compromised in cynomolgus monkeys that were treated with FK506 (10). These findings hamper the clinical use of these agents.

LZ-8 holds promise as a new immunosuppressive agent. Unlike other immunosuppressants, no toxicity toward pancreatic islets could be discerned by histological and blood glucose examinations. Further studies need to be performed to clarify exact modes of action of LZ-8, dosimetric regimens, and potential toxicity of LZ-8.

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