Transpl Int (1996) 9: 184–193

ORIGINAL ARTICLE

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Prolongation of lung xenograft survival in rats with a short course of deoxyspergualin and cyclosporin A

Received: 10 July 1995
Received after revision: 2 November 1995
Accepted: 3 November 1995

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Abstract The efficacy of 15-deoxyspergualin (DSG), cyclosporin A (CyA), and splenectomy – alone or in combination – in prolonging the survival of concordant lung xenotransplants was studied in the hamster-to-rat model. In the untreated group, rejection occurred within 3 days, with an elevation of lymphocytotoxic antibody titers. The rejected lung revealed that ED1+ cells were more prevalent than MRC OX8+ cells in the perivascular infiltrates. In the DSG group, the antibody response was suppressed and median survival increased to 7.5 days. The rejected lungs demonstrated a highly significant depression in ED1+ cellular infiltration and a moderate MRC OX8+ cellular infiltration. When maintenance CyA was combined with a short course of DSG, survival dramatically increased to beyond 100 days. There were no deposits of IgM, IgG, or C3 or of any cell infiltrate in the grafts of two animals sacrificed 107 and 119 days post-transplantation. We conclude that initial treatment with DSG combined with continuous CyA can suppress acute rejection in the hamster-to-rat lung xenograft model, resulting in long-term graft survival.

Key words Xenotransplantation, lung, rat · Rat, lung, xenotransplantation · Splenectomy, deoxyspergualin, lung transplantation · Deoxyspergualin, splenectomy, lung transplantation · Lung transplantation, rat, xenografting

Introduction

The development of immunosuppressive agents and surgical techniques has expanded the indications for organ transplantation. However, a constant shortage of donor organs has become a serious problem. Starzl et al. [24] recently performed two liver xenotransplantations in humans using baboon liver xenografts. It now appears that xenotransplantation may provide a partial solution to the shortage of donor organs.

Recent studies [14, 33] have indicated that rejection of a concordant xenograft in a well-established model of hamster-to-rat heterotopic cardiac xenotransplantation was mediated mainly by humoral immunity. This may differ with lung xenografts because the lung contains a considerable amount of lymphoid tissue in contrast to most other organs [23]. Tavakoli et al. [26] have reported that rejection in the guinea pig-to-rat discordant lung xenograft model is preceded by a very rapid, predominantly humoral, mechanism. However, concordant lung xenograft models have not been reported. We have established a hamster-to-rat orthotopic lung xenograft model, which is a so-called concordant model, to investigate the mechanism of lung xenograft rejection.

The immunosuppressant 15-deoxyspergualin (DSG) has been shown to be effective in xenotransplantation [14, 29]. DSG suppresses the immune response by a mechanism of action that differs from other antirejection drugs such as cyclosporin A (CyA) [33]. We have exam-

ined the immunosuppressive effects of DSG in canine allograft lung models [20]. Katayama et al. reported that DSG was a powerful immunosuppressant that was effective against ongoing rejection in rat lung allotransplantation models [10], and Valdivia et al. [29] demonstrated that DSG prolonged graft survival in the hamster-to-rat cardiac xenograft model. DSG has also been reported to increase organ survival in the same models when used in combination with splenectomy or total lymphoid irradiation [14, 28].

There have been many reports concerning the efficacy and safety of CyA in the prevention of lung allograft rejection, and CyA is considered to be the standard immunosuppressive drug. However, in experimental xenograft rejection, the effects of CyA alone in rat-to-hamster cardiac transplantation have not been satisfactory [1, 16].

The aim of the present study was to investigate the survival and morphological aspects of orthotopic lung xenografts in the concordant hamster-to-rat species combination and to examine the efficacy of DSG and CyA in that model, alone or in combination with splenectomy.

Materials and methods

Animals

Female Golden Syrian hamsters weighing 100–170 g and BN rats weighing 200–300 g were used as donors (SLC, Japan), and inbred male Lewis (LEW/Crj) rats weighing 200–300 g were used as recipients (Charles River, Japan).

Surgical procedure

All animals were anesthetized with an intraperitoneal injection of pentobarbital (Nembutal, 30 mg/kg), intubated, and ventilated at a tidal volume of 10 ml/kg and a respiratory rate of 90 breaths/min using a respirator (Shinano-NS, Japan). Orthotopic left lung transplantation was performed using a cuff technique, as previously described [15]. The pulmonary artery and pulmonary vein were first anastomosed according to the cuff technique; then, the left main bronchus was anastomosed with a 9–0 polypropylene continuous suture for the cartilaginous ring and an interrupted suture for the membranous wall. No antibiotics were given following transplantation. All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals of Nagasaki University".

Splenectomy

Splenectomy (Spx) was performed via a median incision following completion of lung transplantation.

Serial chest roentgenograms

Chest roentgenograms were taken every day following transplantation. Aeration of the left lung was graded as follows: severe infiltrate, score 0; moderate infiltrate, score 1; mild infiltrate, score 2; almost normal, score 3; normal, score 4. The roentgenograms were graded independently by one of the authors (N. Y.) in a blinded fashion.

Graft survival

The post-transplant day on which the ventilation score decreased to less than 1 was considered as the graft survival time.

Experimental groups

The recipient rats were divided into seven treatment groups: group 1 ($n = 10$) received no treatment; group 2 ($n = 6$) underwent Spx; group 3 ($n = 6$) received DSG (5.0 mg/kg per day, i.p., daily, until death or sacrifice); group 4 ($n = 7$) underwent Spx and received DSG (5.0 mg/kg per day, i.p., daily, until death or sacrifice); group 5 ($n = 7$) received CyA (20 mg/kg per day, i.m., daily, until death or sacrifice); group 6 ($n = 8$) received CyA (20 mg/kg from day 0 to day 50; 15 mg/kg from day 51 onwards, i.m., daily, until death or sacrifice) and DSG (5.0 mg/kg/0–3 days, i.p., daily); group 7 ($n = 6$) received CyA (20 mg/kg from day 0 to day 50; 15 mg/kg from day 51 onwards, i.m., daily, until death or sacrifice) and DSG (5.0 mg/kg/0–3 days, i.p., daily) and underwent Spx.

DSG and CyA were administered starting on day 0, immediately after the vascular clamp was removed.

Immunosuppressive agents

DSG was kindly supplied by Nippon Kayaku (Tokyo, Japan). The drug was dissolved in saline and stored at −70 °C prior to use. CyA was purchased from Sandoz (Basel, Switzerland).

Complement-dependent cytotoxicity (CDC) assay

Recipient sera were obtained from the animals in each group on the day of sacrifice and stored at −80 °C until analysis. The concentration of cytotoxic antibodies in the serum of xenografted rats was determined using a CDC assay [21]. Briefly, 25 µl of serially diluted rat sera was incubated with 25 µl (containing 5×10^6/ml) of hamster spleen cells and 25 µl of guinea pig serum in Eagle's MEM for 45 min at 37 °C. The percentage of cells staining with trypan blue (Life Technologies, Grand Island, N.Y., USA) was calculated. The cytotoxic antibody titer was defined as the highest serum dilution with more than 51 % cell lysis. Guinea pig serum diluted 1 : 4 served as the source of complement.

Lymphocyte subpopulations

Peripheral lymphocyte subpopulation were analyzed with monoclonal antibodies (mAbs; Serotec, Oxford) by two-color flow cytometry analysis. Blood samples from recipients were obtained at 3 and 5 days post-transplantation. MAbs used in this study included phycoerythrin (PE)-conjugated anti-CD4 mAb (W3/25), fluorescein isothiocyanate (FITC)-conjugated anti-CD8 mAb (OX-8), PE-conjugated anti-Pan B cells (RLN-9D3), and FITC-conjugated

anti-CD3 (IF4). The surface immunofluorescence of individual cells was determined using a whole-blood labeling technique. Briefly, whole blood (100 µl) was incubated for 60 min on ice in the dark with 10 µl of FITC- and PE-conjugated mAbs. Erythrocytes were subsequently lysed by a 10-min incubation with FACS lysing solution (Becton Dickinson). Cells were suspended in 1.0 ml of 0.5 % paraformaldehyde in phosphate-buffered saline (0.5 % PFA-PBS, pH 7.4). Counts of positively stained cells were computed as a percent of total lymphocytes using CONSORT 30 software on the fluorescence-activated cell sorter (Becton Dickinson).

Histopathology

At autopsy, the lung and heart were removed en bloc and 4 % paraformaldehyde in phosphate-buffered saline (4 % PFA-PBS, pH 7.4) was injected into the trachea until the alveolar spaces were fully expanded. Slices of approximately 0.5 cm thickness were cut from the lungs and stored at –80 °C in (OCT) compound (Tissue-Tek, Miles, Elkhart, USA) for immunohistochemical studies or fixed in 4 % PFA-PBS overnight for hematoxylin-eosin staining.

Immunohistochemistry

The presence of rat complement deposits was determined directly using FITC-conjugated anti-human C3 mAb. The presence of rat IgM and IgG deposits on the vascular endothelium and mononuclear subsets of perivascular infiltrative cells in the xenografts were determined by means of the labeled streptavidin-biotin (LSAB) technique [2]. Five-µm serial cryostat sections were cut with a cryostat microtome, air-dried for 60 min, and incubated in normal goat serum to inhibit nonspecific binding. Mouse affinity-purified mAb to rat IgM (heavy chain), rabbit F (ab')2 anti-rat IgG (whole molecule), mouse mAb to rat CD4 (W3/25), and CD8 (MRC OX-8, Serotec, Oxford) were added at room temperature as primary antibodies. Mouse mAb to rat monocytes and macrophages (ED1) were added at room temperature following deparaffinization of paraffin-embedded material. Following three washes in 0.05 M TRIS-HCl buffer (pH 7.2–7.6), sections were incubated for 10 min with diluted biotinylated antibody solution. Following three washes in 0.05 M TRIS-HCl buffer (pH 7.2–7.6), sections were incubated for 10 min with streptavidin alkaline phosphatase reagent (Dako). Alkaline phosphatase was revealed by staining with the fast red substrate system (Dako). Sections were lightly counterstained with hematoxylin. Sections incubated with 0.05 M TRIS-HCl buffer (pH 7.2–7.6) instead of primary antibody served as negative controls. Estimates of IgM and IgG deposition on vascular endothelium and bronchiolar epithelium were graded from "–" to "+++" in a blinded fashion by one of the authors (T.T.), with "–" meaning none; "+" mild, weakly positive; "++" moderate, clearly positive; and "+++" severe, strongly positive. Within the perivascular areas, 1000 cells in each preparation were analyzed, and the percentage of cells with positive staining was calculated.

Controls consisted of allografted (BN-to-LEW) and nontransplanted animals.

Statistical analysis

Groups were compared using the Mann-Whitney U-test to determine the level of significance of any difference. Any P value below 0.05 was considered statistically significant.

Results

The ventilation scores of the left lungs were assessed via chest roentgenograms following xenotransplantation. In all cases, opalescence was observed in the chest roentgenograms within 2 days after transplantation, which was regarded as a reimplantation response of the transplanted lung. All transplanted lungs in the untreated group showed severe infiltrates during the first 3 postoperative days and, therefore, 3 days was considered the graft survival time. Other groups began to demonstrate good aeration 3 or 4 days following surgery.

Graft survival times

The post-transplant day on which the ventilation score decreased to less than 1 was considered the graft survival time (Table 1). Animals treated with Spx alone had an increased median survival of only 1 or 2 days as compared with untreated animals ($P = 0.0056$ vs group 1). DSG alone increased median survival 2.5-fold to 7.5 days ($P = 0.0003$ vs group 1), but only one of six animals lived beyond 10 days. The effect of DSG was not improved by splenectomy in comparison with DSG alone (Table 1, group 4). CyA alone increased median survival fivefold to 16 days, with two of seven animals surviving beyond 30 days (Table 1, group 5). However, when maintenance CyA was combined with a short course of 5 mg/kg per day DSG, survival was remarkable enhanced, with four of eight animals living beyond 100 days (Table 1, group 6; Fig. 1). Splenectomy did not enhance the survival of animals receiving this combined drug therapy (Table 1, group 7).

All rats treated with DSG (5.0 mg/kg per day) suffered from severe emaciation, losing between 2 % and 26 % of their body weight (data not shown) and developing diarrhea. These toxic reactions gradually disappeared in the DSG short-term treatment groups following cessation of DSG, and body weight was regained by 50 days post-transplantation. However, none of the animals receiving continuous DSG recovered from the drug toxicity. Two of six animals in group 3 and four of seven animals in group 4 died prior to sacrifice. Five animals receiving continuous treatment of CyA (20 mg/kg per day) showed graft lung abscesses histologically (groups 5–7). However, at autopsy, there was no evidence of gross anatomic toxicity in other organs in the recipient rats.

Antibody titers (Fig. 2)

At the time of rejection, CDC assays revealed that the anti-hamster lymphocytotoxic antibody titers varied from 1/16 to 1/256 in the untreated group, while in the

Table 1 Graft survival of left lung xenotransplantation

Group	Treatment	Days after transplantation[a]	Median survival (days)	P^b
1	Untreated	3, 3, 3, 3, 3, 3, 3, 3, 3, 3	3	–
2	Splenectomy	3, 4[c], 5, 5, 5, 6[c]	5	0.0056
3	DSG, 5 mg/kg per day	6, 7, 7, 8, 8[c], 10[c]	7.5	0.0003
4	DSG, 5 mg/kg per day, + splenectomy	4[c], 5, 7[c], 9[c], 9[c], 10, 17	9	0.0004[e]
5	CyA, 20 mg/kg per day	3[c], 6[c], 9, 16, 21, 32, 33	16	0.0013[f]
6	CyA, 20 mg/kg per day[d] + DSG, 5 mg/kg/0–3 days	7[c], 11[c], 22[c], 49, 107, 119, > 100, > 100	78	0.0002[g]
7	CyA, 20 mg/kg per day[d] + DSG, 5 mg/kg/0–3 days + splenectomy	3[c], 7, 9, 32[c], 71, 117	20.5	0.0061[h]

[a] Post-transplant day of ventilation score more than 1 on chest X-ray film
[b] Mann-Whitney U-test vs group 1
[c] Rats died prior to sacrifice
[d] CyA was used at a dose of 20 mg/kg per day for the first 50 days (0–50) and was then continued at 15 mg/kg per day
[e] P = NS vs group 3
[f] P = NS vs groups 3 and 4
[g] P = NS vs groups 5 and 7
[h] P = NS vs groups 3–6

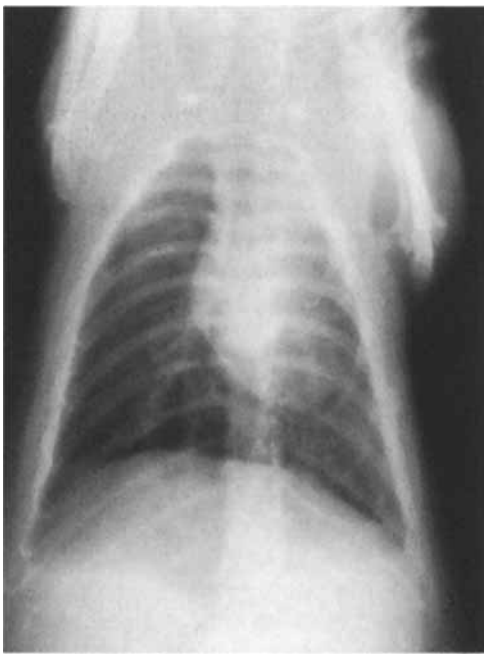

Fig. 1 Chest roentgenogram of one of the CyA/DSG-treated animals showing normal aeration of the left lung at 100 days post-transplantation

Spx group they reached a maximum of 1/32. In the DSG alone group, the antibody response was depressed to a titer of less than 1/8, and in the DSG with Spx (DSG/Spx) treatment group the antibody response was less than 1/2. CyA alone depressed antibody formation to less than 1/2, except in one case in which the titer increased to 1/8 on day 32 post-transplantation. When maintenance CyA was combined with a short course of DSG (CyA/DSG), antibody response was completely suppressed, even at 100 days post-transplantation ($n = 4$). We could not detect any natural antibody titer in naive rats in this xenograft combination.

Lymphocyte subpopulations in peripheral blood lymphocytes (PBLs)

In the untreated group, the CD4+/CD8+ ratio increased significantly at 3 days ($P < 0.05$ vs the nontransplanted group; Fig. 3). At 5 days, the ratio was lower than at 3 days (data not shown). In the DSG alone group at 3 days, the CD4+/CD8+ ratio also increased ($P < 0.05$ vs the untreated group); however, the CD4+/CD8+ ratios were maintained at a high level in comparison with the untreated group (data not shown). There was no significant difference in the CD3+/Pan B ratios between the nontransplanted animals and those in the untreated and Spx groups. In the DSG alone group, the CD3+/Pan B ratios increased at 3 days ($P < 0.02$ vs the untreated group).

Histological findings in the untreated group were characterized by perivascular cellular infiltrates and edema at 3 days posttransplantation (Fig. 4 a). The cells consisted of monocytes and small, round lymphocytes with occasional neutrophils. Binding of the neutrophils and lymphocytes to vascular endothelium, mild lymphocytic bronchiolitis, and moderate alveolar macrophage collections were also observed. Narrowing of the arteriolar lumen, capillary congestion, and intra-alveolar hemorrhage resulting in necrosis of the pulmonary parenchyma were observed at 5 days. In the DSG and DSG/Spx-treated groups, significant perivascular focal

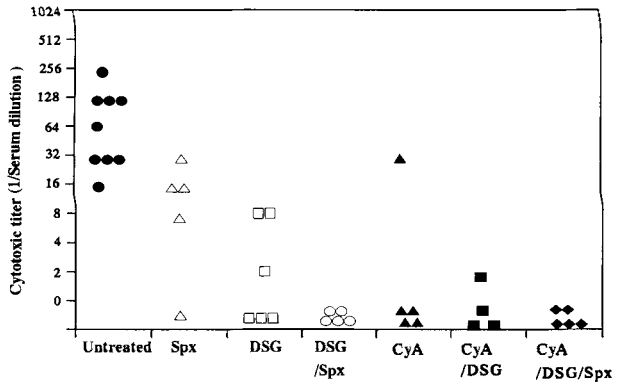

Fig. 2 Lymphocytotoxic antibody response of lung xenograft recipients as measured by a CDC assay. At the time of rejection, cytotoxic titers were measured in all animals. In the DSG/CyA group, cytotoxic titers of four animals with long-term graft survival were measured 100 days post-transplantation [● untreated, △ splenectomy (Spx), □ deoxyspergualin (DSG), ○ DSG/Spx, ▲ cyclosporin A (CyA), ■ DSG/CyA, —— DSG/CyA/Spx]

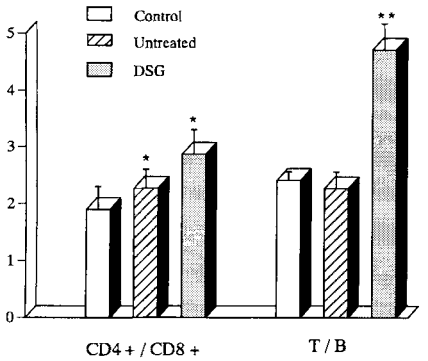

Fig. 3 Peripheral lymphocyte subpopulation at 3 days after transplantation. In the untreated group, the CD4+/CD8+ ratio increased significantly at 3 days ($*P < 0.05$ vs nontransplanted group). In the DSG alone group at 3 days, the CD4+/CD8+ ratio also increased ($*P < 0.05$ vs untreated group). There was no significant difference in the CD3+/Pan B ratios between the nontransplanted animals and those in the untreated group. In the DSG alone group, the CD3+/Pan B ratios increased at 3 days ($**P < 0.02$ vs untreated group)

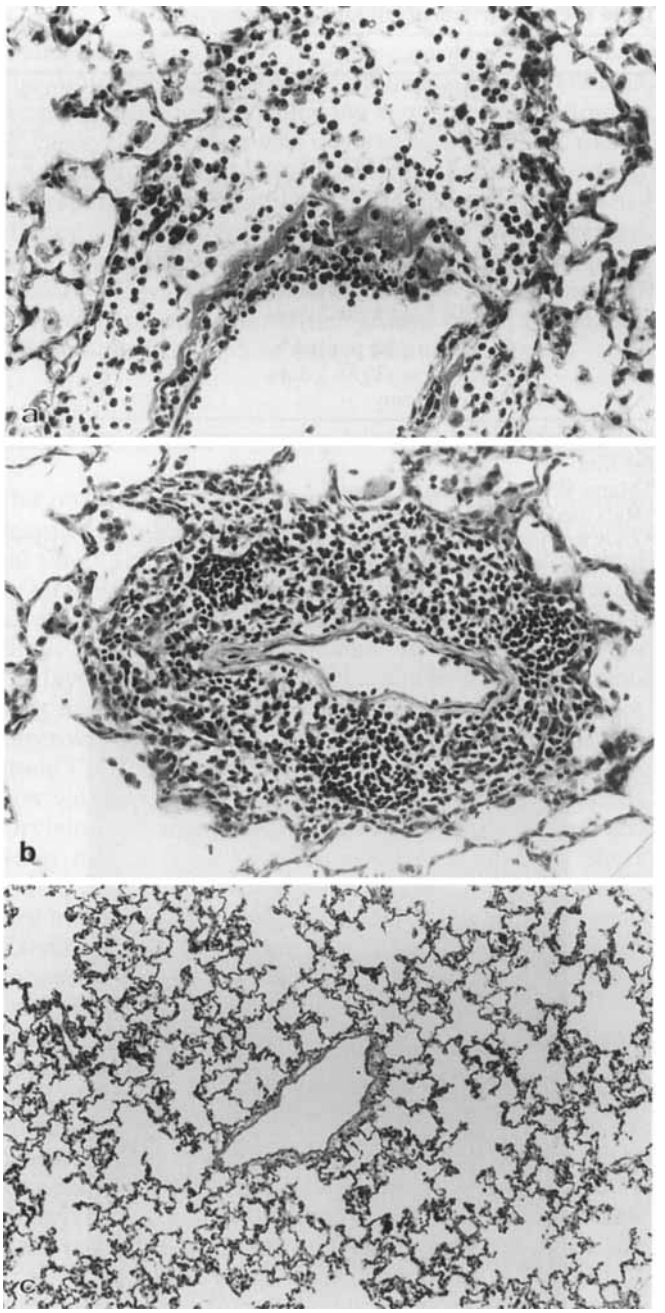

Fig. 4a, b Histology of transplanted lung xenografts: **a** untreated group 3 days post-transplantation. The cellular infiltrate forms a dense cuff around the venules. The cells consisted of monocytes and small, round lymphocytes with occasional neutrophils. Binding of the neutrophils to the vascular endothelium was also observed (H & E, × 480); **b** DSG alone-treated group at 9 days post-transplantation. Significant perivascular focal lymphoid cell infiltration was observed (H & E, × 480); **c** CyA/DSG group at 107 days post-transplantation. There were no abnormal findings (H & E, × 480)

lymphoid cell infiltration was observed at the time of rejection (Fig. 4b). In the animal in the DSG/Spx-treated group that died 4 days after transplantation, there was normal histology of the xenograft. The CyA alone group showed various grades of lymphoid cell infiltration at the time of rejection. Two animals in the CyA/DSG group with long-term survival were sacrificed at 107 and 119 days post-transplantation with ventilation scores of 4; their grafts also revealed normal pulmonary structure (Fig. 4c).

Immunohistochemical analysis of the rejected lungs in the untreated group revealed marked IgM and C3 de-

Table 2 Immunohistochemical analysis of the rejected lungs

Treatment	*n*	Vascular endothelium/ Bronchiolar epithelium[a]		
		IgM	IgG	C3
Untreated	4	+++/–	–/–	+++/–
DSG, 5 mg/kg per day	4	–/–	–/–	–/–
CyA, 20 mg/kg per day + DSG, 5 mg/kg/0–3 days[b]	2	–/–	–/–	–/–
Control[c]	5	–/–	–/–	–/–

[a] Estimates of IgM and IgG deposition were graded from – to +++ in a blinded fashion (– none; + mild, weakly positive; ++ moderate, clearly positive; +++ severe, strongly positive)
[b] Rat was sacrificed at 107 and 119 days post-transplantation with ventilation score of 4
[c] Normal hamster lungs, i.e., nontransplanted lungs

position on the vascular endothelium (Table 2, Fig. 5-a, b), although IgG deposition was absent. The rejected lung in animals in the DSG treatment group at the time of rejection demonstrated no IgM or IgG deposition on the vascular endothelium. No deposits of IgM, IgG, or C3 were found on the bronchiolar epithelium in any of the experimental groups, nor was deposition observed in normal hamster lungs. The distribution of mononuclear cell subsets in the perivascular area is compared in Fig. 6. The control allotransplanted lungs 3 days after transplantation in BN-to-LEW rats revealed a moderate MRC OX8+ (38.6 % ± 0.6 %) and W 3/25 + (28.0 % ± 12.3 %) cellular infiltration, but ED1+ cells (8.0 % ± 3.7 %) were rare. In contrast, in animals in the untreated group 3 days after transplantation, macrophages/monocytes reactive with the ED1 marker (34.2 % ± 16.4 %; $P < 0.01$ vs control; Fig. 7a) were more prevalent than MRC OX8+ (18.0 % ± 16.5 %; $P < 0.05$ vs control) and W 3/25+ (6.2 % ± 6.6 %; $P < 0.05$ vs control) cells. The rejected lungs from animals in the DSG groups demonstrated moderate MRC OX8+ (32.5 % ± 3.7 %) cellular infiltration, but W 3/25+ (3.8 % ± 4.3 %) and ED1+ cells (10.4 % ± 2.3 %; Fig. 7b) were rare. There was a highly significant depression in ED1 ($P < 0.05$ vs untreated group) cellular infiltration in the DSG group when compared with the untreated group. In two CyA/DSG-treated animals, there was no deposition and no infiltrating cells 107 and 119 days post-transplantation.

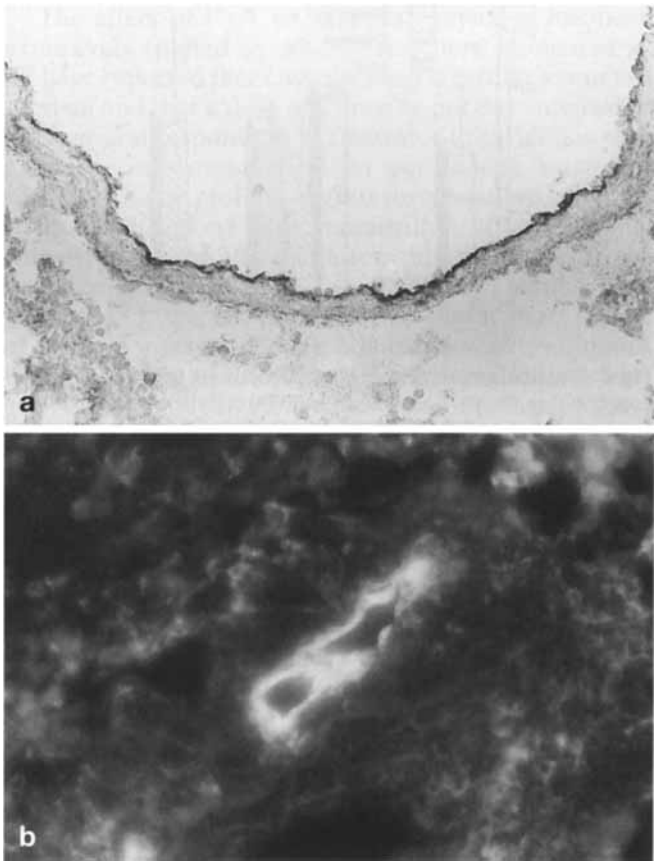

Fig. 5 a, b Immunohistochemical staining of lung xenografts: **a** immunohistochemical analysis of the untreated lung at 3 days post-transplantation showed high levels of IgM deposition in the vascular endothelium (LSAB, × 480); **b** C3 deposition was also detected in the vascular endothelium (LSAB, × 480)

Discussion

Previous research [14, 16, 31] has suggested that antibody-mediated immunity plays an important part in the graft destruction that occurs within 3 days in untreated recipients in the hamster-to-rat cardiac xenograft model. The liver xenograft, however, which is more resistant to antibody-mediated injury, is rejected in about 7 days by combined humoral and cellular immunity [18, 30]. Lung allograft rejection has been investigated using a rat orthotopic transplantation model. In this model, the allograft develops the well-known changes of perivascular, peribronchial, and interstitial lymphocytic infiltration that result in necrosis of the pulmonary parenchyma approximately 7–8 days post-transplantation [13].

In the present study, we demonstrated that the hamster-to-rat lung xenograft was rejected approximately 3 days after transplantation. At the time of rejection, which was determined from chest roentgenograms, grafted lungs showed perivascular cellular infiltration and severe edema. The histologic pattern of infiltrating cells consisted of small, round lymphocytes, macrophages/monocytes, and neutrophils. Immunohistochemically, infiltrative cells consisted of moderate ED1+ and CD8+ cells. In addition, we demonstrated the presence of a moderate titer (1/16 ~ 1/256) of cytotoxic antibodies

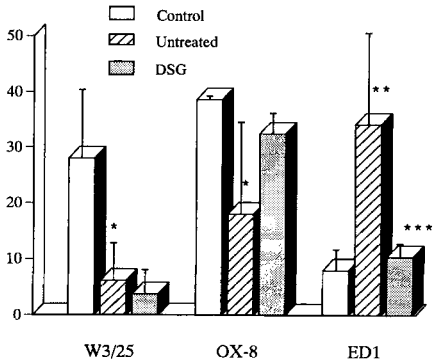

Fig. 6 Distribution of mononuclear cell subsets in the perivascular areas. Within the perivascular areas, 1000 cells in each preparation were analyzed and the percentage of cells with positive staining was calculated (mean ± SD). The control allotransplanted lungs 3 days post-transplantation in BN-to-LEW rats revealed moderate MRC OX8+ (38.6 % ± 0.6 %) and W 3/25+ (28.0 % ± 12.3 %) cellular infiltration, but ED1+ cells (8.0 % ± 3.7 %) were rate. In contrast, animals in the untreated group 3 days post-transplantation revealed that macrophages/monocytes reactive with the ED1 marker (34.2 % ± 16.4 %; $^{**}P < 0.01$ vs control) were more prevalent than MRC OX8+ (18.0 % ± 16.5 %; $^{*}P < 0.05$ vs control) and W 3/25+ (6.2 % ± 6.6 %; $^{*}P < 0.05$ vs control) cells. There was a highly significant depression in ED1 (10.4 % ± 2.3 %; $^{***}P < 0.05$ vs untreated) cellular infiltration in the DSG group at the time of rejection when compared with the untreated group

existing in the recipient serum and definite evidence of IgM and C3 deposits on the vascular endothelium at 3 days after transplantation. There was extension of perivascular cellular infiltration and edema, followed by hemorrhage and destruction of the pulmonary parenchyma beyond 3 days. These findings indicated that both humoral and cellular immune systems were in operation in the acute rejection response in this model.

The efficacy of CyA and DSG, alone or in combination with Spx, was also assessed in the hamster-to-rat lung xenograft model. DSG has been reported to be a useful immunosuppressive agent in allograft [9] and xenograft transplantation models. Current studies indicate that DSG treatment suppresses antispecies antibody formation, thereby significantly prolonging cardiac xenograft survival [19, 25]. Other researchers have studied the combined effect of DSG and other therapies on prolonging xenograft survival. Valdivia et al. [28] have reported that DSG increased graft survival in the hamster-to-rat cardiac xenograft model when used in combination with splenectomy. In our study, splenectomy depressed anti-hamster antibody production in comparison with untreated rats but provoked only a slight prolongation of survival. Although both DSG alone and DSG/Spx depressed the antibody response significantly and prolonged further graft survival in comparison with the untreated or Spx group, long-term graft survival was not achieved because of cellular rejection.

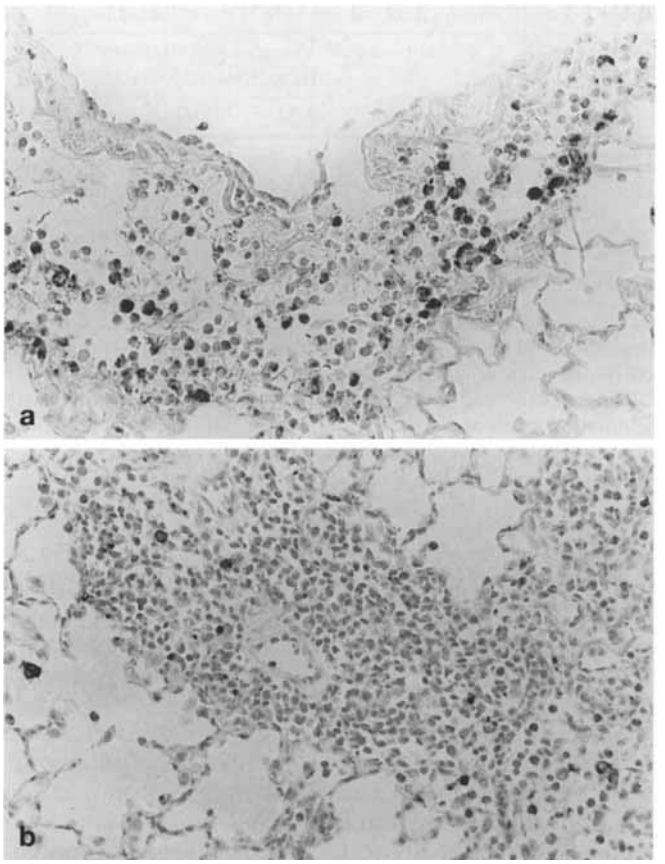

Fig. 7a Untreated graft 3 days post-transplantation. Moderate ED1+ cellular infiltration was observed in the perivascular area (LSAB, × 600). **b** DSG alone-treated graft 7 days post-transplantation. Moderate ED1+ cellular infiltration was rare in the perivascular area (LSAB, × 480)

DSG has a significant inhibitory effect on the development of cytotoxic T lymphocytes but not on natural killer cells or lymphokine-activated killer cells in vitro [11]. In the present study, the CD4+/CD8+ ratio in the peripheral blood lymphocytes (PBLs) at 3 days post-transplantation in the untreated group was significantly greater than it was in the nontransplanted group but decreased to lower than baseline levels 5 days post-transplantation. Conversely, immunohistochemical analysis of cell surface markers on the infiltrating cells revealed that CD8+ cells were more prevalent than CD4+ cells 3 days post-transplantation. This discrepancy suggests that a local cellular immune response more accurately reveals the status of the graft than the general immune response in this model. DSG markedly increased the CD4+/CD8+ ratio of PBLs 3 days post-transplantation and this ratio remained 5 days post-transplantation. Fujii et al. [5] have reported that deoxymethylspergualin, which is a more stable analogue of DSG, resulted in decreased numbers of CD8+ lymphocytes, but that CD4+

lymphocytes were not affected in vitro. We also hypothesize that DSG suppresses CD8+ lymphocytes, as suggested by the observed increase in the CD4+/CD8+ ratio. However, in our lung xenograft model, DSG did not completely abrogate CD8+ mediated cytotoxicity.

The effects of DSG are also directed at the B-lymphocyte level. Morikawa et al. [17] have reported that DSG had a selective immunosuppressive effect on the differentiation pathway of B lymphocytes and suppressed immunoglobulin synthesis in a T-cell-dependent as well as T-cell-independent manner. Their data indicated that DSG had little effect on the proliferative response of resting or activated B lymphocytes. However, other investigators have concluded that DSG also affects the proliferative stage of B cells [4]. Our results show that DSG increased the CD3+/Pan B ratios in PBLs 3 days post-transplantation and inhibited anti-hamster antibody formation.

Macrophages/monocytes and neutrophils play important roles in xenograft rejection. In our models, untreated cases sacrificed 3 days post-transplantation revealed binding of neutrophils to the vascular endothelium, which can cause endothelial damage, and macrophages/monocytes (ED1) were present in the perivascular cellular infiltrates. The histological characteristics of the rejected grafts in the DSG and DSG/Spx treatment groups included marked CD8+ lymphoid cell infiltration in the perivascular and peribronchiolar spaces and scarcity of macrophage/monocyte (ED1) infiltrates, as well as IgM and IgG deposition on the vascular endothelium, a phenomenon similar to the late vascular phase in rat lung allograft rejection [32]. Dickneite et al. [3] have reported that the immunosuppressive action of DSG is predominantly against cells of the monocyte/macrophage group. Kerr et al. [12] also have demonstrated that local macrophage proliferation within the kidney is a prominent feature of acute allograft rejection and that inhibition of this response is one mechanism by which DSG functions. Additionally, Nakajima et al. [19] have reported that neutrophil and macrophage infiltration were characteristic of cardiac xenograft rejection and that these infiltrates were suppressed by treatment with DSG. Our results agree with these findings.

High-dose DSG administration induced significant sublethal body weight loss and diarrhea in recipient rats. Yuh and Morris [33] have evaluated the extent of DSG toxicity and observed no gross anatomical or histopathological evidence of significant tissue toxicity in mouse cardiac allograft recipients treated with daily injections of 5.0 mg/kg per day i.p. from days 1 through 13 that were necropsied on day 14. In our model, the surgical procedure is more invasive than in rat cardiac transplantation [22] and, thus, the side effect of the drug might be enhanced in the continuous, high-dose DSG treatment group.

The effect of CyA on xenograft rejection has been extensively studied by other researchers. Homan et al. [8] have reported that CyA prolonged cardiac xenograft survival and that a dose of 25 mg/kg per day suppressed the humoral response in the hamster-to-rat donor-recipient species combination. In our model, treatment with CyA alone prolonged graft survival more efficiently than DSG alone or a combination of splenectomy with DSG. There have been few reports regarding the synergistic effect of DSG and CyA on rat cardiac allograft [6, 27]. We now demonstrate that a short course of DSG (5 mg/kg per day), combined with continuous CyA (20 mg/kg per day), achieved dramatic long-term graft survival beyond 100 days. These findings indicate that DSG acts synergistically with CyA in this lung xenograft model, and once the initial phase is overcome, optimal CyA dosage can control rejection. Moreover, splenectomy did not enhance prolongation of graft survival in the CyA/DSG-treated animals. Hasan et al. [7] have recently reported that a combination of short-course methotrexate and cyclophosphamide with continuous CyA resulted in consistent survival of greater than 100 days in rat-to-hamster cardiac xenografts.

Infections are a significant cause of morbidity and death in transplantation. The incidence and severity of these infections depend on a number of factors, such as the recipient and organ donor status, the level of immunosuppression used in the basic protocol, the amount of anti-rejection therapy given, and the kind of transplant received. Our study showed that abscess formation, which has a high morbidity, was occasionally seen in animals treated with continuous infusions of CyA or CyA/DSG. Understanding the complications of these treatments will require further research.

In conclusion, our experimental results suggest that both the humoral and cellular immune systems operate in the acute rejection response in the hamster-to-rat lung xenograft model. DSG depresses antidonor antibody formation and macrophage/monocyte infiltration; however, it does not completely abrogate CD8+ cell infiltration. Splenectomy could not enhance the efficacy of DSG in this model. Initial treatment with DSG combined with continuous CyA can depress rejection, resulting in long-term graft survival. This study also indicates the potential for clinical applications of concordant lung xenotransplantation.

Acknowledgements We are grateful to H. Nakao for his excellent technical assistance and also to Dr. R. Takahira and H. Kusano for their advice in the pathological analysis of data.

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