Suppression of Macrophage Function and Prolongation of Graft Survival by the New Guanidinic-Like Structure, 15-Deoxyspergualin

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THE SUBSTANCE (±)-15-deoxyspergualin (15-DS) is a derivative of spergualin, a guanidinic-like structure. Its isolation from *Bacillus laterosporus* and antitumor activity was described by Iwasawa et al.[1] The substance was shown to be effective in experimental transplantation models with pancreas islet cells, heart, and kidney.[2,3] Especially with kidney antigen, specific nonreactivity was achieved by 15-DS in long-term surviving individuals.[4] Its immunopharmacological mode of action was evaluated by testing its effects on lymphocytes and cells of the monocyte/macrophage group after in vivo and in vitro application.

MATERIAL AND METHODS

15-DS

15-DS was kindly supplied by Dr Umezawa, Institute of Microbial Chemistry, Tokyo.

Animals

NMRI mice (H-2^c), F344 (RT1^{lvl}), and LEW (RT1^l) rats were purchased from Charles River/Wiga, Sulzfeld, FRG. DA (RT1^{avl}) and AS (RT1^l) rats were bred and housed at the Department of Immunology, Kiel, FRG. Male rats were used at the age of 9 to 13 weeks.

Cells of the Monocyte/Macrophage Group

Chemiluminescence reaction. Two milliliters of heparinized peripheral venous blood was collected by the puncture of the retroorbital vein plexus of rats. Monocytes were separated by density gradient centrifugation (800 × g, 20 minutes) over Ficoll-Hypaque (density, 1.077 g/L). Monocytes were suspended in TCM 199 containing penicillin, streptomycin (100 U/mL each), and heparin (10 U/mL). Monocytes were stimulated by the addition of immune complexes (tetanus toxoid–antitetanus toxoid, formed at equivalence). The chemiluminescence reaction was performed in a Biolumate (Berthold Co, Wildbad, FRG, model LB9505) in the presence of 20 μg/mL luminol in round-bottomed polystyrene vials (4 × 10^6 monocytes/mL). The integral of relative light units (RLU) was calculated after 15 minutes by an Apple computer.

Expression of major histocompatibility complex (MHC) class II antigens. Splenic and peritoneal macrophages were primarily obtained by plastic adherence with lactalbumin (containing 10% normal rat serum). For enrichment and purification of cells, a second period of adherence on glass cover slips (Ø, 12 mm, placed in 24-well culture plates) followed. Thus purified macrophages were incubated in vitro for one to four days with different concentrations of 15-DS in the presence of 9 units of interferon (added for stabilization of the MHC class II antigen expression of the target cells; the normally occurring rapid loss of these antigens in culture could thus be completely avoided). After this culture period, cells were washed and fixed in 2% formalin before preparing them for immunofluorescence (first antibody, anti-rat MHC class II MRC-OX6, 1:70, 30 minutes, 4 °C in the dark; second antibody, anti-mouse IgG fluorescein isothiocyanate, 1:24, 30 minutes, 4 °C in the dark). Class II–positive cells were evaluated as a percentage of the total cell number.

Lymphocytes

Mitogen-induced proliferation of rat splenic lymphocytes (5 × 10^4/well) was determined in the presence of concanavalin A (Con A, 0.6 μg/mL). The stimulation index was defined as the ratio between mitogen-stimulated ^{14}C-thymidine incorporation and the unstimulated control.

Plaque-forming cells (IgM-secreting cells) were determined with mouse splenic lymphocytes (2.5 × 10^5) after in vitro incubation with 10^6 sheep RBC for 48 hours.

In mixed lymphocyte culture (MLC), LEW responder spleen cells (10^5) were cocultivated with mitomycin C–blocked DA stimulator spleen cells (5 × 10^5) for five days. 15-DS was added to the culture medium in nine

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0041-1345/87/1901-0466$03.00/0

different concentrations ranging from 0.1 to 400 μg/mL culture medium.

Skin Transplantation

Tail skin pieces (0.5 × 1 cm) for LEW rats were grafted to the tails of F344 rats. Rejection was defined as the time the graft was of red-brown color and of hard consistency.

RESULTS

Skin Transplantation

Rejection of skin grafts in the MHC-different LEW-to-F344 system occurred after about 15 days in normal untreated rats. When graft recipients were treated intraperitoneally with 0.5 to 2.0 mg/kg 15-DS, about a doubling of graft survival time was observed. Larger amounts of the drug (5.0 or 10.0 mg/kg) were much less useful or were ineffective (Table 1). *Per os* (PO) administration of 15-DS led to similar or even better results. The optimal dose (about a threefold increase in graft survival) was shown to be in the range of 20 mg/kg. The higher concentrations required with PO administration suggest that only a part of the substance was absorbed unaltered via the enteric route.

Monocyte and Lymphocyte In Vitro Activity After Multiple In Vivo Administration

Under similar conditions, as described for the skin transplantation experiments, the effects of 15-DS on monocyte and lymphocyte functions were examined in vitro. F344 rats were treated ten times with 2 mg/kg 15-DS intraperitoneally (IP). On the days indicated in Table 2, the activities of either monocytes or lymphocytes were examined. The chemiluminescence reaction of peripheral blood monocytes was distinctively suppressed to 25%. Suppression lasted for at least 2 weeks. On the other hand, the mitogen (Con A) induced proliferation of F344 splenic lymphocytes was not affected significantly by 15-DS.

Table 1. Survival Time of Tail Skin Grafts (LEW → F344) After IP or PO Administration of 15-DS

IP		PO	
mg/kg*	Graft Survival Time† (Days, mean ± SD)	mg/kg*	Graft Survival Time† (Days, mean ± SD)
0	14.8 ± 1.5	0	15.4 ± 2.5
0.5	29.4 ± 2.4	2.5	21.5 ± 3.4
2.0	27.4 ± 3.3	5.0	22.2 ± 3.6
5.0	15.0 ± 0	10.0	25.5 ± 4.9
10.0	17.6 ± 2.2	20.0	40.5 ± 8.4

*15-DS was given 10 × (days 0 to 9).

†Rejection time was diagnosed as the day when the graft was of red-brown color and of hard consistency, n = 5.

Expression of MHC Class II Antigens on Macrophages

The variation of MHC class II (Ia$^+$) antigen expression of normal rat macrophages in vitro under the influence of 15-DS was examined by immunofluorescence following one to four days of cultivation of the cells in the presence of 0.01 to 500 μg/mL 15-DS. Figure 1A shows a significant dose-dependent decrease of class II antigen expression on splenic macrophages by 25% in the highest drug concentration in vitro. The effects are time independent as can be seen from the nearly identical patterns of reactivity on days 1, 2, and 4. The immunosuppressive effect of 15-DS is also visible three days after in vivo treatment of the rats with a single IP injection

Table 2. Effect of Multiple In Vivo Administrations of 15-DS on Monocytes and Lymphocytes

Monocytes		Lymphocytes	
Day	Chemiluminescence Reaction* (Percentage of Control)	Day	Con A–Induced Proliferation† (Percentage of Control)
0	100	0	100
3	82	3	82
6	57	5	103
8	56	10	73
10	38	13	99
13	28	21	125

15-DS was administered in doses of 2 mg/kg on days 0 to 9.

Abbreviation: Con A, concanavalin A.

*RLU/15 minutes × 10^3 in the presence of immune complexes (100% = 3,545 ± 499 RLU/15 minutes × 10^3), n = 3 experiments/day.

†Stimulation index control (100%) = 20 ± 4.5 in the presence of 0.6 μg/mL Con A, n = 6 experiments/d.

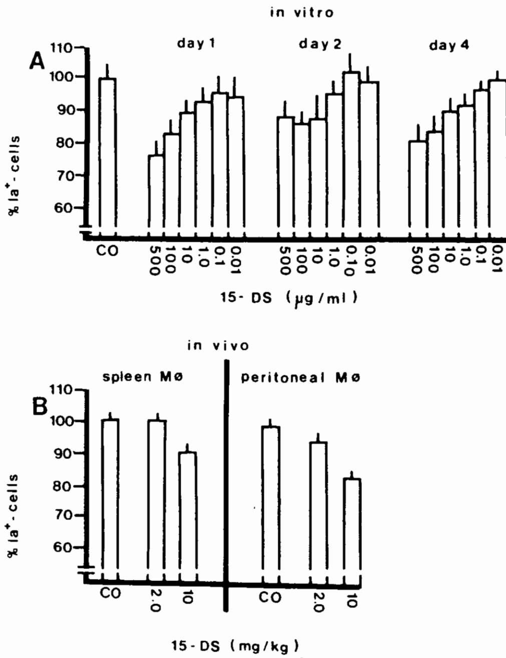

Fig 1. Time- and concentration-dependent inhibition of MHC class II antigen expression of macrophages by 15-DS (A) after in vitro and (B) in vivo application; n = 9 experiments per day and concentration.

of 10 mg/kg 15-DS (Fig 1B). The decrease obtained is more pronounced on peritoneal macrophages (16%, $P \leq .05$) than on splenic macrophages (8%, $P \geq .05$) possibly due to a direct *v* indirect effect of the drug.

In Vitro Influence of 15-DS on Macrophage-Dependent Lymphocyte Function

It was determined whether 15-DS could inhibit the MLC and the plaque-forming cell (PFC) response. Figure 2 shows a dose-dependent decrease of the MLC by 15-DS. Inhibition increasing from >60% to >90% is achieved by drug concentrations ranging from 10 to 400 µg/mL. In the PFC assay, 15-DS was added in four concentrations from 0.1 to 100 µg/mL into the culture medium. Similar to the MLC, a dose-dependent reduction of IgM-secreting lymphocytes was observed; an IC_{50} value of 2 µg/mL was calculated.

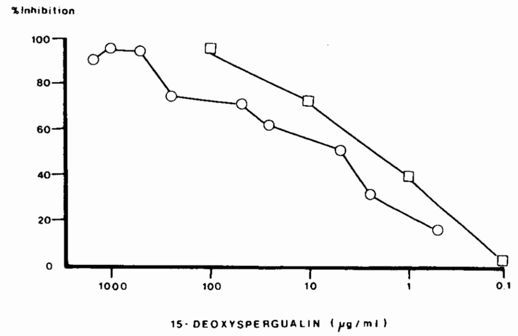

Fig 2. Concentration-dependent inhibition of MLC (○—○) and PFC (□—□) by 15-DS. MLC, N = 5 experiments/concentration; PFC, 100% value (control) ≙ 1,493 ± 61, n = 5 experiments/concentration.

DISCUSSION

15-DS, a new immunosuppressive drug with predominant activity against cells of the monocyte/macrophage group was tested in vivo and in vitro in a variety of experimental models.

Skin graft survival was effectively prolonged in an MHC variant strain combination. Bearing in mind the well-known strong immunogenicity of skin, the data presented here upon IP or PO administration of this drug appear to be remarkable. They are compatible with the data obtained with fully allogeneic kidney grafts suggesting that 15-DS is as powerful as cyclosporine A (CsA).[4]

Although the suppression of chemiluminescence already hints towards an inhibitory influence by 15-DS on cells of the monocyte-macrophage group, the downregulation of MHC class II antigen expression may be regarded as providing a further step towards elucidation of its mode of action. How far this applies to other cells of this group such as the nonphagocytic, highly immunogenic dendritic cells is under investigation.

Although the clearly different effects of 15-DS on functions of monocytes and lymphocytes, analyzed in vitro following in vivo applications of the drug, support the notion of an immunosuppressive activity of 15-DS, predominantly against cells of the monocyte/macrophage group, this requires further in

vitro analysis. The remarkably strong effect of 15-DS on macrophage-dependent lymphocyte functions following in vitro application of the drug will allow us to further clarify this point by separately treating one or the other of the cooperating cells. In particular, MLC inhibition studies may indicate how far MHC class II antigen expression–related graft immunogenicity depression may be involved in addition to a depression of the function of antigen-presenting macrophages on the side of the responder.

Obviously, a preferential effect of 15-DS on antigen-presenting cell functions would lead to the suggestion of combining this drug with another that preferentially acts on T lymphocytes such as CsA. It is the distinct possibility of potentiating immunosuppressive effects by interfering at a different cellular site that appears to make 15-DS interesting.

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