

BLASTOGENIC RESPONSES AND THE
RELEASE OF INTERLEUKINS 1
AND 2 BY SPLEEN CELLS
OBTAINED FROM RAT SKIN
ALLOGRAFT RECIPIENTS
ADMINISTERED WITH
15-DEOXYSPERGUALIN

KYUICHI NEMOTO and FUMINORI ABE

Research Laboratories, Pharmaceuticals Group,
Nippon Kayaku Co., Ltd.,
3-31-12 Shimo, Kita-ku, Tokyo 115, Japan

TERUYA NAKAMURA

Central Research Laboratories,
Takara Shuzo Co., Ltd.,
3-4-1 Seta, Ohtsu-shi, Shiga-ken 520-21, Japan

MASAAKI ISHIZUKA, TOMIO TAKEUCHI
and HAMAO UMEZAWA†

Institute of Microbial Chemistry,
3-14-23 Kamiosaki, Shinagawa-ku,
Tokyo 141, Japan

(Received for publication February 5, 1987)

15-Deoxyspergualin (DSG) is an analogue of spergualin isolated from *Bacillus laterosporus*¹⁾. In mice, DSG strongly inhibits antibody formation and delayed-type hypersensitivity to sheep red blood cells²⁾. In rats, DSG inhibits the rejection of skin³⁾, heart³⁾ and islet cell⁴⁾ allografts. The mechanisms responsible for these immunosuppressive actions of DSG have not been elucidated. To investigate these mechanisms, we administered DSG or cyclosporin A (Cy A), a representative immunosuppressant⁵⁾, to rats transplanted with a skin allograft. We then examined the blastogenic response and the release of interleukins 1 (IL 1) and 2 (IL 2) by spleen cells isolated from these rats.

The transplantation of rat skin was carried out as previously reported⁶⁾. Male 9 weeks old Fisher 344 and SHR rats (Charles River Japan, Kanagawa, Japan) were used as recipients and donors, respectively. DSG was prepared at Takara Shuzo Co., Ltd.⁷⁾. It was dissolved in saline, sterilized by passing through a 0.22 μ m-filter, and stored at -20°C until use. Cy A (Sandimmun) was purchased from Sankyo Co.,

Ltd., Tokyo, Japan, and diluted with olive oil as required. As previously reported⁸⁾, DSG dose-dependently prolonged mean survival time (MST) of the skin allograft at doses of 2 to 12.5 mg/kg, when given ip for the successive 10 days after the grafting. In a separate experiment, when Cy A was given po at doses of 12.5 ($n=6$), 25 ($n=7$) and 50 mg/kg ($n=6$) using the same schedule, the MSTs were 9.3 ± 0.5 , 14.1 ± 3.3 and 16.0 ± 4.4 days (mean \pm SD), respectively (control MST = 7.5 ± 1.5 days; $n=4$). The result is in agreement with the report of DENHAM *et al.*⁹⁾. Therefore, DSG was administered ip into 6 rats at an immunosuppressive dose of 3 mg/kg once a day for 22 days from 1 day (day 1) after the transplantation. Cy A was given orally to 10 rats at a dose of 25 mg/kg following the same schedule. A control group of 7 rats received neither agent. The spleens were removed from 5 animals per group on day 23 and used in the following experiments.

RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), streptomycin (100 μ g/ml) and benzylpenicillin (100 units/ml) was used to incubate spleen cells at 37°C in 5% CO_2 in air. From each group one aliquot of unseparated spleen cells was tested for blastogenic response as reported previously⁸⁾. Another aliquot of cells was used to prepare culture supernatants containing IL 1. These cells were suspended in the RPMI medium (1×10^7 cells/ml), seeded into FCS-coated Falcon 3003 dishes (10 ml/dish) and incubated for 2 hours.

For each group the adherent cells were obtained and pooled. The pooled cells (1×10^6 cells/ml/well) were incubated with 25 μ g/ml of *Escherichia coli* 055: B5 lipopolysaccharide (LPS, Difco, Detroit, U.S.A.) for an additional 36 hours in 24-well Costar culture plates. The supernatants were separated and stored at 4°C . To prepare culture supernatants containing IL 2, a third aliquot of unseparated cells (2×10^6 cells/ml/well) was incubated with or without 10 μ g/ml of concanavalin A (Con A, Sigma, St. Louis, U.S.A.) in the 24-well plates. After 48 hours incubation the supernatants were separated and stored at 4°C . The IL 1 and IL 2 activities in the culture supernatants were determined according to ROSENWASSER and DINARELLO¹⁰⁾ and GILLIS *et al.*¹⁰⁾, respectively. Data were analyzed by Student's t-test.

Tail skin grafts of 4 SHR donors were trans-

† Deceased.

Table 1. Effects of DSG and Cy A administrations on blastogenic responses of spleen cells stimulated with mitogens.

Group	^3H]TdR incorporation (cpm)	
	Con A	LPS
Control	130,833 ± 18,458	24,073 ± 5,967
DSG	359,714 ± 182,209*	26,472 ± 9,650
Cy A	80,381 ± 24,542**	8,177 ± 1,903**

Triplicate 0.2 ml of the unseparated cell (2×10^5 cells) were incubated with 5 $\mu\text{g}/\text{ml}$ of Con A or 100 $\mu\text{g}/\text{ml}$ of LPS in flat bottomed Costar 96-well plates for 72 hours and pulsed with 1 μCi of [^3H]thymidine (^3H]TdR, 15.5 Ci/mmol, New England Nuclear, Boston, U.S.A.) for the last 8 hours of the incubation period. After the cells were harvested, measurement of ^3H]TdR incorporation into the cells was carried out in liquid scintillation counters. Data are shown as the mean \pm SD of five separate determinations.

* $P < 0.05$. ** $P < 0.01$.

planted to 23 Fisher 344 recipients. In the control group ($n=7$), the grafts were rejected at 6.9 ± 1.1 days (mean \pm SD) after grafting. In contrast, DSG ($n=6$) and Cy A ($n=10$) completely inhibited the rejection until day 23 when the spleens were removed.

Unseparated spleen cells prepared from each group were tested for their responses to Con A and LPS. As shown in Table 1, the Con A and LPS-stimulated blastogenic response of cells obtained from Cy A administered rats were significantly lower than the response of cells obtained from those control rats. In contrast, the administration of DSG did not impair the response of the cells to either mitogen. On the contrary, a significantly higher blastogenic response to Con A was observed in spleen cells obtained from the rats treated with DSG.

Adherent spleen cells were tested for their ability to release IL 1 after LPS stimulation. As shown in Table 2, the IL 1 activity was slightly lower in the cells from Cy A-treated rats (no difference statistically significant). In contrast however, a significantly higher IL 1 activity was observed in the cells from DSG-treated rats.

The effects of DSG or Cy A administration on the ability of the unseparated cells incubated with or without Con A to release IL 2 were examined. The results are shown in Table 3. Compared to control, the IL 2 activity released spontaneously and in response to stimulation

Table 2. The ability of adherent spleen cells from DSG and Cy A-treated rats to release IL 1.

Group	^3H]TdR incorporation (cpm)
Control	132,384 ± 14,382
DSG	183,250 ± 23,455*
Cy A	114,319 ± 19,832

Male C3H/HeJ mice purchased from Jackson Laboratory, Bar Harbor, U.S.A., were used as thymocyte donors. The thymocytes (4×10^5 cells) were incubated for 72 hours in 0.15 ml of the incubation medium with 1.25 $\mu\text{g}/\text{ml}$ of Con A with 0.05 ml of 1/32 dilution of the LPS-stimulated supernatants or the incubation medium. The other testing conditions are the same as those described in the legend of Table 1. Data are shown as the mean \pm SD of triplicate determinations. The background level of ^3H]TdR incorporation to the cells stimulated with Con A (1.25 $\mu\text{g}/\text{ml}$) was 33,153 \pm 9,005 cpm.

* $P < 0.05$.

Table 3. Effects of DSG and Cy A administrations on IL 2 release.

Group	^3H]TdR incorporation (cpm)	
	- Con A	+ Con A
Control	16,880 + 4,122	238,329 + 28,524
DSG	23,820 + 6,477*	333,634 + 18,017**
Cy A	10,344 + 2,753**	144,154 + 25,125**

IL 2 activities in the supernatants from the unseparated spleen cells with or without Con A were quantitated in the presence of 1×10^4 CTLL-2 cells in a final volume of 0.2 ml. The cells were incubated for 18 hours, pulsed with 1 μCi of [^3H]TdR, and further incubated for 6 hours. Measurement of the incorporation of [^3H]TdR into the cells was carried out as described in the legend of Table 1. Data correspond to 1/8 dilution of the Con A-free supernatants and 1/256 dilution of the Con A-induced supernatants, respectively, and are shown as the mean \pm SD of five determinations.

* $P < 0.05$. ** $P < 0.01$.

by Con A were significantly lower in the cells from the rats treated with Cy A. The amount of IL 2 released however was found to be significantly higher in the cells from the rats treated with DSG.

These results demonstrated that the administration of Cy A at a dose of 25 mg/kg significantly reduced the blastogenic response and IL 2 re-

lease from the spleen cells of rats receiving skin allografts. The finding on IL 2 release is in agreement with the report of ABBUD-FILHO *et al.*¹¹⁾. However, these functions of the cells were not impaired by the administration of DSG at a dose of 3 mg/kg, with the exception of the blastogenic response to LPS. The findings of this study using an immunosuppressive dose of each agent suggest that the immunosuppressive agent DSG has a different mechanism of action from that of Cy A.

Acknowledgment

We thank Mr. K. TAKAHASHI for his critical discussion and Mrs. M. HAYASHI, Ms. Y. SUGAWARA, Ms. E. HAYASHI and Mr. K. KOTAKE for their technical assistance.

References

- 1) IWASAWA, H.; S. KONDO, D. IKEDA, T. TAKEUCHI & H. UMEZAWA: Synthesis of (–)-15-deoxyspergualin and (–)-spergualin-15-phosphate. *J. Antibiotics* 35: 1665~1669, 1982
- 2) NEMOTO, K.; M. HAYASHI, F. ABE, T. NAKAMURA, M. ISHIZUKA & H. UMEZAWA: Immunosuppressive activities of 15-deoxyspergualin in animals. *J. Antibiotics* 40: 561~562, 1987
- 3) WALTER, P.; J. THIES, G. HARBAUER, G. DICKNEITE, H. H. SEDLACEK & F. VONNAHME: Allogeneic heart transplantation in the rat with a new antitumoral drug — 15-deoxyspergualin. *Transplant. Proc.* 18: 1293~1294, 1986
- 4) DICKNEITE, G.; H. U. SCHORLEMMER, P. WALTER & H. H. SEDLACEK: Graft survival in experimental transplantation could be prolonged by the action of the antitumoral drug 15-deoxyspergualin. *Transplant. Proc.* 18: 1295~1296, 1986
- 5) BOREL, J. F.; C. FEURER, H. U. GUBLER & H. STAHELIN: Biological effects of cyclosporin A: A new antilymphocytic agent. *Agents Actions* 6: 468~475, 1976
- 6) UMEZAWA, H.; M. ISHIZUKA, T. TAKEUCHI, F. ABE, K. NEMOTO, K. SHIBUYA & T. NAKAMURA: Suppression of tissue graft rejection by spergualin. *J. Antibiotics* 38: 283~284, 1985
- 7) UMEDA, Y.; M. MORIGUCHI, H. KURODA, T. NAKAMURA, H. INUMA, T. TAKEUCHI & H. UMEZAWA: Synthesis and antitumor activity of spergualin analogues. I. Chemical modification of 7-guanidino-3-hydroxyacyl moiety. *J. Antibiotics* 38: 886~898, 1985
- 8) DENHAM, S.; J. M. STYLES, R. K. BARFOOT & C. J. DEAN: Reversible suppression of allo-antibody production by cyclosporin A. *Int. Arch. Allergy Appl. Immunol.* 62: 453~458, 1980
- 9) ROSENWASSER, L. J. & C. A. DINARELLO: Ability of human leukocytic pyrogen to enhance phytohemagglutinin-induced murine thymocyte proliferation. *Cell. Immunol.* 63: 134~142, 1981
- 10) GILLIS, S.; M. M. FERM, W. OU & K. A. SMITH: T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* 120: 2027~2032, 1978
- 11) ABBUD-FILHO, M.; J. W. KUPIEC-WEGLINSKI, J. L. ARAUJO, C. D. HEIDECHE, N. L. TILNEY & T. B. STROM: Cyclosporine therapy of rat heart allograft recipients and release of interleukins (IL 1, IL 2, IL 3): A role for IL 3 in graft tolerance? *J. Immunol.* 133: 2582~2586, 1984