PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 145, 669–674 (1974)

Evaluation of Oxisuran as an Immunosuppressive Agent[1] (37872)

BO HUSBERG AND ISRAEL PENN
(Introduced by H. H. Freedman)

Department of Surgery, University of Colorado Medical Center, Denver, Colorado 80220, and Department of Surgery, Veterans Administration Hospital, Denver, Colorado 80220

Oxisuran, 2-[(methylsulfinyl)acetyl] pyridine, is a new immunosuppressive agent which has been reported to have the unique property of inhibiting cell-mediated immunity without any concomitant suppression of humoral antibody formation (1, 2). The mechanism of action is not yet known. It is not cytotoxic nor antiproliferative, and it does not exhibit anti-inflammatory activities. Published studies on its effects on cell-mediated immunity have been performed in allograft models using skin grafts in mice, rats, and dogs, fetal hearts in mice, and mammary glands in rats. Humoral antibody responses have been measured in normal and graft-bearing mice and dogs.

The present investigations were undertaken to further evaluate the effects of oxisuran on humoral and cellular immunity in rats undergoing organ transplantation. The experiments were done in an *in vivo* and *in vitro* kidney transplantation model which permits selective study of cellular immunological response, cytotoxic antibody response, and blocking antibody response (3, 4). In addition, the effects of oxisuran upon functional survival time of heterotopic cardiac allografts in rats were studied.

Materials and Methods. Two groups of experiments were performed in which inbred Wistar–Furth (WF) rats served as donors of kidneys or hearts which were transplanted to inbred, female Brown–Norway (BN) rats. All experiments were performed under anesthesia with pentobarbital sodium (Nembutal), 30 mg/kg intraperitoneally. When necessary, this was supplemented by ether inhalation.

Kidney-Transplant Experiments. The donors were male or female and weighed 150–200 g while the recipients weighed 200–250 g. Two groups of animals were studied: (Group 1) Controls (10) received no immunosuppressive therapy; (Group 2) treated animals (10) received oxisuran 150 mg/kg daily for 14 days, intraperitoneally, commencing 4 hr before transplantation. Renal transplantation was performed by the method of Lauschke and Hermann (5) except that the ureter was not anastomosed to the bladder but was left lying free in the peritoneal cavity. The recipients' own kidney were left undisturbed. The allografts were removed after 5 days. Sections of the kidneys were stained with hematoxylin and eosin and studied by light microscopy. Peripheral WBC and differential counts were performed on Days 0, 5, and 14.

Samples of peripheral blood and thoracic duct lymph were obtained from recipients 14 days after transplantation. Fresh or heat-inactivated serum and/or thoracic duct cells (TD cells) were added to cultured donor-strain kidney cells, which had been grown in monolayers for 20–23 days without subcultivation (3). These target cells were labeled with ^{51}Cr sodium chromate and were suspended in Eagle's medium in flat-bottom

[1] Supported by research grants from the Veterans Administration, by grants RR-00051 and RR-00069 from the General Clinical Research Centers Program of the Division of Research Resources, National Institutes of Health, and by Grants AI-10176-01, AI-10334, AI-AM-08898, AM-07772, and HE-09110 of the United States Public Health Service.

TABLE I. Addition of Thoracic-Duct Lymphoid Cells and/or Serum to 10^5 ^{51}Cr-Labeled WF Kidney Cells.[a]

	Tube No.[b]
Control tubes	
WF cells + 0.3 ml WF normal heat inactivated serum	1
WF cells + BN normal TD cells + 0.3 ml BN normal heat inactivated serum	2[c]
Tubes with test lymphoid cells and/or serum	
WF cells + 0.3 ml BN recipient serum + 0.1 ml BN normal serum	3
WF cells + BN recipientTD cells + 0.3 ml BN recipient heat inactivated serum	4
WF cells + BN recipientTD cells + 0.3 ml BN recipient serum	5
WF cells + BN recipient TD cells + 0.3 ml BN normal heat inactivated serum	6
Complement control tubes	
WF cells + 0.3 ml BN recipient serum + 0.5 ml BN normal serum	7[c]
WF cells + BN recipient TD cells + 0.4 ml BN normal serum	8[c]

[a] The number of thoracic duct (TD) cells was always 10^6. Serum was always fresh except when indicated as heat inactivated.

[b] All sera were added 15 min before the lymphoid cells.

[c] Experiments were performed in five animals in each group.

test tubes to which recipient serum and/or TD cells were added in various combinations as shown in Table I. The volume in each test tube was made up to 1.5 ml by the addition of Eagle's medium. Cytotoxicity was measured by the release of ^{51}Cr from the target cells as previously described (4). The release of radioactivity at different time intervals was measured in 0.5-ml samples of the supernatant removed from each tube. In each instance, the removed fluid was replaced with an equivalent volume of Eagle's medium. The radioactivity measurements were made in a well-type scintillation counter. All tests were done in duplicate. The isotope release values were calculated as percent release of the original total target cell radioactivity and, also, as "corrected release" which was the percent release above the spontaneous release of radioactivity (6). The "corrected release" values in the two experimental groups were compared using the Student's t test when SD_1^2/SD_2^2 was less than 3.2 (Table III).

With the exception of one experiment, the serum and cells which were incubated together with the target cells were taken from animals belonging to the same experimental group. In the exceptional study (Tube X, Table III), 0.3-ml samples of pooled heat-inactivated serum from BN recipients belonging to Group 1 or 2 were each incubated with 10^5 WF target cells and 10^6 thoracic duct cells from 4 different BN recipient rats from Group 1.

Heart-Transplant Experiments. The donors were male or female and weighed 200–250 g; the female recipients weighed 250–300 g. As in the kidney transplant experiments, two groups of animals were studied: (Group 3) Untreated controls (10); (Group 4) oxisuran-treated rats (10) (150 mg/kg per day). The hearts were transplanted heterotopically into the abdomen as described by Ono and Lindsay (7). The graft aorta and pulmonary artery were anastomosed end to side to the aorta and inferior vena cava, respectively. All other major vessels in the donor heart were ligated. Total ischemia time ranged from 45 to 70 min.

The functional status of the homograft was assessed daily by palpation and, if necessary, by electrocardiography under ether anesthesia. When regular heartbeats could no longer be detected by these methods, the abdomen was opened and the heart examined. The presence of cardiac standstill, fibrillation, or very feeble pulsations was indicative of failure of graft function (8).

Peripheral WBC and differential counts were performed on Days 0 and 7.

Results. In the kidney transplant experiments, thoracic-duct lymph flow in Groups

TABLE II. Total White Blood Cell (WBC) and Lymphocyte Counts in Peripheral Blood and Thoracic-Duct Lymph in the Kidney-Transplantation Experiments (per mm³). Mean ± SD.

	Peripheral blood			Lymph
	Pretransplantation	5 Days post-transplantation	14 Days post-transplantation	14 Days post-transplantation
Groups 1				
WBC count	15400 ± 3300	20600 ± 5500	21000 ± 5600	36000 ± 3800
Lymphocyte count	12000 ± 2900	11200 ± 4900	14300 ± 4700	35300 ± 3700
Group 2				
WBC count	17400 ± 3700	20400 ± 6500	22400 ± 7000	33500 ± 5800
Lymphocyte count	13900 ± 2700	13900 ± 4800	14300 ± 6000	32500 ± 5500
t Value[a]				
WBC Groups 1/2	1.22	0.11	0.46	1.15
t Value				
Lymphocytes Groups 1/2	1.40	1.20	0.02	1.26

[a] Homogenous variance, $df = 18$; *** $P < 0.001$, $t > 3.610$; ** $P < 0.01$, $t > 2.552$ (significant); * $P < 0.05$, $t > 1.734$.

1 and 2 was approximately 0.5 ml/hr. The total WBC counts and the numbers of lymphocytes in the peripheral blood and thoracic-duct lymph are shown in Table II. Oxisuran treatment had no effect on the total WBC or lymphocyte counts.

At the time of transplant nephrectomy, the vascular anastomoses were patent in 80–90% of the animals in both groups. Histologic examination of the transplanted kidneys showed that lymphoid cell infiltration was of moderately greater degree in the controls than in the oxisuran-treated rats.

In the *in vitro* cytotoxicity tests, *the control tubes,* which contained ^{51}Cr-labeled WF kidney cells alone or together with normal BN TD cells and heat-inactivated normal BN serum, showed approximately 25% spontaneous release of the isotope into the supernatant in 24 hr in Groups 1 and 2 (Table I, Tubes 1 and 2). After this time, spontaneous cell death occurred at an accelerated rate.

BN recipient TD cells from *Group 1* control animals had a definite cytotoxic effect on the WF target cells after 12 hr of incubation (Table III, Tube 6). This effect was significantly reduced ($P < 0.001$ at 12 hr) when heat-inactivated serum from a BN recipient was added 15 min before the lymphoid cells (Table III, Tube 4). The addition to target cells of complement-containing fresh recipient serum without lymphoid cells also caused a cytotoxic effect which was demonstrable after 2 hr (Table III, Tube 3). It should be noted that all isotope release values in Table III are "corrected ones," i.e., they express percent release above the spontaneous release of radioactivity.

Recipient TD cells from *Group 2* animals caused a diminished isotope release from the target cells as compared with those from Group 1 rats (Table III). According to kinetic studies performed earlier, the change corresponded to a 15–20% decrease in the number of sensitized TD cells that were present (4). The cytotoxic effect of fresh, complement-containing recipient serum was not altered significantly by oxisuran therapy.

In the "exceptional experiment" in which pooled heat-inactivated recipient serum from Group 1 or Group 2 rats was added to the target cells before the introduction of standard samples of sensitized TD cells, the blocking effect of serum on cell-mediated cytotoxicity could be compared better than in the "Tube 4" experiments (Table III, Tube X). The blocking effect was similar when either serum was used. This indicates that the blocking effect of heat-inactivated recipient serum was not altered by oxisuran therapy.

In Groups 1 and 2, when fresh recipient serum and TD cells were added to the target cells in combination (Tube 5), the isotope release pattern in the first 4 hr was similar

TABLE III. Cytotoxicity Test Showing Percent "Corrected Release" of ^{51}Cr from Target Cells to Supernatant.

	Tube 3				Tube 4				Tube 5				Tube 6				Tube X[a]
	Target cells + serum (fresh)				Target cells + TD cells + serum (heat inactivated)				Target cells + TD cells + serum (fresh)				Target cells + TD cells				Target cells + Group 1 TD cells + Group 1 or 2 serum (heat-inactivated)
	2 hr	4 hr	12 hr	24 hr	2 hr	4 hr	12 hr	24 hr	2 hr	4 hr	12 hr	24 hr	2 hr	4 hr	12 hr	24 hr	12 hr
Group 1																	
Mean	23.0	23.5	26.6	20.5	1.5	3.5	27.3	36.3	24.1	26.3	33.6	38.7	1.5	4.0	36.2	43.0	26.5
SD	2.19	1.75	2.78	2.97	—	—	2.22	4.23	2.29	2.35	2.98	4.45	—	—	1.76	4.3	1.52
Group 2																	
Mean	23.1	25.2	25.3	23.4	1.8	2.9	18.3	31.4	25.0	27.4	32.4	39.1	3.1	5.0	27.6	39.3	27.2
SD	1.77	1.56	3.16	3.46	—	—	2.12	3.59	2.97	2.68	2.06	3.44	—	—	2.73	5.16	1.27
t Value[b]																	
Groups 1/2	0.04	2.18*	0.88	1.86*	—	—	8.94***	2.68**	1.05	0.91	0.96	0.21	—	—	7.98***	1.60	0.61

[a] See *Materials and Methods.*

[b] Homogenous variance, $df = 18$; *** $P < 0.001$, $t > 3.610$; ** $P < 0.01$, $t > 2.552$ (significant); * $P < 0.05$, $t > 1.734$.

to that obtained with fresh serum alone. After this time, the release curve resembled that obtained with lymphoid cells alone.

Both in Groups 1 and 2 the addition of fresh, complement-containing serum from normal BN rats did not significantly alter the cytotoxic effects of either fresh recipient serum or recipient TD cells (Table I, Tubes 7 and 8). These results indicate that the need for complement in the tubes with fresh recipient serum was satisfied and that cell-mediated cytotoxic effects were independent of complement.

Heart-Transplant Experiments. The peripheral WBC and lymphocyte counts were not significantly different in the two experimental groups. The functional survival time of the cardiac allografts was 7.8 ± 1.1 days in the controls and 11.9 ± 2.7 days in the oxisuran-treated rats (Table IV). The difference was highly significant ($P < 0.001$) as determined by the rank-sum test (9).

Discussion. The basic kinetics of the test system used in the kidney-transplant experiments in the present study have been described elsewhere (3, 4). The animals treated with oxisuran showed a diminished cellular immune response to the kidney allografts. This was not caused by a decrease in number of lymphoid cells in the peripheral blood or thoracic-duct lymph but by a decreased cytotoxic effect of the cells. This may have resulted from an actual decrease in the number of *sensitized* lymphoid cells or from a decreased ability of all the lymphoid cells to attack the target cells. In contrast, it has been demonstrated previously (3) for cyclophosphamide that there is a "quantitative" rather than "qualitative" depressive effect, with reduction in total number of thoracic-duct lymphocytes and with about the same cytoaggressive effect as for nonimmunosuppressed TD cells when the same numbers of cells are used.

TABLE IV. Prolongation of Functional Survival of Heterotopic Cardiac Allografts by Oxisuran.

Group	Survival (days) (Mean ± SD)
Control	7.8 ± 1.1
Oxisuran treated	11.9 ± 2.7*

* $P < 0.001$.

Oxisuran therapy did not alter the cytotoxic effect of fresh recipient serum nor the blocking activity of heat-inactivated recipient serum, indicating that the antibody-producing cells and their precursors were left undisturbed by this treatment. The results of the kidney-transplant experiments, which showed a selective depression of cellular immunity, are in keeping with the findings in the heart-transplant studies, in which prolongation of graft survival occurred with oxisuran therapy.

These findings confirm the results of previous workers (1, 2) that oxisuran's immunosuppressive qualities are through its effects on cellular immunity. The previous studies showed its efficacy in prolonging skin-graft survival. The present work extends these findings to other organs with the demonstration of decreased *in vitro* cytotoxicity of thoracic duct cells in kidney allograft experiments and prolongation of functional survival time of cardiac transplants.

Compared with other immunosuppressive agents, the effects of oxisuran at the doses used in the rat are relatively weak. In the kidney-transplant model, it has been found that antilymphocyte serum, methotrexate, and corticosteroids all can have more potent suppressive effects on cellular immunity (4). In addition, cyclophosphamide, in a dose of 10 mg/kg/day, caused significantly greater prolongation of WF → BN cardiac allograft survival, 15–16 days as compared with 11.9 days with oxisuran (Husberg, unpublished data). A great virtue of oxisuran, compared with many other immunosuppressive agents, is its lack of toxicity on the bone marrow. It is possible that oxisuran in combination with smaller than usual doses of other immunosuppressive drugs may provide effective and safer control of allograft rejection.

Summary. Oxisuran, 2-[(methylsulfinyl)-acetyl]pyridine, is a new immunosuppressive agent which is reported to act as a differential inhibitor of cell-mediated immunity. Published studies have shown that it prolongs skin graft survival in mice, rats, and dogs. The present experiments demonstrate that it is also an immunosuppressive agent in the transplantation of other organs. Oxisuran produced significant prolongation of func-

tional survival time of heterotopic cardiac allografts in rats. In addition, using a rat kidney-transplant model, there was significant reduction of *in vitro* cytotoxicity of thoracic duct cells against target kidney cells, whereas the humoral antibody response was unchanged.

We thank Dr. H. H. Freedman of Warner–Lambert Research Institute, Morris Plains, NJ, for helpful advice and data concerning oxisuran and for generously supplying us with this immunosuppressive agent.

1. Freedman, H. H., Fox, A. E., Shavel, J. J., and Morrison, G. C., Proc. Soc. Exp. Biol. Med. **139**, 909 (1972).

2. Fox, A. E., Gawlak, D. L., Ballantyne, D. L., Jr., and Freedman, H. H., Transplantation **15**, 389 (1973).

3. Husberg, B., Clin. Exp. Immunol. **10**, 697 (1972).

4. Husberg, B., Clin. Exp. Immunol. **14**, 271 (1973).

5. Lauschke, H., and Hermann, G., Z. Ges. Exp. Med. **147**, 29 (1968).

6. Brunner, K. T., Manuel, J., Cerottini, J. C., and Chapuis, B., Immunology **14**, 181 (1968).

7. Ono, K., and Lindsey, E. S., J. Thor. Cardiovasc. Surg. **57**, 225 (1969).

8. Barker, C. F., and Billingham, R. E., Transplant. Proc. **3**, 172 (1971).

9. Dixon, W. J., and Massey, F. J., "Introduction to Statistical Analysis," McGraw-Hill, New York (1969).

Received July 25, 1973. P.S.E.B.M., 1974, Vol. 145.