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Negative Observations on Immunological Side Effects of Rifampin and Dapsone in Mice[1]

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Rifampin, 3-(4-methyl-1-piperazyl-iminomethyl)-rifamycin SV, is a semi-synthetic derivative of rifamycin which was isolated from *Streptomyces mediterraneus*. It is an orally active, bactericidal antibiotic which exerts remarkable effects on *Mycobacterium leprae* and *M. tuberculosis*. It is known to block bacterial DNA-dependent RNA-polymerase *in vitro* ([10]). Short-period treatment with the drug exerts a prominent effect on patients with leprosy ([12, 20]). However, it is described as an immunosuppressive agent since it prolongs allograft survival in mice ([4]) and rabbits ([19]), suppresses delayed-type hypersensitivity reactions to tuberculin in guinea pigs ([9]), and interferes with the blastic transformation of lymphocytes *in vitro* ([9])

A bacteriostatic sulfone compound, dapsone (4,4'-diaminodiphenyl sulfone, DDS) has also cured many leprosy patients since 1948 ([6]). Some side effects of the drug are reported on cell-mediated immunity, e.g., depression of human peripheral blood lymphocyte transformation by phytohemagglutinin *in vivo* ([18]) and *in vitro* ([3]).

From the point of view of leprosy therapy, it is important to know if these drugs exert immunosuppressive effects at normal clinical doses, i.e., doses equivalent to those used in humans based on body weight. In the present study, we investigated the effects on mice of human clinical doses of rifampin and dapsone on humoral antibody production and delayed hypersensitivity responses to sheep erythrocytes. Since it is reported that 4-hydroperoxycyclophosphamide, a derivate of cyclophosphamide, is more effective on precursors of supressor T cells than on mature ones ([11]), and there is the suggestion that multiplying cells are more sensitive to rifampin than resting ones ([1]), we designed an experimental system to see the effect of rifampin or dapsone on multiplying precursor cells of suppressor T cells. Phagocytic activity by adherent peritoneal cells from mice treated with the drugs was measured since it was reported that rifampin suppressed phagocytosis of sheep erythrocytes by peritoneal macrophages ([2]) and depressed antigen-specific migration inhibition ([17]). The effect of the drugs on tumor growth was also examined in association with a tumor rejection system. In the present study, sheep erythrocytes were chosen as a thymus-dependent antigen instead of *M. leprae* because immunological experimental methods are established and the results can be obtained in a short-term experiment with respect to the antigen. Since no effect was shown with a close equivalent to one clinical dose of the drugs, we also examined the effects with threefold and sixfold higher doses.

MATERIALS AND METHODS

Mice. Mice were either purchased from Shizuoka Laboratory Animals Center, Shizuoka, Japan, or were bred at our institute. Eight to 12-week-old, age- and sex-matched BALB/c mice, 4–9 for each experimental group, were used in all experiments except the one in which 15–30 female C57BL/6 mice, 8–9 weeks old, were utilized as a low-responder strain to sheep erythrocytes.

Drugs. A daily clinical dose (1 CD) of dapsone (4,4'-diaminodiphenyl sulfone; Nakarai Chemicals, Kyoto, Japan) taken to be equivalent to that used in humans was 1.7 mg/kg of body weight. Three g of the drug was mixed with 17 g of starch so that it could be weighed accurately, and 10 mg/kg of the mixture was employed as 1 CD. Ten mg/kg of rifampin (Dai-ichi Seiyaku,

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TABLE 1. *Effect of rifampin and dapsone on antibody production in SRBC.*

Mice	Drug[a]	Dose	Number of PFC[b,c] (mean ± S.D.)	
			Drug treated	Controls
BALB/c	Rifampin	1 CD	2783 ± 560	2641 ± 459
	Dapsone	1 CD	3288 ± 891	3085 ± 327
		3 CD	3298 ± 503	3576 ± 444
C57BL/6	Rifampin	1 CD	1702 ± 586	2002 ± 226
		3 CD	1090 ± 385	981 ± 332
	Dapsone	1 CD	1132 ± 315	1328 ± 679
		3 CD	1493 ± 290	2263 ± 820

[a] Drugs were administered for 10 days.
[b] Number/10^6 viable spleen cells.
[c] In each experiment, control mice that had not received SRBC showed 0 to 9 PFC/10^6 viable spleen cells.

Tokyo, Japan) was given as 1 CD without mixing with starch. The drugs were administered orally by means of a spatula, once daily. The drug administration was started on the day of or, in some experiments, several days before, sensitization of the animals with the antigen or inoculation of the myeloma cells, and continued throughout the entire experimental period. Ten mg/kg of starch was given to the mice in the same manner as above in control experiments.

Antigen. Sheep erythrocytes (SRBC), collected and stored in Alserver's solution, were obtained from our institute, and washed 3 times with phosphate buffered saline (PBS) before use.

Plaque-forming cell (PFC) assay. Antibody-producing cells were detected by direct PFC assay according to Cunningham and Szenberg ([5]). Four days after intraperitoneal injection of 4×10^8 SRBC, the spleens were teased out through a stainless steel screen. The resulting cells were washed 3 times with Eagle's minimum essential medium (MEM), and the viable cell number was determined by trypan blue-dye exclusion. The PFC assay was done in slide-glass chambers using guinea pig serum as a source of complement.

Delayed-type hypersensitivity (DTH) response. Four days after sensitization with 2×10^8 SRBC in PBS by subcutaneous injection into the left hind foot pad, mice were challenged for the DTH response with the same amount of SRBC into the right hand foot pad. The thickness of the right hind foot pad was measured under ether anesthesia, using a dial thickness gauge (Ozaki, Type G, Peacock) immediately before and 24 hr after the challenge injection. We did not measure at 48 hr because the thickness was reduced to about half of that of 24 hr in a preliminary experiment.

Induction of suppression to plaque formation and DTH response. This was done according to Whisler and Stobo ([21]) with a minor modification. Fourteen days after intraperitoneal injection of 4×10^9 SRBC in PBS, the mice were sacrificed and 5×10^7 viable spleen cells, hemolyzed and washed 3 times with MEM, were obtained and transferred intravenously into syngeneic recipient mice. Immediately thereafter, the recipient mice received 4×10^8 SRBC intraperitoneally. Four days later, spleen cells of the recipient mice were collected and washed as above for the PFC assay. For the DTH assay, the recipient animals that had received splenic cells were sensitized immediately with 2×10^8 SRBC, and four days later were challenged with the same amount of antigen.

Phagocytosis. Peritoneal cells were collected from mice used for the above transfer experiments by washing the peritoneal cavity with MEM. Two $\times 10^6$ viable cells in 1 ml of RPMI 1640 with 10% fetal calf serum (FCS) was plated in a tissue-culture Petri dish (35 mm diameter, Falcon #1008), and incubated overnight at 37°C in 5% CO_2. The following day nonadherent cells were removed from the dish by washing 2 times with MEM, and the remaining adherent cells in FCS-free RPMI were fed with 4×10^6 SRBC coated with syngeneic anti-SRBC serum. After a 2-hr incubation at 37°C in 5% CO_2, the SRBC outside the adherent cells were lysed by a brief (1 sec) exposure to distilled water. The percentage of phagocytosing macrophages was determined by

TABLE 2. *Effect of 3 CD of rifampin and dapsone on the production of suppressor cells to antibody production and DTH response.*

Treatment[a]	No. of PFC[b] (mean ± S.D.)	Thickness (mm)[c] (mean ± S.D.)
Rifampin	116 ± 54	0.29 ± 0.02
Dapsone	50 ± 25	0.19 ± 0.02
Starch	79 ± 43	0.28 ± 0.11
No suppressor[d]	4680 ± 483	0.68 ± 0.13

[a] Drugs were administered for 14 days.
[b] Number/10^6 viable spleen cells.
[c] Difference in thickness of right hind foot pad just before and 24 hr after challenge with SRBC.
[d] The mice, sensitized intraperitoneally with 4×10^8 SRBC 4 days before the assay, had not received the suppressor cells.

counting 50 cells in each of 6 different fields of the dish under an inverted-type microscope.

Tumor growth. MOPC 315, a BALB/c mouse myeloma, was maintained by serial passages in the inguinal skin of BALB/c mice. The tumor was obtained from the inguinal site, minced, and teased out through stainless steel mesh. The tumor cells were washed 3 times with MEM. Viable cells (1.2×10^6) were inoculated intradermally into the back of the mouse. Tumor growth was observed continuously thereafter, every 4–5 days, measuring length and width.

RESULTS

Humoral antibody production. The drugs (1 CD and 3 CD) were administered daily, starting 7 days before the SRBC injection, until 3 days after the injection, i.e., until 1 day before the PFC assay. As shown in Table 1, neither rifampin nor dapsone showed any effect on the number of cells producing antibody to SRBC in BALB/c mice. There was no significant difference between the number of PFC in the drug-treated group and the untreated control group. Additionally, 6 CD of rifampin administered as above had no effect on the number of PFC in another experiment (data not shown). No statistically significant difference was observed in C57BL/6 mice, a low-responder strain to SRBC, among the groups treated with 1 CD or 3 CD for 10 days and the untreated control group.

To test the effects of the drugs on the production of suppressor cells, donor mice were immunized with supraoptimal doses of SRBC to induce suppressor cells, and 3 CD of the drugs were administered continuously, beginning on the day of immunization and continuing for 14 days until they were sacrificed for adoptive transfer. As controls, donor mice received starch alone. Splenic suppressor cells were then transferred to recipient mice which were then immunized with SRBC. In Table 2, the production and transfer of suppressor cells are shown by the low numbers of PFC in the drug- or starch-treated groups compared with the high number of PFC in the group which did not receive transferred suppressor cells before immunization. However, the degrees of suppression were equivalent between the drug-treated groups and the starch (control) group. This shows that 3 CD of the drugs did not have any effect on suppressor cells and their induction. A similar result was obtained with 6 CD of rifampin (data not shown).

DTH response. DTH was investigated as a cellular immune response. The swelling of the foot pad was not modified by 1 CD or

TABLE 3. *Effect of rifampin and dapsone on DTH.*

Drug administration[a]	SRBC sensitization	Thickness (mm)[b] (mean ± S.D.)			
		Rifampin		Dapsone	
		1 CD	3 CD	1 CD	3 CD
+	+	0.55 ± 0.12	0.73 ± 0.15	0.77 ± 0.09	0.60 ± 0.10
+	−	0.16[c]	0.12 ± 0.04	0.12[c]	0.10[c]
−	+	0.53 ± 0.09	0.61 ± 0.05	0.67 ± 0.03	0.58 ± 0.06
−	−	0.11[c]	0.12 ± 0.01	0.21[c]	0.14[c]

[a] Drugs were administered for 11 days.
[b] Difference in thickness of right hind foot pad just before and 24 hr after the SRBC challenge.
[c] Only one mouse was used.

TABLE 4. *Effect of rifampin and dapsone on peritoneal cell phagocytic activity.*

Treatment[a]	% Phagocytic cells (mean ± S.D.)	
	Experiment 1[b] (3 CD)	Experiment 2[c] (6 CD)
Rifampin	67.3 ± 6.8	64.0 ± 2.0
Dapsone	57.8 ± 6.9	ND[d]
Starch	71.5 ± 13.3	ND
No drug	ND	63.3 ± 6.1

[a] Drugs were administered for 14 days.
[b] Mice were injected intraperitoneally with 4×10^9 SRBC 14 days before the collection of the adherent cells.
[c] Mice were injected intraperitoneally with 4×10^9 and 4×10^8 SRBC 21 days and 5 days before the collection of the adherent cells, respectively.
[d] Not done.

3 CD drug administration initiated 7 days before the sensitization and continued until 4 days after it (Table 3). Also, 3 CD of the drugs given for 14 days during the suppressor-cell induction period did not seem to affect the suppressor system involved in the DTH response (Table 2) because a similar inhibition of the response was observed between the drug-treated groups and the starch control as compared to the "No suppressor" group which received no suppressor cells.

Phagocytosis. Rifampin or dapsone (3 CD as well as 6 CD) given to the mice for 14 days, starting the day of immunization with SRBS, did not show any functional or populational change of the adherent cells, i.e., the ratio of phagocytic cells in the adherent cells was almost the same among the drug-treated groups and the control groups with starch or without drugs (Table 4).

Tumor growth. As shown in Table 5, 6 CD of the drugs given daily for 19 days did not make any difference in the growth rate of the MOPC 315 tumor compared with the growth rate of the tumor in control animals who received starch. This suggests that the drugs did not affect the tumor rejection system.

DISCUSSION

There are a considerable number of reports on the side effects of rifampin. Păunescu showed that 4 CD of rifampin administered before stimulation with an antigen, bovine serum albumin, inhibited antibody production ([16]). The present results show, however, that 1 CD, 3 CD, and 6 CD of rifampin started 7 days before antigen injection had no effect on the generation of antibody-producing cells.

The low response of C57BL/6 mice to SRBC may be ascribed to specific suppressor T cells, since it is known that this strain is also a low responder to tuberculin and that the low responsiveness in that system is caused by suppressor T cells ([15]). In spite of our expectation that the drugs might exert their effect on the putative suppressor T-cell population involved in the plaque formation and, subsequently, that the PFC number would be increased, the drugs did not alter the number of PFC. Moreover, no influence of the drugs was observed on suppressor cell generation in the adoptive transfer experiment. According to Whisler and Stobo ([21]), this experimental procedure induces a splenic T-cell population capable of specifically suppressing a recipient's PFC and DTH responses to SRBC. All of these findings suggest that both rifampin and dapsone have no side effect detectable by PFC or DTH assays, even on labile, dividing, premature, precursor suppressor T cells.

According to Bellahsene, *et al.* ([4]), 2 CD of the drug given for only 4 days after in-

TABLE 5. *Effect of 6 CD of rifampin and dapsone on MOPC 315 growth.*

Treatment[a]	Tumor size (cm^2)[b] after inoculation (mean ± S.D.)			
	2 days	7 days	11 days	16 days
Rifampin	0	0.73 ± 0.34	2.62 ± 0.62	5.13 ± 0.92
Dapsone	0	0.83 ± 0.52	2.75 ± 0.60	5.90 ± 1.75
Starch	0	0.70 ± 0.12	2.65 ± 0.06	4.63 ± 0.61

[a] Drugs (6 CD) were administered daily, beginning 3 days before the inoculation of MOPC 315 cells, until 16 days after the inoculation.
[b] Length (cm) × width (cm).

jection of the antigen, SRBC, reduced the numbers of PFC and SRBC titer in CBA mice. In that study, the route of administration was not oral but intraperitoneal, and the different results may be due to the different route of drug administration. This explanation may be applied to some of the other reports describing positive effects of the drugs referred to earlier. For example, it has been reported that phagocytosis of SRBC by peritoneal macrophages was suppressed by 7.5 CD of rifampin given intraperitoneally or subcutaneously but not after oral or intravenous administration ([2]). These results coincide with ours.

Cellular immunity is thought to be especially important in preventing *M. leprae* infection. Allograft rejection, DTH to tuberculin, lymphocyte blast transformation, etc., have been reported to be inhibited by rifampin as described earlier. DTH response and tumor rejection were chosen as model systems for the present observations to confirm the above points on cellular immunity. DTH reactions involve effector T cells, antigen-presenting cells, macrophages, etc. MOPC 315 is a mineral-oil-induced BALB/c myeloma secreting an IgA protein specific for 2,4-dinitrophenol and 2,4,6-trinitrophenol, and has often been used in immunological studies. BALB/c mice immunized with the myeloma protein could raise anti-idiotypic antibodies ([13]) and idiotype-specific suppressor T cells ([7]) that reduced the tumor incidence. In addition, cytotoxic T cells, NK cells, and interferon, as well as cell subsets known to be involved in DTH reactions, are likely to participate in the rejection of the tumor. According to the present results, it appears that all of these cell populations are insensitive to rifampin or dapsone.

The therapeutic effect of dapsone has been discussed for many years, since the drug takes a long time to cure leprosy patients. Sengupta, *et al.* ([18]) reported that 1 CD of dapsone taken orally by healthy volunteers for seven days depressed phytohemagglutinin-induced lymphocyte (T cell) blast transformation in *in vitro* culture. They pointed out the possibility that this side effect (immunosuppression) might be responsible for the slow therapeutic effect of dapsone. The drug did not prevent the growth of *M. leprae* inoculated into athymic nude mice at a low dose, while it suppressed the growth in normal mice ([22]). A tempting hypothesis seems to be that dapsone depresses T cell activity. However, no effect of dapsone was detected on the T-cell subsets involved in antibody production and cellular immunity in the present study. These observations are in keeping with those of Gidoh, *et al.* ([8]) on PFC and DTH assays done with an experimental system similar to ours. Other factors, e.g., the metabolism of dapsone in the body, the accessibility of the drug to the bacilli in tissues, the function of macrophages, NK cells, and lymphokines, etc., may be considerably different in nude mice compared with other systems.

Intake of the drugs by the mice in the present experiments is unequivocal and has been shown to be effective ([12]): 1 CD of the drugs administered in the same way as in the present study exerted a remarkable action on inoculated *M. leprae* in the mouse (BALB/c), reducing the number of bacilli and their infectivity.

Oral administration of the powdered drugs was employed in the present study because the purpose of the experiment was to obtain information about the side effects of the drugs used in human therapy. Nowadays, oral therapy has superseded the use of injections, and serum levels of dapsone have been shown to be higher in leprosy patients receiving the drug by mouth than those in patients receiving conventional doses of the drug by injection ([14]). The effects of the drugs given to the animals in doses up to sixfold higher than human clinical doses on the basis of body weight were also studied. As was the case with smaller doses, no drug effects were seen at these higher doses.

SUMMARY

The *in vivo* effects of rifampin and dapsone on immunological responses were investigated using mice immunized with sheep erythrocytes. The number of cells producing antibody was not affected by a clinical (1 CD) or a threefold excess dose (3 CD) of the drugs administered for ten days. A similar result was obtained in an experiment using a mouse strain known to be low responders to the antigen. Induction of suppressor cells acting on antibody production was not influenced by 3 CD or 6 CD of the drugs. Neither delayed-type hypersensitivity nor

induction of the suppressor cell population acting on delayed hypersensitivity was affected by 3 CD of the drugs. Phagocytosis of sheep erythrocytes by peritoneal cells and the growth of a tumor were not altered by 6 CD of the drugs.

RESUMEN

Se investigó el efecto *in vivo* de la dapsona y la rifampina sobre la respuesta inmunológica de ratones inmunizados con eritrocitos de carnero. El número de células productoras de anticuerpo no se afectó por la administración, durante 10 días, de una dosis clínica (1 DC) de las drogas o tres veces esta cantidad (3 DC). Un resultado similar se obtuvo en un experimento donde se usó una cepa de ratón de respuesta baja al antígeno. Ni 3 DC ni 6 DC de las drogas influyeron en la inducción de células supresoras activas en la producción de anticuerpos. Tres DC de las drogas no afectaron ni la hipersensibilidad retardada ni la inducción de células supresoras activas sobre esta respuesta. La fagocitosis de eritocitos de carnero por células peritoneales y el crecimiento de un tumor tampoco fueron afectados por 6 DC de las drogas.

RÉSUMÉ

On a étudié les effets *in vivo* de la rifampicine et de la dapsone sur la réponse immunologique, chez des souris immunisées par des érythrocytes de mouton. Le nombre de cellules produisant des anticorps n'était pas modifié par une dose dépassant la dose clinique (1 CD), ou une dose 3 fois supérieure (3 CD) des médicaments, pendant 10 jours. Un résultat semblable a été obtenu lors d'une expérience menée sur une souche de souris dont on sait qu'elle répond faiblement à l'antigène. L'induction des cellules "suppresseur" agissant sur la production d'anticorps n'a pas été influencée par des doses de médicaments s'élevant à 3 CD ou à 6 CD. De même, l'hypersensibilité de type retardé, non plus que l'induction d'une population de cellules "suppresseur" agissant sur l'hypersensibilité retardée, n'a été affectée par des doses de médicaments s'élevant à 3 CD. La phagocytose des érythrocytes de mouton par les cellules péritonéales, et la croissance d'une tumeur, n'étaient pas davantage modifiées par des doses de médicaments s'élevant à 6 CD.

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