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DISSOCIATION OF THE INHIBITORY EFFECT OF DAPSONE ON THE GENERATION OF OXYGEN INTERMEDIATES—IN COMPARISION WITH THAT OF COLCHICINE AND VARIOUS SCAVENGERS

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Abstract—4,4′-Diaminodiphenyl sulfone (dapsone, DDS) is highly effective in dermatitis herpetiformis and immune complex disease in which polymorphonuclear leukocytes (PMN) play an important role. We studied the dose–response effect of DDS (10–1 mM) on the generation of oxygen intermediates (OI:O_2^-, H_2O_2, OH·) and chemiluminescence using PMN and the xanthine–xanthine oxidase system. The effect of colchicine and five oxygen radical scavengers on OI production in both systems was also examined and compared with that of DDS. We found that, except for O_2^- generation, which was slightly enhanced, DDS decreased the levels of OI measured as effectively as scavengers. Scavengers also capable of degrading OH· and $^1O_2$ markedly reduced the levels of OH· and chemiluminescence while slightly increasing the level of O_2^-. Therefore, DDS was considered to exert effects similar to H_2O_2, and OH· and $^1O_2$ scavengers. Colchicine did not decrease OI values produced in the xanthine–xanthine oxidase system but affected PMN-mediated OI generation, possibly by its cytotoxic effect. Our results suggest that DDS, in contrast to colchicine brings about rapid clinical improvement in PMN-mediated autooxidative disorders by quenching reactive OI such as H_2O_2, OH· and chemiluminescence.

Colchicine and 4,4′-diaminodiphenyl sulfone (dapsone, DDS) exert an anti-inflammatory effect by inhibiting neutrophilic functions. Colchicine suppresses polymorphonuclear leukocyte (PMN) chemotaxis [1, 2] and the Arthus reaction [3]; dapsone inhibits myeloperoxidase (MPO)–H_2O_2–halide-mediated cytotoxicity [4]. Such actions may explain the effectiveness of these agents in the treatment of disorders such as Beheçet's disease [3, 5, 6], dermatitis herpetiformis [4, 7, 8] and necrotizing vasculitis [4, 9], in which immune complexes and/or neutrophil amplification play an important role.

Stendahl *et al.* [4], who examined the effect of DDS on the generation of superoxide anion (O_2^-) by neutrophils, ascribed the anti-inflammatory effect of the drug to its inhibition of MPO–H_2O_2–halide-mediated cytotoxicity. However, we have found that DDS has scavenger-like function, markedly decreasing the concentrations of oxygen intermediates (OI) except for O_2^-. We have investigated the effect of DDS on the generation of OI (O_2^-, H_2O_2, OH·) and chemiluminescence, comparing DDS with colchicine and five scavengers with different action sites, using stimulated human PMNs and the xanthine–xanthine oxidase system. The five scavengers were as follows: SOD for O_2^-, catalase for H_2O_2, benzoate for OH·, β-carotene for $^1O_2$ and xanthine for both OH· and $^1O_2$ [10]. The mechanism which may be involved in the effect of DDS on OI generation is discussed.

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MATERIALS AND METHODS

Drug for OI assay. Cholchicine, SOD, benzoate, and xanthine were purchased from Sigma Chemical Co. (St. Louis, MO, USA), catalase from Washington Biochemical Corp. (Freehold, NJ, USA), β-carotene from Nakarai Chemicals (Kyoto, Japan) and DDS from Yoshitomi Pharmaceuticals (Osaka, Japan). The following drug concentrations were used in OI generation assays: colchicine and DDS, 1 mM; SOD, 1200 U/ml; catalase, 1200 U/ml; benzoate, 10 mM; β-carotene, 1 mM; and xanthine, 4 mM. For dose–response experiments, 1/10 and 1/100 of the above concentrations of each drug were simultaneously prepared. To examine the possible mechanism of DDS on enzyme activity, we used the xanthine–xanthine oxidase system.

Preparation of PMNs for OI assay. PMNs were isolated from the peripheral blood of 16 healthy volunteers using a previously described technique [11]. For OI-generation assay, these cells were suspended in Krebs–Ringer phosphate buffer (KRP) [12] containing 5 mM glucose and gelatin (1 mg/ml). KRP containing only glucose was used for OH· generation assay; Hanks solution (Nissui Pharmaceutical Co., Tokyo, Japan) containing only gelatin was used for the determination of chemiluminescence.

OI-generation by PMNs. In studies on O_2^- formation, 4×10^6 PMNs were preincubated at 37° for 10 min with opsonized zymosan (Sigma), then 0.1 mM ferricytochrome *c* (type III, Sigma) was added. The PMNs were incubated for another 30 min. Immediately after sedimentation of the

PMNs and opsonized zymosan by centrifugation, 0.1 ml of the supernatant was assayed for reduced cytochrome *c* by measuring the absorbance at 550 nm [13, 14] in 2 ml of 100 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA (pH 7.8). The results were converted to nmole of cytochrome *c* reduced, using $\Delta E550\,nm = 2.1 \times 10^4/M/cm$ [14].

H_2O_2-generation was measured by quantitating the decrease in fluorescence intensity of scopoletin (Sigma) due to its peroxidase-mediated oxidation by H_2O_2 [13, 15]. After 10 min of room temperature incubation of 2.5×10^6 PMNs in KRP containing glucose and gelatin in the presence of opsonized zymosan, 0.1 ml of 50 mM scopoletin in KRP and 0.1 ml of 1 mg/ml horseradish peroxidase (type II; Sigma) in phosphate-buffered saline (PBS) were added. The H_2O_2-plus-peroxidase-induced rate of decrease in fluorescence intensity of the scopoletin within 30 min was quantitated using a fluorescence spectrometer (Hitachi Co., Ltd., Tokyo, Japan). To calculate H_2O_2 concentration we assumed that 1 mole of H_2O_2 oxidized 1 mole of scopoletin [15].

OH· was quantitated by the amount of ethylene formed from α-keto-methiol butylic acid (KMB) (Sigma) plus OH· generated by PMNs [16]. PMNs (2×10^6) in 2 ml KRP containing glucose were preincubated with 1 mM KMB in a stoppered tube and gently mixed in 37° shaker bath for 5 min. Then, opsonized zymosan was added and the cells were incubated for 10 min. Thereafter, aliquots of gas in the tube were sampled using a gas-tight syringe, and the ethylene content was determined by a gas chromatograph (Hitachi). The total amount of ethylene formed for 10, 20 and 30 min served as the OH· value.

Since ethylene formation from α-keto-methiol butylic acid can potentially be mediated by radicals other than OH·, the specificity of OH· assay was confirmed by the depletion of OH· levels by benzoate, a specific scavenger for OH·, 10 mM of which reduced peak OH· generation by 75%. In addition, simultaneously with our ethylene formation method, another assay method to confirm OH· generation was performed [17]; formaldehyde formation method from Me_2SO and *t*-butyl alcohol, as well as the *N*-dimethylation of aminopyrine [18], was determined according to Nash [19]. OH· generation by both methods showed similar behaviour.

Chemiluminescence was measured in a scintillation spectrometer (Packard, Illinois, U.S.A.), according to Allen and Loose [20] with slight modification. PMNs (5×10^6) in 3 ml colorless Hank's solution containing gelatin were incubated at 37° for 10 min with opsonized zymosan in the absence of luminol. Chemiluminescence was monitored on the spectrometer which was operated in an out-of-coincidence summation mode. All procedures were performed in the dark.

OI generation assay in the xanthine–xanthine oxidase system. All OIs were also measured in the xanthine–xanthine oxidase system. Instead of adding PMNs and opsonized zymosan, 0.1 ml of 13.5 mg hypoxanthine in 50 ml physiological saline plus 0.05 ml of 50 mM EDTA were diluted in 2 ml of KRP (pH 7.2–7.4). Thereafter, 0.1 ml of 0.1 unit/ml dialyzed xanthine oxidase was added to generate O_2^-. Taking into consideration the inhibitory effect of hypoxanthine on OI production in this assay system, we also substituted acetaldehyde (Nakarai Chemicals, Kyoto, Japan) for the hypoxanthine substrate [21].

Viability and phagocytic function of PMNs. PMN viability after incubation with the agents was determined by means of the trypan-blue dye test; phagocytic function was measured by zymosan-induced stimulation of [^{14}C]inulin uptake [22]. When over 2% of the PMNs were stained with trypan-blue, or when PMNs showed less than a 600 dpm [^{14}C]inulin uptake/mg protein, their function was considered to have been impaired and the results were discarded.

Triplicate assays were performed in each experiment; the results are expressed as the mean ± S.E. of replicate assays. Statistical significance was ascertained by Student's *t*-test.

RESULTS

DDS significantly decreased OI levels in a dose-dependent manner in both PMN-mediated and xanthine–xanthine oxidase systems, with the exception of O_2^- in both systems and chemiluminescence in xanthine–xanthine oxidase system (Tables 1 and 2). In both systems, DDS decreased the H_2O_2 levels as strongly as catalase, a H_2O_2 scavenger (12 and 120 U; $P < 0.01$, 1 mM; $P < 0.001$). Among all agents tested, DDS manifested the strongest effect on OI values; it was effective even at the lowest concentration used (0.01 mM), which is considered to be under the therapeutic dose (0.1 mM). Five scavengers for OIs, except for SOD, manifested similar effects, significantly removing corresponding OI with subsequent decrease in later components (OH· and $^1O_2$) in the OI generation pathway in both systems. Scavengers for later components had little effect on the concentration of the early component H_2O_2. On the other hand, the concentrations of the early component O_2^- were slightly but significantly raised by DDS and the scavengers for later components, especially carotene and xanthine at 1 mM. Although colchicine at 1 mM inhibited the generation of all OIs except for H_2O_2 in PMN-mediated system (O_2^- and chemiluminescence, $0.01 < P < 0.05$; OH·, 1 mM $P < 0.001$), at no concentration tested did the drug reduce the values of OI produced in xanthine–xanthine oxidase system ($P > 0.05$). The effect of colchicine on H_2O_2 generation was not tested because of the decrease in the fluorescence intensity of scopoletin by the drug per se in the absence of peroxidase. Among the OIs examined, OH· was most markedly decreased by DDS, colchicine (only in PMN system) and OH·, and the OH· and $^1O_2$ scavengers (benzoate and xanthine). SOD and catalase also markedly reduced OH· formation (both at 1200 U; $P < 0.001$).

In the zymosan-stimulated PMN system, DDS and OH· and/or $^1O_2$ scavengers, brought about a significant reduction in chemiluminescence, virtually irrespective of the applied concentration (Table 1), while, in the xanthine–xanthine oxidase system, chemiluminescence values were very low (Table 2) and comparable with those generated by non-stimulated PMNs [23]. These results suggest that chemiluminescence is

Table 1. Effect of the agents on oxygen intermediate levels generated by zymosan stimulated PMNs

Agents	Doses†	O_2^- (nmole/min/4 × 10^6)	H_2O_2 (pmole × 10^2/min/2.5 × 10^6)	OH· (ethylene) (pmole × 10^2/2 × 10^6)	Chemiluminescence (cpm × 10^4/5 × 10^6)
	0.01	0.50 ± 0.02	2.43 ± 0.08**	8.81 ± 0.18**	8.81 ± 0.53**
DDS	0.1	0.52 ± 0.03	1.85 ± 0.08**	4.78 ± 0.20***	5.78 ± 0.55***
	1	0.55 ± 0.03*	1.40 ± 0.09***	2.52 ± 0.06***	4.38 ± 0.57***
	0.01	0.46 ± 0.02		11.50 ± 0.18	18.31 ± 0.68
Colchicine	0.1	0.42 ± 0.04	n.d.	10.72 ± 0.20	17.66 ± 0.80
	1	0.37 ± 0.04*		5.28 ± 0.18***	15.41 ± 0.68*
	0.01	0.43 ± 0.02	8.13 ± 0.13**	13.16 ± 0.20	19.80 ± 0.81
SOD	0.1	0.39 ± 0.02	10.08 ± 0.14****	13.80 ± 0.16	20.54 ± 0.78
	1	0.18 ± 0.03***	12.00 ± 0.12****	4.78 ± 0.12***	12.41 ± 0.66*
	0.01	0.45 ± 0.04	3.30 ± 0.06*	10.23 ± 0.12	16.92 ± 0.69
Catalase	0.1	0.47 ± 0.03	2.11 ± 0.08**	7.91 ± 0.10*	15.00 ± 0.54
	1	0.43 ± 0.03	1.63 ± 0.08***	4.58 ± 0.11***	12.86 ± 0.75*
	0.01	0.47 ± 0.03	4.08 ± 0.10	11.31 ± 0.20	18.18 ± 0.80
Benzoate	0.1	0.47 ± 0.03	3.82 ± 0.08	9.90 ± 0.15	16.72 ± 0.74
	1	0.49 ± 0.02	3.70 ± 0.08	5.34 ± 0.11***	12.76 ± 0.64*
	0.01	0.59 ± 0.03	4.30 ± 0.52	12.36 ± 0.81	10.48 ± 0.75**
β-Carotene	0.1	0.62 ± 0.02	4.05 ± 0.68	11.81 ± 0.74	8.91 ± 0.53**
	1	0.67 ± 0.05*	3.62 ± 0.31	10.05 ± 0.63	4.99 ± 0.51***
	0.01	0.53 ± 0.04	4.34 ± 0.09	10.83 ± 0.18	15.98 ± 0.75
Xanthine	0.1	0.60 ±-0.03*	4.21 ± 0.08	9.61 ± 0.15	14.48 ± 0.58*
	1	0.64 ± 0.04*	3.49 ± 0.10*	3.72 ± 0.08***	5.86 ± 0.50***
Control‡	0	0.45 ± 0.02	4.46 ± 0.07	12.56 ± 0.15	19.46 ± 0.81

* $0.01<P<0.05$ ** $P<0.01$ *** $P<0.001$ ****$P<0.0001$ vs. control. n.d.: not done.

† Each concentration corresponding to 1, 0.1 and 0.01 in the Table is described in detail under Materials and Methods.

‡ Control denotes control assay in medium without agents.

Table 2. Effect of the agents on oxygen intermediate levels generated in the xanthine–xanthine oxidase system

Agents	Doses†	O_2^- (nmole)	H_2O_2 (pmole × 10^2)	OH· (ethylene) (pmole × 10^2)	Chemiluminescence (cpm × 10^4)
	0.01	0.24 ± 0.03	6.08 ± 0.18	8.02 ± 0.61***	4.43 ± 0.18
DDS	0.1	0.26 ± 0.02	5.12 ± 0.16*	5.01 ± 0.55***	4.08 ± 0.15
	1	0.27 ± 0.02*	3.45 ± 0.15**	3.85 ± 0.50***	3.80 ± 0.11
	0.01	0.18 ± 0.02		19.01 ± 0.78	5.96 ± 0.20
Colchicine	0.1	0.21 ± 0.02	n.d.	20.24 ± 0.66	5.10 ± 0.21
	1	0.19 ± 0.03		19.21 ± 0.71	5.23 ± 0.16
	0.01	0.15 ± 0.02	10.41 ± 0.20*	16.77 ± 0.68	4.57 ± 0.18
SOD	0.1	0.12 ± 0.04*	14.30 ± 0.21***	14.23 ± 0.69*	4.10 ± 0.16
	1	0.08 ± 0.02***	17.35 ± 0.20****	8.62 ± 0.75***	3.91 ± 0.15
	0.01	0.22 ± 0.04	6.41 ± 0.18	14.55 ± 0.80*	4.82 ± 0.13
Catalase	0.1	0.22 ± 0.03	5.37 ± 0.15*	6.91 ± 0.64***	4.23 ± 0.17
	1	0.24 ± 0.03	2.71 ± 0.15***	4.78 ± 0.53***	3.99 ± 0.13
	0.01	0.24 ± 0.03	7.35 ± 0.20	18.45 ± 0.73	4.86 ± 0.19
Benzoate	0.1	0.25 ± 0.02	6.58 ± 0.18	14.75 ± 0.71*	4.38 ± 0.17
	1	0.26 ± 0.02*	6.45 ± 0.16	4.44 ± 0.58***	4.25 ± 0.15
	0.01	0.20 ± 0.04	7.05 ± 0.61	19.03 ± 1.12	4.71 ± 0.23
β-Carotene	0.1	0.23 ± 0.04	6.68 ± 0.75	18.56 ± 0.09	4.58 ± 0.19
	1	0.34 ± 0.03*	6.04 ± 0.52	18.04 ± 0.28	4.02 ± 0.25
	0.01	0.24 ± 0.03	7.20 ± 0.15	15.98 ± 0.69*	5.00 ± 0.19
Xanthine	0.1	0.26 ± 0.03*	6.92 ± 0.16	13.66 ± 0.75*	4.52 ± 0.16
	1	0.36 ± 0.03**	6.56 ± 0.20	4.18 ± 0.57***	4.33 ± 0.16
Control‡	0	0.20 ± 0.02	7.44 ± 0.19	20.00 ± 0.80	5.24 ± 0.20

* $0.01<P<0.05$ ** $P<0.01$ ***$P<0.001$ **** $P<0.0001$ vs control. n.d.: not done

† See the legend for Table 1.

‡ Control denotes control assay in medium without agents.

not generated significantly in the xanthine–xanthine oxidase system.

When we used acetaldehyde rather than hypoxanthine as the substrate in the xanthine oxidase system, we obtained comparable results (data not shown), thereby ruling out an inhibitory effect of hypoxanthine on OI generation. Furthermore, neither the viability nor the phagocytic function of PMN was affected by adding the test agents, except for colchicine. Less than 2% of the PMNs were stained with trypan blue or exhibited less than 655 dpm/mg protein [^{14}C]inulin uptake [22] in medium containing any drugs, except for colchicine. Upon adding colchicine, a slight decline in phagocytic function (623 ± 28 dpm/mg protein, $0.01 < P < 0.05$ vs controls) was noted. This indicates that a decrease of OI values induced by the drugs except for colchicine in this study is not due to a toxic effect on PMNs.

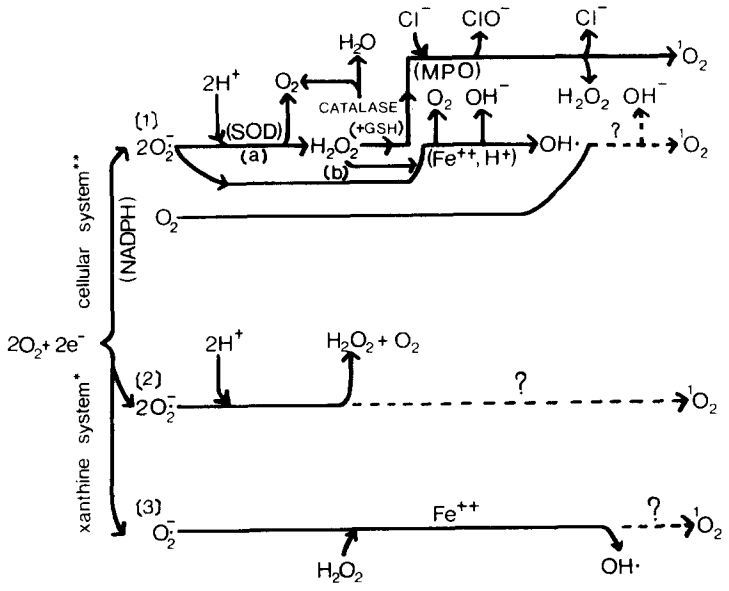

Fig. 1. Three disposal pathways of oxygen intermediates. [1]: dismutation reaction and Haber Weiss reaction. [2]: spontaneous dismutation reaction. *, xanthine–xanthine oxidase system. **, PMN system.

DISCUSSION

Our present results show that DDS quenched all the OIs measured except O_2^-. Thus, DDS at therapeutic doses (0.1 mM) was as effective as the scavengers at the concentrations usually used *in vitro*. DDS appears to act like the scavengers, presumably by reacting directly with H_2O_2 with resultant decrease in OH· and $^1O_2$. The possibility that DDS might act by inhibiting SOD activity was also ruled out by our following findings; the drug effectively reduced OI levels produced by not only PMN but also by xanthine oxidase system, in which both cells and SOD are absent. The data that both DDS, and H_2O_2, and OH· and $^1O_2$ scavengers induced a slight increase in O_2^- seem to suggest that the removal of latter components of OI generation pathway increase the levels of an earlier component (O_2^-). The beneficial clinical effects of DDS [4, 7–9] may be ascribable to this scavenger-like function, since H_2O_2, OH· and $^1O_2$ are known to be the potent OI [24–28]. The inhibition of the MPO–H_2O_2 cytotoxicity system reported by Stendahl *et al.* [4] could also be induced by the marked decline in H_2O_2 production induced by the scavenger-like function of DDS. O_2^- levels in their study [4] were also slightly raised, just as was the case in our study, although they assessed only O_2^- among OIs. In addition, the cytotoxic activity in the MPO–H_2O_2–halide system may not be highly potent, as patients with MPO deficiency are not unduly susceptible to infection [10, 29]. Therefore, DDS seems not only to induce the inhibition of the MPO–H_2O_2–halide system but also to remove potent OIs (H_2O_2, OH· and $^1O_2$) [24–28]. Besides these effects, upon long-term, high-dose administration, DDS is reported to suppress the Arthus reaction [30] and lysosomal enzyme release [31]. Similarly the Arthus reaction has been reported to be inhibited by OI scavengers [32].

Colchicine slightly reduced PMN-mediated OI production without affecting xanthine–xanthine OI generation system. Colchicine seems to interfere with the initial steps of cell metabolism and/or oxygen burst with little scavenger effect. This is supported by the reports that the drug inhibits PMN chemotaxis [1, 2], phagocytosis [33] and lysosomal degranulation [33]. In view of these data and reports, the different effects of scavengers, DDS and colchicine, on various functions of neutrophils are summarised in Table 3.

Recently, it has been reported that O_2^- and OH· concentrations are linked by a mechanism mediated by iron ions [34–36]. It may therefore seem strange that in the present study, O_2^- behaved differently from the other three OI. However, the removal of later components of the OI system ($^1O_2$, OH·) may induce an increase in the levels of an earlier components system (O_2^-) without mediation of iron anion. The effects of trace SOD, Fe^{2+}, SO_4^{2-}, phosphate, lipids and protein also need to be taken into consideration [34–39], and the time-related changes of OI generation need to be assessed to determine the precise effect of these drugs. We suggest that it is necessary to perform all possible OI assays before drawing conclusions regarding the increase or decrease of OI levels, since O_2^-, H_2O_2, OH· and chemiluminescence do not always behave in an identical manner.

Table 3. Effect of colchicine, scavengers, and DDS on neutrophil function

	Chemotaxis	Phagocytosis	Arthus	Lysosomal enzyme release	OI generation	MPO–H_2O_2 system
Colchicine [1–3,5,6,33,40,41]	++	+	++	+	+ (cytotoxic effect)	?
Scavengers [32,42]	–*	–	+	–	++ (quenching effect)	–
DDS [4,43,44]	–	–	±†	±†	++‡	++

* Inhibited by SOD, but not by catalase (in high doses).
† Long-term administration of high doses is required.
‡ Oxygen intermediates except for O_2^-.
++, strong effect.
+, moderate effect.
±, weak effect.
–, no effect.

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