Pergamon

Biochemical Pharmacology, Vol. 48, No. 7, pp. 1349–1354, 1994.
Elsevier Science Ltd
Printed in Great Britain.
0006-2952/94 $7.00 + 0.00

0006-2952(94)00317-3

REDUCTION OF DAPSONE HYDROXYLAMINE TO DAPSONE DURING METHAEMOGLOBIN FORMATION IN HUMAN ERYTHROCYTES *IN VITRO* IV: IMPLICATIONS FOR THE DEVELOPMENT OF AGRANULOCYTOSIS*

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(*Received* 15 *March* 1994; *accepted* 28 *June* 1994)

Abstract—We have studied the efflux of dapsone hydroxylamine from normal and diabetic erythrocytes by the use of a two-compartment (1 and 2) *in vitro* dialysis system, in order to model the *in vivo* blood supply to the bone marrow. When both types of erythrocytes were dialysed against mononuclear leucocytes, the hydroxylamine crossed the membrane and caused significantly greater white cell death compared with dialysis of leucocytes against untreated erythrocytes. However, in the case of both normal and diabetic cells, the presence of the glutathione depletor diethyl maleate (DEM) caused a marked reduction in movement of hydroxylamine from compartment 1 to 2. Diethyl dithiocarbamate (DDC), a methaemoglobin accelerant, caused a marked reduction in movement of hydroxylamine from erythrocytes (diabetic and normal) in compartment 1 to 2 which led to a significant reduction in white cell death compared with the absence of DDC (18.3 ± 5.5 vs 34.8 ± 8.1%, $P < 0.05$). Dapsone recovery from compartment 1 rose significantly in the presence of DDC compared with control in both erythrocyte types. In contrast, recovery of dapsone from normal erythrocytes incubated in compartment 1 was significantly reduced by the presence of DEM compared with control, although there was no difference between control and DEM-treated diabetic cells. Dapsone analysis in compartment 2 revealed a significant increase in dapsone recovery in both diabetic (11.3 ± 1.1%) and normal (11.9 ± 1.1%) erythrocytes in the presence of DDC compared with diabetic (3.3 ± 0.4%) and normal control (4.8 ± 2.0%, $P < 0.001$). The presence of DEM in compartment 1 caused a significant fall in dapsone recovery in compartment 2 (3.7 ± 0.26) compared with control (4.7 ± 0.36%, $P < 0.05$). Hence, dapsone hydroxylamine is capable of leeching out of normal and diabetic erythrocytes, traversing a semi-permeable membrane and causing toxicity to human mononucleocyte cells *in vitro*. This process may be one of the first stages in immune-mediated agranulocytosis.

Key words: mononucleocyte; diethyl maleate; sulphone; diethyldithiocarbamate

Dapsone is effective in many inflammatory and infectious disease states, despite its wide-ranging adverse effects which may be life-threatening [1]. Although it is well documented that the hydroxylamines of dapsone are toxic to erythrocytes, [2], the potentially damaging effect of the drug on circulating granulocyte populations is unpredictable, difficult to study and poorly understood [3]. However, the causes of granulocyte loss are probably linked to the formation and binding of derivatives of the hydroxylamine metabolites to the granulocytes, leading to haptenation and an immune response [3, 4]. It has been shown that erythrocytes may reduce the toxic hydroxylamine metabolite of dapsone to the parent drug, a process which is glutathione-mediated [5]. Dapsone thus produced is capable of leaving the erythrocytes and traversing a semi-permeable membrane to enter other erythrocytes [6]. It was also apparent that in the two-compartment system, the hydroxylamine could leave the cells in one compartment, cross the membrane and cause detectable methaemoglobinaemia in the second compartment [6]. It is conceivable that erythrocytes may act as a delivery system enabling the hydroxylamine to reach the bone marrow and cause haptenation, which in some individuals, may lead to agranulocytosis. Therefore, we wish to evaluate the factors which may influence hydroxylamine efflux from erythrocytes and the effect of the metabolite on human mononuclear leucocytes *in vitro*.

MATERIALS AND METHODS

Dapsone hydroxylamine was provided by the Jacobus Pharmaceutical Company Inc. (Princeton, NJ, U.S.A.), and was found to be 97% pure by HPLC. Dapsone, DEM§ and DDC were obtained from the Sigma Chemical Co. (Poole, U.K.). The internal standard for the HPLC assay (3,3′diaminodiphenyl sulphone) and diethyl maleate

* This work is dedicated to the memory of Mark J. Winn, PhD.

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§ Abbreviations: PBGS, phosphate-buffered (pH 7.4) 0.9% saline containing 10 mM glucose; DDC, diethyldithiocarbamate; DEM, diethyl maleate.

Table 1. Recovery (%) of dapsone hydroxylamine (DDS-NHOH) from PBGS (containing 5 mM ascorbate) in compartment 2 following dialysis with erythrocytes exposed to dapsone hydroxylamine (150 μM) in compartment 1 in the presence of DDC (5 mM) or DEM (10 mM)

Compartment 1 contents	Recovery of hydroxylamine (%)
Normal cells/DDS-NHOH	3.0 ± 0.8*
Normal cells/DDS-NHOH/DDC	0.7 ± 0.05*
Diabetic cells/DDS-NHOH	2.3 ± 0.5†
Diabetic cells/DDS-NHOH/DDC	1.0 ± 0.06†
Normal cells/DDS-NHOH	2.8 ± 0.2**
Normal cells/DDS-NHOH/DEM	0.6 ± 0.2**
Diabetic cells/DDS-NHOH	2.7 ± 0.06††
Diabetic cells/DDS-NHOH/DEM	0.6 ± 0.06††

N = 4, mean ± SD; levels of significance apply to similarly indicated data.
**, * $P < 0.001$.
††, † $P < 0.01$.

were obtained from the Aldrich Chemical Co. (Poole, U.K.). All HPLC solvents were supplied by Fisons Ltd (Loughborough, U.K.) Whole human diabetic and normal blood was obtained and washed as previously described [5]. Erythrocyte incubations (0.6 mL) each contained 1.2 μmol of haemoglobin and were placed on ice and equilibrated for 10 min in uncapped tubes. Experiment 1 consisted of 18 tubes: 1–8 contained normal erythrocytes, while 9–16 contained diabetic erythrocytes. Tubes 17 and 18 were normal and diabetic untreated cells, respectively. Prior to the addition of dapsone hydroxylamine (0.075 μmol in 5 μL acetone) to tubes 1–16, DDC was dissolved in PBGS and added to tubes 5–8 (normal cells) and 13–16 (diabetic) to give a final concentration of 5 mM. DDC is a potent accelerator of both erythrocytic processes of dapsone hydroxylamine-induced methaemoglobin formation and hydroxylamine detoxification [5] but did not affect mononucleocyte viability. Experiment 2 differed from experiment 1 as follows: DEM instead of DDC was added to the cells in methanol (5 μL; final DEM concentration 10 mM), which were warmed to 37° and incubated for 30 min prior to cooling to 4° and the addition of dapsone hydroxylamine. Experiment 3 was identical to experiment 1 and experiment 4 was identical to experiment 2. As the process of methaemoglobin generation is extremely rapid and temperature sensitive [5], experiments 1–4 were then placed in a water bath at 37° for 75 sec to allow for uptake of the hydroxylamine into the erythrocytes without appreciable methaemoglobin formation. The cells were then removed and placed on ice. After cooling to 4°, the cells were centrifuged for 1 min at 10,000 *g* in a Beckman microfuge. The supernatant (270 μL) was removed and discarded. PBGS was added to the cells (270 μL), which were then carefully resuspended. This process was repeated twice more to give a total of three washes. The aliquots were transferred to a Dianorm equilibrium dialysis system (Dianorm-Gerate, Maierhofer, Munich, Germany). The apparatus consisted of a series of bi-compartmental Teflon cells, where each cell was divided into two compartments, 1 and 2 by a cellulose dialysis membrane (molecular weight cut-off, 5000 Da). The washed samples were inserted into compartment 1 of 20 Dianorm cells. In experiments 1 and 3 DDC was again added to samples 1–4 to give a final concentration of 5 mM. In both experiments 1 and 2 PBGS buffer containing 5 mM ascorbic acid was added to compartment 2 for each sample to prevent hydroxylamine degradation before HPLC measurement. In experiments 3 and 4 mononuclear leucocytes (1×10^6 per incubation) isolated from the blood of two healthy normal volunteers as previously described [7] were added to compartment 2. Mononucleocyte viability was 99% prior to use. Ascorbate was not added to these samples, as it would protect the mononucleocytes from hydroxylamine toxicity [8]. In experiments 3 and 4, a total of four cell controls for each experiment were set up, two with diabetic and two with normal erythrocytes. The entire apparatus containing either 18 or 20 cells was then incubated in a waterbath at 37°, each cell rotating at 8 rpm. At 30 min, the apparatus was removed from the waterbath and the contents of compartments 1 and 2 of all the cells were expelled into individual microcap tubes. Each erythrocyte sample was immediately assayed for methaemoglobin formation using an IL-482 CO-oximeter (Instrumentation Laboratory, Warrington, U.K.). Background methaemoglobin levels were below 0.6% and were subtracted from the test levels. The mononuclear leucocytes from experiments 3 and 4 were washed once with HEPES buffer [7] and resuspended in HEPES containing 5 mg/mL BSA. The cells were incubated for 18 hr overnight and dead cells were then counted using the Trypan Blue exclusion method. The contents of compartment 2 in experiments 1 and 2 were stored at −20° until assayed for dapsone hydroxylamine and dapsone by direct injection onto the HPLC. The remainder of the samples were frozen at −20° until assayed for dapsone alone by the HPLC method of Grossman and Jollow [9]. Analysis of vehicle control incubations containing erythrocytes or mononuclear leucocytes and either 5 μL of methanol or acetone revealed no

Table 2. Recovery (%) of dapsone (containing 5 mM ascorbate) in compartments 1 and 2 following dialysis with erythrocytes exposed to dapsone hydroxylamine (DDS-NHOH, 150 μM) in compartment 1 in the presence of DDC (5 mM) or DEM (10 mM)

Incubation contents	Dapsone recovery (%) Compartment 1	Compartment 2
Normal cells/DDS-NHOH	8.3 ± 1.1*	4.8 ± 2.0*
Normal cells/DDS-NHOH/DDC	25.4 ± 9.2*	11.9 ± 1.1*
Diabetic cells/DDS-NHOH	8.9 ± 0.9†	3.3 ± 0.4**
Diabetic cells/DDS-NHOH/DDC	15.5 ± 5.3†	11.3 ± 1.1**
Normal cells/DDS-NHOH	16.2 ± 2.1†	4.7 ± 0.3†
Normal cells/DDS-NHOH/DEM	9.5 ± 0.9†	3.7 ± 0.3†
Diabetic cells/DDS-NHOH	13.3 ± 2.9	5.3 ± 0.2
Diabetic cells/DDS-NHOH/DEM	10.0 ± 3.3	5.1 ± 0.6

N = 4, mean ± SD; levels of significance apply to similarly indicated data.
**, * $P < 0.01$.
† $P < 0.05$.

effect on methaemoglobin formation or white cell viability compared with cells alone. Statistical comparisons were made using Student's *t*-test accepting $P < 0.05$ as significant. Recovery of dapsone and dapsone hydroxylamine from the compartments was measured by the HPLC assay in microgrammes, then converted to μmoles and expressed as a percentage of the number of μmoles of dapsone hydroxylamine originally added to the samples. Where more than one comparison was made with the same data, the acceptable level of significance was reduced to 0.05/k (where k is the number of tests) to compensate for the increased likelihood of reaching $P < 0.05$ during multiple testing. Data is presented graphically as mean ± SD.

RESULTS

Dapsone hydroxylamine concentrations in compartment 2 buffer after dialysis with either normal or diabetic erythrocytes were not significantly different (Table 1). However, in both cell types, the presence of either DDC or DEM was associated with significant fall in hydroxylamine concentrations measured in compartment 2 (Table 1). Recovery of dapsone from compartment 1 was significantly increased in the presence of DDC compared with control in both types of erythrocytes (Table 2). In contrast, recovery of dapsone from normal erythrocytes incubated in compartment 1 was significantly reduced by the presence of DEM compared with control ($P < 0.05$; Table 2), although there was no significant difference between control diabetic cells and those treated with DEM.

Dapsone analysis in compartment 2 revealed a significant increase in dapsone recovery in both diabetic and normal erythrocytes in the presence of DDC compared with diabetic and normal control ($P < 0.01$, Table 2). The presence of DEM in compartment 1 caused a significant fall in dapsone recovery in compartment 2 compared with control (Table 2, $P < 0.05$). The presence of DEM in the first compartment caused no significant difference in dapsone concentrations to occur in compartment 2 using diabetic erythrocytes (Table 2).

Analysis of methaemoglobin levels in compartment 1 indicated that significantly greater dapsone hydroxylamine-mediated haemoglobin oxidation occurred in normal compared with diabetic cells (Table 3). Methaemoglobin formation in the presence of DDC was significantly reduced compared with control for both normal and diabetic erythrocytes (Table 3). Similarly, in the presence of DEM, there was a significant reduction in methaemoglobin formation in both normal and diabetic erythrocytes compared with normal and diabetic control cells.

Examination of the mononuclear leucocytes revealed that significantly greater cell death occurred where dapsone hydroxylamine was present in both normal and diabetic erythrocytes compared with dialysis of mononuclear leucocytes against untreated erythrocytes (Table 4). Dialysis of normal erythrocytes which had been incubated with dapsone hydroxylamine against mononucleocytes in the presence of DDC caused a significant reduction in white cell death compared with the absence of DDC (Table 4). This was also the case with diabetic erythrocytes, with and without DDC (Table 4). Differences between erythrocytes incubated with and without DEM did not attain significance. Direct incubation of 10 mM DEM with healthy mononucleocytes for 1 hr at 37° caused a reduction of mononucleocyte viability from 99 to 80%.

DISCUSSION

The liver is the major site of dapsone hydroxylamine formation, a metabolite which is known to be toxic to human mononuclear leucocytes *in vitro* [8] as well as to bone marrow [10]. However, it is unlikely that peripheral destruction of mononucleocytes can entirely account for the characteristic delay in onset seen in agranulocytosis, which is often between 6 and 8 weeks after therapy commencement [3]. Hydroxylamine made in the liver cannot be detected in human plasma and is avidly removed from the circulation by erythrocytes [5, 11], which will

Table 3. Methaemoglobin formation with and without DDC (5 mM) and DEM (10 mM) in the presence of dapsone hydroxylamine (DDS-NHOH, 150 μM) in compartment 1 in normal and diabetic erythrocytes (N = 4 per incubation, mean ± SD); levels of significance apply to similarly indicated data

Treatment group	Methaemoglobin (%) Normal erythrocytes	Diabetic erythrocytes
DDS-NHOH	54.8 ± 1.3†	44.5 ± 0.5*
DDS-NHOH/DDC	51.2 ± 0.6†	33.5 ± 0.2*
DDS-NHOH	70.6 ± 0.7*	66.9 ± 0.4**
DDS-NHOH/DEM	59.8 ± 4.7*	59.1 ± 0.7**

**, * $P < 0.001$.
† $P < 0.001$.

Table 4. Dapsone hydroxylamine (DDS-NHOH)-mediated mononucleocyte cell death in compartment 2 (ascorbate free) following dialysis of mononucleocytes against erythrocytes in compartment 1 treated with 5 mM DDC or 10 mM DEM

Compartment 1 contents	Compartment 2 mononucleocyte cell death (%)
Normal erythrocytes alone	11.7 ± 2.3*
Diabetic erythrocytes alone	8.7 ± 1.3§
Normal erythrocytes/DDS-NHOH	34.0 ± 7.2*†
Normal erythrocytes/DDS-NHOH/DDC	18.7 ± 5.1†
Diabetic erythrocytes/DDS-NHOH	31.7 ± 7.1‖§
Diabetic erythrocytes/DDS-NHOH/DDC	12.7 ± 5.1‖
Normal erythrocytes alone	1.2 ± 0.6‡
Diabetic erythrocytes alone	1.1 ± 1.2¶
Normal cells/DDS-NHOH	4.7 ± 2.1‡
Normal cells/DDS-NHOH/DEM	2.2 ± 0.5
Diabetic cells/DDS-NHOH	4.5 ± 1.3¶
Diabetic cells/DDS-NHOH/DEM	4.1 ± 0.7

N = 4, mean ± SD; levels of significance apply to similarly indicated data.
*,§ $P < 0.001$.
†,‖ $P < 0.01$.
‡,¶ $P < 0.05$.

preferentially take up the metabolite and protect white cells from toxicity *in vitro* [12]. Hence it is unlikely that hydroxylamine from the liver makes a significant direct contribution to the disappearance of granulocytes from the circulation. Several studies have shown that activated neutrophils may oxidize aryl amines to reactive metabolites [13, 14]. It has been hypothesised that covalent binding of aryl amine metabolites produced by neutrophils may either cause cell death, or act as haptens, leading to immune targetting and subsequent cell death [3, 4]. This would account for the specificity of the loss of only one cell type seen in agranulocytosis. In addition, the rapid resumption of granulocyte production after dapsone therapy is stopped, would indicate that the structure of the bone marrow is undamaged [3]. Aryl amine oxidation by neutrophils has been ascribed to the "compound I" activated form of myeloperoxidase [15], which is the form of the enzyme which ultimately produces the most powerful neutrophil-mediated oxidant, hypochlorous acid [16]. However, dapsone has recently been shown to act as a poor substrate for compound I and is thought to prevent hypochlorous acid formation, thus abolishing tissue damage seen in neutrophil-mediated inflammatory conditions such as dermatitis herpetiformis [16]. The apparently contradictory nature of these studies appear to indicate that neutrophils are not necessarily the sole source of the hydroxylamine metabolites which lead to the haptenation of the granulocytes. Although other enzyme systems present in bone marrow may oxidise xenobiotics, such as cytochrome P450 [17], data from the present study suggests a possible role for erythrocytes in the earliest stages of agranulocytosis.

In a previous study using the two-compartment system, dapsone hydroxylamine was shown to escape the erythrocyte and cause methaemoglobinaemia in erythrocytes in the second compartment [6]. In this study, after the erythrocytes were spiked with the hydroxylamine and briefly incubated, they were then washed three times in buffer compared with twice in the previous study [6], to reduce the possibility

of the metabolite crossing the membrane which had not been taken up by the cells in compartment 1. In agreement with previous work, hydroxylamine-dependent methaemoglobin formation in compartment 1 was lower in diabetic erythrocytes compared with normal cells [18]. However, there was no difference between the diabetic and normal cells in the movement of the hydroxylamine from compartment 1 to the buffer in compartment 2. There was sufficient hydroxylamine present in the buffer to cause significant leucocyte cytotoxicity in compartment 2. It is conceivable that *in vivo* hydroxylamine formed in the liver may be taken up by human erythrocytes which may reach the capillaries of the bone marrow and release the hydroxylamine. However, in the present work, mononucleocyte incubations contained no protective ascorbate to facilitate measurement of hydroxylamine-mediated cytoxicity. In order to measure actual hydroxylamine concentrations, cell free incubations contained ascorbate to preserve the metabolite. This experimental model is greatly over simplified, as the cellular environment of the bone marrow contains endogenous anti-oxidants which would normally stabilize the hydroxylamine escaping from the erythrocyte and direct cytotoxicity would probably be unlikely, as agranulocytosis is infrequent and usually only occurs several weeks after dapsone treatment is initiated. If this condition is linked with the immune system, haptenation may occur when low levels of hydroxylamine are oxidised to the reactive nitroso derivative [13] when cellular anti-oxidant capability is compromised.

The process by which the hydroxylamine undergoes reduction to the parent drug and subsequent escape from the erythrocyte is thought to be mediated by intracellular thiols such as glutathione as well as haemoglobin oxidation [5]. Although DDC contains a sulfhydryl group, it does not stabilize the dapsone hydroxylamine and is unlikely to directly prevent hydroxylamine-mediated monoleucocyte toxicity. The protective effect of DDC seen in this study is probably due to its acceleration of hydroxylamine-dependent methaemoglobin formation and hydroxylamine detoxification to dapsone [5, 6]. The fall in hydroxylamine movement across the membrane thus produced which was accompanied by an increase in parent drug formation and reduced leucocyte cytotoxicity. In the absence of compounds such as DDC, it is likely that hydroxylamine egress from the erythrocyte is dependent on the level of glutathione-mediated reduction of metabolite to parent drug.

The glutathione depleting effect of DEM has been shown previously using human erythrocytes [5] and in this study the glutathione-dependent process of hydroxylamine-mediated methaemoglobin formation was significantly reduced in the presence of DEM in both diabetic and normal erythrocytes. The presence of DEM led to a fall in hydroxylamine levels in compartment 2, but in contrast to the effect of DDC, a reduction in parent drug recovery was seen in both compartments. The fall in dapsone recovery could have been due to the DEM-mediated depletion of glutathione which diminished hydroxylamine conversion to dapsone within the erythrocytes. However, the data also suggest that hydroxylamine egress from the erythrocyte is also dependent on a full cellular complement of glutathione. The hydroxylamine is thought to react with haemoglobin to form methaemoglobin and the nitroso derivative, which is then reduced to the hydroxylamine by glutathione and thus continues the redox cycle [19, 20]. Therefore, of the hydroxylamine formed from the glutathione-dependent reduction of the nitroso derivative, a small proportion must normally evade both the redox cycle of reaction as well as the process of glutathione-mediated reduction to dapsone and diffuse out of the erythrocyte. DEM-mediated glutathione depletion might therefore diminish the egress of hydroxylamine as well as dapsone from the erythrocyte. In contrast to DDC, pretreatment of the erythrocytes with DEM did not protect the mononucleocytes. This may be due to the direct cytotoxic effect of DEM which leeched out of the erythrocytes into the compartment containing the monoleucocytes.

The accelerating effect of DDC on hydroxylamine reduction to dapsone has been shown in two previous studies [5, 6] as well as the present work. However, in contrast to static incubations [5], using the two-compartment system in the present and in an earlier report [6] caused a net fall in methaemoglobin formation in the presence of DDC. Reduction of oxidized haemoglobin is routinely achieved by methaemoglobin reductases, which are almost entirely NADH-dependent [21, 22]. It is possible that the process of rotation of the two-compartment system leads to a more rapid reversal of methaemoglobinaemia mediated by the reductase enzymes.

In this study, the ability to release the hydroxylamine and the parent drug from the first to the second compartment was shown in diabetic as well as normal erythrocytes. The glutathione-dependent effect of DDC was also similar in both cell types. Indeed, glutathione concentrations were similar to those of normal erythrocytes in previous studies [18, 23]. However, the effect of DEM in reducing dapsone formation from the hydroxylamine shown in normal erythrocytes was not evident in diabetic cells. As glutathione metabolism is thought to be impaired in diabetic cells [24] it would be expected that DEM might be more effective in glutathione depletion compared with normal cells. The effect of DEM may have been less clear cut in diabetic cells due to the combination of non-specific cellular binding which is known to occur when DEM is used experimentally [25] and the characteristic glycation seen in diabetic erythrocytes [26].

The oxidative metabolites of dapsone are known to bind to human tissue *in vitro* [8]. The cytochrome P450 A4 which N-hydroxylates dapsone as well as metabolising endogenous steroids and host of different drugs, is a constitutive enzyme in human liver [27, 28]. If haptenation of granulocytes and other white cells occurs *in vivo* through the putative erythrocytic delivery system described in this work, it is likely to occur in all patients and vary according to the extent of hepatic hydroxylamine formation, which is itself thought to be subject to a polymorphism in man [29]. Agranulocytosis is most prevalent in

patients with excessively sensitive immune systems such as in dermatitis herpetiformis, and least common in immunocompromised leprosy patients [30]. Therefore, a complex interaction of risk factors such as extent of hepatic metabolite formation, erythrocyte glutathione status, immune reactivity and disease state may predispose an individual to agranulocytosis.

In summary, dapsone hydroxylamine is capable of escaping normal and diabetic erythrocytes, traversing a semi-permeable membrane and causing toxicity to human mononucleocyte cells *in vitro*. This process may be one of the first stages in immune mediated agranulocytosis.

Acknowledgements—This work was supported by the Nuffield Foundation and the Jacobus Pharmaceutical Company.

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