INTERACTION OF CHEMICAL CARCINOGENS WITH PLASMA MEMBRANES: THE EFFECT OF DIMETHYLAMINOAZOBENZENE ON ERYTHROCYTE OSMOTIC FRAGILITY

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Summary

The capacity of the hepatocarcinogen dimethylaminoazobenzene and two of its analogs to protect erythrocytes against hemolysis is demonstrated. The aminoazo dye compounds exhibit stabilization at relatively low final concentrations, suggestive of an efficient partitioning of the dye molecules into the erythrocyte membrane. Methyl groups at either the 2 or 3' position of dimethylaminoazobenzene confer even greater stabilization potential to the basic dye structure. The application of the erythrocyte osmotic hemolysis assay in analysis of carcinogen-membrane interactions is discussed.

INTRODUCTION

While our understanding of the processes involved in the biotransformation and metabolism of chemical carcinogens has been developing rapidly during the past several decades (1,2), relatively little is known of the interactions occurring between carcinogens and target cell plasma membranes. A comprehension of the physiochemical basis of this interaction is necessary in order to evaluate the role of membrane in carcinogen-tissue specificity and penetration, as well as the possible effect of carcinogen binding on membrane related function. In this communication we have attempted to analyze chemical carcinogen-membrane interactions by estimating the capacity of an aromatic amine carcinogen

[1] Abbreviations: DAB, dimethylaminoazobenzene; 3' Me DAB, 3' methyl dimethylaminoazobenzene; 2 Me DAB, 2 methyl dimethylaminoazobenzene.

to protect against osmotic lysis of erythrocytes. The studies illustrate the general features of the carcinogen-erythrocyte membrane interaction and quantitatively demonstrate the stabilization effect of DAB[1] and two of its analogs on the red blood cell membrane.

MATERIALS AND METHODS

DAB and 3' Me DAB were obtained from Aldrich Chemicals Inc. (Cedar Knolls, N.J.) and recrystallized from benzene-petroleum ether. 2 Me DAB was synthesized by reacting diazotized aniline with dimethyl-m-toluidine (3). The aminoazo dye was isolated by chromatography on neutral alumina oxide in benzene and was recrystallized twice from benzene-petroleum ether. The melting point of the resulting crystals was 64-66° C. All three compounds behaved as single products when analyzed by thin layer chromatography on alumina oxide or silica gel in benzene or benzene:ethanol (98:2, v/v).

Stock solutions of the carcinogens were prepared at a concentration of 1.0×10^{-3} M in absolute ethanol. The concentrations of the actual working solutions were determined from the absorption spectra of the dye solutions in 1.2 N HCl, 50% ethanol, employing the ε_M, λ values of 35,500,514 nm; 37,600,522 nm; 48,200,514 nm, for DAB, 3' Me DAB and 2 Me DAB respectively (4). Stock solutions of pregnanolone (5 β-pregnane-3 β-ol-20-one) and progesterone (Δ^4-pregnene-3,20-dione) in ethanol were prepared immediately prior to introduction into erythrocyte suspensions.

The osmotic hemolysis assays were performed essentially as described (5) and recent recommendations (6) for improvement of experimental precision were followed. An additional control consisting of erythrocytes, buffered saline and carcinogen was incubated and centrifuged in parallel with the test samples. The OD_{543} of the supernate of this reaction mix-

ture containing varying amounts of unabsorbed dye was subtracted from the OD_{543} values of test supernates initially containing equivalent concentrations of dye. Relative hemolysis relates the lysis occurring in the presence of a given drug to the lysis occurring in the absence of the drug; percent hemolysis relates the lysis in hypotonic solution occurring in the presence or absence of drug to absolute hemolysis (7,8). All experimental values reported were determined 6-8 times.

RESULTS

Figure 1 illustrates the antihemolytic effect of DAB on human erythrocytes. The protective effect extended approximately over a twenty-five fold range in final DAB concentration within the 0.36-0.40 grams NaCl/100 cc range of the osmotic fragility curve. Increasing the assay temperature to 37° C introduced a significant shift in the fragility curve as previously reported (7) and reduced the antihemolytic effect of DAB. The experiments presented were performed at 21-22° C.

Figure 2 compares the antihemolytic effect of DAB to two steroids previously shown to exhibit high and intermediate protective capacities

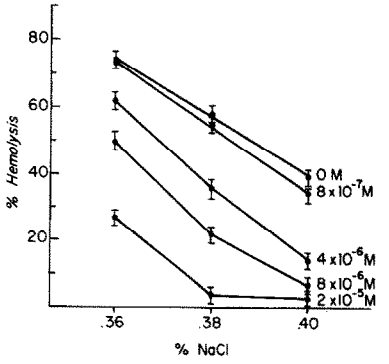

FIGURE 1 Illustrates the capacity of various concentrations of DAB to reduce the osmotic fragility of erythrocytes. Final concentrations of carcinogen are as indicated.

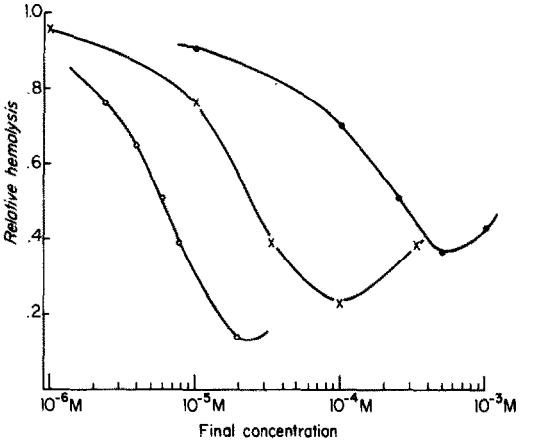

FIGURE 2 Comparison of antihemolytic effect of DAB -o-o-o-, pregnanolone -x-x-x- and progesterone -●-●-●-. The upturn in the curve of DAB indicates that hemolysis occurs at higher concentrations of carcinogen; pronounced unbound dye absorption at 543 nm and hemoglobin precipitation precludes assignment of precise relative hemolysis values at these higher concentrations of dye. The relative hemolysis of 1.0 corresponds to an absolute hemolysis of approximately 70%.

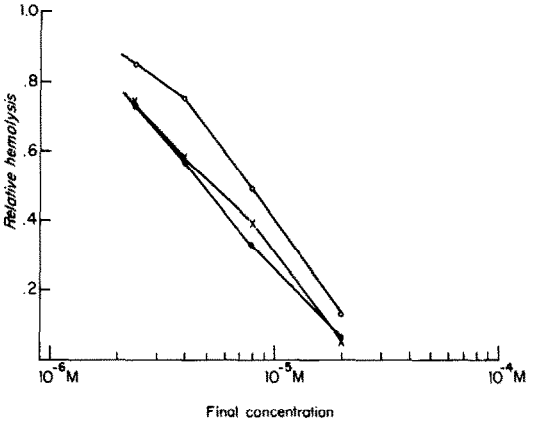

FIGURE 3 Comparison of antihemolytic effects of DAB -o-o-o-, 3' Me DAB -x-x-x- and 2 Me DAB -●-●-●-. The relative hemolysis of 1.0 corresponds to an absolute hemolysis of approximately 70%.

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in osmotic fragility assays. It can be seen that in this assay DAB is more efficient than pregnanolone, the most protective of a series of nine steroid compounds previously analyzed (8). The upward turn of

the fragility curve at higher DAB concentrations indicates that DAB, like the steroids, exhibits a direct lytic effect at higher concentrations of dye, in addition to exhibiting a concentration-dependent protective effect.

Figure 3 compares the antihemolytic effect of DAB, 3' Me DAB, its more carcinogenic derivative, and non-carcinogenic 2 Me DAB. The single methyl substitution in either the prime or non-prime ring of DAB confers a notably higher antihemolytic capacity over the eight-fold concentration range of dye compounds analyzed.

DISCUSSION

The capacity of a variety of compounds including anesthetics, tranquilizers, alcohols, steroids (9) and lipids (10) to stabilize erythrocytes against osmotic hemolysis is well documented. The most plausible explanation for the protective phenomenon is that lipophilic substances expand the membrane, thereby increasing the cell's surface area/volume ratio; erythrocytes with higher surface area/volume ratios exhibit lower osmotic fragility. In several instances, correlations can be drawn between pharmacologic potency of a drug, its membrane binding capacity, octanol/water partition coefficient and capacity to stabilize against osmotic hemolysis (9). By comparing antihemolytic effects within families of structurally related drugs, it has been possible to ascertain the influence of different structural substitutions on the behavior of a given compound towards the plasma membrane. Thus, while the erythrocyte need not be the actual target of a given agent, it has proven a useful model for evaluating the effects of small molecules of varying hydrophobic nature on membrane structure.

The results presented in this study indicate that the erythrocyte can serve as a model for examination of membrane effects with yet another class of compounds, the aminoazobenzene dyes. The general features of the carcinogen-erythrocyte interaction differ little from drug-erythrocyte

interactions where significant protective capacity has been achieved at relatively low final concentrations of drug. In these studies, the elevated protective capacity has been correlated with a high octanol/water or olive oil/water partition coefficient, P. P values for the three dye compounds examined in this study are not available; however, azobenzene itself exhibits a relatively high log P octanol/water of 3.82 (11).

The antihemolytic effects of 3' Me DAB, a more potent carcinogen than DAB (12) and 2 Me DAB, a non-carcinogen in standard dye feeding experiments, (3) were compared to the antihemolytic effects of DAB. At present it is felt that the different carcinogenic properties of these two compounds result from a direct influence of the 2 methyl or 3' methyl group on the initial chemical activation and ultimate targeting of the activated dye product, although the molecular basis for this difference in biologic activity has not been firmly established (3,13,14). The data presented in these studies suggest that 3' Me DAB or 2 Me DAB may either partition more effectively to the cell membrane or be more effective than DAB in expanding the membrane. The former, more likely alternative, would be consistent with a more efficient uptake of these compounds by the plasma membranes of target cells. Thus, 2 Me DAB may lack carcinogenic potential, owing to a direct influence of the 2 methyl group on the various steps in the biotransformation of the dye, e.g. N-demethylation, N-hydroxylation and azoreduction, while the enhanced carcinogenicity of 3' Me DAB over DAB may be influenced by the behavior of the latter towards the liver cell plasma membrane. This general conclusion is supported in the earlier theoretical work of Hansch and Fujita (15) in which the effect of a 3' methyl group on the lipophilic properties of DAB was estimated from a substituent constant, π, obtained from estimation of P values in a series of substituted phenoxyacetic acids.

While metabolic activation is the essential step in defining the

carcinogenic potential of a compound, other aspects of carcinogen metabolism, such as the initial relationships of carcinogen with membrane as mentioned here should be taken into consideration, particularly where the influences of structural substituents on ultimate carcinogenicity are not easily reconciled. The erythrocyte osmotic fragility assay may prove a useful tool in understanding the interactions between the various classes of chemical carcinogens, their analogs and plasma membranes.

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