THE CORRELATION BETWEEN DRUG BINDING TO THE HUMAN ERYTHROCYTE AND ITS HEMOLYTIC ACTIVITY

TOSHIHARU HORIE,* YUICHI SUGIYAMA, SHOJI AWAZU* AND MANABU HANANO

Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo, 113, Japan

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Cationic phenothiazine derivatives, anionic anthranilic acid derivatives and fluorescent probe 1-anilino-8-naphthalene sulfonate (ANS) which had hemolytic activities were used to investigate hemolysis of human erythrocyte. The observed hemolytic activities of drugs could divided into two categories: (1) the difference of the binding activity of drugs to the erythrocyte and (2) the difference of the membrane perturbation activity of drugs bound to the erythrocyte.

The human erythrocyte had two kinds of binding sites for any drug used. The first site of them was already saturated before hemolysis occurred and the second site of them may play an important role in hemolysis by these drugs.

Keywords—phenothiazine derivatives; anthranilic acid derivatives; 1-anilino-8-naphthalene sulfonate; hemolysis; drug binding; binding site; erythrocyte

INTRODUCTION

It has been reported that several agents, such as some of anesthetics, tranquilizers and non-steroidal anti-inflammatories, induce various changes in erythrocytes depending on their concentrations. For example, they protect erythrocytes from osmotic hemolysis at low concentration, but cause hemolysis at high concentration and morphological changes. Hitherto, these phenomena have been investigated from the total drug concentration (the value divided the initial amounts of drug in the erythrocyte suspension by the total volume). However, it will be reasonable to investigate these phenomena from the amount of drugs bound to the erythrocyte since they are induced as a result of the interaction between drug and erythrocyte. Seeman quantitated the uptake of chlorpromazine by the erythrocyte ghosts at the concentration where hemolysis did not occur. Mohandas et al. investigated in detail the relationship between morphological changes of erythrocyte and uptake of cationic and anionic phenothiazine derivatives and showed that the erythrocyte morphological changes were dependent on the cellular concentration of them. We reported that the fluorescence of fluorescent probe, 1-anilino-8-naphthalene sulfonate (ANS) in the erythrocyte ghosts was changed by hemolytic drugs and that there existed a correlation between the degree of ANS fluorescence changes and hemolytic activity of drugs. This implied that the binding of hemolytic drugs to the erythrocyte was very important in hemolysis. Weltzien et al. introduced the amount of lysophospholipid which was absorbed to the erythrocytes at 50% lysis to describe the actual "membrane disturbing activity" of phosphatides.

In this study, the relationship between hemolytic activities and binding activities of various drugs to the human erythrocytes was investigated in order to make clear the mechanism of hemolysis by drugs. Hemolysis by drugs was compared not only from the apparent hemolysis as usual but also from the stoichiometry of binding to the erythrocyte. Phenothiazine deri-
natives which are tranquilizers as cationic compounds, anthranilic acid derivatives which are nonsteroidal anti-inflammatories and ANS as anionic compounds were used.

MATERIALS AND METHODS

Drugs — Chlorpromazine HCl (CPZ), chlorpromazine sulfoxide HCl (CPZSO), prochlorperazine dimaleate (PCP), thioridazine HCl (TRZ), diethazine HCl (DTZ), promethazine HCl (PMZ) were obtained from Yoshitomi Co. Ltd.; flufenamic acid sodium salt (FA), mefenamic acid (MA) were supplied by Sankyo Co. Ltd.; the sodium salt of 1-anilino-8-naphthalene sulfonate as the research grade for protein structures from Tokyo Kasei Co. Ltd., was used without further purification. Other chemicals were obtained from the usual commercial sources. Anthranilic acid derivatives were dissolved in dimethylsulfoxide (DMSO) and diluted with 10 mM phosphate buffered isotonic saline, pH 7.4. The low final concentration of DMSO did not cause the release of hemoglobin from erythrocyte.

Hemolysis — Human erythrocytes were the gifts from Japanese Red Cross Central Blood Center. Human erythrocytes were separated by centrifugation at 1000 × g for 10 min in Hitachi 20PR-5 and washed with 10 mM phosphate buffered isotonic saline, pH 7.4 and centrifuged at 3000 × g for 10 min. This washing process for human erythrocyte was repeated three times at 4°C. Drug solution was added to 5 ml of 2% erythrocyte suspension in 10 mM phosphate buffered isotonic saline by microsyringe. The mixtures were incubated for 90 min at 37°C and then centrifuged at 1000 × g for 5 min in Kubota KC-70. The supernatant was separated and its optical density was determined at 543 nm in Hitachi 124 Spectrophotometer. Per cent hemolysis is expressed by the ratio of optical density at 543 nm of hemoglobin released from the erythrocyte by drug to the optical density after the complete hemolysis of the erythrocyte in water.

Binding Kinetics of Drugs to the Erythrocyte — The erythrocyte suspension was the same as used in hemolysis experiment. Drug solution was added to it and the mixtures were incubated for 90 min at 37°C and then centrifuged at 1000 × g for 5 min in Kubota KC-70. The bound amount of drugs to the intact erythrocyte was measured.

**FIG. 1. Hemolytic Effects of Cationic Compounds on the Human Erythrocyte**

The final concentration of the erythrocyte was 2% in 10 mM phosphate buffered isotonic saline, pH 6.5. Temp. 37°C ● TRZ; ○ PCP; ■ CPZ; □ DTZ; ▲ PMZ; △ CPZSO.

**FIG. 2. Hemolytic Effects of Anionic Compounds on the Human Erythrocyte**

The final concentration of the erythrocyte was 2% in 10 mM phosphate buffered isotonic saline, pH 7.4. Temp. 37°C ● FA; ○ ANS; ▲ MA.
from the difference between the initial concentration and that in the supernatant of the erythrocyte suspension which was centrifuged at the concentration where released hemoglobin did not interfere the measurement of drug concentration, using Hitachi 356 Spectrophotometer by dual wavelength to eliminate the blank absorption due to released hemoglobin. The number of erythrocyte in each suspension was measured by Coulter Counter Model F. The amount of the bound drugs to the erythrocyte was calculated from this value.

Protection against Osmotic Hemolysis by Drugs — TRZ as a cationic compound and ANS as an anionic compound were added to 3 ml of 4% erythrocyte suspension in 10 mM phosphate buffered isotonic saline, pH 6.5 and pH 7.4, respectively. They were incubated for 30 min at 37°. Then 3 ml of 10 mM phosphate buffer containing the same concentration of drug as the above erythrocyte suspension was added to induce osmotic hemolysis. The mixture was incubated for 60 min at 37° and then centrifuged at 1000 × g for 5 min. The supernatant was separated and its optical density was determined at 543 nm. Per cent hemolysis was expressed as that in hemolysis.

All the experiments were performed in pH 6.5 for phenothiazine derivatives and pH 7.4 for anionic compounds to study hemolysis by ionized forms of drugs.

RESULTS

Hemolysis by Drugs and Their Binding to the Human Erythrocytes

Hemolytic activities of drugs and their binding to the erythrocyte were measured using the same lot of erythrocyte. The results were shown in Fig. 1, 2 (hemolysis) and in Fig. 3, 4 (binding). In phenothiazine derivatives, the order of apparent hemolytic activities was TRZ, PCP, CPZ, PMZ, DTZ. The order of the bound amount of drugs per erythrocyte was TRZ, PCP, CPZ, DTZ, PMZ. Although CPZSO bound to the erythrocyte, it did not cause hemolysis within these concentrations. In anionic drugs, the order of apparent hemolytic activities was FA, ANS, MA, but there was no marked difference as that in cationic drugs.

Correlation between Hemolysis and Bound Amount of Drugs to the Human Erythrocyte

Since erythrocytes have a distribution of aging, it will be reasonable to compare the amount of drugs bound to the erythrocyte in which 50%
Drug Binding and Its Hemolytic Activity

Hemolysis occurs. Hemolysis (%) and the amount of drugs bound to an erythrocyte \( (r) \) were plotted against total drug concentration \( (C) \). The total drug concentration \( (C_{50}) \) which caused 50% hemolysis was obtained from hemolytic curve and the bound amount per erythrocyte \( (r_{50}) \) at \( C_{50} \) was obtained from binding curve. Hemolytic activities of drugs were compared using \( C_{50} \) and \( r_{50} \) (Table I). In phenothiazine derivatives, \( C_{50} \) of PMZ was larger than those of TRZ, PCP and CPZ, but each \( r_{50} \) of these four drugs was nearly equal. However, there were exceptions; DTZ and CPZSO were different from the above four drugs.

DTZ caused 50% hemolysis when it bound to the erythrocyte about three times as much as other drugs. CPZSO did not cause hemolysis even at \( C = 23.2 \) mM, \( r = 3.15 \times 10^{-16} \) mol/cell. Both \( C_{50} \) and \( r_{50} \) of anionic drugs were larger than those of cationic ones.

**Analysis of Binding Kinetics of Drugs to the Human Erythrocyte**

The data obtained from binding kinetics were analyzed by Scatchard plots (Fig. 5, 6). As the plot was curvilinear, the data were fitted by non-linear least square method to a two-term Langmuir-type equation:

**FIG. 5.** The Scatchard Plots of the Amount of Cationic Compounds bound to the Human Erythrocyte

- \( r: \) number of mol of drug bound per cell, \( C_r: \) concentration of free drug.
- a): ◦ TRZ; ○ PCP; ■ CPZ. b): □ DTZ; ▲ PMZ; △ CPZSO.

**FIG. 6.** The Scatchard Plots of the Amount of Anionic Compounds bound to the Erythrocyte
- ◦ FA; ○ ANS; ▲ MA.

<table>
<thead>
<tr>
<th></th>
<th>( C_{50} ) (mM)</th>
<th>( r_{50} ) (10(^{-16}) mol/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRZ</td>
<td>0.223</td>
<td>2.18</td>
</tr>
<tr>
<td>PCP</td>
<td>0.212</td>
<td>2.23</td>
</tr>
<tr>
<td>CPZ</td>
<td>0.552</td>
<td>3.06</td>
</tr>
<tr>
<td>PMZ</td>
<td>1.83</td>
<td>2.29</td>
</tr>
<tr>
<td>DTZ</td>
<td>1.98</td>
<td>7.50</td>
</tr>
<tr>
<td>FA</td>
<td>1.55</td>
<td>7.70</td>
</tr>
<tr>
<td>ANS</td>
<td>2.00</td>
<td>13.6</td>
</tr>
<tr>
<td>MA</td>
<td>3.37</td>
<td>6.30</td>
</tr>
</tbody>
</table>

a) These data were obtained from Fig. 1—4.

\( C_{50}: \) total drug concentration which caused 50% hemolysis.

\( r_{50}: \) the amount of drug bound to the erythrocyte at \( C_{50} \).
TABLE II. Binding Parameters analyzed by the Scatchard Plots of the Amount of Cationic and Anionic Compounds bound to the Erythrocyte

<table>
<thead>
<tr>
<th></th>
<th>$K_1$ (M$^{-1}$)</th>
<th>$K_2$ (M$^{-1}$)</th>
<th>$n_1$</th>
<th>$n_2$</th>
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</thead>
<tbody>
<tr>
<td>TRZ</td>
<td>$1.19 \times 10^6$</td>
<td>$7.70 \times 10^3$</td>
<td>0.122</td>
<td>3.02</td>
</tr>
<tr>
<td>PCP</td>
<td>$5.21 \times 10^8$</td>
<td>$5.24 \times 10^3$</td>
<td>0.116</td>
<td>4.46</td>
</tr>
<tr>
<td>CPZ</td>
<td>$4.20 \times 10^5$</td>
<td>$3.10 \times 10^3$</td>
<td>0.106</td>
<td>4.67</td>
</tr>
<tr>
<td>PMZ</td>
<td>$1.58 \times 10^4$</td>
<td>$1.17 \times 10^2$</td>
<td>0.347</td>
<td>13.4</td>
</tr>
<tr>
<td>DTZ</td>
<td>$1.34 \times 10^4$</td>
<td>$9.25 \times 10^1$</td>
<td>0.366</td>
<td>50.9</td>
</tr>
<tr>
<td>CPZSO</td>
<td>$2.05 \times 10^5$</td>
<td>$1.80 \times 10^2$</td>
<td>0.025</td>
<td>3.88</td>
</tr>
<tr>
<td>FA</td>
<td>$9.40 \times 10^4$</td>
<td>$1.80 \times 10^3$</td>
<td>0.845</td>
<td>9.86</td>
</tr>
<tr>
<td>ANS</td>
<td>$1.39 \times 10^5$</td>
<td>$1.40 \times 10^3$</td>
<td>0.249</td>
<td>8.18</td>
</tr>
<tr>
<td>MA</td>
<td>$1.57 \times 10^5$</td>
<td>$3.10 \times 10^3$</td>
<td>0.279</td>
<td>6.66</td>
</tr>
</tbody>
</table>

$\text{a})$ $10^{-16}$ mol/cell. $n$: number of binding site. $K$: binding constant.

Subscripts, 1, 2: binding site one and two, respectively.

FIG. 7. Stoichiometry of Cationic Drug Actions and Its binding to the Human Erythrocyte

- $\bigcirc$: protective effect against osmotic hemolysis in hypotonic buffer by cationic drug, TRZ.
- $\bullet$: hemolysis in isotonic buffer by TRZ.
- $\longrightarrow$: the degree of saturation of TRZ binding site 1 and 2 of the human erythrocyte; They were calculated from binding parameters in Table II.

$$r = r_1 + r_2 = \frac{n_1K_1C_f}{1+K_1C_f} + \frac{n_2K_2C_f}{1+K_2C_f}$$  \hspace{1cm} (1)

$r$: number of mol of drug bound per cell
$n$: number of binding site
$K$: binding constant
$C_f$: concentration of free drug

Subscripts, 1, 2: binding site one and two, respectively.

Binding parameters were obtained after the fitting (Table II). TRZ, PCP and CPZ having high hemolytic activities had larger binding constants ($K_1$). The numbers of binding site $n_1$, $n_2$, respectively, were almost equal among them.

FIG. 8. Stoichiometry of Anionic Compound Actions and Its binding to the Human Erythrocyte

- $\bigcirc$: protection by anionic compound, ANS.
- $\bullet$: hemolysis by ANS.
- $\longrightarrow$: the degree of saturation of ANS binding site 1 and 2.

Hemolysis and protective effect against osmotic hemolysis by TRZ as a cation and ANS as an anion were investigated. Hemolysis was tested in isotonic medium. This shows hemolysis by drugs. Protective effect of drugs against osmotic hemolysis was tested in hypotonic medium. In hypotonic medium, in the absence of drugs,
hemolysis occurs, but in the presence of drugs, they protect erythrocytes against hemolysis. The ratio of $r_i$ to $n_i$, which indicated the degree of saturation at binding site $i$ ($i = 1, 2$) was calculated. Figures 7 and 8 showed the relationship among protective effect, hemolysis and the degree of saturation at binding site $i$, which was calculated as $r_i/n_i$.

**DISCUSSION**

Comparing apparent hemolytic activities of drugs,$^{15,16}$ cationic drugs could be divided into two groups: one had high apparent hemolytic activities (TRZ, PCP and CPZ) and the other had low one (PMZ and DTZ). The difference among anionic drugs were not so clear as that among cationic ones. Anionic drugs caused hemolysis at a concentration higher than cationic ones with high hemolytic activities. Evidently, drugs having high apparent hemolytic activities bound strongly to the erythrocyte. Phenothiazine derivatives changed ANS fluorescence in the ghost membrane, except CPZSO which did not change it at all.$^{11}$ So CPZSO might have different binding sites for ghost membrane from other phenothiazine derivatives or cause weaker damages to the membrane than others after binding. And there was a qualitative relationship between apparent hemolytic activities and the bound amounts of drugs to the erythrocyte.

It is considered that hemolysis by drugs would occur generally through the following steps: binding $\rightarrow$ membrane perturbation $\rightarrow$ hemolysis. From these results, the observed hemolytic activities of drugs can be divided into two categories. The one is the binding activity of drugs to the erythrocyte and the other is the membrane perturbation activity of drugs bound to the erythrocyte. For example, PMZ has larger $C_{50}$ than TRZ, PCP and CPZ, but it has similar $r_{50}$ to those of the three phenothiazines. This shows that the low hemolytic activity of PMZ is ascribed to the low binding activity, but not to the low perturbation activity. On the other hand, DTZ has similar $C_{50}$ to that of PMZ, but the former has larger $r_{50}$ than the latter. This suggests that DTZ has the lower perturbation activity than PMZ and the other phenothiazines tested (TRZ, PCP and CPZ). As for the anionic drugs, they have low binding and perturbation activities. The difference of perturbation activity might come from the difference in binding site since DTZ with the highest $r_{50}$ among the phenothiazines has the highest $n_2$ and the anionic drugs has, needless to say, an opposite charge to the phenothiazines.

From the results of binding kinetics of drug-erythrocyte, it was shown that drugs used had two kinds of binding sites for the erythrocyte (Fig. 5, 6). The uptake of cationic phenothiazines by intact erythrocyte was investigated. Cationic phenothiazines reached an equilibrium rapidly in the exterior and interior of the erythrocyte (in preparation). This may imply that cationic phenothiazines bind to the erythrocyte membrane. So from the results of binding kinetics, the membrane mass per mol of binding site for each drug is given as the reciprocal value of $n^*$, and about $10^4 - 10^5$ g/mol and about 100-2000 g/mol were obtained for the first and second binding site, respectively. The value for the first site corresponds to the usual molecular weight of proteins and is much larger than that of lipids. Therefore, the first site might be composed of proteins. And the similar consideration suggests that the second site might be composed of lipids. Concerning anionic compounds, from the results of binding kinetics of ANS-erythrocyte ghost membrane, the similar discussion was carried out.$^{11}$ These considerations about binding sites have been supported by the direct informations about the binding site, obtained by measuring ANS fluorescence lifetime and polarization using ANS fluorescence (submitted). That is, measuring the fluorescence lifetime of ANS in the human erythrocyte ghost membrane suspension, two kinds of fluorescence lifetime were obtained:

\* $n^*$ value is expressed as mol/g protein, since the membrane mass is reported as $6.0 \times 10^{-13}$ g protein/cell by Dodge et al.$^{17}$
\[ \tau_1 = 15 \text{ nsec at low ANS concentration and } \tau_2 = 8.4 \text{ nsec at high ANS concentration. And the rotational relaxation times obtained from the ANS polarization in the erythrocyte membrane suspension at low and high ANS concentration resembled those of proteins and lipids, respectively.} \]

Drug actions, i.e., hemolysis and protective effects against osmotic hemolysis, were compared with the degree of saturation of the drug binding site in the erythrocyte (Fig. 7, 8). It is known that phenothiazine derivatives have an antihemolytic and membrane-expanding effect on erythrocyte membranes. These effects occur at the concentrations which are almost identical to those which exhibit pharmacological effect.\(^{14} \) When hemolysis by TRZ and ANS occurred, the first binding site was already saturated by them, but the second site was about 50% saturated. Any drugs used showed a similar relationship between hemolysis and the degree of saturation at the binding sites of them in the ghost membrane. These results show that the second site, which may be composed of lipids, may play an important role in hemolysis.

It will be necessary to make clear the nature of the second binding site and membrane perturbation by drugs in order to elucidate the mechanism of hemolysis.\(^{10} \) Furthermore, it will be important to consider how the saturation of the first binding site influences the states of membrane lipids.

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