Diisopropylfluorophosphate Is Not a Specific Label for the Red Cell Membrane

By David A. Sears and Robert I. Weed

Since the investigations of Grob et al. and the later studies of Cohen and Warringa, diisopropylfluorophosphate (DFP), containing either radioactive phosphorus ($^{32}$P) or tritium, has been widely and effectively used as an agent to tag erythrocytes, leukocytes, and platelets. Its advantages as a firmly-bound label for red cells have been well discussed by Garby. DFP is a potent and irreversible inhibitor of acetylcholinesterase (ACHE), probably exerting its effect by binding to a serine hydroxyl group at the active center of the enzyme. Red cell ACHE is firmly associated with and probably confined to the cell membrane. For these reasons it is frequently assumed, though seldom explicitly stated, that DFP labels red cells by binding specifically to membrane ACHE. Because of the potential usefulness of a specific membrane label and the lack of definitive experimental evidence on the distribution of DFP within red cells, the studies described herein were performed.

Materials and Methods

Blood was drawn from normal adults and anticoagulated with acidified citrate-dextrose (ACD-A). Red cells were labeled in vitro by the dropwise addition of DFP$^{32}$P and incubation for 60 minutes at room temperature. The blood was centrifuged with gradually increasing force as described by Chapman to permit good separation of white blood cells and platelets from red cells, and the red cells were washed at least 6 times with 5-10 volumes of saline. The red cells were suspended in an equal volume of saline, and aliquots were removed for determination of hemoglobin, microhematocrit, and red cell count in a Coulter counter, and for measurement of radioactivity. The remainder of the red cell suspension was then divided into two parts. The red cells from one aliquot were hemolyzed with an equal volume of distilled water. The hemolysate...
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was frozen and thawed once and used for hemoglobin determination and radioactivity measurement. From the second aliquot hemoglobin-free red cell ghosts were prepared as previously described.\textsuperscript{12} After a final wash with isotonic 0.01 M phosphate-buffered saline (pH 7.4), the ghosts were suspended in the buffer for enumeration in the Coulter counter and for measurement of radioactivity. In one experiment the red cells were washed three times with saline and resuspended in saline to the original volume. Labeling was then carried out as described above.

For the single experiment in vivo 0.5 ml. DF\textsuperscript{32}P (0.28 mg. DFP, 98 micro-curies \textsuperscript{32}P) was mixed with 10 ml. sterile saline in a syringe and immediately injected intravenously in a normal subject over a 5 minute period. Blood samples were drawn at intervals for the next 64 days and anticoagulated with EDTA (1.5 mg./ml. blood). The red cells were separated by centrifugation as described above, washed three times with saline, and suspended in an equal volume of saline. The samples were then handled as outlined above for the in vitro experiments.

Radioactivity of the DF\textsuperscript{32}P-labeled red cells, hemolysates, and ghosts was measured in a low-background, thin window, gas-flow detector system.\textsuperscript{*} The cell suspensions and hemolysates were pipetted in duplicate onto filter paper disks in disposable aluminum planchets, dried overnight at room temperature, covered with transparent tape, and counted for sufficient time to reduce counting error to 1 per cent (except for the last two samples in the in vivo experiment in which the maximum error was under 3.2 per cent). Sample volumes pipetted were either 0.5 or 1.0 ml. and were constant in any one experiment. For the in vivo labeling experiment two of the initial samples were counted with each subsequent set of samples to correct for isotope decay and detector variation, so that a survival curve could be constructed. For all calculations the mean value of the two counts was used. The percent of total red cell radioactivity associated with the cell membrane was determined from the red cell and ghost counts and radioactivity measurements.

The red cell survival curve was fitted to the experimental points by the method of least squares.\textsuperscript{13}

RESULTS

Data from the in vitro labeling studies are shown in Table 1. In nine separate blood samples from five subjects the proportion of the total red cell DF\textsuperscript{32}P associated with the cell membrane averaged 10.5 per cent with a range of 6.5 to 12.9 per cent. The percent was independent of the amount of DFP used and was the same whether whole blood or washed red cells were labeled.

Data from the in vivo labeling experiment are shown in Figure 1. Counts per 10\textsuperscript{9} red cells and ghosts are plotted against time, and the least squares line for red cell radioactivity is shown. The extrapolated red cell life span was 121 days. Similar curves were drawn plotting counts per ml. red cells in the red cell suspensions and counts per Gm. of hemoglobin in the red cell suspensions and in the hemolysates against time. These resulted in similar good fits with extrapolated values for red cell life span of 114, 118, and 118 days respectively. The proportion of total red cell counts associated with the cell membrane varied from 2.9 to 19.0 per cent over the course of the study with a mean value of 8.6 per cent. There is no obvious explanation for the higher values in the midportion of the study.

Table 1.—Red Cells Labeled in vitro with DF$^{32}$P

<table>
<thead>
<tr>
<th>Subject</th>
<th>$^{32}$DFP Added to</th>
<th>$\mu$gm. DFP per ml. blood</th>
<th>Per cent of DFP associated with membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>GB</td>
<td>whole blood</td>
<td>0.28</td>
<td>6.5</td>
</tr>
<tr>
<td>GB</td>
<td>”</td>
<td>0.56</td>
<td>12.9</td>
</tr>
<tr>
<td>RH</td>
<td>”</td>
<td>0.56</td>
<td>9.8</td>
</tr>
<tr>
<td>MM</td>
<td>”</td>
<td>0.28</td>
<td>8.2</td>
</tr>
<tr>
<td>TT</td>
<td>”</td>
<td>0.28</td>
<td>7.7</td>
</tr>
<tr>
<td>CB</td>
<td>”</td>
<td>2.00</td>
<td>11.7</td>
</tr>
<tr>
<td>CB</td>
<td>”</td>
<td>1.00</td>
<td>12.5</td>
</tr>
<tr>
<td>CB</td>
<td>”</td>
<td>0.50</td>
<td>12.6</td>
</tr>
<tr>
<td>CB</td>
<td>washed red cells</td>
<td>0.50</td>
<td>12.6</td>
</tr>
</tbody>
</table>

Fig. 1.—Radioactivity of whole red cells and ghosts after in vivo labeling with $^{32}$DFP.

Conclusions

It is clear that when red cells are labeled with DF$^{32}$P either in vitro or in vivo, only a small percentage of the bound material is attached to the cell membrane, and the majority is attached to intracellular sites. Though ACHE was not assayed in these experiments, it is very likely that DFP was bound to that membrane enzyme. Its affinity for ACHE may account for the relatively large concentration on the membrane, which contains less than 2 per cent of the cellular protein. It is entirely possible that DFP was bound to non-ACHE membrane sites in addition to non-ACHE intracellular sites. It is well known that DFP can react with other enzymes and probably with a variety of protein molecules. In view of its relatively non-polar structure, it is not surprising that DFP penetrates the red cell with ease.
When red cells were labeled either in vitro or in vivo with DF\textsuperscript{32}P, the label was attached primarily at intracellular, not membrane, sites. Thus, despite the fact that it binds to and inactivates the red cell membrane enzyme, acetylcholinesterase, DF\textsuperscript{32}P is not a specific label for the red cell membrane.

SUMMARIO IN INTERLINGUA
Quando erythrocytos esseva marcate in vitro o in vivo con DF\textsuperscript{32}P, le marca esseva attachate primarimente a sitos intracellular e non membranal. Assi, le facto que DF\textsuperscript{32}P liga e inactiva le erythrocytic enzyma membranal acetylcholinesterase non significa que illo es un marca specific pro le membrana erythrocytic.

ACKNOWLEDGMENT
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REFERENCES
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