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NOTES

Reduced Parasitemia Observed with Erythrocytes Containing Inositol Hexaphosphate

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Chemicals entrapped in erythrocytes by hypotonic hemolysis can be assessed for possible antiparasitic activity both in vivo and in vitro, regardless of whether they are able to diffuse into erythrocytes readily. Inositol hexaphosphate, a highly charged compound, produced a dramatic lowering of the percentage of cells infected by *Babesia microti* in vivo and both *B. microti* and *Plasmodium falciparum* in vitro. Several possible mechanisms for this observation are discussed.

To act directly on intracellular parasites during the intracellular phase of their life cycle, drugs must first diffuse or be transported into the cell. Many chemicals do not enter cells readily, for example, polar or charged compounds which cannot diffuse readily through hydrophobic membranes. Direct assay of a chemical for antiparasitic activity is therefore complicated because its chemotherapeutic effects cannot be readily differentiated from the properties that permit it to gain entry into the host cell. Probably many chemicals with antiparasitic properties exist which are unrecognized because they do not readily enter host cells. If their antiparasitic properties were known, chemicals with the necessary antiparasitic properties could, in some cases, be converted into effective drugs by introduction of chemical modifications to increase hydrophobicity or by synthesis of analogs of compounds whose entry is mediated by specific transport proteins. Or, if the mechanism by which a chemical acts were known, drugs which produce a similar effect on the target but are more readily transported could be sought.

For these reasons, it would be useful to develop a system for testing the intracellular antiparasitic properties of chemicals which are not able to enter cells readily. Chemicals or drugs normally unable to be transported across erythrocyte membranes can be entrapped in erythrocytes by hypotonic lysis, followed by subsequent resealing of the plasma membrane. Erythrocytes containing drugs loaded in this way might be useful as circulating drug carriers or for delivery of drugs to erythrophagocytic cells (reviewed by Ihler and Tsang [9]). Certain chemicals loaded into erythrocytes can produce substantial alterations in the properties of the erythrocytes themselves. Because inositol hexaphosphate (IHP) is a much stronger allosteric modifier of hemoglobin than 2,3-diphosphoglycerate (2), IHP-loaded erythrocytes release oxygen more readily than do normal cells and therefore have an oxygen dissociation curve which is shifted to higher P_{O_2} values (reviewed by Nicolau et al. [13]). Avian erythrocytes, but not mammalian erythrocytes, utilize inositol pentaphosphate as a modifier of hemoglobin affinity for oxygen, presumably as an adaptation to permit greater oxygen delivery to flight musculature. IHP-loaded erythrocytes are functional in mammals, as demonstrated by the survival of piglets after complete exchange transfusion, but significant and potentially useful and important physiological changes were found after infusion of IHP-containing erythrocytes, including a marked reduction in coronary blood flow in isolated rat heart (21) and increased arterial P_{O_2} and enhanced O_2 extraction with decreased cardiac output in piglets (22, 23).

Drugs entrapped in erythrocytes might not have direct access to erythrocyte parasites such as plasmodia or *Bartonella bacilliformis*, which enter by a process analogous to endocytosis and are, at least initially, found in intracellular vacuoles separated from the erythrocyte cytoplasm by a membrane (reviewed by Moulder in reference 11). However, the plasma membrane which initially encloses *Babesia microti* in a vacuole disintegrates after invasion and is lost (20), and so this organism was selected for the initial experiments since it is more likely to be susceptible to poorly transported chemicals introduced into the erythrocyte cytoplasm by hypotonic lysis.

Hypotonic lysis and resealing, if carried out with certain precautions, may have little or no adverse effect on the survival of the resealed cells in vivo. Although there is early loss of some cells after reinfusion, the loss is generally less than 20 to 40% and often is not much greater than that observed for washed, unlysed cells. It has been shown in humans (19) and animals (reviewed by Ihler [8]) that the cells which survive the first 24 to 48 h in a carefully resealed erythrocyte preparation can have a normal life span.

We developed a procedure for studying the effect of entrapped drugs on hemotrophic parasites, either in vivo or in vitro. The novel features of this procedure are introduction of drugs by hypotonic lysis and simultaneous entrapment of fluorescent compounds in the erythrocytes, which permits the drug-containing erythrocytes to be easily distinguished from the host erythrocytes, either while they are circulating (24) or on slides. This, in turn, permits the number of drug-containing erythrocytes infused to be a small

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TABLE 1. Infection by *B. microti* in vivo[a]

Erythrocytes infusion	Mean ± SEM (range) % of:	
	Total erythrocytes infected	Fluorescent erythrocytes infected
IHP–FITC-BSA	67 ± 9 (50–79)	10 ± 4 (6–19)
FITC-BSA	76 ± 8 (64–87)	60 ± 4 (39–79)

[a] A total of 10 mice were used for the IHP–FITC-BSA experiments, and 8 mice were used with cells containing only FITC-BSA.

fraction of the total erythrocytes, so that their presence should not perturb the course of the infection. Erythrocytes from BALB/c mice were loaded with a fluorescent molecule (24), either fluorescein isothiocyanate-bovine serum albumin (FITC-BSA) or calcein, by using a modification (7) of the preswell lysis procedure (17). Erythrocytes in heparin were washed three times with phosphate-buffered saline containing 1.4 mM glucose and 5 mM inosine and then hypotonically swollen without lysis by resuspension twice in 0.6× buffer after gentle centrifugation, and the packed cells were lysed by addition of 0.5 volume of 8 mg of FITC-BSA (or 1 mM calcein) per ml and, where desired, 5 mM IHP or 1.2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). After gentle mixing, the cells were incubated at room temperature for 5 min, resealed by addition of 1.54 M KCl (0.15 volume of the lysing solution), and then incubated for 30 min at 37°C, and washed several times. Generally a higher percentage of erythrocytes was fluorescent when IHP was omitted than when it was present; probably, this effect is due to the contribution of IHP to the osmolarity of the solution. When injected into uninfected mice, fluorescent resealed erythrocytes continued to circulate for several days. Twenty-four-hour survival was about 50% (43% for cells containing IHP and 61% for cells not containing IHP), which is similar to that previously reported for mice (4, 6, 7). An additional one-third to one-half of the infused erythrocytes were lost during the next 24 h, with the remaining cells surviving with a more extended half-life. Long-term survival was not routinely determined in these experiments, but after 3 days 18% of the fluorescent IHP-containing cells were still circulating, as were 33% of the fluorescent cells not containing IHP. It has been reported that in mice the half-life of resealed erythrocytes which survive the first day or two was 4 days for virus-containing erythrocytes loaded by either dialysis or the preswell dilution procedure (7) and 7 days when loaded by dialysis (4).

Mice which had been injected intraperitoneally 6 days previously with 10^6 *B. microti* and had about 60% parasitemia were injected in the tail veins or intraperitoneally with 0.2 to 0.3 ml of resealed erythrocytes (15% hematocrit) containing FITC-BSA (90 to 95% fluorescent cells) or FITC-BSA plus IHP (65 to 70% fluorescent cells). For the latter cells, IHP and FITC-BSA were entrapped simultaneously, permitting IHP-containing cells to be identified by fluorescence of the entrapped FITC-BSA. The percentage of infected cells overall was determined from Giemsa-stained smears. The percentage of fluorescent (FITC-BSA-containing) cells which were infected was determined by fluorescence microscopy. Slides were stained with ethidium bromide, a fluorescent DNA-intercalating agent, to render the parasites fluorescent (16), and so both parasites and erythrocytes could be directly visualized by fluorescence microscopy. Within 18 to 20 h, *B. microti* had invaded the fluorescent cells not containing IHP nearly to the same extent as it had invaded the host cells (Table 1). Fluorescent erythrocytes containing calcein or FITC-BSA plus EGTA were parasitized to approximately the same extent as FITC-BSA-containing cells, as were erythrocytes incubated with 5 mM IHP without entrapment before or after loading of FITC-BSA.

A much lower percentage of IHP-containing fluorescent cells than fluorescent control cells was parasitized. Only 10% of the fluorescent IHP-containing cells were parasitized, as compared with 67% of the host cells. When the resealed erythrocytes did not contain IHP, a mean of 60% of the fluorescent erythrocytes contained parasites as determined by staining with ethidium bromide; 76% of the host cells contained parasites. This result demonstrates that in vivo IHP-loaded fluorescent erythrocytes were only one-fifth as likely to contain *B. microti* as were similar cells not containing IHP. Since the number of IHP-loaded cells is only a small percentage of the total endogenous cells, reduced parasitemia in IHP-containing cells does not reduce the overall observed parasitemia, although if large numbers of IHP-containing cells were infused the overall parasitemia should presumably drop and possibly the mice could be saved from eventual death by a partial or complete exchange transfusion. We interpret the finding of reduced parasitemia in IHP-containing cells to indicate either that *B. microti* fails to enter IHP-containing cells effectively or, if *B. microti* does enter, that the parasite either degenerates so that its presence cannot be detected by staining or that parasite-containing erythrocytes are effectively removed from the circulation.

The same system using hypotonic loading of chemicals and fluorescent compounds developed for the in vivo experiments can also be used in vitro, where in principle it might be simpler to determine whether an antiparasitic drug affected entry or subsequent development. We were unsuccessful in setting up a continuous cultivation system for *B. microti* by using mouse cells because the mouse erythrocytes lysed after incubation (however, a continuous culture system has been reported for *B. bovis* [10]). We were able to achieve in vitro infection of human erythrocytes added to a mouse erythrocyte inoculum, although *B. microti* development was arrested in the human erythrocytes at an early stage and so the cultures were not self-sustaining. Although this disadvantage eliminated the possibility of determining whether IHP had an effect on *B. microti* development, the results suggest that IHP has an effect on the entry process and that IHP loaded in human erythrocytes could have an antiparasitic effect.

B. microti was cultured in vitro by a modification of the procedure of Palmer et al. (14), in 60% medium 199 with Earle modified salts (Irvine Scientific), 40% human plasma, 2 mM glutamine, 4 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, 10 mM inositol, and 0.03 mM glutathion, starting with 20 μl of packed erythrocytes per ml from mice with 50 to 60% infected erythrocytes which were mixed with 60 μl of packed, washed human erythrocytes or packed resealed fluorescent human cells, either containing or not containing IHP, per ml. Penicillin-streptomycin-Fungizone (GIBCO Laboratories) was added to the final packed-erythrocyte suspensions. Samples of 200 μl were pipetted into wells of a 96-well culture plate and incubated at 37°C in a candle jar. The cells were allowed to settle, and every 24 h 150 μl of supernatant was replaced with 150 μl of fresh medium without disturbing the cells. In three experiments using washed human erythrocytes added to the infected mouse erythrocytes, initially 17 to 25% of the erythrocytes

TABLE 2. Infection by *P. falciparum* in vitro[a]

Erythrocyte treatment	% Erythrocytes infected			% Fluorescent erythrocytes infected		
	I	N	H	I	N	H
IHP–FITC-BSA	0.66	0.53	2.87	0.00[b]	0.00	0.00
EGTA–FITC-BSA	1.88	1.36	—	0.55	0.60	—
FITC-BSA	1.56	1.54	3.81	0.95	0.80	0.56
Resealing	1.43	1.20	3.28	—	—	—
Washing	1.10	0.98	4.62	—	—	—

[a] Human erythrocytes, loaded with FITC-BSA with or without IHP or EGTA, were sent by Air Express to the Centers for Disease Control identified only with codes. In various experiments, three strains of *P. falciparum* were used: Indochina 1 (I), Nigeria 3-CDC (N), and Honduras 1/CDC (clone b3) (H). Initially, FITC-BSA erythrocytes contained 82% (I and N) or 70% (H) fluorescent cells, and IHP–FITC-BSA cells contained 65% (I and N) or 40% (H) fluorescent cells. Slides were examined at 24, 48, and 96 h of culture; data are given for the 96-h Indochina 1 and Nigeria (I and N) cultures and for the 48-h Honduras (H) cultures. The percentages of fluorescent cells in the 96-h FITC-BSA culture were 20% (I and N) and 3.7% in the 96-h IHP–FITC-BSA culture. The percentage of fluorescent cells in the 48-h (H) culture was 30% for both FITC-BSA and IHP–FITC-BSA cells. —, Not done.

[b] No *P. falciparum* organisms were seen in IHP-containing cells in any experiment, despite routine scanning of more than 10-fold more fluorescent erythrocytes (n = 20,000) than erythrocytes not containing IHP (n = 2,000).

TABLE 3. Inhibitory effect of external IHP

Erythrocyte treatment and concn (mM)	No. of cells[a] with:			
	Rings	Trophozoites	2 Nucleus schizonts	>2 Nucleus schizonts
None (control)	152	20	10	22
IHP				
5	0	2	1	2
0.5	0	2	0	1
0.05	128	16	10	28
0.005	166	19	10	30
myo-Inositol				
5	98	19	8	14
0.5	159	26	7	18
0.05	139	18	9	18
0.005	157	20	8	21

[a] Values are means for two duplicate cultures, except for the control, which had four duplicate cultures. Parasites were counted against 10,000 erythrocytes.

overall were infected, but this dropped rapidly to 3.9 to 7.6% in 24 h and to 1.2 to 2.2% at 48 h because of lysis of the mouse cells. On the basis of cell size, by 48 h most of the cells remaining appeared to be human erythrocytes.

In a series of three experiments, the percentages of cells infected with parasites at 48 h were 1.8 ± 0.3% (range, 1.35 to 2.15%) for cultures to which washed erythrocytes had been added, 2.2 ± 0.5% (range, 1.9 to 2.9%) for preparations of resealed erythrocytes with a mean of 54% fluorescent cells containing FITC-BSA, and 1.6 ± 0.1% (range, 1.5 to 2.3%) for resealed erythrocytes with a mean of 28% fluorescent cells containing FITC-BSA and IHP. For cells resealed in the absence of IHP, 1.3 ± 0.2% of the fluorescent cells contained parasites, and for cells resealed in the presence of IHP, 0.32 ± 0.15% of the fluorescent cells contained parasites. This indicates that in vitro IHP-containing resealed fluorescent cells were invaded to about one-third of the level of fluorescent cells resealed in the absence of IHP. The results observed were similar whether the erythrocytes contained FITC-BSA or calcein. These results are consistent with those obtained in vivo and suggest that one inhibitory action of IHP is to prevent entry of the parasite. Although infrequent, human infections by *B. microti* are known and there is unfortunately no good chemotherapeutic agent available. The demonstration that human cells containing IHP display reduced parasitemia suggests some directions for further research. It is possible that IHP-containing human erythrocytes will be available in the future as a new blood transfusion product (18).

It is possible to maintain continuous in vitro cultures of *Plasmodium falciparum* with human erythrocytes, and the effect of intracellular IHP on infection by *P. falciparum* in in vitro cultures was determined. The results (Table 2) indicated that IHP-loaded erythrocytes might be completely resistant to invasion by *P. falciparum*. In fact, no *P. falciparum* organisms were seen in IHP-containing erythrocytes in any experiment, despite extensive scanning of the erythrocytes.

Addition of IHP to the medium in concentrations of 5 or 0.5, but not 0.05, mM proved markedly inhibitory in this in vitro system (Table 3). *myo*-Inositol was not inhibitory at the same concentration. The inhibitory activity was assessed by the 48-h reinvasion test (12) using clone B3 of the Honduras 1/CDC strain. Cultures contained a 5% cell suspension with about 0.2% infected cells and were incubated in duplicate at 37°C in a candle jar for 48 h. The effect of external IHP is probably due to chelation of calcium in the external medium. Wasserman et al. (26) have shown that addition of 1 mM EGTA to the culture blocks the cell cycle by two mechanisms, one of which prevents reinvasion of fresh erythrocytes. Similar observations were made by Vial et al. (25). Thus, it is likely that external IHP, a very potent Ca^{2+} chelator, acts by a similar mechanism. The effect of entrapped IHP is unlikely to be due to lysis of some of the cells and release of the IHP, since that hypothesis cannot explain the results obtained in vivo. We had anticipated that calcium chelators would have an antiparasitic effect, which was one reason why IHP was included as a test chemical, but IHP may not act through chelation of Ca^{2+}, since entrapped EGTA or calcein had no effect on the entry of *B. microti*. Dluzewski et al. (5) have reported that EGTA introduced into resealed ghosts has no effect on invasion by *P. falciparum*. Entrapped IHP (or EGTA or calcein) had no inhibitory effect (data not shown) on the entry of another hemotrophic parasite, *Bartonella bacilliformis* (3).

IHP is a powerful allosteric effector of the oxygen affinity of hemoglobin, much more so than 2,3-diphosphoglycerate or ATP. The sensitivity of these parasites to oxygen is well known, as indicated, for example, by the requirement for diminished oxygen concentrations in vitro and the protection provided by erythrocytes containing hemoglobin (1, 15). It seems possible that the allosteric effects of IHP on hemoglobin is responsible for the antiparasitic effect of IHP.

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