oncology wards where good communication among staff is the norm, precise drug timing cannot be achieved. However, implantable and extracorporeal programmable drug delivery systems will make clinical "chronotherapy" both possible and economical. Devices now available time therapy with an external clock, but a third generation of "closed loop" devices will eventually time each dose to the individual patient's internal circadian clock.

The most crucial element in the successful application of cancer chemotherapy is optimization of the antineoplastic effects and minimization of secondary toxicity to normal tissues, that is, selectivity (*17*). One approach to increasing this selectivity is administration of these highly toxic drugs at times associated with their best tolerance.

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References and Notes

1. F. Halberg, *Am. J. Anat.* **168**, 545 (1983).
2. L. E. Scheving, in *Cell Cycle Clocks*, L. N. Edmunds, Ed. (Dekker, New York, 1984), pp. 455–499.
3. W. J. M. Hrushesky, *Am. J. Anat.* **168**, 519 (1983).
4. W. J. M. Hrushesky *et al.*, in *Chronobiology: Principles and Applications to Shifts in Schedules*, L. E. Scheving and F. Halberg, Eds. (Sijthoff & Noordhoff, Alphen aan der Rijn, Netherlands, 1980), pp. 513–533.
5. F. Levi, W. Hrushesky, E. Haus, F. Halberg, L. E. Scheving, B. J. Kennedy, in *Chronobiology: Principles and Applications to Shifts in Schedules*, L. E. Scheving and F. Halberg, Eds. (Sijthoff and Noordhoff, Alphen aan der Rijn, Netherlands, 1980), pp. 481–511.
6. E. Haus *et al.*, *Science* **177**, 80 (1972).
7. R. B. Sothern, F. Halberg, H. H. Zinneman, B. J. Kennedy, in *Chronopharmacology and Chronotherapeutics*, C. A. Walker, C. M. Winget, K. F. A. Soliman, Eds. (Florida A&M University Foundation, Tallahassee, 1981), pp. 269–273.
8. W. J. Hrushesky, F. A. Levi, F. Halberg, B. J. Kennedy, *Cancer Res.* **42**, 945 (1982).
9. F. A. Levi *et al.*, *ibid.*, p. 950.
10. F. Levi, W. Hrushesky, F. Halberg, E. Haus, T. Langevin, B. J. Kennedy, *Eur. J. Cancer Clin. Oncol.* **18**, 471 (1982).
11. F. Levi *et al.*, *Cancer Treat. Rep.* **66**, 1933 (1982).
12. W. J. M. Hrushesky, *Ann. N.Y. Acad. Sci.* **397**, 293 (1982).
13. ———, R. Borch, F. Levi, *Clin. Pharmacol. Ther.* **32**, 330 (1982).
14. W. J. M. Hrushesky, in *Platinum Coordination Complexes in Cancer Chemotherapy*, M. Hacker, E. B. Douple, I. H. Krakoff, Eds. (Nijhoff, The Hague, Netherlands, 1984), pp. 165–186.
15. W. J. M. Hrushesky, in *Toxicity of Chemotherapy*, M. Perry and J. Yarbro, Eds. (Grune & Stratton, New York, 1984), pp. 449–477.
16. V. DeVita, *Cancer Lett.* **10**, 1 (1984); W. Hryniuk and H. Bush, *J. Clin. Oncol.* **2**, 1281 (1984).
17. G. Elion, address at the meeting of the American Association of Cancer Research, Toronto, Canada, 9 May 1984.
18. I thank V. DeVita, S. Carter, and M. Slavik for bringing the work of F. Halberg to my attention; F. Halberg for guidance and advice; and B. J. Kennedy for constant support. Supported by the NCI grant R01 CA 31635 and the Clinical Research Center grant 5M-01RR00400.

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Evidence for a Malarial Parasite Interaction Site on the Major Transmembrane Protein of the Human Erythrocyte

Abstract. *Soluble oligosaccharides derived from the surface of human erythrocytes were tested for their ability to competitively inhibit invasion of erythrocytes by* Plasmodium falciparum, *a malarial parasite. Invasion was most effectively inhibited by erythroglycan, a carbohydrate component of the band 3 transmembrane protein. The lactosamine chains of erythroglycan contributed much of the inhibitory activity. This indication of a primary parasite interaction site on band 3 supports a role for this protein in mediating the radical alterations of the erythrocyte cytoskeleton that accompany invasion.*

The molecular interactions at the cell surface which determine and control the infection of a human erythrocyte by the falciparum malaria parasite are critical to the life cycle of the parasite and the course of disease (*1*). During invasion the parasite binds to and deforms the erythrocyte membrane and enters the cell by a process resembling endocytosis. In so doing, it subverts the elements of the erythrocyte membrane responsible for maintaining cell shape and integrity. Because the mechanism of this subversion is unknown, we undertook to identity the components of the erythrocyte membrane with which the parasite interacts. We report that the oligosaccharide portion of the band 3 protein, erythroglycan (*2, 3*), is a primary interaction site for the *Plasmodium falciparum* merozoite (*4*).

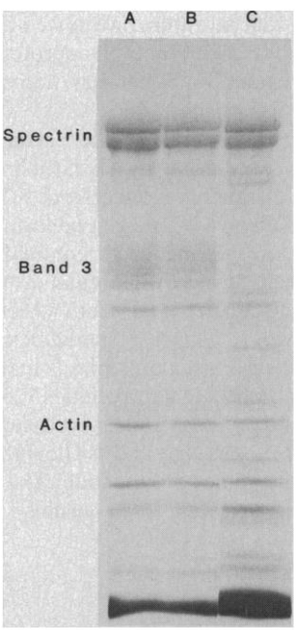

Fig. 1. (A to C) Effect of trypsin on erythrocyte membrane proteins. Washed human erythrocytes (10 percent by volume) were incubated for 60 minutes at 37°C with shaking in 25 m*M* Hepes, 135 m*M* NaCl, 5 m*M* $CaCl_2$ (*p*H 7.4), and tosylamide phenylethyl chloromethyl ketone–trypsin (Worthington). After two washes in 100 volumes of 25 m*M* Hepes and 135 m*M* NaCl (*p*H 7.4) (HBS), the cells were treated with 1 m*M* phenylmethylsulfonyl fluoride (Sigma) in HBS for 20 minutes at 22°C and washed two more times. Membranes from 10^8 cells were prepared by hypotonic lysis in 50 volumes of 10 m*M* tris-HCl (*p*H 7.8), washed two times in the same buffer, and analyzed by SDS-PAGE (*22*). The effects of trypsin at 0, 0.075, and 1 mg/ml are shown in (A), (B), and (C), respectively.

Ultrastructural (*5*) and biochemical studies of encounters between malarial parasite merozoites and erythrocytes reveal two distinct interactions: (i) initial attachment, mediated by erythrocyte surface sialic acid (*6*), and (ii) subsequent formation of a tight junction between the anterior end of the merozoite and the erythrocyte membrane. Freeze-fracture studies of the junction zone have shown that junction formation is associated with a closely packed assembly of intramembranous particles in the erythrocyte membrane. During invasion the junction zone changes from a cap to a constricting ring through which the merozoite enters an endocytotic vacuole of the host cell.

The cytoskeleton of the erythrocyte membrane is a network of structural proteins with unique dynamic interactions that allow the cell to first resist but then accommodate external forces (*7*). A spectrin-actin mesh underlies the lipid bilayer (*8*) and is in part responsible for preventing internalization of membrane by endocytosis. Thus antibody to spectrin within the cell can prevent drug-induced endocytosis by strengthening the spectrin network and preventing formation of the spectrin-free domain associated with endocytotic events (*9*). The spectrin-actin network is anchored to the membrane by the transmembrane proteins of the erythrocyte. Band 3, the major transmembrane protein, is exposed to the outside, crosses the bilayer several times, and is firmly bound to the cytoskeleton (*10*). Glycophorin A is normally attached weakly or not at all, but if the externally exposed domain of glycophorin A is constrained by antibody or lectin binding, this glycoprotein also becomes firmly bound to the skeleton (*11*).

It is reasonable to guess that the tight

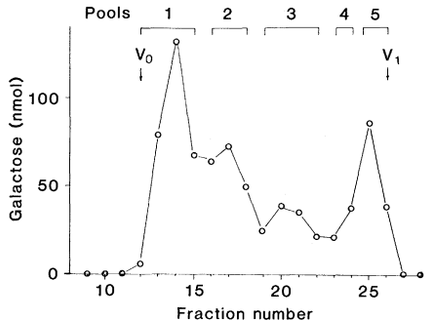

Fig. 2. Sephadex G-50 gel permeation chromatogram of glycopeptides from human erythrocyte membrane prepared by pronase digestion of intact cells (*13*). Fractions of 2.2 ml were collected from a 1.5 by 30 cm column. Sugars were determined by gas-liquid chromatography of the trimethylsilyl methylglycosides after methanolysis of a portion of each fraction. Only galactose content is indicated (*2*). Pool 1 contains the erythroglycan fraction (4,000 to 11,000 daltons); pool 2, glycopeptides of the *N*-linked complex type (3000 to 4000 daltons); pool 3, smaller *N*-linked complex glycopeptides of the biantennary and triantennary types (1500 to 3000 daltons); and pools 4 and 5, the *O*-linked, GalNac-containing molecules (500 to 1500 daltons). V_o and V_i are the exclusion and inclusion volumes, respectively. Oligosaccharide pools were dialyzed against distilled water and lyophilized.

junction complex formed by the malarial merozoite involves interactions with one of the erythrocyte transmembrane proteins and is responsible for creating a spectrin-free domain at the point of merozoite entry. This hypothesis is supported by the observation that the invaginated membrane domain is free of intramembranous particles (*5*) and that introduction of antibody to spectrin (but not the Fab portion thereof) into erythrocyte ghosts is sufficient to prevent parasitic invasion of the cells (*12*).

In our earlier study of invasion of the human red cell by *P. falciparum* (*6*), we found that, in addition to the sialic acid needed for attachment, invasion required an erythrocyte membrane component removed by treatment with trypsin at 1 mg/ml but not 0.075 mg/ml. In the present study sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of the membrane proteins of trypsin-treated human erythrocytes (Fig. 1) showed that trypsin at 1 mg/ml caused alterations in the proteins that were not found after treatment with the lower amount. Along with the appearance of several new bands, band 3 was virtually eliminated (*13*).

The correlation of band 3 hydrolysis with loss of infectivity and the association of band 3 with intramembranous particles (*14*) indicate a role for band 3 in junction formation, but there has been no evidence that merozoites interact with band 3 directly. It has been demonstrated that *N*-acetyl glucosamine (GlcNac), either alone or attached to albumin, can effectively inhibit *P. falciparum* invasion (*15*), suggesting that a carbohydrate structure on the erythrocyte is recognized by the parasite. To identify that structure, we tested all the erythrocyte membrane glycoprotein oligosaccharides for inhibitory activity in an invasion assay.

The glycoproteins of the erythrocyte surface carry an array of carbohydrate structures ranging in size from 1,200 to 12,000 daltons (*2*). The glycophorins carry primarily serine or threonine-linked short-chain structures containing *N*-acetyl galactosamine (GalNac) (<1500 daltons) with a single asparagine-linked complex structure containing a mannose core and five GlcNac residues (2500 to 4000 daltons) (*16*). Thus, glycophorin A contains 15 GalNac and 5 GlcNac residues per mole of polypeptide. The band 3 protein carries an unusually large (8,000- to 12,000-dalton) carbohydrate called erythroglycan (*2, 3*), which is a polylactosamine (galactose-GlcNac polymer) linked to a mannose core and containing 15 to 30 GlcNac residues per molecule (*17*). All these oligosaccharides can be prepared as glycopeptides by pronase digestion of the erythrocyte surface and separated by gel chromatography (Fig. 2) (*2*).

Table 1. Inhibition of invasion by the endo-β-galactosidase core of erythroglycan.

Concentration (μM)	Percentage of inhibition* (mean ± standard error; n = 3)
Erythroglycan	
50	83 ± 2
17	39 ± 2
5.7	21 ± 1
1.9	10 ± 5
Erythroglycan core 4† (4000 to 5000 daltons)	
150	55 ± 2
50	17 ± 3
17	4 ± 3
5.7	0
Erythroglycan core 1† (2500 to 3000 daltons)	
150	26 ± 4
50	17 ± 3
17	6 ± 4
5.7	2 ± 2
Heparin‡	
22	96 ± 2
7.4	80 ± 5
2.4	52 ± 7
0.8	14 ± 4

*Determined as described in the legend to Fig. 3. †Erythroglycan was digested with endo-β-galactosidase (1.25 U/ml) at 37°C for 48 hours and the core glycopeptides were isolated and fractionated into two peaks (cores 1 and 4) by Sephadex G-50 gel filtration (*19*). ‡Included as a positive inhibitor control (*1*).

Pool 1, containing the major fraction of erythroglycan, was the most effective inhibitor (Fig. 3). The concentration at which pool 1 inhibited invasion 50 percent was 12 μM, 40 times lower than any other pool and much lower than the major oligosaccharides of glycophorin (pools 4 and 5). The low inhibitory concentration of erythroglycan indicates its strong interaction with the merozoite surface.

Endo-β-galactosidase from *Escherichia freundii* specifically hydrolyzes the β-galactosidic bond of repeating lactosamine structures and therefore is a highly specific hydrolase for the erythroglycan component of band 3 (*2, 18*). When erythroglycan is digested with endo-β-galactosidase in solution, a core structure remains that retains a variable amount of lactosamine (*19*). Two size fractions of the core structure were tested for inhibitory activity (Table 1). Both

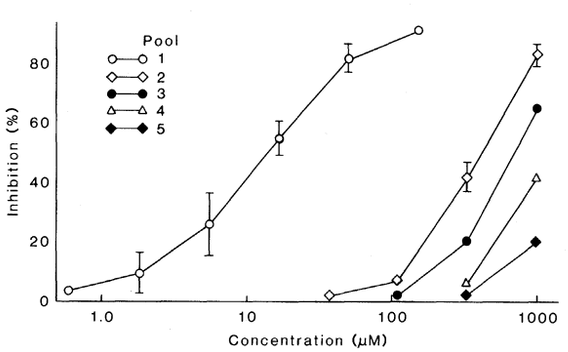

Fig. 3. Effect of erythrocyte glycoprotein oligosaccharides on invasion of human erythrocytes by *P. falciparum*. The rate of invasion was determined by overnight incubation of 2×10^7 erythrocytes containing approximately 1 percent late-stage parasites (*6*). Pooled fractions of glycoprotein oligosaccharides were dissolved in RPMI 1640 medium (Gibco) and added to cultures in duplicate to make a final volume of 0.1 ml. Molarity of oligosaccharides was calculated from the dry weight and average molecular weight was determined by compositional analysis. The extent of invasion was determined by [^{3}H]hypoxanthine incorporation during the 24 hours after invasion (*23*). None of the pools inhibited ^{3}H incorporation when added after invasion was complete. Inhibition was calculated as the percentage of decrease in incorporation compared to a control culture without additions. Each point represents the mean ± standard error for four cultures.

retained significant ability to competitively inhibit invasion. They had less binding affinity than the intact molecule, however, and the smaller structures were the least effective in inhibiting invasion. These observations suggest that the lactosamine chains of erythroglycan may be the primary interaction binding site, but that the core, after endo-β-galactosidase treatment, retains enough lactosamine structures to be active. Indeed, erythrocytes treated with endo-β-galactosidase are still susceptible to infection.

The ability of band 3 eythroglycan to strongly inhibit invasion implicates this structure as an important parasite-binding site and supports a role for band 3 in mediating the transmembrane modulation of the erythrocyte cytoskeleton during invasion. It has been observed that chymotrypsin cleaves band 3 between the erythroglycan attachment site and the cytoplasmic domain but has no effect on infectibility (*20*); however, neither of the cleaved fragments is released from the cell membrane and both are still strongly associated even after solubilization of the membrane with nonionic detergent (*21*). Thus chymotrypsin cleavage of band 3 would not interfere with communication between erythroglycan and the cytoplasmic domain.

In malaria the merozoite binds to the erythrocyte surface and disrupts the forces that maintain the shape of the cell and that ordinarily prevent the entry of foreign bodies. The lactosamine oligomers of band 3 erythroglycan appear to mediate part of this process. We showed earlier that α_1-acid glycoprotein blocks merozoite attachment as part of the natural protective response (*1*). Similarly, synthetic chemotherapeutic agents resembling polylactosamine may prove effective against this disease.

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References and Notes

1. M. J. Friedman, *Trends Biochem. Sci.* **7**, 332 (1982); *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5424 (1983).
2. J. Jarnefelt, J. Rush, Y.-T. Li, R. A. Laine, *J. Biol. Chem.* **253**, 8006 (1978).
3. M. Fukuda, M. N. Fukuda, S. J. Hakomori, *ibid.* **254**, 3700 (1979); C. G. Gahmberg, G. Myllyla, J. Leikola, A. Pirkola, S. Nordling, *ibid.* **251**, 6108 (1976); T. Krusius, J. Finne, H. Rauvala, *Eur. J. Biochem.* **92**, 289 (1978).
4. In a recent report L. H. Miller *et al.* [*J. Clin. Invest.* **72**, 1357 (1983)] implicated this same protein in *P. knowlesi* infection of rhesus erythrocytes.
5. L. H. Miller, M. Aikawa, J. G. Johnston, T. Shiroishi, *J. Exp. Med.* **149**, 172 (1979); M. Aikawa, L. H. Miller, J. R. Rabbege, N. Epstein, *J. Cell Biol.* **91**, 55 (1981).
6. M. J. Friedman, T. Blankenberg, G. Sensabaugh, T. S. Tenforde, *J. Cell Biol.* **98**, 1672 (1984).
7. N. Mohandas, J. A. Chasis, S. B. Shohet, *Semin. Hematol.* **20**, 225 (1983).
8. D. Branton, C. Cohen, J. Tyler, *Cell* **24**, 24 (1981); S. E. Lux, *Semin. Hematol.* **16**, 21 (1979).
9. B. Hardy and S. L. Shrier, *Biochem. Biophys. Res. Commun.* **81**, 1153 (1978).
10. M. Ramjeesingh, A. Gaarn, A. Rothstein, *Biochim. Biophys. Acta* **729**, 150 (1983); V. Bennett, *J. Cell. Biochem.* **18**, 49 (1982).
11. J. A. Chasis, N. Mohandas, S. B. Shohet, *J. Cell Biol.* **95**, 261a (1982).
12. J. A. Olson and A. Kilejian, *ibid.*, p. 757; A. R. Dluzewski, K. Rangachari, W. B. Gratzer, R. J. M. Wilson, *Br. J. Haematol.* **55**, 629 (1983).
13. Band 3 is usually regarded as not being affected by trypsin; however, the incubation conditions used here exceed those of previous studies.
14. J. Yu and D. Branton, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3891 (1976).
15. M. M. Weiss, J. D. Oppenheim, J. P. Vanderberg, *Exp. Parasitol.* **51**, 400 (1981); M. Jungery, G. Pasvol, C. I. Newbold, D. J. Weatherall, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1018 (1983).
16. D. B. Thomas and R. J. Winzler, *J. Biol. Chem.* **244**, 5943 (1969); H. Yoshima, H. Furthmayr, A. Kobata, *ibid.* **255**, 9713 (1980).
17. A similar carbohydrate structure is also found on band 4.5 [(*18*); T. J. Mueller *et al.*, *J. Biol. Chem.* **254**, 8103 (1979)], the glucose transport protein, but while there are about 2×10^5 band 4.5 molecules per cell [D. C. Sogin and P. C. Hinkle, *Biochemistry* **19**, 5417 (1980)], there are about 10^6 molecules of band 3. Erythrocyte glycosphingolipid also carries a polylactosamine molecule [A. Gardas, *Eur. J. Biochem.* **68**, 177 (1976)], but in even smaller numbers.
18. M. N. Fukuda, M. Fukuda, S. Hakomori, *J. Biol. Chem.* **254**, 5458 (1979).
19. M. Fukuda, A. Dell, J. E. Oates, M. N. Fukuda, *ibid.* **259**, 8260 (1984).
20. L. H. Miller *et al.*, *J. Exp. Med.* **146**, 277 (1977).
21. R. A. F. Reithmeier, *J. Biol. Chem.* **254**, 3054 (1979).
22. U. K. Laemmli, *Nature (London)* **227**, 680 (1970).
23. R. E. Desjardins, C. J. Canfield, J. D. Haynes, J. D. Chulay, *Antimicrob. Agents Chemother.* **16**, 710 (1979).

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Gene Transfer and Expression of Human Phenylalanine Hydroxylase

Abstract. *Phenylketonuria (PKU) is caused by a genetic deficiency of the enzyme phenylalanine hydroxylase (PAH). A full-length complementary DNA clone of human PAH was inserted into a eukaryotic expression vector and transferred into mouse NIH3T3 cells which do not normally express PAH. The transformed mouse cells expressed PAH messenger RNA, immunoreactive protein, and enzymatic activity that are characteristic of the normal human liver products, demonstrating that a single gene contains all of the necessary genetic information to code for functional PAH. These results support the use of the human PAH probe in prenatal diagnosis and detection of carriers, to provide new opportunities for the biochemical characterization of normal and mutant enzymes, and in the investigation of alternative genetic therapies for PKU.*

Phenylketonuria, the most common inborn error of amino acid metabolism, is caused by a deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH) (*1*). Most of the biochemical characterization of PAH has been done with enzyme purified from rat liver, which contains 20 to 30 times more PAH activity than the human liver. The purified rat enzyme has an apparent molecular size of 100 kilodaltons (kD) and consists of two subunits of approximately 50 kD each (*2, 3*). The human enzyme has been purified and also appears to be a dimer of 50 kD subunits (*4, 5*). There has been considerable controversy concerning the identity of the monomeric subunits. Several investigators have reported finding multiple forms of the enzyme which differ in size, charge, activity, and antigenicity, and two distinct bands representing the monomeric protein can be separated

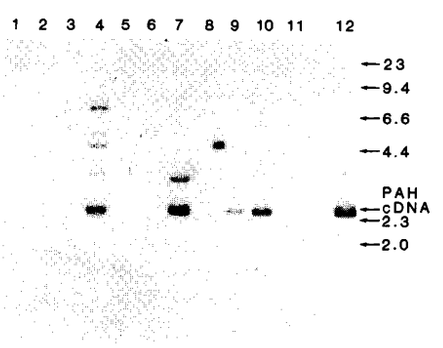

Fig. 1. Southern blot analysis of NIH3T3 cells transformed with MPAH(+) and pSV2NEO. (Lanes 1 to 10) DNA from transfected MPAH(+) and SV2NEO cell lines MPN1 to MPN10 (10 μg); (lane 11) DNA from cells transfected with SV2NEO alone (10 μg); (lane 12) MPAH(+) plasmid DNA (20 pg). DNA was digested with Eco RI, run on a 0.8 percent agarose gel, transferred to nitrocellulose paper, and hybridized with the purified Eco RI insert from phPAH247 (*8*). Filters were washed in 2× SSC (standard saline citrate) and 0.1 percent sodium dodecyl sulfate (SDS) for 1 hour at room temperature and 1 hour at 68°C, and then in 0.2× SSC and 0.1 percent SDS for 1 hour at 68°C.