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COMMENTARY

TRANSPORT PATHWAYS IN THE MALARIA-INFECTED ERYTHROCYTE

THEIR CHARACTERIZATION AND THEIR USE AS POTENTIAL TARGETS FOR CHEMOTHERAPY

HAGAI GINSBURG*

Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

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During the last decade, malaria research has been intensified at both the applied and the basic biological level. Although most of the resources have been devoted to vaccine development, some novel chemotherapeutic approaches have been entertained, based on a better understanding of the biology of the parasite and its interaction with the host. Since the pathogenicity of malaria is provoked mostly by the synchronous asexual development of the parasites inside the erythrocytes of the vertebrate host (schizogony), this stage of parasite development certainly deserves to be examined with a high priority in order to provide new rationales for therapeutic intervention. In this brief essay, I shall review recent developments in the understanding of transport in and out of the malaria-infected cell and, wherever possible, indicate the feasibility of using this knowledge for the design of new drugs.

Sequential and parallel pathways for the movement of solutes into and out of the malaria-infected red blood cell

During the schizogony of *Plasmodium falciparum*, the most lethal human parasite species and hence the one responsible for most cases of mortality, a single intraerythrocytic parasite would produce asexually 16–32 offspring within 48 hr. Although this is a relatively slow rate of multiplication compared with other unicellular organisms, it nevertheless requires a considerable traffic of solutes in and out of the parasite and its host cell. This movement happens in a host cell whose innate metabolic activity is rather slight, although some metabolic pathways, e.g. glycolysis, the hexose monophosphate shunt, or some parts of purine salvage pathways, can be considerably accelerated given proper triggering. In some cases, the native capacities of the host cell membrane probably suffice to meet the needs of the parasite, e.g. glucose transport (in human but not in murine red cells), and accelerated uptake is due to a larger metabolic sink. However, the permeability of the host cell membrane for other solutes, either substrates or waste products of host and/or parasite, may not be adequate for the malaria-infected cell. Such is the case, probably, for *myo*-inositol and for choline needed for phospholipid synthesis, for purine bases required for the synthesis of nucleic acids, and for some amino acids such as isoleucine, which is relatively scarce in hemoglobin (digested by the parasite to provide amino acids), or others that must be supplied from the outside to maintain parasite growth, at least in culture conditions [1]. The high rate of lactate production in IRBC† [2] surpasses the capacity of the native carboxylate and anion transport systems, and ways must be secured to prevent extensive acidification of both parasite and host cell. Notably, murine RBC are only poorly permeable to glucose, whereas their permeability to lactate is significantly higher compared with human RBC. Yet, mice and rats serve as hosts for various malaria species, suggesting that a universal strategy may be employed by the parasite to secure its physiological access to the extracellular world.

The parasite also links with the extracellular environment through proteins that it synthesizes, which could account for the phenomena of cytoadherence, erythrophagocytosis and immune suppression, and through endocytosed proteins.

Morphologically, the parasite is seen surrounded concentrically by the HCM, the PVM and its own cell PPM. Various stratagems have been evolved by the parasite in order to secure an adequate traffic of solutes through these barriers:

(a) The permeability of the HCM is vastly increased to almost all small solutes investigated thus far, including anions, cations, carbohydrates, amino acids and nucleosides, although to a different extent in each case.

(b) Non-selective and relatively large pores, resembling those found in the outer membrane of mitochondria, have been identified in the PVM.

(c) The parasitophorous space has been shown to

* Correspondence. Tel. 972-2-585539; FAX 972-2-666804.

† Abbreviations: IRBC, infected red blood cells; HCM, host cell membrane; PVM, parasitophorous vacuole membrane; and PPM, parasite plasma membrane.

be connected directly to the extracellular milieu by means of a relatively large duct, which allows even proteins, let alone smaller solutes, contact with the parasite membrane, and to be endocytosed subsequently into the parasite. The same pathway could be used for the export of similar solutes.

(d) The PPM is highly permeable to calcium, lactate, glucose and purine bases and nucleosides, and is equipped with various specific carrier-mediated transport systems. An active proton extruder has also been suggested, and a Na^+-K^+-ATPase must also be invoked in order to account for the ability of the parasite to control a high K^+:Na^+ ratio in its cytosol.

(e) The transmembrane movement of phospholipids, which is usually very limited in the membrane of normal RBC, is increased significantly in IRBC, thus, in addition to the innate high permeability to fatty acids, permitting the extensive biosynthesis of parasite membranes.

(f) Extensive and diverse mechanisms have been identified to export various proteins from the parasite through the two concentric membranes that surround it and the host cell cytosol, to be either associated with the host cell membrane or transported through it.

(g) Finally, the parasite demonstrably endocytoses the cytosol of its host cell into its acidic food vacuole, where the cytosol is digested. In this way the parasite secures the provision of most amino acids and iron, although the contribution of other host cell solutes has not been investigated. The digestion of host cell hemoglobin also results in the production of reactive oxidative species, e.g. superoxide, hydrogen peroxide and OH radicals [3]. A partial protection against these oxidants is provided by ingested host cell superoxide dismutase, which is claimed to be partially resistant to proteolysis. However, it seems that this oxidative stress may be instrumental for the biochemical and structural modifications that the parasite must induce in the host cell for its own physiological advantage [4].

Mechanism (e) has been addressed by several authors in recent years [5–11], and mechanisms (f) and (g) have been extensively reviewed recently [12–16] and will not be discussed here. In what follows, pathways (a) to (d) will be discussed briefly in concert with their possible use as targets for chemotherapy.

Increased permeability of the host cell membrane to polar solutes

Although the membrane of normal erythrocytes contains many, diverse transport agencies [17], the intense metabolism of the parasite may not be satisfied by their capacity to transport the relevant solutes. For example, whereas the glucose carrier of human red cells has a high transport capacity (100-fold higher than the glucose consumption of the normal cell), the ability of the monocarboxylate carrier may not suffice to discharge all the lactate that is produced by the glycolytic activity of the parasite. The situation in murine red cells is diametrically opposed: low glucose and high lactate transport. Another example is that the transport of amino acids in the uninfected red cell is mediated by a family of carriers of limited capacity. Yet the parasite produces enormous amounts of amino acids during the digestion of the host cell cytosol [18]: 40% of the host cell cytosol (essentially 8 mmol of hemoglobin monomers/L) is digested during about 10 hr of the trophozoite stage. If all the globin is hydrolysed to its constituent amino acids, this will result in the production of 114.4 mmol of amino acids/L cells/hr. If the egress of these acids is not sufficiently rapid, they should achieve high osmotic concentrations that would draw water into the parasite and/or the host cell with ensuing osmotic lysis of these compartments. That this does not actually occur indicates that the transport of amino acids through the various membranes, including that of the food vacuole, must match the rate of host cell cytosol digestion. Hence, understanding the details of membrane transport in the infected cell is of major importance for the perception of the adaptation of the parasite to its erythrocytic habitat.

The most informative way of studying transport across membranes is the use of radiotracers, since this permits the full elucidation of the various kinetic parameters and of the biophysical nature (carrier, pore, simple diffusion) of the system studied [19]. However, the structural complexity of alternative transport pathways in IRBC may pose formidable theoretical and experimental difficulties in using radiotracers: (1) If the permeability of the HCM is similar to that of both the PVM and the PPM, the use of radiotracers may be worthless for the study of the specific properties of either membrane. (2) Another complication may stem from the functional presence of the parasitophorous duct (see below): labelled solutes could gain direct access into the parasite membrane, and the time-dependent measurement of uptake may reflect solute transport by both the trans-erythrocytic path and the duct bypass. This difficulty could have been solved by measuring the volume of distribution of the transported solute as a function of time: a solute transported exclusively through the duct could distribute only into the parasite compartment, whereas one that translocates across the host cell membrane and then into the parasite should distribute into the whole infected cell. But if non-specific channels exist in the PPM (see below), a solute may enter exclusively through the parasitophorous duct, but distribute in both the host cell and the parasite compartments. These aspects are often overlooked or are experimentally impractical due to metabolization (metabolic trapping, usually phosphorylation) of the substrate.

In spite of these difficulties, for many solutes, notably non-electrolytes, zwitterions and anions, an alternative methodology is available. When RBC are suspended in an isosmotic solution of a permeating solute (the ammonium salt in the case of anions), they lyse because of solute entry and the ensuing osmotic swelling exceeding the lytic volume. Such lysis should occur only if the relative permeability of intracellular osmotically active solutes, usually cations, is low. The half-time ($t_{\frac{1}{2}}$) of cell rupture is inversely proportional to the permeability coefficient of the solute [20]. The unique advantage of the osmotic lysis technique is

that it monitors exclusively the permeability of the host cell membrane, irrespective of whether a parasitophorous duct (see below) exists.

Characterization of parasite-induced pathways using the isosmotic lysis technique

Using the isosmotic lysis technique, it has been shown that the host cell membrane is highly permeable to pyrimidine bases, most amino acids and to relatively small carbohydrates such as pentitols, pentoses, hexitols, hexoses (including D-glucose), but not di- or trisaccharides [21–23]. Such lack of solute specificity and a relatively low enthalpy of activation of transport (10–11 kcal/mol) suggest that the parasite induces a non-specific leak pathway in its host cell membrane, or that it inserts in this membrane an aqueous channel of a relatively large diameter. This explanation is consistent with the total refractoriness of the pathway to specific inhibitors of the native RBC hexose transport system, and, on the other hand, its blocking by non-specific inhibitors such as phloretin [24], analogues of cinnamic acid [25], or various non-specific inhibitors of ion channels [23, 26].

All the available data on the lysis induced by carbohydrates and amino acids were subjected to a rigorous biophysical analysis [27]. The body of data neither fit the model of an aqueous pore, nor did it seem to fit a model of "non-Stokesian diffusion" [28]. However, the relative permeability was found to be correlated with the number of hydrogen bonds that the solutes can potentially form with water molecules. Hence, it has been suggested that the increased permeability could result from structural defects initiated in the host cell membrane due to the insertion and/or translocation of polypeptides produced by the intracellular parasite. Reconstitution of proteoliposomes from non-matching phospholipids and proteins yields leaky membranes [29, 30], as could also happen if parasite proteins do not fit the lipid composition of the host cell membrane. Interestingly, a mismatch of protein and lipid can also result in an increased transbilayer movement of phospholipids [29], a phenomenon also observed in the membrane of malaria-infected red cells [31]. It should be noted that a similar analysis has led to the conclusion that separate channels for carbohydrates and amino acids may be present [17]. However, closer inspection of the raw data used for this analysis shows some disparity with the data used in the previous analysis, probably because they were obtained by a different experimental protocol.

The hydrophobic interaction between parasite proteins and host membrane lipids is supposedly weak, and the phospholipid head-group region, having a low dielectric constant, provides the selectivity filter, which determines the membrane selectivity by determining solute partitioning in the membrane [19]. The solubility of solutes in apolar solvents decreases with the polarity of the solute. That the partitioning of the tested solutes in a solvent that mimics the polarity of the polar head-group region and the relative permeability of the same solutes both depend (inversely) to the same extent on the number of hydrogen bonds that the solute can form with water is expected from the mechanism of permeation. This mode of permeabilization would be compatible with the relative low specificity of the new pathways (no discrimination between optical enantiomers [21, 23]), with the lack of saturability [22, 23, 32] and with the insensitivity to chemical modifiers of proteins [33]. However, the insertion of specific, parasite-derived transport agencies [17, 34] and the activation of silent but innate volume-sensitive anion channels that mediate the passage of *all* solutes [23] have also been suggested.

The new permeability pattern observed in infected cells offers interesting prospects for the chemotherapy of malaria: drugs or pro-drugs could be designed to fit the permselectivity properties of the host cell membrane. Thus, although they have a cytotoxic effect, such compounds would not be able to penetrate into the cells of the host and would hit only infected erythrocytes. Such inhibitors could be directed against physiological processes in the host cell that ensure the integrity of the parasite's intraerythrocytic haven, thus reducing the probability of evolution of drug resistance.

Alterations of constitutive transport systems in the host cell membrane

Activation of constitutive transporters could result from a K_m effect (lower K_m, i.e. increased affinity to the substrate) and/or a V_{max} effect (recruitment of silent carriers, change in turnover rate). In either case, one should not expect *a priori* the altered transporter to preserve its susceptibility to specific inhibitors. The transport of tryptophan [35], glutamine and glutamate [36], nucleosides [37–39] and glucose [40, 41] was found to be accelerated considerably in malaria-infected RBC following the maturation of the parasite. Interestingly, no acceleration was found with *p*-aminobenzoic acid (*p*ABA), which serves as a substrate for *de novo* folate synthesis in the parasite [42]. However, most of the innate transport of *p*ABA in erythrocytes occurs by rapid simple diffusion (and is not expected to be influenced greatly by structural defects) and is high enough to supply the needs of the parasite.

In all these experiments, performed with radiolabelled substrates, two components of substrate uptake have been observed, one saturable and one non-saturable, and it was concluded that the constitutive host cell transport systems have been activated and that a parallel permeability pathway has been induced by the parasite in the host cell membrane. However, since the time-dependence of volume distribution has not been determined in these experiments, it is impossible to conclude whether the parallel transport pathway was present in the host cell membrane, or whether the non-saturable transport took place through the parasitophorous duct. A deeper insight into the data on 2-deoxyglucose transport in *P. yoelii*-infected mouse erythrocytes [40] indicates that the rate of translocation of the free carrier in the latter cells is 18.8-fold faster than in uninfected RBC. According to the kinetic analysis of the simple carrier [19], the translocation of the unloaded carrier is the rate-limiting step in most carrier-mediated transport systems, and its increase results in a rise in both V_{max} and K_m.

The transport of nucleosides is also amplified in malaria-infected cells [39]. The constitutive RBC system is rather non-specific and mediates the uptake of both purine and pyrimidine nucleosides. While the parasite relies on its own synthetic capability for pyrimidines, it depends on the host cell for the supply of purines [43]. One of the major roles of RBC is the detoxification of adenosine released by some tissues into the blood stream. This is done by rapid uptake of adenosine and its metabolization to hypoxanthine by the purine salvage pathway. The normal serum levels of adenosine ($<1\ \mu M$) and hypoxanthine ($\approx 25\ \mu M$) reflect the steady state of these speedy processes. In IRBC, the rate of adenosine transport is increased further, and only part of it can be reduced by specific inhibitors of the native system [38, 39]. Unfortunately, whether the increased uptake is saturable (a K_m effect) has not been tested. An increase in the number of transporters has been demonstrated in *P. falciparum*-infected human RBC (a V_{max} effect?) but not in *P. yoelii*-infected mouse RBC. Since the permeability to tubericidine, a cytotoxic purine nucleoside analogue, is also increased in infected cells, it has been suggested that it be used in combination with nitrobenzylthio-purine analogues to treat malaria [44]. The rationale for this combination is that the analogues will inhibit the influx of tubericidine into non-infected RBC and other host cells, thereby protecting them from the toxic effect of the drug, while its entry into infected cells will proceed unhampered, with consequential parasite death. Although this rationale works perfectly well *in vitro*, it only reduces parasitemia but does not provide a radical cure in *P. berghei*- or *P. yoelii*-infected mice [45]. It is rather unlikely that this tactic could be extended to therapeutic application in view of the role of RBC in the detoxification of adenosine.

Inhibition of the new permeability pathways

Classical inhibitors of the native systems had no effect on the non-saturable component of uptake and a reduced efficacy in blocking the saturable component. Other compounds of a very different chemical nature, such as the bioflavonoids phloretin and phlorizin [46], cinnamic acid derivatives, and pyridoxalhydrazone [25], affect mostly the non-saturable component. It has been reported recently that the cystic fibrosis transmembrane conductance regulator (CFTR) inhibitors glibenclamide and meglitinide (but not the analogue tolbutamide) block the new permeability pathways to sorbitol, threonine, choline and Cl^- [26]. Since the pathway is also inhibited by other CFTR inhibitors such as niflumate, furosemide and 5-nitro-2-(3-phenylpropylamin) benzoic acid (NPPB) [23], it has been suggested that the new permeability pathway shares common properties with Cl^- channels in other cell types. However, none of the inhibitors used is absolutely specific in that it exclusively affects channels, and other techniques are needed to identify the new pathways as genuine channels. The case of phloretin is most instructive in this respect (see Ref. 47 for a detailed analysis). Phloretin has been shown to inhibit the increased leakiness of red cells due to membrane barrier defects induced by oxidative stress. Although oxidative damage mostly affects the membrane proteins, the resulting defects are thought to be localized in the lipid domain. Phloretin is a dipolar molecule, like many of the other compounds shown to inhibit the new pathways, and its transport-inhibiting effect has been related to its ability to perturb and dehydrate the head group region of the membrane phospholipids by replacing, and thus displacing, the dipolar water molecules. The increased hydrophobicity of the polar head group region is expected to decrease the solubility of polar solutes in this region, which has been suggested above to be the selectivity filter of the parasite-induced permeability pathways. Hence, inhibition of the new pathways by this compound indirectly confirms that membrane structural defects may underlie the permeabilization of the host cell membrane.

Increase of cation permeability

Malaria-infected cells are also more permeable to potassium (probed by ^{86}Rb) [48, 49]. As mentioned above, the use of radiotracers does not disclose the rate-limiting barrier for solute transport, and no attempt has been made in these investigations to assess the volume of distribution of ^{86}Rb. Hence, the inhibition of this pathway by piperine and quinine does not allow one to distinguish the affected membrane and/or target. The increased leak was inhibited partially by the Na:K:Cl cotransporter inhibitor bumetanide. The Na:K:Cl system in itself is more active in infected cells, but the reasons for this activation have not been investigated. The K^+ leak is not due to Ca^{2+}-dependent activation of the K^+ channel [50], although the Ca^{2+} content of the RBC is increased substantially upon parasitization [51]. The inhibition of the K^+ leak by purported inhibitors of anion channels has been suggested as evidence that a non-specific channel that appears in the membrane of the infected cell also mediates cation transport [23]. However, other mechanisms can be suggested. For example, heme has been shown previously to increase the K^+ leak in normal erythrocytes [52, 53] by an unresolved mechanism, and to increase the peroxidation of membrane lipids with consequent effects on ion pumps [54]. It has been demonstrated recently that membranes of infected cells contain high levels of heme [55] that could originate from host cell hemoglobin digestion or from hemichromes produced in the host cell due to oxidative stress. It is not known yet whether membrane heme is confined to the host cell membrane or if it is also associated with the parasite membrane(s). In the latter case, it may be interesting to investigate the cation homeostasis of the parasite and the means by which it overcomes the heme-induced K^+ leak. In the host cell membrane, the increased K^+ leak, in combination with the inhibition of the Na^+-K^+-ATPase [56], accounts for the large decrease in K^+ and the increase in Na^+ levels in the host cell compartment [57, 58]. It remains to be shown how the host cell retains its integrity faced with such disruption of the double-Donnan system [59].

The Ca^{2+} content of normal RBC is very low and probably insufficient for parasite growth. Upon

invasion of the RBC by the merozoite, there is a transient rise in Ca^{2+} influx, but the excess calcium is cleared within the next 1–2 hr [60]. As the parasite matures, infected cells become more permeable to Ca^{2+}, and their Ca^{2+} increases [51, 61] may be due, in part, to the demonstrable inhibition of the Ca^{2+} pump by heme [62]. Here again, the native transport of RBC is saturable, while that of the infected cell is not [51]. No inhibitors have been identified thus far that could specifically inhibit the K^+, Na^+ or Ca^{2+} pathways induced by the parasite.

Choline is an indispensable substrate for parasite phospholipid synthesis [63]. Normal RBC have a constitutive choline carrier, and infected cells display enhanced choline uptake. According to one report on *P. knowlesi*-infected simian erythrocytes, it is saturable, displaying similar K_m and 10-fold larger V_{max} compared with uninfected RBC (possibly due to accelerated translocation of the unloaded carrier), and susceptibility to specific inhibitors [64]. In *P. falciparum*-infected human RBC, no saturation was observed, and sensitivity to specific inhibitors has not been tested [49]. The choline analogues decyltrimethylammonium, decamethonium and hemacholinium 3, which are effective inhibitors of choline transport, also efficiently inhibited the growth of *P. falciparum* in culture and the incorporation of choline into *P. knowlesi* phosphatidylcholine [65], suggesting that choline transport could serve as a target for novel antimalarials, but since these inhibitors adversely affect cholinergic synapses, other less detrimental analogues should be found. Overall, it seems from the above deliberations that when the permeability of the infected cell is increased only slightly to a certain solute, it may be the result of alteration of the constitutive transporter, which is still amenable to inhibition by its specific inhibitors.

Modulation of anion transport in IRBC

The capacity of RBC to transport anions is inherently very high [66]. Nevertheless, the presence of a malaria parasite within these cells alters anion transport considerably [67–69]. Monitoring continuously the efflux of the fluorescent anion analogue NBD-taurine from infected cells, we have demonstrated [69] that the rate of efflux increases with parasite maturation and that, in parallel, the susceptibility of transport to 4,4′-dinitro-2,2′-stilbene disulfonic acid (DNDS) decreases to nil in cells harboring mature stages of parasite development. It could not be ascertained whether the native system is modified or if new permeability pathways are induced by the parasite in the host cell membrane. The number of native transport agencies is not altered due to parasite activity, and the integrity of band 3, the RBC membrane polypeptide mediating anion transport, is preserved throughout parasite development [67]. The pitfall in using tracers was observed in a later series of experiments [33], where influx was found to be several orders of magnitude faster than efflux. Logically, the two experimental protocols were monitoring different rate-limiting events, efflux revealing the transport across the parasite membrane and influx reflecting the permeability of the host cell membrane, both being insensitive to classical inhibitors of anion transport. Nevertheless, for yet unknown reasons, the specific anion inhibitors suppressed parasite growth and propagation with high correlation to their potency as inhibitors of the native anion transport of RBCs [29].

The possibility that inhibition was exerted on lactate efflux (lactate being the major end-product of parasite and host cell glucose oxidation) can be refuted. Lactate transport was shown to be 600-fold higher in infected cells despite the presence of the native transporter inhibitor DNDS and only partially sensitive to specific inhibitors (cinnamic acid derivatives) of the native lactate transporter [25, 70, 71]. Although the various derivatives of cinnamic acid were found to inhibit effectively parasite growth in culture [25], they probably do so by blocking the parasite-induced pathways in the HCM. It is rather unlikely that these compounds could serve as antimalarials because of their effect on the lactate transport across the plasma and the mitochondrial membranes of various host cells [72].

Possible reasons for transport-related alterations of the host cell membrane

Alterations of the native erythrocyte membrane transporters and/or the induction of new (leak?) pathways could stem from various processes occurring in the parasitized cell: (a) Oxidative stress demonstrably alters the permselectivity of RBC [73] and increases the flip-flop of phospholipids. Oxidative stress is known to be exerted by the parasite on its host cell [74], and increased flip-flop has been observed in the membrane of parasitized red cells [31]. However, the permselectivity pattern of mildly oxidized erythrocytes is essentially different from that found in infected cells [27], and blocking of the endogenous oxidative stress in infected cells for 2 hr does not affect their sensitivity to sorbitol-induced lysis. (b) The host cell membrane is depleted of cholesterol [75] with consequent fluidization [76–78]. These alterations could modify either the basal permeability through an overall fluidization, or affect the carrier-mediated processes that may require cholesterol specifically or that are influenced by membrane fluidity [79]. (c) The parasite produces and exports polypeptides into and through the host cell membrane (see Refs. 12–16 for reviews). These polypeptides could alter the kinetic properties of native transporters, or introduce structural defects into the host membrane. (d) Conceivably, some of the polypeptides inserted by the parasite could act as specific transport systems [23, 24]. Isolation of the host cell membrane, its fractionation into lipid and protein components, and reconstitution of the various components could resolve these different alternatives. (e) Heme derived from the digestion of host cell hemoglobin or from hemichromes produced in the host cell cytosol by the parasite-induced oxidative stress could alter the host cell membrane permeability either through phospholipid peroxidation [54], or through its disordering effect on the lipid bilayer structure [80].

Possible pharmacological implications of the parasite-induced transport pathways

Undeniably, the permselectivity properties of the HCM are distinctly different from those on uninfected RBC or any other type of mammalian cell. Only in the cases of nucleoside and choline transport have these alterations been shown to provide targets for new antimalarial chemotherapy, though even in these cases *in vivo* tests are far from being conclusive. However, the unique properties of the new pathways could provide for the targeting of tailor-made pharmacological agents specifically into IRBC. Such agents could be aimed against targets either in the host cell or in the parasite, the former being preferable because they would not provide for the evolution of drug resistance through selection. A straightforward approach would be to glycosylate a toxic agent, thus rendering it less permeable into host cells [81]. The glycosylated compounds should be able to penetrate through the new permeability pathways, and act either directly or after hydrolysis by red cell and/or parasite glucosidase(s).

This tactic for drug targeting has been demonstrated experimentally. Thus, phloridzin and its analogues penetrate into IRBC through the new pathways and inhibit parasite growth both by blocking these same pathways and by affecting parasite targets [82]. Since phloridzin is a glycosuric drug due to its inhibition of Na^+-glucose cotransport in epithelial cells, its use as a potential antimalarial may be limited. However, phloridzin analogues that are more potent antimalarials and of lesser glycosuric potency have been identified [46], but this avenue has not been pursued further. The zinc chelator dipicolinic acid does not enter into uninfected RBC, but readily permeates into IRBC and inhibits parasite growth [83]. Sometimes no direct effect of a drug can be observed on the parasite, yet the treated infected cell is eliminated. The pyridine glucosides of fava beans, vicine and convicine, penetrate into IRBC where they are hydrolyzed by host cell and parasite β-glucosidase to yield the highly oxidative aglycones divicine and isouramil. Whereas parasite growth is not inhibited by the glucosides, probably because the antioxidant defense capacity of the parasite suffices to counteract the deleterious effect of the aglycones, the phagocytosis of the IRBC is enhanced considerably, plausibly by the oxidative alteration of the host cell membrane (Ginsburg *et al.*, unpublished observations). These three examples clearly demonstrate the feasibility of targeting drugs or pro-drugs, and could be extended by a strategic choice of more specific and/or active and potentially chemotherapeutic agents.

The parasitophorous duct

Exposure of IRBC to fluorescently labelled phospholipids results in a marked pattern of labelling, involving vesicular and tubular structures in the host cell cytosol and the parasite membranes. A direct tubular connection between host cell surface and the parasitophorous space has been demonstrated recently [84], which admits also proteinaceous macromolecules. Fluoresceinated dextrans, rhodaminated protein A and fluorescently labelled latex beads (30 nm) were shown by confocal microscopy to fill a continuous tubular structure bridging the host cell membrane and the parasitophorous space. Rhodamine-dextran was eventually endocytosed by the parasite in an energy-dependent process, e.g. requiring physiological temperature and adequate ATP levels. Pre-accumulated label was similarly released from infected cells, implying an exocytotic process. These results suggest a direct contact of the parasite membrane to the extracellular milieu by means of a parasitophorous duct. However, macromolecular uptake could not be demonstrated by similar techniques in other investigations [7, 8], and it has been contended recently that the manifestation of the duct may be an experimental artifact [85]. But, on the other hand, physiological and pharmacological evidence is accumulating in favor of the parasitophorous duct, in which direct access of extracellular solutes to the parasite membrane is implied [86].

The endocytosis of proteins that penetrate through the duct proceeds along a pathway that is distinctly different from that which operates in the translocation of host cell cytosol to the food vacuole of the parasite. While the latter involves the formation of endocytic vesicles, which are surrounded by both the parasitophorous and the parasite membranes [87], the first should involve only the parasite cell membrane. The fact that labelled proteins ingested via the duct are not processed further implies that two distinct endocytotic pathways may exist in the parasite. The lack of processing raises the question as to how the ingested compounds can be utilized by the parasite. Obviously, additional work is warranted to establish the physiological role of the duct. The possibility that it can be used as a new path for the targeting of antimalarial drugs directly to the PPM is very attractive, because this barrier has already been shown to contain several transport agencies that are important for the cellular homeostasis of the parasite and its interaction with the host cell (see below), and more may be found in the future.

Permeability of the parasitophorous membrane

The parasitophorous membrane is the next barrier that must be hurdled by solutes that ingress across the host cell membrane. Since it is tightly juxtaposed to the parasite cell membrane, it is hard (and presently impossible) to isolate, and investigating its permeability properties poses a formidable task. Nevertheless, using the cell attached patch-clamp technique, it has been shown recently to hold relatively wide channels [88]. The channel has a high conductivity (140 pS); admits equally well cations and anions, including lysine and glucuronate; is present at high density; and is open 98% of the time. It seems to represent the major transport thoroughfare between the host cell cytosol and the parasite membrane, and is reminiscent of the porin channels of Gram-negative and mitochondrial outer membranes. These cells and organelles also possess two juxtaposed concentric membranes. With these channels, the PVM can be viewed as a sieve that prevents the loss of enzymes and metabolites that are indispensable for host cell function, yet secures

the translocation of solutes that must enter the parasite or exit from it. However, unlike the outer membranes of mitochondria and Gram-negative bacteria, which are permanent structures, the PVM is subjected to a considerable turnover because of its involvement in the endocytosis of host cell cytosol [87]. During the trophozoite stage, it can be calculated that the PVM turns over 3–5 times in 8–10 hr. It is not yet established whether the PVM is reformed by membrane recycling or by *de novo* synthesis.

Inability of the parasitophorous duct and the PVM channels to coexist

While the presence of the PVM channels is physiologically sensible, they cannot coexist with the parasitophorous duct, at least not by experimental criteria. Several examples will be considered. The high permeability of the channels to Na^+ and the connection of the parasitophorous space to the extracellular medium should have resulted in an eventual osmotic lysis of infected cells, because the contribution of the Na^+ gradient that is essential to the double-Donnan system, which preserves the osmotic integrity of the host cell [59], should be obliterated. Following similar biophysical considerations, infected cells should not lyse when suspended in isosmotic medium as they do, because such lysis depends on the impermeability of the host cell compartment membrane (of which the PVM can be viewed as an integral part) to the major cations that constitute the osmotically active ingredients of the host cell cytosol [21]. The PVM channels are permeable to Ca^{2+}, and hence the host cell cytosol Ca^{2+} concentration should equilibrate with that of the extracellular medium. Yet, the host cell cytosol free Ca^{2+} in *P. falciparum*-infected RBC is 1–2 μM [51] or less [89], while that of the culture medium is 400 μM. The PVM channel is also permeable to Na^+ and K^+, and the levels of these cations in the host cell cytosol should be equal to that of the bathing medium if the duct exists. This is indeed observed at the most advanced stages of parasite development [58] but not at the trophozoite stage [57] when the duct is perceived. Decrease in K^+ and increase in Na^+ could be fully explained by the increase in the leakiness of the host cell membrane to these cations and the inhibition of the sodium pump and the increased K^+ leak discussed above. The permeability of the PVM channel to small phosphorylated solutes has not been tested, but the channel is highly permeable to Tris whose molecular radius is of the size of phosphate. The PVM channels must also be permeable to ATP in order to allow this compound to reach the ATP/ADP antiporter (see below), and indirect evidence suggests that it is also permeable to adenosine and inosine monophosphate (Ginsburg *et al.*, unpublished observations). The high conductivity and lack of charge discrimination of the channel, as well as its permeability to phosphorylated compounds, would therefore suggest that intermediates of host cell metabolic processes [glycolysis, hexose monophosphate shunt (HMS), purine salvage] could also translocate through the channel and exit the infected cell through the duct. If this were true, one would expect total depletion of the host cell compartment from glutathione (its synthesis should be blocked) and massive oxidation of hemoglobin to methemoglobin (due to lack of NADH required as a cofactor for methemoglobin reductase). Both expectations are not met by direct measurements, and the HMS activity of the host cell compartment is actually increased several-fold [90]. It is clear from these deliberations that the PVM channels and the parasitophorous duct cannot be present simultaneously in the infected cell. As inferred from experimental observations and from teleological considerations, one of these transport paths must be an experimental artifact, or some intricate mechanism(s) must exist to overcome their unrealistic mutual presence. Desai [91] has attempted recently to reconcile this controversy by suggesting that gap junction-like channels may be spanning both the PVM and the PPM, and that these were the entities recorded in his on-cell patch-clamp experiments. However, the demonstrable presence of several antiporters and symporters on the host cell–parasite interface and the Na^+ and K^+ gradients observed across it (see next section) are incompatible with this suggestion. The first could not be detected with the parallel company of such gap junctions that are expected to be permeable to the same solutes whose passage is mediated by specific translocators. Appreciable cation gradients cannot exist across a membrane that has highly abundant 140 pS channels that are non-selective for monovalent cations. Hence, the parasite cytosol cannot be directly connected to the host cell cytosol via gap junctions.

Permeability of the parasite membrane

The knowledge of the transport characteristics of the parasite membrane (actually the PVM–PPM couple) is very limited; most of it has been obtained by permeabilizing the membrane of the host cell to solutes of up to 1600 molecular weight by treating the cells with Sendai virus [92], thus allowing direct access of extracellular solutes to the parasite membrane. Using Sendai virus-treated infected RBC, it has been demonstrated that the parasite membrane contains an ADP/ATP antiporter displaying the characteristics of the classical antiporter of mitochondria [92, 93], and it was suggested that by means of this system the parasite supplies ATP to the host cell whose glycolysis is impaired due to acidification and reduction of K^+ levels. This antiporter is inhibited by the same specific inhibitors that affect the antiporter of eukaryote mitochondria, and therefore it cannot serve as a target for new drug design. Similarly, a Na^+/H^+ antiporter has been demonstrated and assigned a role in pH homeostasis of the parasite [93] using the Na^+-gradient across the parasite membrane (probably maintained by a Na^+-K^+-ATPase) for the extrusion of protons. Although amiloride inhibits the antiporter as well as parasite growth, it is probably impossible to consider this drug as a potential antimalarial. Using an indirect approach of measuring the distribution of 2-deoxyglucose [40] and calcium [61] as affected by protonophores, it has been suggested that the parasite membrane also includes a glucose/H^+ symporter and a Ca^{2+}/H^+ antiporter, regulating

glucose uptake and Ca^{2+} egress, respectively. Since a proton motive force is required for these processes to be of any physiological significance, an active H^+-pump has been assigned to the parasite membrane, but its function has been questioned [94] and its presence has not been demonstrated directly. In fact, the Na^+/H^+ could suffice to generate the necessary pH gradient for glucose uptake and Ca^{2+} extrusion.

Using the same preparation, the transport of lactate [70] and Ca^{2+} [51] across the parasite membrane has been investigated. The transport of both solutes was found to be non-saturable, and while the rate of lactate translocation was similar to that of the infected cell membrane, that of Ca^{2+} was substantially more rapid. At the present time, no specific inhibitors of solute transport across the parasite membrane have been found (except those inhibiting the antiporters), and the nature of these transport systems is virtually unknown. Further investigations are required to clarify the PPM transporters in the hope that specific inhibitors may be found and eventually tested for their antimalarial potential.

Summary

The intraerythrocytic malarial parasite is involved in an extremely intensive anabolic activity while it resides in its metabolically quiescent host cell. The necessary fast uptake of nutrients and the discharge of waste products are guaranteed by parasite-induced alterations of the constitutive transporters of the host cell and the production of new parallel pathways. The membrane of the host cell thus becomes permeable to phospholipids, purine bases and nucleosides, small non-electrolytes, anions and cations. While the new pathways are quantitatively unimportant for the translocation of a particular solute, classical inhibitors of native transporters can be used to inhibit parasite growth. Several compounds were found to inhibit effectively the new pathways and, consequently, parasite growth. The pathways have also been used to introduce cytotoxic agents. The parasitophorous membrane consists of channels that are highly permeable to small solutes and display no ion selectivity. Transport of some cations and anions across the parasite membrane is rapid and insensitive to classical inhibitors, and in some cases it is mediated by specific antiporters that respond to their respective inhibitors. Macromolecules have been shown to reach the parasitophorous space through a duct contiguous with the host cell membrane, and subsequently to be endocytosed at the parasite membrane. The simultaneous presence of the parasitophorous membrane channels and the duct, however, is incompatible with experimental evidence. No specific inhibitors have been found as yet that would efficiently inhibit transport through the channels or the duct.

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