Cell surface receptor directed targeting of toxin to human malaria parasite, *Plasmodium falciparum*

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Abstract Gelonin (a toxin and type II ribosome inactivating protein) when linked to human transferrin can be targeted to *Plasmodium falciparum*. The transferrin toxin conjugate is significantly toxic to parasite growth and is 25 times more potent than toxin alone in inhibiting parasite protein synthesis. The mechanism of its entry into the intraerythrocytic parasite is discussed.

Key words: *Plasmodium falciparum*; Toxin; Ribosome inactivating protein; Gelonin-transferrin conjugate; Targeting

1. Introduction

Plasmodium falciparum malaria is responsible for the morbidity and mortality of several million children under the age of 5, in Asia and Africa alone. The rapid and unprecedented rise of multidrug resistance and the recent pessimism about the prospects of a malaria vaccine make new therapeutic approaches of paramount significance [1]. Antigens synthesized by plasmodial trophozoites and schizonts, present on the surface of IRBCs of the host, can be envisaged for targeting chemotherapeutic agents to the intracellular parasites [2].

RIPs are a family of toxins, produced by plants, that inactivate eukaryotic ribosomes [3]. These toxins have catalytic properties and only a small number of molecules need to reach cytosol to inactivate ribosomes to kill the cells. These toxins can be divided into two groups, those composed of two subunits, termed type I RIPs (viz. ricin) and those having only the toxic chain, termed type II RIPs (viz. gelonin). Ricin, ricin A chain and gelonin upon internalization irreversibly inactivate the protein synthesis through their A chains, by release of a single adenine 4324 from the 28S rRNA found in the 60S subunit of eukaryotic ribosomes leading to cell death [4]. Transferrin is the donor of non-heme iron (Fe^{3+}), essential for the survival of the malaria parasite [5–7]. Internalization of transferrin by a mechanism reminiscent of receptor mediated endocytosis indicates the existence of its receptor on the surface of IRBCs [8,9]. The intracellular parasites synthesize and translocate this receptor to the membrane of mature erythrocytes, which are otherwise devoid of it. The parasite transferrin receptor, unlike its human counterpart is not a disulfide linked dimer of subunits of M_r 95 000 each, but is made of single polypeptide of M_r 102 000 [10]. Data questioning the existence of this receptor as well as its involvement in iron transport have also been reported [11].

That targeted toxins represent a rational approach to cytocidal therapy in certain infectious diseases, envisaged earlier, has indeed been attested to, by the success of anti CD4-toxin and CD4-toxin conjugates for combating human immunodeficiency virus [12,13]. Moreover, such hybrid molecules prepared by chemical conjugation, linked to either antireceptor antibodies or ligands, have been demonstrated to selectively kill cells, displaying the complementary determinants (antigen or receptor) on their surfaces [14].

In this report we present evidence that this approach can be used for directed cytotoxicity towards the malaria parasite. When conjugate of human sero-transferrin to gelonin (or ricin A chain) is used, it selectively binds to IRBCs, by virtue of the presence of transferrin receptors on their cell surface, followed by the release of the toxic subunit into the cytosol of the parasite, leading to inhibition of parasite protein synthesis and eventual death.

2. Materials and methods

2.1. Materials

Human sero-transferrin, *Vibrio cholerae* neuraminidase, iminothiolane and anti-transferrin antibodies were from Sigma, St. Louis, MO, USA. Sephadex G-25 and Sephadex G-100 were the products of Pharmacia, Iodobeads and *N*-succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) were obtained from Pierce. Carrier-free Na[^{125}I] (200 Ci/mmol) was obtained from Bhabha Atomic Research Centre, Bombay, India. [^{35}S]Methionine (800 Ci/mmol) was from Amersham. The anti-gelonin antibody Sepharose was available from a previous study [15].

2.2. Maintenance of parasite culture and synchronization of different stages of parasite

P. falciparum FCK2 strain was maintained as described in [16] using the method of Trager [17]. Parasites (8–10% parasitemia) were synchronized using 5% D-sorbitol [18]. Trophozoite stage was used for most of the experiments unless otherwise stated.

2.3. Parasite protein synthesis and growth inhibition assays

Cell-free parasite protein synthesizing system was prepared as described by Surolia and Padmanaban [16]. Effects of various toxins and their conjugates on parasite growth were measured by microscopic examination of thin smears stained with Giemsa stain as well as by monitoring short-term [^{35}S]methionine incorporation by parasites into the trichloroacetic acid precipitable fraction after toxin/conjugate treatment [19]. Cell viability was also monitored by counting the infected cells and by the trypan blue exclusion method.

2.4. Preparation of ricin, ricin A chain and gelonin

Ricinus agglutinin (RCA I) and ricin were purified according to [20]. Ricin was separated from agglutinin by gel filtration on Sephadex G-100 as described in [21]. Ricin A and its B chains were prepared according to the method of Shimizu et al. [22]. Gelonin was purified

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Abbreviations: IRBC, infected red blood cells; URBC, uninfected red blood cells; RIP, ribosome inactivating protein; SMCC, *N*-succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; EMEM, Eagle's minimal essential medium; Tf, transferrin; [^{125}I]Tf[Fe^{2+}], iodinated holotransferrin; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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according to [3]. Activities of ricin, ricin A chain and gelonin were assayed as described in [3] using a rabbit reticulocyte lysate system prepared according to Jagus [23].

2.5. Preparation of gelonin-transferrin conjugate

Gelonin-transferrin conjugate was prepared as described in [24]. Transferrin (10 mg) in 0.1 M sodium phosphate buffer (pH 7.4) was treated with a 25-fold molar excess of SMCC for 1 h at 25°C. Subsequent to the removal of excess SMCC by gel filtration on Sephadex G-25 (1×25 cm), 1.8–2.1 molecules of SMCC were incorporated per molecule of transferrin. Gelonin (25 mg) was thiolated with a 25-fold molar excess of 2-iminothiolane according to [25] under nitrogen atmosphere at 25°C. This led to the incorporation of 0.9–1.1 sulfhydryl groups per gelonin molecule. The conjugate was then prepared by incubating a 5-fold molar excess of derivatized gelonin over SMCC-transferrin for 24 h at 4°C in 0.1 M sodium phosphate buffer pH 7.4, under nitrogen atmosphere. The remaining maleimido groups were saturated with 1.5 mM 2-mercaptoethanol. Gelonin-transferrin conjugate was separated from free gelonin and transferrin by gel filtration on a superfine Sephadex G-100 column (1.4×100 cm), equilibrated and eluted with 0.1 M sodium phosphate buffer (pH 7.4). Ricin A and B chain-transferrin conjugates were also prepared and characterized as above except that the thiolation step was omitted, as both of these chains contain sulfhydryl groups [26].

2.6. Binding studies

Transferrin was saturated (≥95%) with iron according to the method of Larrick and Cresswell [27]. Transferrin and gelonin-transferrin conjugates were iodinated by the chloramine T method [28] and separated from free [^{125}I] by gel filtration on Sephadex G-25. IRBCs were incubated with EMEM with Earle's salts for 20 min to ensure that the receptor-bound transferrin was dissociated. To measure binding, defined concentrations of iodinated holotransferrin [^{125}I]Tf[Fe^{2+}] (4.2×10^5 cpm/μg protein, prepared in our laboratory) or iodinated gelonin-transferrin(Fe^{2+}) conjugate (1.1×10^6 cpm/μg protein), were added to IRBCs and URBCs in a final volume of 0.2 ml EMEM containing 4 mg/ml bovine serum albumin for 20 min at 15°C. Subsequently, the cells were washed four times with 1 ml chilled PBS and the cell-associated radioactivity quantified in a gamma counter. The binding data were analyzed according to Steck and Wallach [29].

3. Results

3.1. Immunoprecipitation of the parasite transferrin receptor by anti-transferrin antibody

A monospecific antibody purified from polyclonal sera raised against IRBCs in rabbit immunoprecipitated a 100 kDa band in IRBCs but not in URBCs. Conjugate of gelonin with this antibody was found to be a more potent cytotoxic agent than gelonin to the malaria parasite [30]. Detailed examination of the antigen on the IRBCs revealed it to be the transferrin receptor.

3.2. Characterization of gelonin-transferrin conjugate

The conjugate was separated from free transferrin and gelonin by gel filtration on a Sephadex G-100 column (Fig. 1). On rechromatography of the conjugate (peak I), peaks II and III did not reappear. SDS-PAGE of the conjugate showed a single band with M_r 110000 which corresponds to the molecular masses of the hybrid of transferrin and gelonin with a stoichiometry close to 1:1 (data not shown). As transferrin contains the biantennary *N*-linked oligosaccharide chain which on treatment with neuraminidase exposes its terminal β-galactosyl residues, affinity chromatography of an aliquot (1 mg) of the conjugate on a RCA I-Sepharose (1×5 cm; 10 mg RCA I/ml gel) column was carried out to evaluate the presence of free gelonin in the preparation [31,32]. All of the desialylated conjugate was retained on the RCA I-Sepharose column and no free gelonin was observed in the washes. Likewise no free transferrin was detected in the washes when 500 μg of the conjugate was loaded on anti-gelonin-Sepharose column (1×5 cm; 8 mg antibody/ml gel). Based on these results, it is concluded that the conjugate thus prepared is devoid of free gelonin or transferrin. Under reducing conditions, two protein peaks, II and III, corresponding to transferrin and gelonin respectively, were obtained on the above column. Based on the protein content of each peak the molar ratio of transferrin and gelonin in the conjugate was found to be close to 1:1.05.

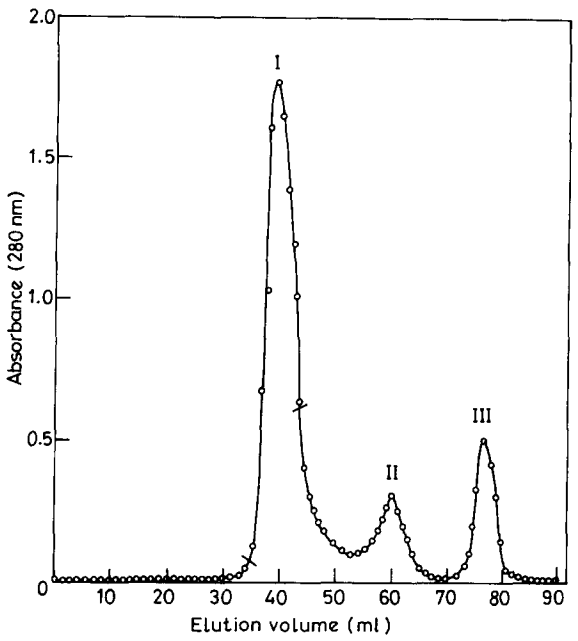

Fig. 1. Gel filtration of transferrin-gelonin conjugate on Sephadex G-100. Subsequent to the coupling step of conjugation the reaction mixture was loaded on the column equilibrated and eluted with 0.1 M phosphate buffer, pH 7.4. Peak I, II and III indicate peaks for conjugate, transferrin and gelonin respectively. Peak I was pooled at positions indicated by slashes in the figure.

3.3. Characterization of transferrin receptor sites on IRBCs

[^{125}I]Transferrin fails to bind to URBCs even at concentrations where its binding to IRBCs is saturated. The quantity of [^{125}I]transferrin bound to synchronized IRBCs is shown in Fig. 2A. These binding data were utilized to calculate the number of transferrin receptor sites and their association constants according to [29]. About 97000 (±15000) transferrin receptors were found per IRBC with an association constant of 5×10^6 M^{-1}. Gelonin-transferrin or ricin-transferrin conjugates also bound to IRBCs but not to URBCs. Moreover, the number of binding sites and their affinities for binding to the IRBCs were identical to those observed for transferrin itself, indicating that these hybrids bind to the same receptor.

3.4. Inhibition of cell-free parasite protein synthesis by gelonin, ricin A chain and gelonin-transferrin conjugate

As shown in Fig. 3 gelonin and ricin A chain, at 10–12 ng/ml, inhibited the parasite protein synthesis by 50% within 10 min of incubation with the lysate, which corresponds to the linear region of parasite protein synthesis [16]. Parasite protein synthesis virtually ceases (≈80%) at 40–45 ng/ml of these toxins. Since both gelonin and ricin A chain were found to be

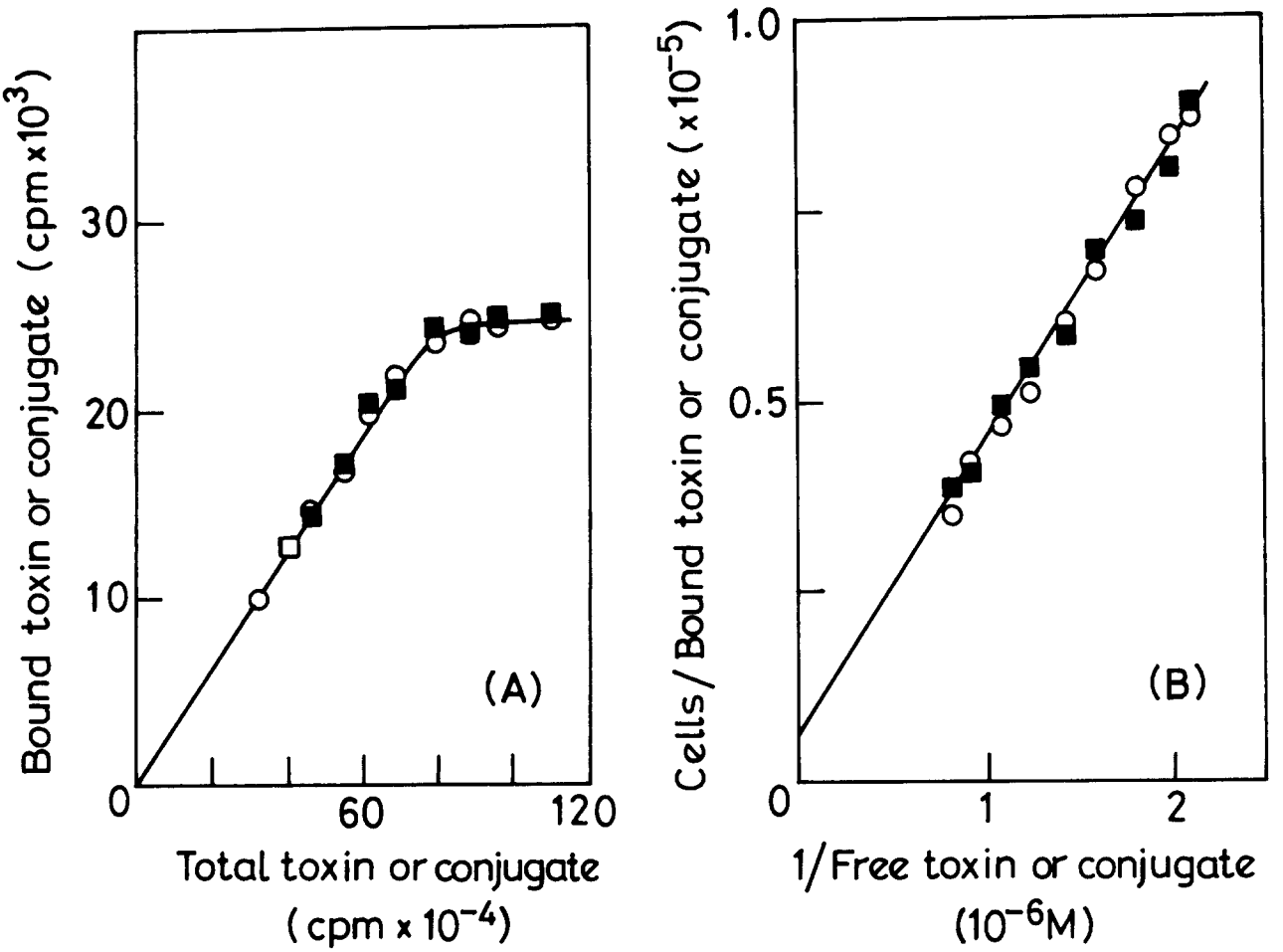

Fig. 2. Quantification of transferrin receptor sites on IRBCs. A: [^{125}I]Fe^{2+}-Transferrin (■) or [^{125}I]conjugate of Fe^{+2}-transferrin with gelonin (●) was incubated for 20 min at 15°C with synchronized cultures (8% parasitemia, trophozoite stage) in EMEM. Subsequently the cells were washed 4 times with chilled PBS and the cell associated radioactivity counted on a gamma counter. URBCs were treated in an identical manner and were used as controls. B: Scatchard analysis of data in A.

equally effective in inhibiting protein synthesis, gelonin was used for most of the studies unless otherwise stated. The gelonin-transferrin conjugate at 150 ng/ml inhibited protein synthesis up to 82%. Thus the toxicity of the conjugate is similar to that of gelonin alone, in molar terms.

3.5. *Effect of gelonin and gelonin-transferrin conjugate on parasite growth and viability*

The toxic effect of gelonin on the growth of the malaria parasite is observed at micromolar concentrations (Fig. 4). Thus, 1 μM gelonin inhibits parasite growth by 50%, while 5 μM is inhibitory up to 90%. Ricin A chain and ricin were equally potent in inhibiting the parasite growth (data not shown). These toxins are relatively less toxic to the intracellular parasites as compared to eukaryotic cells [3,26]. Gelonin-transferrin conjugate was 20–25 times more toxic to cells than gelonin alone. Incubation of cultures (8% parasitemia, trophozoite stage) with 50–100 nM conjugate led to 50% and 95% inhibition of parasite growth respectively as monitored by [^{35}S]methionine uptake, microscopic examination of the

Table 1
Effect of conjugates in combination with antibodies or NH_4Cl on *P. falciparum* growth

Conjugate	Addition	Cell growth (%)
Gelonin-transferrin (50 nM)	–	43
Anti-gelonin antibody (10 μM)	–	93
Gelonin-transferrin (50 nM)	Anti-gelonin antibody (10 μM)	90
Gelonin-transferrin (50 nM)	Anti-transferrin antibody (10 μM)	94
Gelonin-transferrin (50 nM)	NH_4Cl (2 mM)	45
	NH_4Cl (2 mM)	90
Ricin A chain-transferrin (50 nM)	–	53
Ricin A chain-transferrin (50 nM)	Ricin B chain-transferrin (100 nM)	51
Ricin B chain-transferrin (100 nM)	–	97

Cultures in microtiter plates (8% parasitemia, trophozoites) were incubated with conjugates or conjugates in combination with other compounds (coincubation) as described for Fig. 4. Incubation with NH_4Cl was for 24 h prior to [^{35}S]methionine addition. Details of incubation with [^{35}S]methionine and measurement of radioactivity are given in the legend to Fig. 4. Samples were analyzed in triplicate.

smears and the trypan blue exclusion method. Results obtained by microscopic examination of parasites and [^{35}S]methionine incorporation were quantitatively similar. Microscopy makes it possible to score the parasite number and morphology, whereas [^{35}S]methionine uptake measures the parasite's ability to synthesize protein, utilizing a precursor. By 72 h after toxin treatment schizonts are seen to degenerate completely (Fig. 5). Coincubation of the conjugate with anti-gelonin antibodies (10 μM) or anti-transferrin antibodies (10 μM) blocked the toxicity (Table 1). It may be noted that coincubation of ricin A chain-transferrin (50 nM) and ricin B chain-transferrin (100 nM) conjugates does not have any effect over and above that observed for ricin A chain-transferrin alone (Table 1) [33]. Likewise the addition of 2 mM NH_4Cl does not enhance the toxicity of the gelonin-transferrin conjugate.

4. Discussion

These studies were undertaken to confirm the presence of transferrin receptors on IRBCs and to examine the feasibility of targeting toxin-transferrin conjugate to the intracellular malaria parasite as an alternative chemotherapeutic approach. Our studies clearly establish that the concentration dependent and saturable binding of transferrin and gelonin-transferrin conjugate to IRBCs is due to transferrin-receptors on these cells. Transferrin, gelonin-transferrin conjugate and gelonin by itself are unable to bind to URBCs, whereas gelonin did not bind to infected erythrocytes.

Unlike eukaryotes, prokaryotes are completely resistant to these toxins. Malaria parasites, as shown here, are susceptible to their cytotoxic effects. In this respect the plasmodial ribosomes differ from those of another protozoan, *Tetrahymena pyriformis*, which is totally resistant to these toxins [34]. An analysis of the structures of rRNAs of *P. falciparum* and *T. pyriformis* should reveal the basis for differences in their susceptibilities towards these RIPs. In accordance with these results, these proteins (ricin, ricin A chain, gelonin and gelonin-transferrin conjugate) are found to be growth inhibitory to

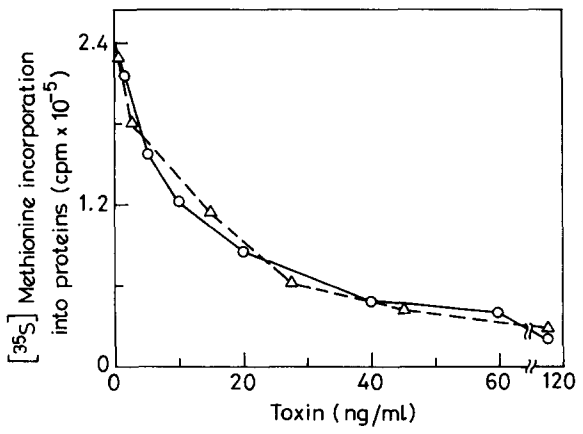

Fig. 3. Effect of toxins on in vitro protein-synthesizing system of *P. falciparum*. The assay was initiated by the addition of [^{35}S]methionine to the S-30 preparations [18] with or without (control) the toxin and terminated after 10 min. The [^{35}S]methionine incorporation was measured by spotting an aliquot on filter disks followed by washing with hot trichloroacetic acid–H_2O_2, alcohol:ether and ether. Gelonin ○, ricin △. Ricin A chain showed activity similar to ricin.

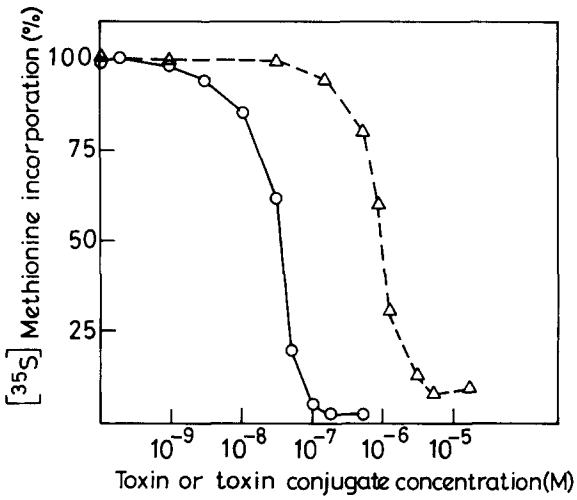

Fig. 4. Dose-response of gelonin and gelonin-transferrin conjugate on *P. falciparum* growth. *P. falciparum* growth was monitored by [^{35}S]methionine incorporation into parasite proteins as mentioned in the text. Cultures (8% parasitemia, trophozoites) were incubated with the toxin or its conjugate at 37°C for 15 min, in incomplete RPMI 1640 medium, which was then replaced with complete RPMI. Cultures were further incubated at 37°C. The medium was changed every 24 h. To monitor parasite growth, smears as well as [^{35}S] incorporation into the trichloroacetic acid-precipitabe fraction was monitored at 24 h, 48 h and 72 h. At these time points, the parasite pellet was lysed and an aliquot used for measurement of radioactivity on filter disk as described in Fig. 3. [^{35}S]Methionine was added 2 h prior to monitoring the incorporation, i.e. at 22 h, 44 h and 70 h. Trypan blue exclusion assays were also carried out at these time points. Gelonin (△) and conjugate (○) treated, after 72 h.

this parasite and inhibit cell-free parasite protein synthesis significantly. A finding of considerable interest is the stage specific effects of the toxin and its transferrin conjugate on *Plasmodium* growth. The conjugate-treated parasites do not infect fresh erythrocytes at all, as the parasite growth is totally arrested at the schizont stage, which are completely degenerated and are seen as debris outside the cells (Fig. 4). In this respect the effect of the conjugate is schizontocidal, like another antimalarial agent, chloroquine, though the mode of action may be different.

In contrast to its highly cytotoxic effect on many cell lines and eukaryotic cells, ricin was much less toxic to parasites compared to gelonin or ricin A chain, which is probably due to its poor endocytosis. Moreover, the uptake of the conjugate does not appear to follow the conventional route of receptor-mediated endocytosis and processing in an acidic compartment, as the addition of a high concentration of the lysosomotropic amine NH_4Cl, along with the conjugate does not potentiate the cytotoxic effect of the conjugate (Table 1) [35].

Immunotoxins (conjugate of toxins and their antibodies) made up of type II RIPs are much less toxic than those made up of type I RIPs. Coincubation of the A chain conjugate with B chain conjugate enhances cytotoxicity of the former, which is explained by assuming that such conjugates are endocytosed independently and recombine in an intracellular compartment, where the B chain promotes the entry of the A chain to the cytosol, enhancing its cytotoxicity. In our studies, the absence of a stimulatory effect of the B chain conjugate on the A chain conjugate further proves that these

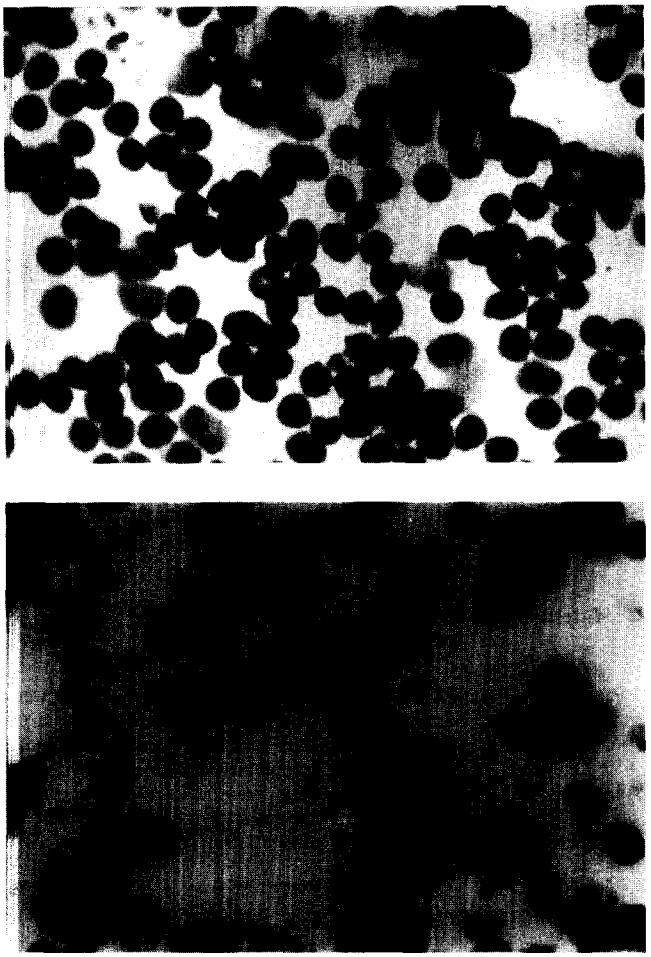

Fig. 5. Effect of transferrin-gelonin conjugate on parasite morphology. The conjugate (100 nM) was incubated with cultures (8% parasitemia, trophozoites) as described in legend for Fig. 3. Giemsa-stained smears were examined after 72 h. A: Control (without conjugate) after 72 h; B: conjugate treated, after 72 h.

hybrids are not being endocytosed in this parasite, as in other eukaryotes [33]. That transferrin-ricin conjugate was as toxic as its A chain counterpart or gelonin-transferrin conjugate and the non-toxicity of transferrin-ricin B chain conjugate further corroborate these observations.

Given such a scenario, what could be the mechanism for the enhanced cytotoxicity of the conjugate. Questions regarding the accessibility and uptake of macromolecules and nutrients in malaria parasite have been a subject of intense investigations. Two divergent views about the uptake are direct access to the parasite plasma membrane by parasitophorous duct [36] in the IRBC throughout the life cycle of the parasite in the erythrocytes and a phospholipid/lipid recruitment pathway [37]. Irrespective of its mechanism of entry, the toxin-transferrin conjugate is shown here to be a potent cytotoxic agent and its targeted delivery and alternative approach for chemotherapy to malaria parasite.

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