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J Clin Invest. 1990;86(2):618-624. <https://doi.org/10.1172/JCI114753>.

Research Article

The human malaria parasite *Plasmodium falciparum* demonstrates variability in its dependence upon erythrocyte sialic acid residues for invasion. Some lines of *P. falciparum* invade neuraminidase-treated or glycophorin-deficient red blood cells poorly, or not at all, while other lines invade such cells at substantial rates. To explore the molecular basis of non-sialic acid dependent invasion, we selected parasite lines from a clone (Dd2) that initially exhibited low invasion of neuraminidase-treated erythrocytes. After maintaining Dd2 for several cycles in neuraminidase-treated erythrocytes, parasite lines were recovered that invaded both untreated and neuraminidase-treated erythrocytes at equivalently high rates (Dd2/NM). The change in phenotype was maintained after removal of selection pressure. Four subclones of Dd2 were isolated and each readily converted from sialic acid dependence to non-sialic acid dependence during continuous propagation in neuraminidase-treated erythrocytes. The neuraminidase-selected lines and the Dd2 clone demonstrated identical restriction fragment length polymorphism markers indicating that the Dd2 clone was not contaminated during the selection process. Parasite proteins that bound to neuraminidase-treated and untreated erythrocytes were indistinguishable among the parent Dd2 clone and the neuraminidase-selected lines. The ability of the Dd2 parasite to change its invasion requirements for erythrocyte sialic acid suggests a switch mechanism permitting invasion by alternative pathways.

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Evidence for a Switching Mechanism in the Invasion of Erythrocytes by *Plasmodium falciparum*

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Abstract

The human malaria parasite *Plasmodium falciparum* demonstrates variability in its dependence upon erythrocyte sialic acid residues for invasion. Some lines of *P. falciparum* invade neuraminidase-treated or glycophorin-deficient red blood cells poorly, or not at all, while other lines invade such cells at substantial rates. To explore the molecular basis of non-sialic acid dependent invasion, we selected parasite lines from a clone (Dd2) that initially exhibited low invasion of neuraminidase-treated erythrocytes. After maintaining Dd2 for several cycles in neuraminidase-treated erythrocytes, parasite lines were recovered that invaded both untreated and neuraminidase-treated erythrocytes at equivalently high rates (Dd2/NM). The change in phenotype was maintained after removal of selection pressure. Four subclones of Dd2 were isolated and each readily converted from sialic acid dependence to non-sialic acid dependence during continuous propagation in neuraminidase-treated erythrocytes. The neuraminidase-selected lines and the Dd2 clone demonstrated identical restriction fragment length polymorphism markers indicating that the Dd2 clone was not contaminated during the selection process. Parasite proteins that bound to neuraminidase-treated and untreated erythrocytes were indistinguishable among the parent Dd2 clone and the neuraminidase-selected lines. The ability of the Dd2 parasite to change its invasion requirements for erythrocyte sialic acid suggests a switch mechanism permitting invasion by alternative pathways. (J. Clin. Invest. 1990; 86:618-624.) Key words: malaria • erythrocytes • sialic acid

Introduction

Recently several studies have focused on erythrocyte sialic acid and its role in invasion of erythrocytes by the human malaria parasite *Plasmodium falciparum* (1-7). Some studies have shown that different lines of *P. falciparum* vary in their dependence on erythrocyte sialic acid for effective invasion of red blood cells (8-10). Cleavage of sialic acid from erythrocytes with neuraminidase treatment greatly reduces erythrocyte invasion by some parasite lines, but produces only modest reduction by others (1, 2, 6, 8-10). Erythrocytes genetically deficient in glycophorin molecules, which contain the majority of erythrocyte sialic acid (11), have confirmed this diversity in the invasion characteristics of *P. falciparum* (1, 5, 9, 12).

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Received for publication 22 January 1990 and in revised form 21 March 1990.

The Journal of Clinical Investigation, Inc.
Volume 86, August 1990, 618-624

Specific proteins synthesized by the parasite have been found to bind to neuraminidase-sensitive residues on the red cell surface (13, 14). The binding of these proteins has been correlated with the ability of the sialic acid-dependent parasite to invade susceptible erythrocytes. Parasite or erythrocyte molecules involved in non-sialic acid dependent pathways have not been identified. A trypsin-sensitive erythrocyte molecule, distinct from glycophorin A, has been implicated as being involved in invasion by some parasites (9). To this end, we have begun to explore the effect of selection pressure on parasite invasion in vitro.

In this report we show that *P. falciparum* clones can be grouped into two categories according to their ability to invade sialic acid-deficient erythrocytes. Type I parasites invade and grow in neuraminidase-treated erythrocytes, although at reduced rates as compared with untreated red cells. Type II parasites can be subdivided into two categories, II-A and II-B. With continued cultivation in neuraminidase-treated erythrocytes, type II-A parasites disappear from culture, demonstrating their complete dependence on sialic acid for erythrocyte invasion. Type II-B parasites eventually produce a population that is able to effectively invade neuraminidase-treated erythrocytes; this subpopulation emerges during continuous propagation in neuraminidase-treated erythrocytes. The type II-B phenotypic change is reproducible in the parent clone and in multiple subclones of the parent. Furthermore, the change persists in the absence of selection pressure, suggesting that a switch occurs in the expression of a gene or group of genes of type II-B parasites.

Methods

P. falciparum clones. *P. falciparum* clones Dd2 (15), HB3 (16), 3D7 (17), 7G8 (18), Camp/A1 (8, 9), and FCR-3/A2 (19) were cultivated in vitro according to the methods of Trager and Jensen (20). Subclones of Dd2 were obtained by limiting dilution (21). To avoid variability in erythrocytes from different individuals, O⁺ erythrocytes from a single individual (S.A.D.) were used in all cultures and experiments. Cultures were maintained at 5% hematocrit in RPMI 1640 (Gibco Laboratories, Grand Island, NY) to which 24 mM Hepes, 360 µM hypoxanthine, 24 mM NaHCO₃, 10 µg/ml of gentamicin and 10% heat-inactivated, pooled, human A⁺ serum were added. Incubation was at 37°C under 3% O₂/6% CO₂/91% N₂.

Neuraminidase treatment of erythrocytes. Erythrocytes were collected by venipuncture into citrate-phosphate-dextrose solution, pelleted from the plasma, washed in 5 vol of RPMI 1640, centrifuged, and resuspended to 50% hematocrit in either complete medium or RPMI 1640 at 4°C. Neuraminidase treatment of erythrocytes was performed as described (8, 9). Neuraminidase (from *Vibrio cholera*; Gibco Laboratories) was added directly to the washed erythrocytes at 10-50 U/ml. Erythrocytes were incubated rocking at 37°C for 60-90 min, pelleted, washed in 10 vol of RPMI 1640, resuspended at 50% hematocrit with either complete medium or RPMI 1640, and stored at 4°C.

Invasion assays. Parasite cultures were synchronized with 5% sorbitol (22) and expanded to 3-5% late trophozoite forms in 25-50-ml

cultures. The erythrocytes were pelleted, resuspended to 50% hematocrit in complete medium, and layered over either a 65% Percoll/complete medium cushion or a Percoll/sorbitol gradient (23, 24). Late stage forms were recovered (50–90% infected red blood cells), washed with RPMI 1640, counted with a hemocytometer, and resuspended in complete medium at 1.5×10^7 parasites/ml. Invasion assays were performed in 96-well, flat-bottomed microtiter plates (Costar, Cambridge, MA). 100 μ l of the parasite suspension was inoculated into 100 μ l of target erythrocytes at 2.5×10^8 erythrocytes/ml in complete medium. A 1:10–1:20 ratio of parasites/target erythrocytes was used. Assays were incubated at 37°C for 18–24 h. The number of ring forms per 1,000 erythrocytes was estimated from blood films. Individual assays were performed in duplicate and the invasion rates were taken as the average result. Invasion rates for neuraminidase-treated erythrocytes were recorded as percent of control erythrocyte invasion rates.

Growth assays. Parasite cultures were grown to ~5% asynchronous parasitemia in normal erythrocytes (5-ml volume). From this culture an inoculum was delivered to fresh 5-ml cultures containing either untreated or neuraminidase-treated erythrocytes (initial parasitemia 0.1–0.5%). Parasitemia was estimated daily from the number of parasitized erythrocytes per 1,000 erythrocytes.

Erythrocyte binding assays. 25–50-ml cultures were synchronized with sorbitol and allowed to reach 3–5% late trophozoite forms. After enrichment on a Percoll gradient, the trophozoites were returned to methionine-cysteine-deficient complete medium (Select-Amine Kit; Gibco Laboratories) at 1.3×10^8 parasites/ml and labeled with 100–200 μ Ci/ml of radiolabeled methionine and cysteine (Trans 3 S-Label; ICN Radiochemicals, Irvine, CA). After incubation for 12–20 h the culture was centrifuged (12,000 g for 10 min at 4°C). The supernatant was frozen at -70°C. The pellet, containing schizonts, free merozoites, and cellular debris, was resuspended in 3 ml of RPMI 1640 in a 15-ml Corex centrifuge tube and sonicated on a wet ice bath. Three cycles of sonication (30 s on/30 s off) were completed at an output control setting of 100 (sonic disruptor; Tekmar Co., Cincinnati, OH). The sonicate was centrifuged at 12,000 g for 20 min at 4°C. The supernatant was frozen at -70°C until use. 500 μ l of supernatant and 250 μ l of sonicate were mixed with 100 μ l of packed erythrocytes. Erythrocyte binding assays were performed as described by Haynes et al. except protease inhibitors were not included (25).

Southern blot analyses. DNA was prepared from saponin-lysed, parasitized erythrocytes (26) and separated over cesium chloride gradients according to standard procedures (27). Restriction endonuclease digests were performed with enzymes from Bethesda Research Laboratories (Gaithersburg, MD) and New England Biolabs (Beverly, MA) according to manufacturers' recommendations. Restriction fragments were fractionated in agarose gels and transferred to nylon membranes for hybridization (28). The pC4.H32 probe, obtained from a size-fractionated Hinf I *P. falciparum* library (29), was used to "fingerprint" repetitive elements in the genome.

Results

Variation among clones in sialic acid requirements. To assess sialic acid requirements for invasion, six *P. falciparum* clones were evaluated for their ability to invade neuraminidase-treated erythrocytes (Fig. 1). These invasion assays revealed that parasites could be classified into two groups according to their dependence on sialic acid residues. Type I parasites (HB3, 3D7, and 7G8) invaded neuraminidase-treated erythrocytes at 30–60% of the rate in control erythrocytes. Type II parasites (FCR-3/A2, Camp/A1, and Dd2) invaded neuraminidase-treated erythrocytes at low rates compared with control (5–15%). Low residual invasion rates of Dd2, FCR-3/A2, and Camp/A1 into neuraminidase-treated erythrocytes may, in part, be accounted for by the presence of untreated erythrocytes that were carried with the parasite inoculum after enrich-

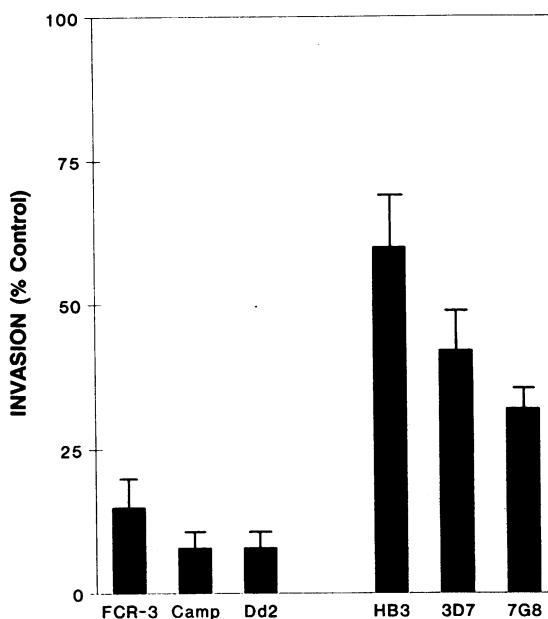


Figure 1. Invasion of neuraminidase-treated erythrocytes by type I and type II *P. falciparum* parasites. Results are an average of three separate invasion assays and are presented as the percentage of invasion into neuraminidase-treated erythrocytes relative to invasion of untreated erythrocytes. In all experiments the invasion rate of untreated erythrocytes was > 3.5%. Error bars indicate SEM.

ment. Preabsorption of the sera used in the culture medium with neuraminidase-treated erythrocytes did not affect results of the invasion assays with Dd2 and HB3. The decreased invasion, therefore, was not related to antibody binding of neuraminidase-treated erythrocytes.

To study the growth characteristics of type I and type II parasites, the six parasite clones were each placed into parallel cultures with untreated and neuraminidase-treated erythrocytes and propagated for several days (Fig. 2). Type I parasites (HB3, 3D7, and 7G8) grew in neuraminidase-treated erythrocytes at approximately one-half to one-third of the rate in control cells. In contrast, type II parasites subdivided based on their growth in neuraminidase-treated erythrocytes. Type II-A parasites (FCR-3/A2 and Camp/A1) exhibited complete dependence on erythrocyte sialic acid residues. When attempts were made to propagate type II-A parasites in neuraminidase-treated red blood cells, the parasites were lost from culture and could not be recovered despite maintenance of the culture for 4 wk. However, clone Dd2 (type II-B) exhibited novel behavior when propagated in neuraminidase-treated erythrocytes. After the inoculation of Dd2 into neuraminidase-treated erythrocytes, parasites were no longer detectable by thin-film blood films after two to three growth cycles. However, after 10–12 d in continuous culture, a population of parasites emerged that was capable of invasion and growth in neuraminidase-treated erythrocytes (Dd2/NM1). The experiment was repeated and a second line of parasites capable of invasion and growth in neuraminidase-treated red cells was again obtained (Dd2/NM2).

Invasion rates of Dd2/NM1 and Dd2/NM2 into neuraminidase-treated erythrocytes were nearly equivalent to the rates of invasion into untreated erythrocytes (Fig. 3 A). Fur-

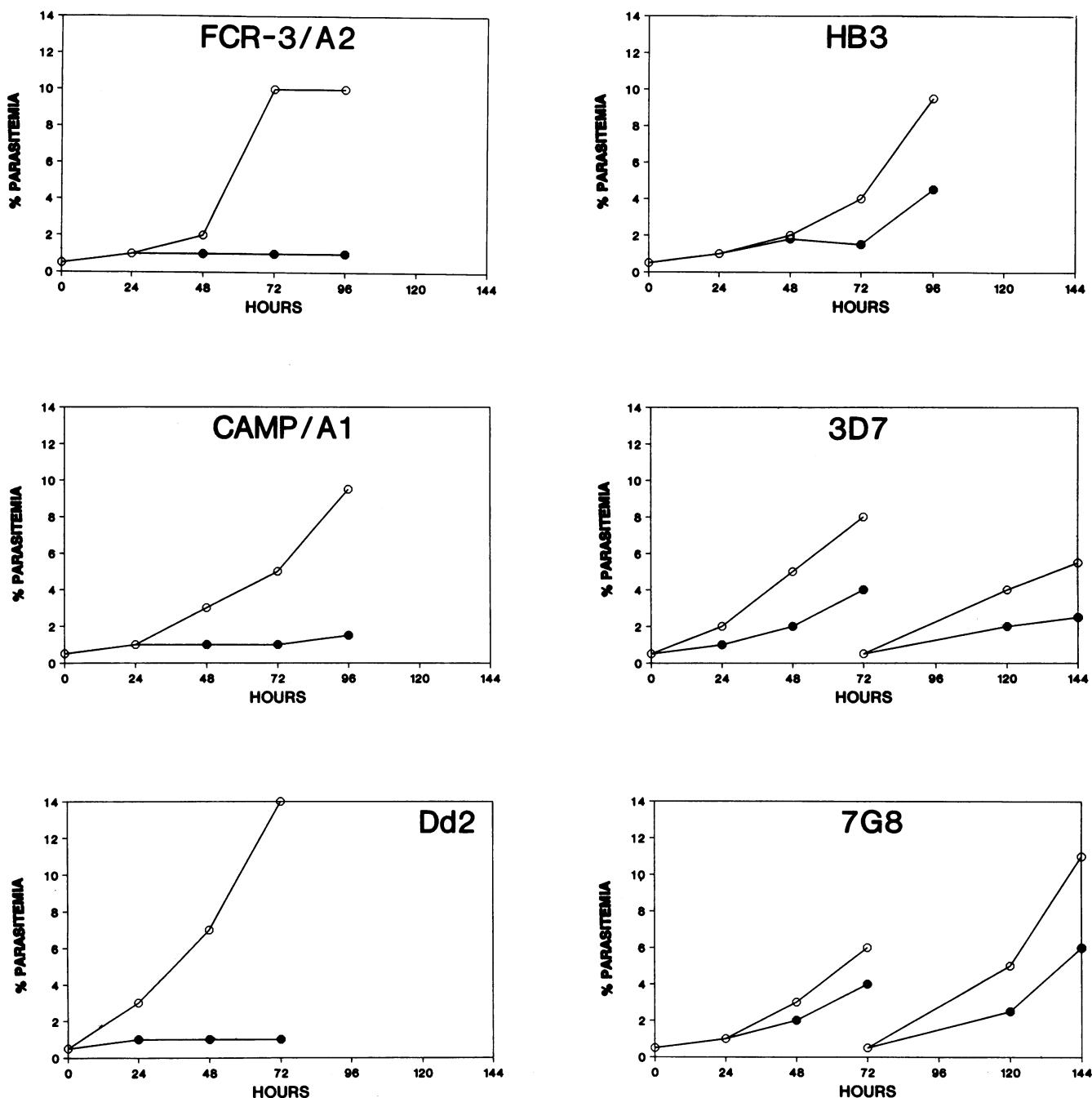


Figure 2. Propagation of *P. falciparum* clones in untreated and neuraminidase-treated erythrocytes. Clones 3D7 and 7G8 were diluted to 0.5% parasitemia at 72 h and cultivated for 144 h. ●, Neuraminidase-treated erythrocytes; ○, untreated erythrocytes.

thermore, in both untreated and neuraminidase-treated erythrocytes, Dd2/NM1 was able to grow at rates comparable to those of Dd2 in untreated erythrocytes (Fig. 3 B). Such behavior by Dd2/NM1 and Dd2/NM2 contrasts with the lower rates observed for type I clones in neuraminidase-treated erythrocytes (Fig. 1).

The Dd2/NM1 and Dd2/NM2 phenotypes were found to be stable in culture with untreated normal erythrocytes. After their selection in neuraminidase-treated erythrocytes, both lines were propagated in untreated erythrocytes for at least 2 wk (eight cycles) and then reexamined for invasion into neur-

aminidase-treated erythrocytes. Despite removal of selection pressure, the Dd2/NM1 and Dd2/NM2 lines continued to invade neuraminidase-treated erythrocytes and untreated erythrocytes at similar rates.

To confirm that the Dd2/NM1 and Dd2/NM2 lines had been selected from a clonal population, Dd2 was subcloned by limiting dilution. Four subclones were examined and each of these subclones (Dd2/R7, Dd2/R8, Dd2/R9, and Dd2/R14) exhibited the same phenotypic switch as the Dd2 clone. Growth assays revealed an initial inability of the inocula to expand in neuraminidase-treated erythrocytes; however,

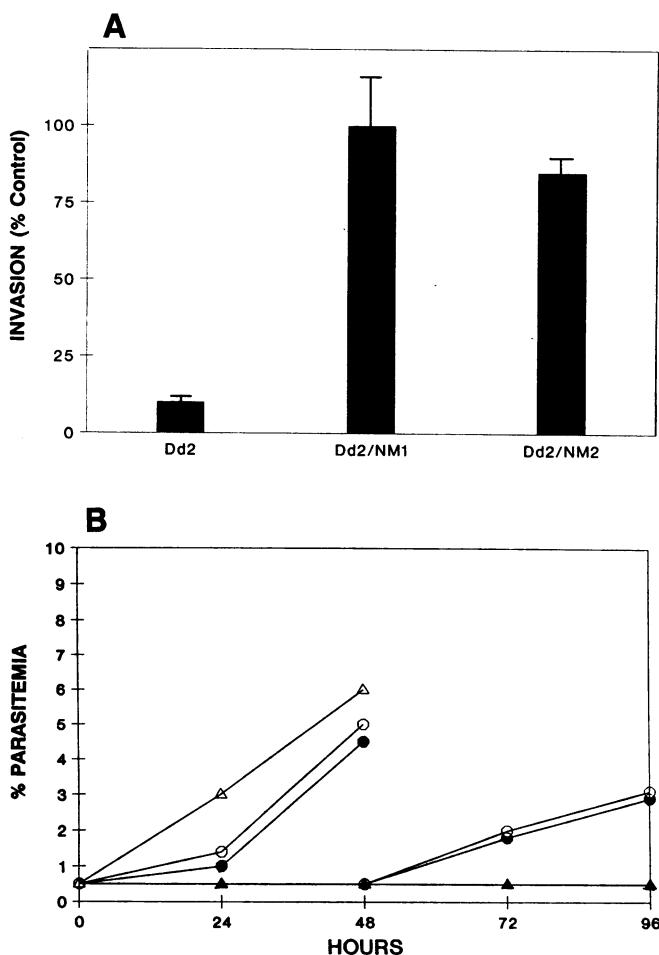


Figure 3. Invasion and growth of the Dd2/NM1 and Dd2/NM2 lines. *A*, Invasion rates of Dd2, Dd2/NM1, and Dd2/NM2 into neuraminidase-treated and untreated erythrocytes. The results are an average of three separate invasion assays for Dd2 and Dd2/NM1 and two separate invasion assays for Dd2/NM2 presented as the percentage of invasion into neuraminidase-treated erythrocytes relative to invasion into untreated erythrocytes. In all experiments the invasion rate of untreated erythrocytes was > 3.5%. Error bars indicate SEM. *B*, Growth of Dd2 and Dd2/NM1 in neuraminidase-treated and untreated erythrocytes. Dd2/NM1 parasites were diluted 1:10 at 48 h. Propagation of Dd2 in untreated cells was discontinued at 48 h. Triangles, Dd2; octagons, Dd2/NM1; open symbols, untreated erythrocytes; solid symbols, neuraminidase-treated erythrocytes.

maintenance in culture for 10–12 d produced lines from each subclone capable of growth in neuraminidase-treated erythrocytes (Dd2/NM7, Dd2/NM8, Dd2/NM9, and Dd2/NM14). Therefore, each subclone from Dd2 had the intrinsic ability to convert from sialic acid dependence to sialic acid independence.

To confirm the clonal integrity of Dd2 and the Dd2/NM lines, the genomic structure of the parasites was examined by probing restriction digests with single-copy and repetitive sequences from the genome. Fig. 4 shows hybridization patterns obtained with pC4.H32, a recombinant clone that detects interspersed repetitive elements on chromosomes of the parasite. Clones HB3, 3D7, 7G8, FCR-3/A2, Camp/A1, and Dd2 each exhibited unique patterns of hybridization ("fingerprints"). By

comparison, the subclones of Dd2 and the NM lines all produced patterns identical to that of the parent Dd2 clone. Restriction fragment length polymorphism likewise revealed no detectable differences in the Dd2 family. 30 separate plasmid and oligonucleotide probes hybridized against 18 separate restriction endonuclease digests revealed no polymorphisms among Dd2, Dd2/NM1, and Dd2/NM2 (data not shown).

Erythrocyte binding assays. Erythrocyte binding assays with soluble proteins from culture supernatants and parasite-infected cellular sonicates of Dd2, Dd2/NM1, and Dd2/NM2 parasites were performed. Comparison of protein binding to untreated and neuraminidase-treated erythrocytes was made in an attempt to explain the phenotypic differences among the parasites. Culture supernatant mixed with normal erythrocytes and neuraminidase-treated erythrocytes revealed similar patterns of bound radiolabeled proteins among the three lines (Fig. 5 *A*, lanes 2, 5, 8). Some radiolabeled proteins, such as a 175-, a 165-, and a 125-kD bound preferentially to untreated erythrocytes. With neuraminidase-treated erythrocytes, however, a 140-kD protein produced the most prominent eluted band and bound more strongly to neuraminidase-treated erythrocytes than untreated erythrocytes. Neither Dd2/NM1 nor Dd2/NM2 demonstrated alterations in protein-binding profiles that could account for their acquired ability to invade neuraminidase-treated erythrocytes.

Sonicates of parasite-infected cells demonstrated patterns of bound radiolabeled proteins to either untreated or neuraminidase-treated erythrocytes that were also similar among the three lines (Fig. 5 *B*). The most prominent protein from the cellular sonicate bound to untreated erythrocytes was at 165 kD; less intense bands were seen at 175, 140, and 125 kD. As with the culture supernatants, a 140-kD radiolabeled protein was the most prominent protein eluted from neuraminidase-treated erythrocytes. The sonicates did not show any differences in the patterns of radiolabeled protein bound by Dd2/NM1, Dd2/NM2, and Dd2.

Discussion

In this work *P. falciparum* parasites have been classified into two categories based on their requirements for erythrocyte sialic acid residues for invasion. Type I parasites, represented by clones HB3, 3D7, and 7G8, effectively invade sialic acid-deficient erythrocytes. Type II parasites, represented by clones FCR-3/A2, Camp/A1, and Dd2, are dependent on erythrocyte sialic acid residues for invasion. These findings concur with the results of previous studies (8–10, 13).

In the course of the present investigations we have found that type II parasites are separable into two distinct subtypes (types II-A and II-B). Type II-A parasites are unable to survive when propagated in neuraminidase-treated erythrocytes. Type II-B parasites produce parasite lines that are able to invade and grow equally well in untreated and neuraminidase-treated erythrocytes. Clone Dd2, a type II-B parasite, produced such populations in repeated experiments of propagation in neuraminidase-treated erythrocytes. Further, four subclones of Dd2 likewise yielded sialic acid-independent lines. From these data we infer that each and every parasite of the Dd2 population carries the potential to produce non-sialic acid dependent lines under selection pressure. Unlike the type I parasites that

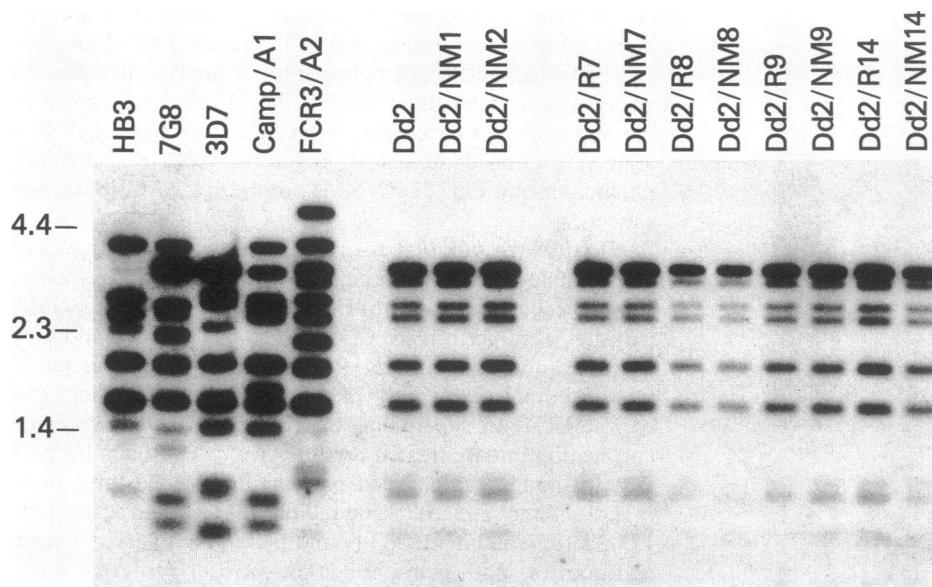


Figure 4. Patterns of interspersed repetitive DNA sequences in *P. falciparum* clones. Lanes 1–6 show the hybridization patterns of the clones used in the study (HB3, 7G8, 3D7, Camp/A1, FCR3/A2, and Dd2). Each yields a unique fingerprint. Lanes 6–8 show the hybridization patterns of clone Dd2 and the lines selected by growth in neuraminidase-treated erythrocytes (Dd2/NM1 and Dd2/NM2). Lanes 9–16 show the hybridization patterns of the four subclones of Dd2 (Dd2/R7, Dd2/R8, Dd2/R9, and Dd2/R14) and the corresponding lines selected by growth in neuraminidase-treated erythrocytes (Dd2/NM7, Dd2/NM8, Dd2/NM9, and Dd2/NM14).

invade neuraminidase-treated erythrocytes at subnormal rates, non-sialic acid dependent lines derived from Dd2 invade both untreated and neuraminidase-treated erythrocytes equally well.

Experiments with an uncloned isolate, Thai-2, previously demonstrated that non-sialic acid dependent parasites could be selected from a parasite population (8). Propagation of the Thai-2 isolate for a period of time in Tn erythrocytes (deficient in the galactose and sialic acid components of the glycophorin oligosaccharide side chain [11]) yielded "Thai-Tn" parasites that were characterized by an enhanced ability to invade neuraminidase-treated erythrocytes. In the course of the present work, we found Thai-2 by restriction fragment length polymorphism analysis to be polyclonal in character (data not shown). Hence, it is uncertain if type II-B parasites were present in Thai-2 or if propagation in neuraminidase-treated erythrocytes selected a subpopulation of type I parasites.

Erythrocyte binding assays were performed in an attempt to identify any differentially expressed proteins that might be associated with the ability to invade neuraminidase-treated erythrocytes. We were unable to demonstrate any differences among Dd2 and Dd2/NM1 or Dd2/NM2. Several possible explanations might account for this lack of discrimination, including inability to solubilize parasite components with our procedures, insufficient protein labeling, or involvement of nonprotein parasite components in the invasion process. A 175-kD binding protein described by Camus and Hadley (13) was able to bind untreated erythrocytes but not neuraminidase-treated erythrocytes. Mutational events involving this protein are not likely to explain the non-sialic acid dependent invasion of Dd2/NM.

One hypothesis that may account for the adaptation process is that the Dd2 population is continually switching between non-sialic acid dependent and sialic acid-dependent phenotypes. The ability of organisms to switch phenotypes is a well-described biologic phenomenon including antigenic variation in bacteria and protozoa, mating types in yeast, and immunoglobulin heavy chains in vertebrates (30–34). Genetic switch mechanisms expand the biologic repertoire of organ-

isms and permit adaptive capacity in changing environments.

The suggestion that Dd2/NM might result from a genetic switch mechanism is based on two observations. First, the switched parasite acquires a positive attribute, the ability to invade an erythrocyte that the majority of the starting population is unable to invade. Second, all four subclones of Dd2 produced non-sialic acid dependent lines when propagated in neuraminidase-treated erythrocytes. These observations are reminiscent of the surface protein variations that have been described for *Borrelia*, *Salmonella*, and *Trypanosome* species (30–32). In these instances variation of surface proteins has been demonstrated to be the result of genetic switch mechanisms.

During cultivation in normal erythrocytes the Dd2 population may be switching at low frequency between the Dd2 and Dd2/NM phenotypes, but Dd2/NM phenotype would not develop because of the absence of selection pressure. The Dd2/NM phenotype is seen when conditions, neuraminidase-treated erythrocytes, confer an advantage. Conversely, one might anticipate that there is switching between Dd2/NM and Dd2 when Dd2/NM is propagated in untreated erythrocytes. However, since Dd2 and Dd2/NM grow at equivalent rates in untreated erythrocytes, there is no apparent advantage to Dd2. One could speculate that the removal of the non-sialic dependent pathway would result in the reappearance of the Dd2 phenotype. Bacterial and yeast plating techniques permit direct verification and quantitation of switching phenomenon. These techniques are not adaptable to malaria parasite cultures. Definitive proof of a switch mechanism must, therefore, await identification of the molecules involved in the non-sialic acid dependent pathway of invasion.

It will be of interest to examine freshly cloned clinical isolates for invasion pathway heterogeneity and the ability to switch phenotypes. To date investigations of *P. falciparum* invasion have used uncloned parasite populations or parasite clones that have been propagated for prolonged periods of time under *in vitro* culture conditions. Alterations in cytoadherence phenotype and the deletion of a parasite gene associated with cytoadherence in the human host have been asso-

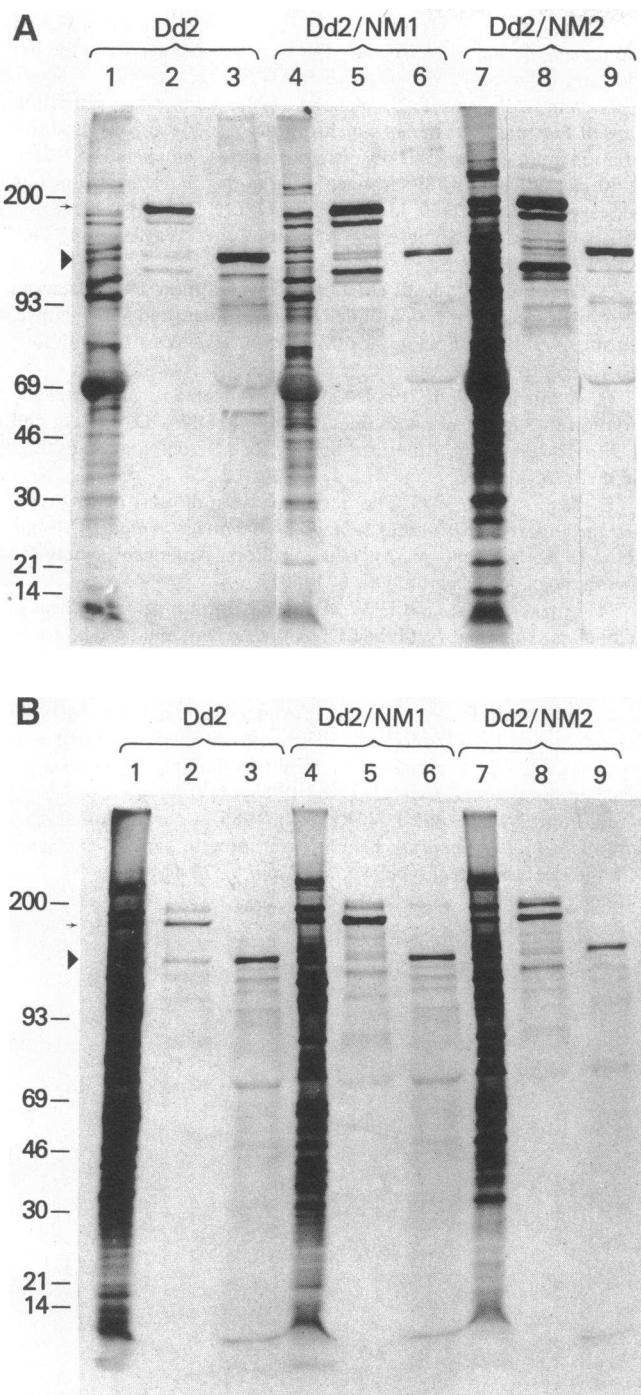


Figure 5. Erythrocyte binding assays of Dd2, Dd2/NM1, and Dd2/NM2. **A**, Binding assays of soluble proteins from culture supernatants. Lanes 1, 4, and 7 show total radiolabeled proteins from culture supernatants of Dd2, Dd2/NM1, and Dd2/NM2, respectively. Lanes 2, 5, and 8 show radiolabeled protein bound to untreated erythrocytes. Lanes 3, 6, and 9 show radiolabeled protein bound to neuraminidase-treated erythrocytes. Arrow indicates 175-kD band and arrowhead indicates 140-kD band. **B**, Binding assays of proteins from corresponding parasite-infected cellular sonicates displayed as in **A**. Arrow indicates 165-kD band and arrowhead indicates 140-kD band.

ciated with in vitro propagation (35, 36). Similar phenomena may have occurred in the multi-step invasion process during in vitro propagation.

Dd2 and Dd2/NM parasites should be useful in delineating non-sialic acid dependent pathways of invasion. Identification of the molecules involved in this pathway may be possible through differential analysis, and would be predicted to contribute to our growing knowledge of the molecular events involving invasion.

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