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Attachment of *Toxoplasma gondii* to Host Cells Is Host Cell Cycle Dependent

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The initial attachment of *Toxoplasma* tachyzoites to target host cells is an important event in the life cycle of the parasite and hence critical in the pathogenesis of this infection. The efficiency of *Toxoplasma* attachment to synchronized populations of Chinese hamster ovary cells and bovine kidney cells was investigated by using a glutaraldehyde-fixed host cell assay system. For both cell lines, parasite attachment increased as the synchronized host cells proceeded from the G1 phase to the mid-S phase and then decreased as the cells entered the G2-M boundary. Postulating that these differences in attachment reflect the upregulation of a specific receptor, polyclonal antibodies were generated against whole MDBK antigen at 0 and 4 h into the S phase. Both antisera were shown to inhibit parasite attachment to both synchronous and asynchronous host cell populations. However, the attachment blockade observed with the 4-h antiserum was significantly greater than that with the 0-h antiserum, completely abolishing the cell cycle-dependent increase in attachment found in control samples. These findings suggest that *Toxoplasma* tachyzoites bind specifically to a host cell receptor which is upregulated in the mid-S phase of the cell cycle.

Toxoplasma gondii is an obligate intracellular parasite in which host cell invasion is an essential component of its life cycle. In addition to having an extremely broad host range (6), *T. gondii* is uniquely promiscuous in vitro, infecting every mammalian cell as well as insect and primary fish cell lines (3, 39). The invasion process is highly ordered, initiated by the binding of the apical end of the tachyzoite to the host cell plasma membrane. As it enters, the parasite is visibly constricted at the site of a tight junction with the host cell plasma membrane. This constriction moves posteriorly along the length of the parasite as it moves into the host cell (1, 25). Short cytoplasmic projections reseal the host membrane (8, 30), leaving the parasite inside a specialized parasitophorous vacuole (36).

Attachment to the host cell is thus the first step required for the process of invasion and hence is critical for its continued survival. Although the process of attachment was described more than 30 years ago, the identification of parasite ligands and cell surface receptors remains incomplete (16). Previous work from our laboratories has demonstrated an important role for the major tachyzoite surface protein SAG1 as a parasite ligand (13, 14, 27). With a fixed host cell system, attachment can be inhibited by using some anti-SAG1 monoclonal antibodies and, in addition, attachment is quantitatively reduced in chemically mutagenized parasite mutants lacking antigenic SAG1 (26).

The broad host cell range of *T. gondii* implies either the presence of a highly conserved host cell receptor or that the parasite can utilize a variety of different receptors. Specificity is suggested in that attachment is saturable in competition assays (26) and can be inhibited with the neoglycoprotein bovine serum albumin (BSA) glucosamide (18, 33). It has been suggested that parasites may bind to host cell laminin (10, 11). In addition, previous work has suggested that *Toxoplasma* infection of host cells is modulated by the vertebrate cell cycle (7, 41). In this report, we demonstrate that parasite attachment to the host cell includes a specific receptor-mediated interaction that occurs with higher frequency during the mid-S phase of the host cell cycle.

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MATERIALS AND METHODS

Parasite strains. The RH strain of *T. gondii* was used in all experiments. This strain has been propagated by serial passage both in vitro and in vivo (34). The parasites were maintained by serial passage in human foreskin fibroblast (HF) monolayers as described previously (17). For use in an assay, the tachyzoites were released from host fibroblasts by forced extrusion through a 27-gauge needle, centrifuged, and resuspended in minimum Eagle medium containing 0.2% BSA. Tachyzoites were used in assays within 30 min of harvest.

Host cells. Adult bovine kidney cells (MDBK; ATCC CCL-22; American Type Culture Collection, Rockville, Md.) (22) were maintained in Ham's F12 medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, Utah). Chinese hamster ovary cells (CHO Pro5; ATCC CRL-1781) were maintained in alpha minimum Eagle medium supplemented with 10% fetal calf serum. Both cell lines were subcultured routinely twice weekly and incubated at 37°C and in 5% CO_2 in air.

Cell synchronization. Host cells were synchronized by serum starvation and hydroxyurea block to halt cell cycle progression at the G1-S phase boundary (37). Cells were seeded at low density onto 18-mm-diameter glass coverslips and incubated overnight. The monolayer cultures were subsequently incubated in medium containing 0.1% fetal bovine serum for 48 h and then for 12 h in medium plus 10% fetal bovine serum containing 1 mM hydroxyurea (Sigma Chemical Co., St. Louis, Mo.). Cells were released from the hydroxyurea block by washing and progressed in synchrony into the S phase.

Cell synchronization and passage through the S phase were monitored by thymidine incorporation. At the appropriate time points, [^{3}H]thymidine (DuPont NEN, Boston, Mass.) was added to the culture medium at a final concentration of 5 μCi/ml and the cells were incubated for 30 min. After washing with ice-cold phosphate-buffered saline (PBS), the cells were fixed overnight at 4°C in 10% trichloroacetic acid. The fixed monolayers were solubilized in 0.1 N sodium hydroxide, and incorporated thymidine was counted by scintillation.

Invasion assay. Parasite invasion levels were assayed by using live host cells grown on floating coverslips. Nonconfluent asynchronous host cell cultures or synchronous cultures at the appropriate stage in the cell cycle were washed twice in medium. For the inhibition assays, host cells were preincubated in a 1/100 dilution of antibody or the appropriate concentration of purified immunoglobulin G (IgG) for 30 min prior to the addition of the parasites. The antibody remained in the culture medium for the entire experiment.

Parasites (3×10^6) were added to the cultures, and the cultures were incubated at 37°C for 30 min. The coverslips were then washed to remove extracellular parasites by immersion 10 times in warm medium. Host cells, adherent parasites, and intracellular parasites were fixed for 20 min with Bouin's fixative, dehydrated, and stained with Giemsa stain. Infection was quantitated by count-

ing the number of intracellular and attached parasites per 200 host cells on at least three replicate coverslips.

Results were compared for statistical significance between control and test groups by use of a two-tailed *t* test.

Attachment assay. The attachment of parasites to host cells was evaluated as described previously (26). Floating coverslips of nonconfluent asynchronous host cells or synchronous cultures at the appropriate stage in the cell cycle were washed twice in PBS and treated for 5 min at 4°C with 2% glutaraldehyde (grade 1; Sigma) in PBS. After fixation, the cells were immediately washed three times with PBS and incubated overnight in 0.16 M ethanolamine (pH 8.3) to saturate nonspecific binding sites created by the fixation procedure. Prior to incubation with parasites, the fixed cells were again washed three times in PBS, once with minimum Eagle medium–0.2% BSA, and equilibrated at 37°C for at least 30 min. An assay with RH strain parasites was performed as described above.

Preparation of host cell antigen and polyclonal antibody. MDBK cells were synchronized as described above. At 0 and 4 h into the S phase, the cells were washed twice with homogenization buffer (50 mM Tris-HCl, 25 mM KCl, 5 mM $MgCl_2$, 25 mM sucrose [pH 7.4]) and scraped into homogenization buffer containing phenylmethylsulfonyl fluoride (1 mM), leupeptin (25 μg/ml), and antipain (25 μg/ml). The cells were disrupted by nitrogen cavitation (600 lb/in^2 for 10 min) followed by slow release, and the remaining cell debris was removed by centrifugation at 2,000 × *g*. The protein concentration was determined as described previously with BSA as a standard (2). Antigen for electrophoresis was prepared as described above with asynchronous cultures of MDBK, CHO, and HF cells.

Polyclonal antibody against synchronized MDBK antigen was prepared by immunization of New Zealand White rabbits. Rabbits were immunized with whole MDBK cell antigen (at 0 and 4 h into the S phase) by using Freund's complete adjuvant for the first injection and Freund's incomplete adjuvant for subsequent injections. Animals received a total of 280 μg of protein over a 6-week period. One week after the seventh immunization, the rabbits were bled, and the serum was separated and stored at −20°C. The titer of anti-MDBK antibodies by enzyme-linked immunosorbent assay was greater than 1:12,800 for both sera (termed MDBK0 and MDBK4). The IgG fraction was purified by affinity chromatography on protein G coupled to Sepharose (Pharmacia, Piscataway, N.J.).

RESULTS

Host cell synchronization. MDBK and CHO cells can be readily synchronized by serum starvation and a hydroxyurea block (Fig. 1A and B). After the hydroxyurea block is removed (0 h), the cells enter the synthetic phase in synchrony. DNA synthesis, as measured by [^{3}H]thymidine incorporation, reaches a peak at mid-S phase, before dropping off as cells enter the G2 phase and mitosis. [^{3}H]thymidine incorporation in control cultures which have not been serum starved or treated with hydroxyurea remains at a steady state throughout the incubation period.

Increased binding of *T. gondii* to synchronized host cells. To determine the effect of the host cell cycle on *Toxoplasma* infectivity, synchronized host cell populations were infected with parasites by use of our in vitro assay systems. A representative example of such an assay using live MDBK cells can be seen in Fig. 1C. A live assay system allows parasites to both attach to the host cells and proceed through the invasion step. Cells were released from the hydroxyurea block and allowed to progress into the S phase for the appropriate time interval; at each time point, *Toxoplasma* parasites were added to the cultures and allowed to interact for 30 min. Cells at the G1-S phase boundary have a low susceptibility to infection. Invasion rates increase as the cells progress through the S phase, reaching a peak at mid-S phase. Invasion rates decrease back to baseline levels as the cells pass further into the S phase and into the G2 phase. Similar results are obtained with synchronized populations of CHO cells (Fig. 1D). In the example shown, although the peak in DNA synthesis is delayed until 6 h into the S phase, cells at mid-S phase are three times more susceptible to invasion than cells at the G1-S boundary. For both MDBK and CHO asynchronous cultures, no differences in the invasion rates were found, rates being similar in both cases and remaining constant throughout the assay. Differences were noted in the ratio of invasion of asynchronous versus synchronized cells, invasion rates increasing 0.5-fold above asynchronous baseline in MDBK cells and 2.2-fold above baseline in CHO cells, at 4 h into the S phase.

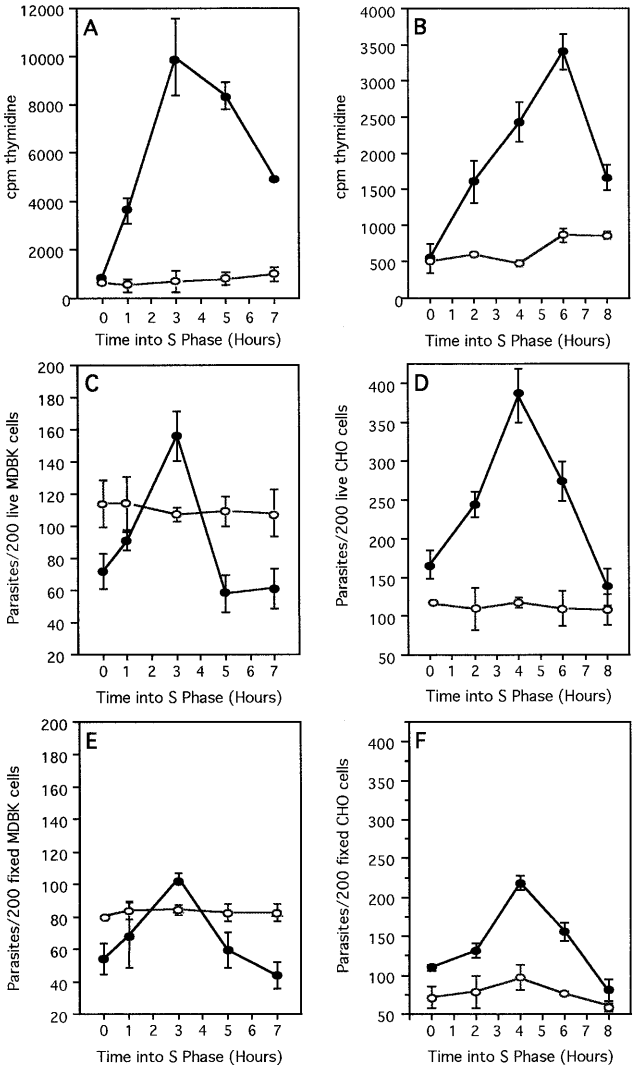

FIG. 1. Kinetics of attachment of RH strain *Toxoplasma* tachyzoites to synchronous (●) and asynchronous (○) MDBK (A, C, and E) and CHO (B, D, and F) cells. MDBK (A) and CHO (B) cells were synchronized by serum starvation and hydroxyurea treatment, resulting in a halt in cell cycle progression at the G1-S boundary. Synchrony was then monitored through the S phase by thymidine incorporation. The resulting cultures were infected with *Toxoplasma* tachyzoites for 30 min at 37°C by either a live (C and D) or fixed (E and F) in vitro assay system. Results are expressed as the number of parasites per 200 host cells. Each point represents the mean of three preparations ± standard deviation.

By using a glutaraldehyde-fixed assay system, it is possible to separate the attachment step from the subsequent invasion step (26). Synchronized host cells were released from the hydroxyurea block for the appropriate time period, fixed with glutaraldehyde, and then used in an assay with *Toxoplasma* parasites. The number of parasites bound to the fixed host cells was reduced by about 40% as compared to that of the corresponding live cultures (Fig. 1E and F). However, a pattern identical to that of the live cultures was found, with attachment rates increasing to a peak at mid-S phase and then decreasing as cells entered the G2-M boundary.

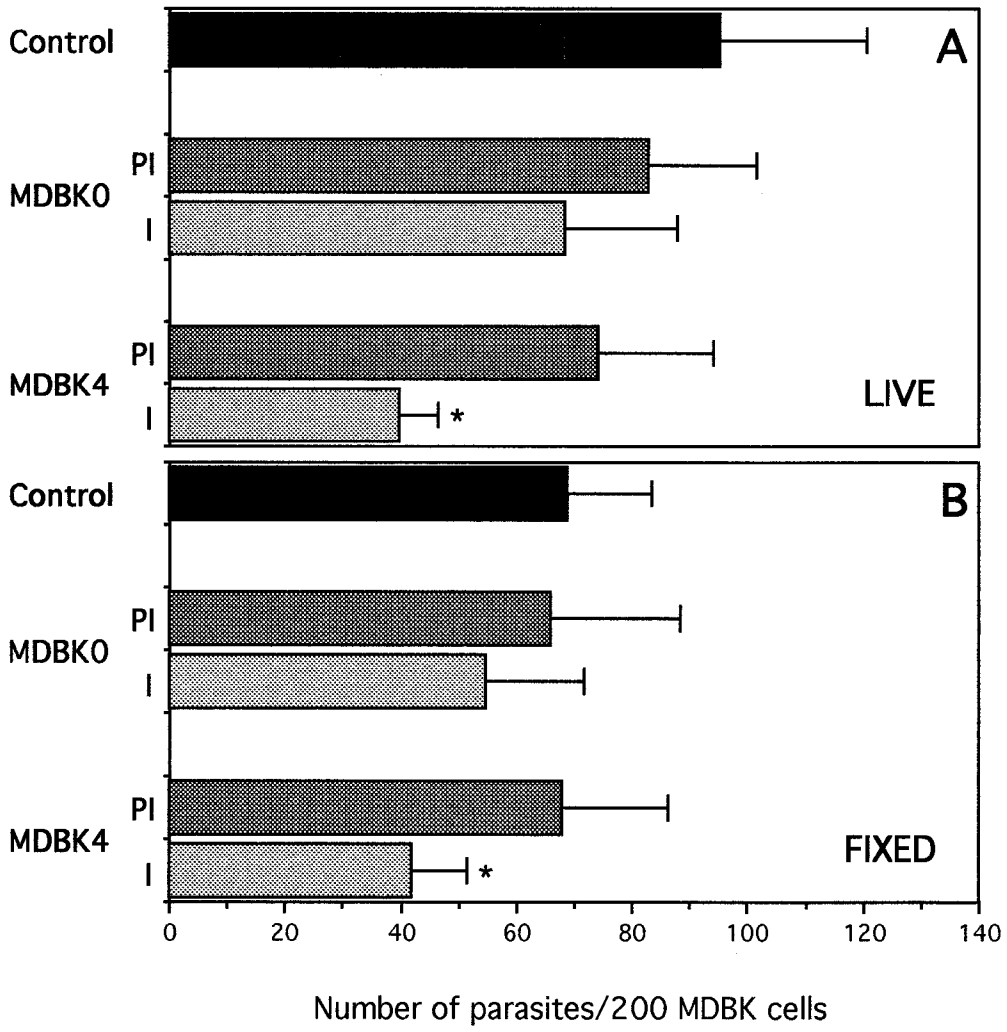

FIG. 2. Effect of MDBK0 and MDBK4 polyclonal antisera on attachment of RH strain *Toxoplasma* tachyzoites to asynchronous live (A) or fixed (B) MDBK cells over a 30-min time period. Host cells were used untreated or preincubated in preimmune (PI) or immune (I) test antiserum for 30 min prior to the addition of the parasites. Results are expressed as the number of parasites (mean ± standard deviation) attached to 200 host cells for three replicate cultures ($n = 3$ for each treatment). *, significantly inhibited compared with preimmune control ($P < 0.05$).

Polyclonal antibody to host cells inhibits parasite attachment. If the differences in infectivity at the different phases of the cell cycle are due to the upregulation of certain cell surface receptors, we postulated that antibodies generated against these receptors should inhibit attachment. Polyclonal antisera were raised against MDBK cells at 0 (MDBK0) and 4 (MDBK4) h into the S phase. Both antisera were reactive against whole MDBK antigen when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting (immunoblotting), recognizing multiple bands (data not shown). Although some variation was apparent in the recognition of MDBK antigens by the two antisera, there were no striking differences. Both antisera also cross-reacted with CHO and HF antigen by Western blot analysis. Both control preimmune sera reacted with low affinity to a limited repertoire of antigens.

We evaluated the ability of the MDBK0 and MDBK4 polyclonal antisera to block *Toxoplasma* attachment to asynchronous host cell populations. MDBK cells were preincubated in antisera for 30 min prior to the addition of the tachyzoites. In this assay, both antisera were able to inhibit the invasion of tachyzoites in a live assay as well as the attachment of tachyzoites in a fixed assay (Fig. 2). When compared with controls, the MDBK0 antiserum blocked infection by 17.6 and 16.9% in live and fixed cultures, respectively. However, the inhibition found for the cells preincubated in MDBK4 antiserum was significant in both cases, being 46.2 and 38.6% in live and fixed cultures, respectively.

The effect of the blocking antisera on parasite attachment to synchronized host cells was also evaluated. MDBK and CHO cells were synchronized by using serum starvation and hydroxyurea and fixed at various time points into the S phase. The fixed cells were then preincubated in antisera prior to the addition of the tachyzoites for assay. The results of such an assay using MDBK host cells are shown in Table 1. Both antisera blocked attachment at all time points. The greatest effect on attachment was seen at 4 h into the S phase. In all cases, the greatest inhibition of binding was noted with the MDBK4 antiserum.

Interestingly, a similar effect was found when CHO cells were used as the host. The percentages of inhibition of attachment observed with MDBK0 and MDBK4 antisera at 4 h into

TABLE 1. Effect of polyclonal antibodies against MDBK cells at 0 (MDBK0) and 4 (MDBK4) h into the S phase on the attachment of *Toxoplasma* tachyzoites to synchronized host cells

Time[a]	Control (no. of parasites attached)[b]	MDBK0		MDBK4	
		No. of parasites attached[b,c]	% Inhibition[d]	No. of parasites attached[b,c]	% Inhibition[d]
0	71.71 ± 1.2	71.02 ± 3.4	1	49.58 ± 14.2	30.9
2	108.08 ± 14.4	92.69 ± 2.5	14.2	44.81 ± 2.7	58.5*
4	126.40 ± 18.7	97.33 ± 12.3	23	40.04 ± 8.2	68.3*
6	89.10 ± 9.1	80.47 ± 19.2	9.7	40.74 ± 2.7	54.3*
8	67.57 ± 4.7	65.52 ± 5.6	3.0	50.62 ± 10.1	25.1

[a] Hours into the S phase of fixed host MDBK cells.
[b] Results are expressed as the number of parasites attached to 200 host cells (mean ± standard deviation; $n = 3$ for each treatment).
[c] Synchronized host cells were preincubated in antiserum for 30 min prior to the addition of parasites.
[d] The percentage inhibitory effect of the antibodies relative to control. *, $P < 0.05$.

the S phase were 35.9 and 71%, respectively (Fig. 3). Neither of the preimmune control sera had any significant effect at any of the time points.

To confirm the blocking efficiencies of the MDBK0 and MDBK4 antisera, IgG was purified by affinity chromatography and then used at various concentrations in a blocking assay (Fig. 4). The inhibition of attachment was shown to be dose dependent with both antisera, ranging from 5.1 to 27.9% for the MDBK0 antiserum and 44.9 to 76.1% for the MDBK4 antiserum (range of 15.8 to 250 μg/ml in both cases).

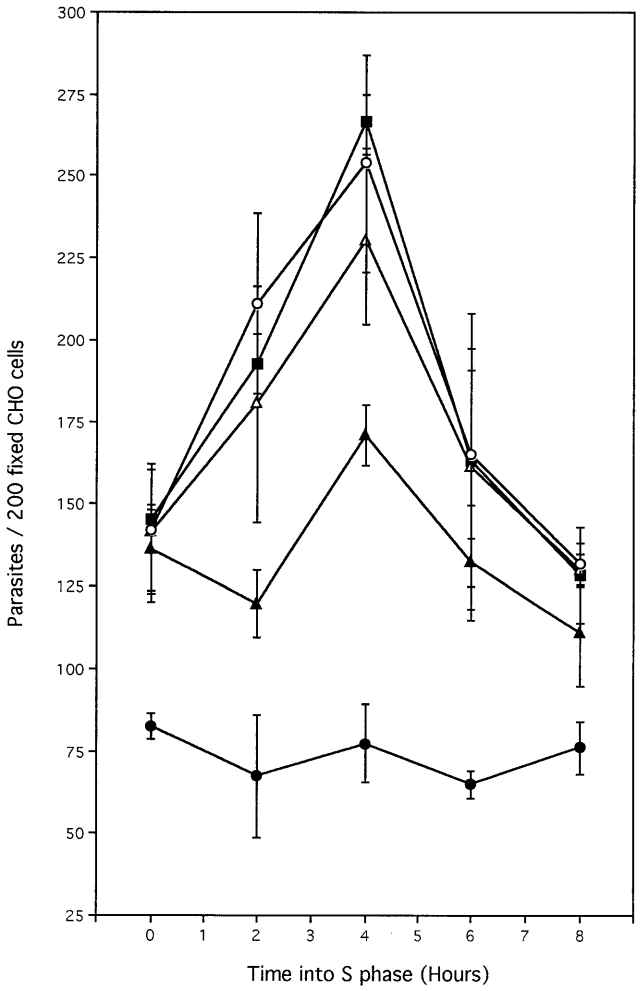

FIG. 3. Kinetics of the attachment of *T. gondii* to synchronous CHO cells, fixed at various time points into the S phase, in the presence of polyclonal antibody against synchronized MDBK cells or control preimmune sera. Host cells were preincubated in the test sera for 30 min prior to the addition of the parasites. Results are expressed as the number of parasites (mean ± standard deviation) attached to 200 host cells in control cultures (■) or in the presence of preimmune MDBK0 (△), MDBK0 (▲), preimmune MDBK4 (○), or MDBK4 (●) antiserum ($n = 3$ for each time point).

DISCUSSION

Treatment of cells with hydroxyurea inhibits RNase reductase (20), which results in a deletion of intracellular deoxynucleotide pools and inhibition of DNA repair (40). During a short exposure and at a 1 mM concentration, it is selectively lethal to S-phase cells, while cells in the G1, M, and G2 phases of the cell cycle are not affected and accumulate at the G1-S boundary. Highly synchronized populations of host cells can be obtained with a hydroxyurea block used in combination with serum starvation.

Our results indicate that the attachment of *T. gondii* to its host cell is a specific receptor-ligand interaction. By using synchronized host cells and a fixed in vitro assay system, the efficiency of parasite attachment was shown to be directly related to the cell cycle phase of the target host cells. In two different host cell lines, parasite attachment was maximal at the mid-S phase of the host cell cycle. Our postulation that this difference in infectivity was due to an upregulation of specific cell surface receptors was supported by the finding that antibody generated against MDBK cells at the mid-S phase blocked parasite attachment to both fixed and live host cells. The antibody also blocked parasite attachment to CHO host cells, suggesting cross-recognition by the blocking antiserum of a conserved cell surface receptor.

Parasite invasion modulated by the vertebrate cell cycle has been demonstrated previously (7). However, in the previous study, the investigators used live host cell systems and were therefore studying both parasite attachment and active penetration. The study of receptor-ligand interactions can be complicated in a live system. Plasma membrane fluidity, for example, has been shown to vary with the cell cycle phase (5) and could potentially affect *Toxoplasma* binding or penetration. The use of our fixed host cell assay system reduces the likelihood of this variability. Although the binding of parasites to a fixed cell system is less efficient than that to live cells (15, 24, 26, 35), the system allows the quantitation of *Toxoplasma* attachment without active penetration. Cell volume and dry mass are known to increase continuously throughout the cell cycle (28). Although the corresponding increase in surface area and receptor number could explain the increase in attachment to mid-S-phase cells, it does not explain the subsequent decrease in attachment levels as the host cells progress to G2. We

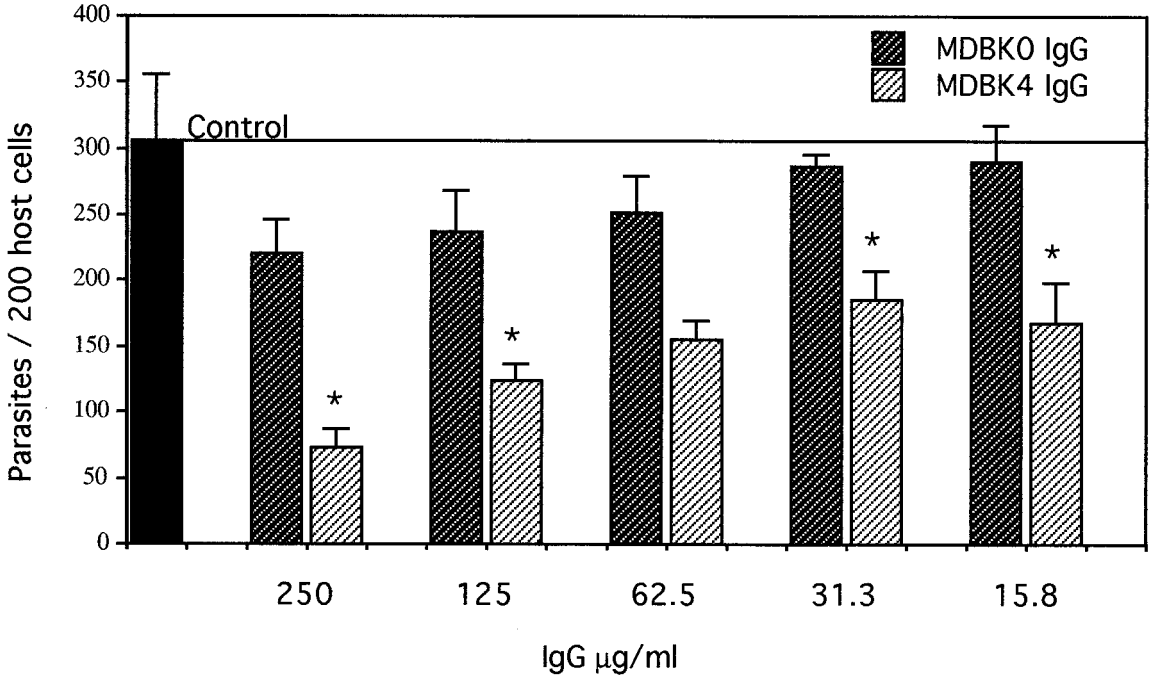

FIG. 4. Effect of MDBK0 IgG or MDBK4 IgG concentration on attachment of RH strain *Toxoplasma* tachyzoites to fixed synchronized MDBK cells over a 30-min time period. Host cells were synchronized by serum starvation and hydroxyurea block and fixed with glutaraldehyde 4 h into the S phase. They were then preincubated in various concentrations of purified IgG for 30 min prior to the addition of parasites. Results are expressed as the number of parasites (mean ± standard deviation) attached to 200 host cells ($n = 3$). *, significantly inhibited compared with control ($P < 0.05$).

postulate, therefore, that the increase in parasite attachment at the mid-S phase results either from a redistribution of surface components, such that previously covert receptors become exposed, or from the de novo synthesis and upregulation of specific cell surface molecules. The difference in the rates of attachment in the mid-S phase with the MDBK cells and CHO cells could reflect differences in the number of receptors expressed on the different cell types.

It is a frequent observation that *Toxoplasma* tachyzoites bind in a nonhomogeneous pattern to a monolayer of cells; i.e., some host cells bind several parasites, and others bind none (7, 26). In addition, confluent cultures are less well infected than nonconfluent monolayers (12). Both of these observations can be explained by our findings. The majority of cells in a confluent culture are in the G1 or resting phase, whereas in a nonconfluent culture, 30 to 60% of the cells are in the S phase (7).

Modulation of parasite invasion by the host cell cycle has also been reported for the protozoan parasite *Trypanosoma cruzi* (7) and for the viruses human immunodeficiency virus (21, 23) and encephalomyocarditis virus (29). It is probable that these observations reflect the upregulation of different cell surface receptors. Renewal of proteins may occur in either a continuous or a discontinuous process (28). Although many cell surface receptors have been shown to be expressed continuously throughout the growth of a cell, others have been demonstrated to have growth phase-related expression (4). Examples of quantitative changes in expression include membrane-bound immunoglobulin (19), CD4 (23), murine leukemia virus major envelope protein (32), β2-integrins (38), the tumor necrosis factor receptor (31), and CD40 (9). From this selection, the tumor necrosis factor receptor and the beta 2-integrins are upregulated on the cell surface during the S phase of their cell cycle. Work is currently in progress to identify such a molecule which functions as the receptor for *Toxoplasma* attachment.

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