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DIFFERENTIAL SURFACE DEPOSITION OF COMPLEMENT PROTEINS ON LOGARITHMIC AND STATIONARY PHASE *LEISHMANIA CHAGASI* PROMASTIGOTES

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Abstract

Previous works demonstrated that various species of *Leishmania* promastigotes exhibit differential sensitivity to complement-mediated lysis (CML) during development. Upon exposure to normal human serum (NHS), cultures of *Leishmania chagasi* promastigotes recently isolated from infected hamsters (fewer than 5 in vitro passages) are CML-sensitive when in the logarithmic growth phase but become CML-resistant upon transition to the stationary culture phase. Visualization by light and electron microscopy revealed dramatic morphological differences between promastigotes from the 2 culture phases following exposure to NHS. Flow cytometric analysis demonstrated that surface deposition of the complement components C3, C5, and C9 correlated inversely with promastigote CML-resistance. The highest levels of complement protein surface accumulation were observed for logarithmic phase promastigotes, while stationary phase promastigotes adsorbed the least amount of complement proteins. Additionally, fluorescence microscopy revealed that C3 and C5 localized in a fairly uniform pattern to the plasma membrane of promastigotes from logarithmic phase cultures, while the staining of promastigotes from stationary phase cultures was indistinguishable from background. By Western blot analysis, high levels of the complement proteins C3, C5, and C9 were detected in the total lysates of NHS-exposed logarithmic phase *L. chagasi* promastigotes, relative to NHS-exposed stationary phase promastigotes; this finding indicates that the low levels of C3 and C5 seen on the surface of stationary phase promastigotes were not due to protein uptake/internalization. Together, these data demonstrate the differential deposition of complement proteins on the surfaces of logarithmic and stationary phase *L. chagasi* promastigotes. The data support a model wherein stationary phase *L. chagasi* promastigotes resist CML by limiting the deposition of C3 and its derivatives, which, in turn, limit surface levels of complement proteins (including C5 and C9) that form the lytic membrane attack complex.

Leishmania spp. are the etiologic agents of leishmaniasis, a disease group ranging in severity from self-healing cutaneous lesions to potentially fatal visceral infections. The heteroxenous life cycle of this protozoan parasite includes an intracellular stage wherein the amastigote parasite form infects and replicates within phagocytic cells of the vertebrate host, and an extracellular stage in which the promastigote form undergoes replication and maturation within the midgut lumen of the female sandfly vector. A blood feeding fly becomes infected via ingestion of blood that contains intracellular amastigotes. Ingested amastigotes quickly

differentiate into procyclic stage promastigotes that attach to the midgut wall and, over a period of 1 to several weeks, replicate and progress through a series of developmental stages, ultimately yielding the metacyclic promastigote stage. Metacyclic promastigotes are characterized by their lack of replication, infectivity to mammals, and resistance to lysis by the complement component of serum, while the other promastigote forms are sensitive to lysis by complement (Sacks and Perkins, 1984; Franke et al., 1985; Pinto-da-Silva et al., 2002; Rogers et al., 2002). A naive vertebrate host becomes infected when metacyclic promastigotes are inoculated into the vertebrate by a blood feeding female sandfly that bears a mature infection. The inoculated parasites are quickly opsonized via attachment of complement proteins (Dominguez and Torano, 1999; Dominguez et al., 2002) and then subjected to receptor-mediated phagocytosis by cells of the immune system, predominantly macrophages, but also including dendritic and other cells. Once inside the macrophage, parasites remain within a parasitophorous vacuole in which they differentiate into the replicative amastigote form.

Elucidating the process by which metacyclic parasites resist complement-mediated lysis (CML) is important for understanding the early events associated with successful transmission and infection into the vertebrate. Results from a number of CML studies indicate the required involvement of both general and specific interactions between the parasite surface and the blood. Surface expression of Major Surface Protein (MSP; also known as gp63), a zinc protease that in vitro cleaves C3b to the CML-inactive form iC3b, positively correlates with increased deposition of iC3b on the parasite surface (Brittingham et al., 1995). Surface lipophosphoglycan (LPG) of some *Leishmania* species increases 2-fold in length on metacyclic versus procyclic promastigotes (Saraiva et al., 1995) and is thought to either provide CML-protection by positioning functional membrane attack complexes (MAC) at a location too distal to penetrate the parasite membrane and cause cell lysis (McConville et al., 1992) or provide protection by allowing shedding of the attack complex (Puentes et al., 1990). In protein add-back experiments with *Leishmania chagasi*, parasite surface antigen (PSA; also known as gp46) has also been implicated in CML-resistance (Lincoln et al., 2004).

A great deal of CML research has capitalized upon the observation that differential sensitivity of *Leishmania* spp. promastigotes to CML during development can also be observed during development in vitro culture (Franke et al., 1985; Joshi et al., 1998; Noronha et al., 1998; Pinto-da-Silva et al., 2002). In a specific example of this phenomenon with *L. chagasi*, we showed that upon exposure to normal human serum (NHS), cultures of promastigotes are CML-sensitive when in the logarithmic growth phase but become CML-resistant upon transition to the stationary growth phase (Lincoln et al., 2004; Dahlin-Laborde et al., 2005, 2008). Additionally, we showed that the parasite population within cultures at the logarithmic growth phase is comprised almost completely of non-metacyclic promastigotes, whereas metacyclic form parasites predominate within cultures at stationary growth state (Lei et al., 2010). Given these recent characterizations of CML-phenotype and parasite form within cultured *L. chagasi*, the study reported here sought to extend our understanding of the interaction between *L. chagasi* and the complement component of human serum.

MATERIALS AND METHODS

Parasites

Infectious *L. chagasi* amastigotes (strain MHOM/BR/00/1669, originally isolated in Brazil from a patient with visceral leishmaniasis) were maintained in golden Syrian hamsters as previously described (Pearson and Steigbigel, 1980). Axenic promastigote cultures in supplemented modified minimum essential media (HOMEM) were initiated with

amastigotes isolated from hamster spleens and subsequently passaged as described (Pearson and Steigbigel, 1980; Zarley et al., 1991; Ramamoorthy et al., 1992; Dahlin-Laborde et al., 2005). Briefly, axenic promastigote culture densities increased throughout logarithmic culture phase until reaching a maximum concentration of 2×10^7 to 5×10^7 parasites ml^{-1} at about day 5 of culture (stationary phase); cultures were passaged by dilution to 1×10^6 parasites ml^{-1} 48 hr after reaching stationary phase. For the experiments described in this paper, logarithmic phase promastigotes were grown for 2 to 3 days in culture to a density between 1×10^7 and 2×10^7 parasites ml^{-1} , and stationary phase promastigotes were grown 6 to 7 days in culture to a density between 4×10^7 and 5×10^7 parasites ml^{-1} . All parasites were from cultures that were serially passaged for less than 5 wk.

Human serum and complement assays

Human serum was pooled from multiple naive donors, processed, and stored in aliquots at -80°C , and complement assays were performed as previously described (Dahlin-Laborde et al., 2005). Briefly, promastigotes were pelleted by centrifugation at $1,250\text{ g}$ for 5 min, resuspended in phosphate buffered saline (PBS), and plated in 96-well flat-bottom plates at a density of 3.5×10^6 promastigotes in $50\text{ }\mu\text{l}$. An equivalent volume of either PBS or 24% NHS (for a final concentration of 12% NHS) was added to each well; parasites were incubated at 37°C for 30 min. Parasites were then diluted 1:10 or 1:50 in PBS, and motile cells were counted on a hemocytometer. CML-sensitive cells were distinguishable as non-motile and granular “ghost cells” and were not counted as survivors. The percentage of cell survival was calculated as the ratio of motile cells present in incubations with, versus without, serum. Photomicrographs of parasites subjected to complement assays were taken by spotting $10\text{ }\mu\text{l}$ of assay mixture onto glass slides, air drying the slides, staining with a HEMA 3 stain set (Thermo Fisher Scientific, Waltham, Massachusetts), and visualizing via light microscopy.

Electron microscopy

Parasites were incubated in PBS supplemented with 0% or 12% in NHS for 30 min at 37°C , and then washed 2 times in cold (4°C) PBS via pelleting (at $1,200\text{ g}$, 2 min at 4°C). The resulting cell pellet was resuspended in cold (4°C) fixative (0.1 M cacodylate pH 7.2, 2% glutaraldehyde, 2% paraformaldehyde) and incubated for 24 hr at 4°C . Cells were washed 3 times in 0.1 M cacodylate buffer pH 7.2 and then incubated 1 hr at room temperature ($23\text{--}27^\circ\text{C}$) in buffer (1% osmium tetroxide, 0.1 M cacodylate, pH 7.2). Cells were then washed with de-ionized water and stained with aqueous 2% uranyl acetate for 1 hr. After dehydration in an ethanol/ acetone series, cells were infiltrated and embedded in epoxy resin then subjected to ultrathin sectioning. Ultrathin sections were stained in aqueous 2% uranyl acetate then imaged at 200 kV with a JEOL 2100 transmission and scanning electron microscope (Japan Electron Optics Laboratories, Peabody, Massachusetts). Images were captured with a $2\text{ k} \times 2\text{ k}$ Gatan (Warrendale, Pennsylvania) digital camera.

Flow cytometry analysis

Parasites were exposed to either 12% NHS or an equivalent volume of PBS (for 0% NHS) for 30 min at 37°C in 96-well U-bottom plates, then pelleted in the plate at $1,200\text{ g}$ for 2 min. Supernatants were aspirated and parasites resuspended in primary antibodies diluted 1:100 in fluorescence activated cell sorting (FACS) buffer (0.1% sodium azide and 0.1% bovine serum albumin in PBS) and incubated for 5 min. Primary polyclonal antisera used included fluorescein isothiocyanate (FITC)–anti-human C3 (MP Biomedicals, Solon, Ohio) and unlabeled goat anti-human C5 and C9 (Complement Technology, Tyler, Texas). Parasites were pelleted in the plate at $1,200\text{ g}$ for 2 min, supernatants were aspirated, and then parasites being stained for C5 or C9 were resuspended in FITC-mouse anti-goat secondary antibody diluted 1:100 in FACS buffer and incubated for 5 min. Parasites were pelleted in

the plate at 1,200 *g* for 2 min; supernatants were aspirated and parasites resuspended in FACS buffer for acquisition on a FACScan flow cytometer (Becton Dickinson, San Jose, California); and 50,000 events were collected per sample. Data were analyzed using FlowJo software (Tree Star, Ashland, Oregon).

C5 labeling and fluorescence microscopy

Purified C5 (Complement Technology) was labeled using the Molecular Probes Alexa Fluor 488 Monoclonal Antibody Labeling Kit (Life Technology, Carlsbad, California). Parasites were incubated in 12% NHS, 12% NHS supplemented with Alexa Fluor 488-labeled C5 (NHS + C5), or an equivalent volume of PBS for 30 min at 37 C. Parasites were then fixed with 4% paraformaldehyde in PBS, pH 7.4, for 10 min, pelleted (1,200 *g*, 2 min), washed twice with cold (4 C) PBS, and resuspended in 50 μ l PBS. Twenty microliters of the resuspended cells were applied to a microscope slide and mounted by adding 1 drop of ProLong[®] Gold antifade reagent with DAPI (Life Technologies). Fluorescence microscopy was performed using an Olympus IX-61 inverted microscope equipped with blue (DAPI) and green (Alexa Fluor 488) filter sets and a cooled CCD camera. Final grayscale images were prepared using ImageJ v1.36b image analysis software (Rasband, 1997).

Protein detection

Parasites were exposed to either 12% NHS or an equivalent volume of PBS (for 0% NHS) for 30 min at 37 C. Parasites were pelleted at 1,200 *g* for 2 min, resuspended in PBS, then pelleted and resuspended a second time. The total lysate from 1×10^6 parasites (including “ghost cells”) or purified complement proteins (Complement Technology) were separated via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electro–semi-dry–transferred to polyvinylidene difluoride membranes using standard buffers. Blots were probed with polyclonal antisera to either C3, C5, or C9 (Complement Technology) diluted 1:2,000. A horseradish peroxidase-conjugated anti-goat secondary antibody (Pierce Chemical, Rockford, Illinois) was used at a 1:20,000 dilution. Antibody binding was visualized via enzyme-linked chemiluminescence as per the manufacturer’s instructions (SuperSignal[®], Pierce). Densitometry values were determined using AlphaView software (Alpha Innotech, San Leandro, California).

Statistical analysis

Statistical analysis was performed using Prism 5 (GraphPad Software, La Jolla, California). Data were analyzed using a paired *t*-test. Differences were considered significant when *P* < 0.05.

RESULTS

Parasites progressively change in serum sensitivity, size, complexity, and C3 adsorption during axenic culture

To confirm the differential sensitivity of *L. chagasi* promastigotes to CML, parasites within cultures at logarithmic (2–3 days in culture) and stationary (day 7 in culture) growth phases were exposed to 12% NHS. Consistent with previous work, promastigote resistance to CML increased over time as culture density increased, with promastigotes present at stationary phase exhibiting significantly more resistance to CML than those present at logarithmic phase (Fig. 1A) (Lincoln et al., 2004; Dahlin-Laborde et al., 2005; Lei et al., 2010).

Visualization by light and electron microscopy (Figs. 1B, D) revealed dramatic morphological differences between promastigotes from the 2 culture phases following exposure to NHS. Within stationary phase cultures, the predominant promastigote developmental form was the metacyclic form (form determination was based upon

characteristics of morphology and serum resistance as previously described) (Lei et al., 2010). These metacyclic cells exhibited no change in morphology following incubation in NHS (Figs. 1B, D, labeled “M”). The population of cells within these same stationary phase cultures also included a much smaller number of non-metacyclic form cells; exposure of these non-metacyclic form cells to NHS resulted in “ghost cells,” i.e., cells exhibiting reduced intracellular complexity presumably due to loss of membrane integrity (Fig. 1D, labeled “G”). In sharp contrast to the situation observed with metacyclic form parasites, NHS exposure of non-metacyclic parasites from cultures at logarithmic growth phase had a dramatic effect on morphology, turning essentially all the cells into enlarged “ghost cells” (Figs. 1B, D). Consistent with a previous report (Lei et al., 2010), the parasite population within the cultures at logarithmic growth phase was more heterogeneous than at stationary growth phase, was comprised primarily of nectomonad and leptomonad form promastigotes, and included few procyclic or metacyclic form cells (data not shown).

The NHS-induced morphological changes to cells from cultures at logarithmic growth phase could also be detected via flow cytometry (Fig. 1C), with the parasites exhibiting a large decrease in their forward scatter (a measure proportional to size) as well as a smaller but distinguishable increase in their side scatter (a measure proportional to intracellular complexity/granularity). Promastigotes from cultures at stationary growth phase exhibited similar forward (size) and side (complexity) scatter profiles regardless of exposure to NHS (Fig. 1C).

The amount of complement component C3 deposited upon the promastigote surface during exposure to NHS was evaluated via flow cytometric analysis of intact (non-permeabilized) cells using a fluorescent antibody against human C3. C3 deposition (Fig. 1E) correlated inversely with promastigote CML-resistance (Fig. 1A). The highest levels of C3 surface accumulation were observed on logarithmic phase promastigotes at days 3 and 4 in culture. C3 deposition continued to decrease over time, with stationary phase promastigotes (day 7 in culture) adsorbing the least amount of C3.

Stationary phase parasites exhibit decreased surface deposition of complement proteins C3, C5, and C9 as compared with logarithmic phase parasites

Additional experiments using flow cytometric analysis of non-permeabilized cells demonstrated that more logarithmic phase *L. chagasi* promastigotes bind C3 on their surface than do stationary phase promastigotes. The results from a single but representative experiment are shown in Figure 2A, while the graphical representations of results from 18 independent experiments are depicted in Figures 2B and C. As shown in Figure 2B, 81.2% versus 29.3% of the population of parasites within cultures at logarithmic versus stationary phase were positive for C3. Moreover, the amount of C3 deposited on the surface of the logarithmic *L. chagasi* parasites, as measured in units of mean fluorescence intensity, was significantly greater than that bound to stationary phase parasites (Fig. 2C, left). To determine whether *L. chagasi* promastigotes differentially bind other complement components that normally bind after C3, a flow cytometric analysis of C5 and C9 surface deposition was performed. Consistent with the C3 deposition results, a higher percentage of logarithmic phase promastigotes bound C5 and C9 on their surface as compared with stationary phase promastigotes (Fig. 2A, B). The amount of C5 and C9 detected on the surface of logarithmic phase parasites was also significantly increased as compared with stationary phase parasites (Fig. 2C, middle and right panels).

Distribution of complement proteins on/in logarithmic and stationary phase promastigotes

Using fluorescent microscopy and NHS supplemented with fluorescently labeled complement protein C5, we assessed the general distribution of complement proteins present

on, or in, serum-exposed promastigotes. Microscopic evaluation revealed that C5 localized in a fairly uniform pattern to the plasma membrane of promastigotes from logarithmic phase cultures, while membranes of promastigotes from stationary phase cultures were absent of C5 staining (Fig. 3A). When cells exposed to NHS supplemented with labeled C5 protein were assessed by flow cytometry without the use of any other fluorochromes, the results were equivalent to those reported in Figure 2 for cells exposed to non-supplemented NHS and then labeled through antibody tagging. Specifically, log cells exhibited high C5 levels and stationary cells exhibited low C5 levels (Fig. 3B). These data were comparable to equivalent analyses using fluorescently labeled C3 (data not shown).

Western blot assessment of complement proteins C3, C5, and C9 on parasites at logarithmic and stationary culture phases

Internalization of complement proteins or membrane recycling by stationary phase promastigotes could explain the differential complement deposition patterns observed between *L. chagasi* parasites at logarithmic and stationary culture phases (Figs. 1–3). To assess these possibilities, total cell lysates derived from equivalent numbers of parasites exposed to either PBS or NHS were analyzed via Western blotting. Consistent with our flow cytometric and fluorescent microscopy evaluations, and relative to serum-incubated stationary phase promastigotes, high levels of complement proteins C3, C5, and C9 were detected in lysates of logarithmic phase *L. chagasi* promastigotes incubated in NHS (Fig. 4A). Similar patterns of deposition were observed when multiple parasite cultures were analyzed (Fig. 4B). Densitometric analysis revealed a 2- to 2.5-fold increase in the amount of C3, C5, and C9 complement proteins present in the lysates from parasites at logarithmic culture phase as compared with parasites at stationary culture phase (Fig. 4C). Together, these Western blot data indicate that (as compared with logarithmic phase cells) the relatively low amounts of complement proteins detectable on the surface of stationary phase promastigotes (Figs. 1–3) are unlikely to be due to the parasite having internalized those proteins.

DISCUSSION

The work on *L. chagasi* presented here demonstrates that (1) the promastigotes that predominate in logarithmic growth phase cultures bind much greater levels of the complement proteins C3, C5, and C9 than do the metacyclic promastigotes that predominate in stationary growth phase cultures; (2) the bound complement proteins primarily localize proximal to the plasma membrane and are most likely to be on the outer cell surface; and (3) resistance to CML associates with reduced levels of bound C3, C5, and C9. Considering that C3 binding and activation are required for assembly of the MAC (comprised of C5b, C7, C8, and C9) and subsequent cell lysis, our data support a model in which *L. chagasi* metacyclic cells resist CML via reduced levels of cell-associated C3 that results in reduced levels of MAC.

Previous studies of various *Leishmania* species characterized the existence of multiple CML-resistance mechanisms and indicated substantial variation among species in the mechanism primarily employed by particular species. One mechanism that is similar to the “low C3 binding” model proposed here for *L. chagasi* has been proposed for *Leishmania mexicana* based upon experiments showing greater C3 deposition on the surface of CML-sensitive versus CML-resistant parasites (Russell, 1987). Proteolytic inactivation of C3 is a second CML-resistance mechanism and was experimentally supported by studies demonstrating the presence of CML-resistance even in the absence of reduced C3 deposition on the parasite surface (Joiner, 1988). For example, with *Leishmania donovani* promastigotes, CML-resistant and -sensitive cells bind equivalent amounts of C3 (Wozencraft et al., 1986; Wozencraft and Blackwell, 1987; Puentes et al., 1989); however,

the CML-resistant cells possess protease activity that degrades surface-bound C3 into a form (iC3b) that does not support the downstream events required for MAC formation (Puentes et al., 1989). Shedding of complement proteins, including MAC, is a third mechanism of complement resistance; studies with *Leishmania major* promastigotes demonstrate that CML-resistant cells activate complement but then shed the C5b-C9 MAC (Puentes et al., 1990). It has been speculated (Puentes et al., 1990) that such shedding may be enabled in those species that build a thicker glycan coat during metacyclogenesis, e.g., *L. major*, whose surface LPG coat increases about 2-fold in thickness on metacyclic versus procyclic promastigotes (Saraiva et al., 1995). The increased surface LPG thickness may also directly confer CML-resistance by causing MAC to be assembled so far from the cell membrane that the parasite is not lysed (Sacks et al., 1990).

A number of proteins and other surface macromolecules on the surface of *L. chagasi* are known to undergo stage-specific expression or modification and are likely to be involved in the mechanism by which metacyclic promastigotes display lower levels of complement proteins. The primary surface macromolecule (LPG) on metacyclic promastigotes has far fewer glucose substitutions than on non-infectious parasite forms (Soares et al., 2002). Yao et al. (2010) used a proteomics approach to identify a number of proteins that are upregulated in metacyclic promastigotes. Two predominant surface glycoproteins, Major Surface Protease (Wilson et al., 1993) and Promastigote Surface Antigen (Beetham et al., 1997, 2003), also undergo stage-specific regulation and have been implicated to be involved in CML-resistance. Experimental overexpression of Major Surface Protease resulted in cells that possessed increased proteolytic activity against C3 (Brittingham et al., 1995), while overexpression of Promastigote Surface Antigen resulted in partial CML-resistance (Lincoln et al., 2004).

The C3 Western blotting analysis presented in Figure 4 for both logarithmic and stationary phase *L. chagasi* parasites demonstrates the presence of both C3b and a cleaved alpha chain, 2 of the C3-derived products formed when C3 is fixed on a cell surface. These analyses also demonstrate that serum-exposed parasites from both culture phases acquire cell-associated iC3b, a C3 derivative that functions as an opsonogen, but that fails to support MAC assembly. These data signify that *L. chagasi* promastigotes can inhibit C3-mediated MAC formation even as they maintain the opsonogenic activity conferred by the presence of C3 derivatives. In a number of *Leishmania* species, the opsonogenic activity of C3 derivatives has been shown to be important for gaining proper entry into macrophages via complement receptors (Mosser and Edelson, 1985) and for influencing parasite survival within the macrophage (Mosser and Edelson, 1987). We surmise that the levels of C3 products fixed to parasites from stationary phase cultures, while low relative to that seen in logarithmic phase cells, are sufficient to facilitate internalization by macrophages via opsonophagocytosis and intracellular survival.

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Figure 1.

Parasites progressively change in serum sensitivity, size, complexity, and C3 adsorption during axenic culture. (A) Parasite culture density (“Density”; closed circles) and serum sensitivity (“Survival”; open bars) were monitored daily. Parasites were exposed to 12% normal human serum (NHS) diluted in phosphate buffered saline (PBS), or to PBS alone, for 30 min at 37 C. Survival was determined by via hemacytometer-enumeration of intact, motile parasites. Data are pooled from 8 independent experiments. (B–E) Parasites from cultures at logarithmic (“Log,” 2–3 days) and stationary (“Stat,” 6–7 days) growth phases were exposed to 0% or 12% NHS for 30 min at 37 C and then (B) Giemsa stained and

photographed at 100 $\times$, or (C) assayed via flow cytometry to determine forward scatter and side scatter, or (D) visualized via transmission electron microscopy, or (E) stained with fluorescein isothiocyanate (FITC)–anti-human C3 polyclonal antibody and assessed by flow cytometry. The additional images contained within the bottom left panel in (B) depict “ghost cells” (see “Results”). (E) Gray or black lines represent parasites incubated in 0% or 12% NHS, respectively. X-axis (labeled “C3”) represents fluorescence, with intensity increasing from left to right. Y-axis (labeled “Percent”) represents the parasite count at each value of fluorescence divided by the parasite count at the fluorescence value that contained the largest number of parasites, multiplied by 100.

Figure 2.

Stationary phase parasites exhibit decreased surface deposition of complement proteins C3, C5, and C9 as compared with logarithmic phase parasites. Promastigotes from cultures at logarithmic ("Log," 2–3 days) and stationary ("Stat," 6–7 days) growth phases were exposed to 0% or 12% normal human serum (NHS) for 30 min at 37 C. To assess C3, C5, and C9 deposition on the parasite surface, parasites were stained with either fluorescein isothiocyanate (FITC)–anti-human C3 polyclonal antisera or unlabeled anti-human C5 or C9 polyclonal antisera followed by a FITC–anti-goat secondary antibody and assayed via a fluorescence activated cell sorting (FACScan) flow cytometer. (A) Histograms depict C3, C5, or C9 staining, with gray or black lines representing parasites incubated in 0% or 12% NHS, respectively. X-axis (labeled "C3," "C5," or "C9") represents fluorescence, with intensity increasing from left to right. Y-axis (labeled "Percent") represents the parasite count at each value of fluorescence divided by the parasite count at the fluorescence value that contained the largest number of parasites, multiplied by 100. Histograms are representative of 18 independent experiments. (B) Summary of the percentage of parasites positive for C3, C5, or C9 from all experiments. (C) Summary of the mean fluorescence intensity (MFI) of C3, C5, or C9 on parasites from all experiments. An asterisk represents a statistically significant difference between Log and Stat at $P < 0.05$.

A **B** **Figure 3.**

Distribution of complement proteins on/in logarithmic and stationary phase promastigotes. Promastigotes from cultures at logarithmic (“Log”) and stationary (“Stat”) growth phase were exposed to either phosphate buffered saline (PBS), 12% normal human serum (NHS), or 12% NHS containing C5 labeled with Alexa Fluor 488 ($A^{488}C5$) for 30 min at 37 C. (A) Cells were imaged to reveal nuclei (DAPI—top row) and $A^{488}C5$ (bottom row). Photomicrographs were taken at 100 $\times$ and are representative of 3 independent experiments.

(B) A portion of the parasite–NHS mixture imaged in (A) was analyzed via flow cytometry. Histograms depict $A^{488}C5$ labeling of parasites incubated in PBS alone (gray lines) or in 12% NHS (black lines). X-axis (“C5 Fluorescence”) represents fluorescence with intensity increasing from left to right. Y-axis (labeled “Percent”) represents the parasite count at each value of fluorescence divided by the parasite count at the fluorescence value that contained the largest number of parasites, multiplied by 100. Histograms are representative of 3 independent experiments.

Figure 4.

Western blot assessment of complement proteins C3, C5, and C9 on parasites at logarithmic and stationary culture phases. Logarithmic and stationary phase promastigotes were exposed to 0% or 12% normal human serum (NHS) for 30 min at 37 C. (A) Total lysates derived from 1×10^6 parasites (at logarithmic, "L," or stationary, "S") or purified complement protein subunits (C3, C3b, iC3b, C5, C5b,6, C9, SC5b-9) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) then detected by Western blotting. Labels on left indicate specific subunits of the complement protein standards; brackets on the right denote the size-region examined among experiments that are depicted in Panel B. (B) Parasite lysates (as described in (A)) from 6 independent experiments (labeled "1" to "6") were separated by SDS-PAGE and detected by Western blotting using polyclonal antisera against C3 or monoclonal antibodies specific for C5 or C9. An asterisk indicates the C3

band that was assessed by densitometry in Panel C. (C) Fold increase in C3, C5, or C9 observed on logarithmic versus stationary cells among the 6 experiments depicted in (B).