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Monoclonal antibodies that recognize distinct epitopes of the macrophage type three complement receptor differ in their ability to inhibit binding of *Leishmania* promastigotes harvested at different phases of their growth cycle

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SUMMARY

The macrophage receptor CR3 has been shown by several investigators to be involved in the binding of *Leishmania* promastigotes to host macrophages. This receptor is known to recognize iC3b and to mediate direct lectin-like attachment of particles such as yeast zymosan. In the present study, two anti-CR3 monoclonal antibodies, M1/70 and 5C6, which ligate different epitopes of murine CR3, have been used in conjunction with sodium salicyl hydroxamate (Saha; inhibits covalent ester linkages of C3 to an activator surface) to block binding of *L. donovani* and *L. major* promastigotes harvested at different phases of their growth cycle. M1/70 inhibited all promastigote binding. 5C6 and Saha blocked in parallel only the binding of peanut agglutinin (PNA)-positive late log and early stationary phase parasites. These results suggest that the binding PNA-positive parasites to CR3 is iC3b-mediated, while entry of the more infective PNA-negative late stationary phase promastigotes into host macrophages may involve direct lectin-like binding to CR3.

INTRODUCTION

Several studies (Blackwell *et al.*, 1985; Mosser & Edelson, 1985; Wilson & Pearson, 1987) have demonstrated that monoclonal antibodies (mAbs) directed against the type three complement receptor (CR3) block the binding and ingestion of *Leishmania* promastigotes by host macrophages. This receptor is known to recognize the inactivated form of complement factor 3 (iC3b) (Beller, Springer & Schreiber, 1982) and to mediate direct lectin-like attachment of yeast zymosan to human polymorphonuclear leucocytes (Ross, Cain & Lachmann, 1985). In our earlier studies (Blackwell *et al.*, 1985) we demonstrated that the anti-CR3 mAb M1/70, Fab–anti-C3, and the nucleophile sodium salicyl hydroxamate (Saha), which inhibits covalent ester linkages of C3 (Sim *et al.*, 1981) to the parasite surface (Russell, 1987), all inhibited the serum-independent uptake of *L. donovani* promastigotes by murine resident peritoneal macrophages to equivalent degrees. This suggested that, as had been demonstrated earlier for the binding of zymosan to murine macrophages (Ezekowitz *et al.*, 1984), binding of promastigotes to CR3 was mediated by locally secreted macrophage complement proteins. This was supported by our subsequent demonstration of Saha-inhibitable deposition of macrophage-derived C3 on the promastigote surface using EM–gold techniques (Wozencraft, Sayers & Blackwell, 1986). A role for CR3 in direct lectin-like binding of the parasite to macrophages could not, however, be excluded. Studies by Sacks and co-workers (Sacks & Perkins, 1984; Sacks, Hieny & Sher, 1985) for *L. major* and Howard, Sayers & Miles (1987) for *L. donovani*, demonstrating a strong association between infectivity of logarithmic and stationary (metacyclic) phase promastigotes and developmentally regulated cell surface carbohydrate/antigenic changes measurable in terms of the ability of the parasite to bind the lectin peanut agglutanin (PNA), also suggest that the parasite side of the interaction may have been ill-defined in the earlier macrophage studies. In our own laboratory, for example, *L. donovani* promastigotes have been maintained by routine weekly subculture following initial transformation from amastigotes isolated from hamster spleen. Parasites used in macrophage-binding experiments (Blackwell *et al.*, 1985; Wozencraft *et al.*, 1986) were harvested anywhere between Days 3 and 7 from subcultures 3–17.

In the present study we have attempted to define both the parasite and the macrophage side of the interaction between *Leishmania* promastigotes and CR3 more precisely. *L. donovani* and *L. major* promastigotes harvested at different phases of their growth cycle have been characterized according to their ability to bind PNA, and inhibition of binding to murine macrophages compared using two different anti-CR3 mAbs, as well as the nucleophile Saha. The results suggest that, while the binding of

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PNA-positive promastigotes to macrophages involves Saha-inhibitable complement-dependent binding to CR3, the entry of the more infective PNA-negative organisms into host macrophages is mediated by direct lectin-like binding to CR3.

MATERIALS AND METHODS

Parasite isolation and culture in vitro

Amastigotes of *L. donovani* (LV9) and *L. major* (LV39) were isolated from hamster spleens or BALB/c footpads as described previously (Channon, Roberts & Blackwell, 1984; Alexander & Vickerman, 1975), seeded at 5×10^6 amastigotes/ml in RPMI containing 20% heat-inactivated fetal calf serum (FCS), 20 mM L-glutamine, 10 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and 75 μg/ml gentamicin, and allowed to transform at 24° for 72 hr. Primary subcultures (5 × 100 ml per parasite strain) were then seeded with 1×10^6/ml motile flagellated transformed promastigotes obtained by differential centrifugation at 650 *g*, non-transformed parasites remaining in the supernatant. Cultures were counted daily using a Helber bacteriological counting chamber. For use in experiments parasites were harvested by centrifugation at 650 *g* and washed (twice) in RPMI without FCS. The proportion of promastigotes binding the lectin PNA was determined by fluorescence microscopy following incubation with tetramethylrhodamine isothyonate (TRITC)-labelled PNA (Sigma L-3766; Poole, Dorset) at 50 μg/ml for 5–10 min. Parasite viability was monitored using acridine orange and ethidium bromide as described (Channon *et al.*, 1984).

Inhibition of promastigote binding to macrophages

Promastigotes harvested at Days 2, 4, 6, 9, 11 and 13 of culture *in vitro* were used to infect murine resident peritoneal macrophages (5×10^6 promastigotes/coverslip culture) *in vitro*. Twenty-minute binding inhibition assays using anti-CR3 mAbs, M1/70 (Springer *et al.*, 1979) and 5C6 (Rosen & Gordon, 1987), and Saha were carried out as described previously (Blackwell *et al.*, 1985). The rat anti-mouse CR3 mAb M1/70 was prepared from hybridoma supernatants by ammonium sulphate precipitation and dialysis against phosphate-buffered saline. A stock solution at 0·4 mg/ml total protein was used at 1 : 5 in inhibition assays. Control P3NS1 myeloma supernatants treated in the same manner produced no inhibition of parasite binding. The rat anti-mouse CR3 mAb 5C6 (2·8 mg/ml) was purified from ascites by sodium sulphate precipitation, anion exchange chromatography on DEAE–Sephacel and gel filtration chromotography on S-300 Sephadex as described previously (Rosen & Gordon, 1987) and used at a concentration of 7 μg/ml in binding assays. Saha was prepared and used as described elsewhere (Blackwell *et al.*, 1985).

Promastigote infectivity in vivo

Promastigotes harvested at Days 2, 4, 6, 9, 11 and 13 were also used to infect mice either i.v. via the lateral tail vein (2×10^7/mouse, 6–8-week-old male C57BL/10ScSn) or s.c. into the footpad (10^6/mouse; 6–8-week-old female BALB/c). The sex and strain of mice was chosen according to previous observations (Mock *et al.*, 1985) of susceptibility to infection with *L. donovani* versus *L. major* given i.v. or s.c. Mice infected i.v. were killed after 14 days and parasite loads determined from liver impression smears ($\log_{10}$LDU/liver) as described elsewhere (Bradley & Kirkley, 1977). For s.c. infected mice, footpad thickness was measured weekly for 6 weeks using a direct reading vernier calliper. To allow direct comparison of s.c. infectivity of promastigotes harvested at different times, only the Week 4 footpad thicknesses are shown.

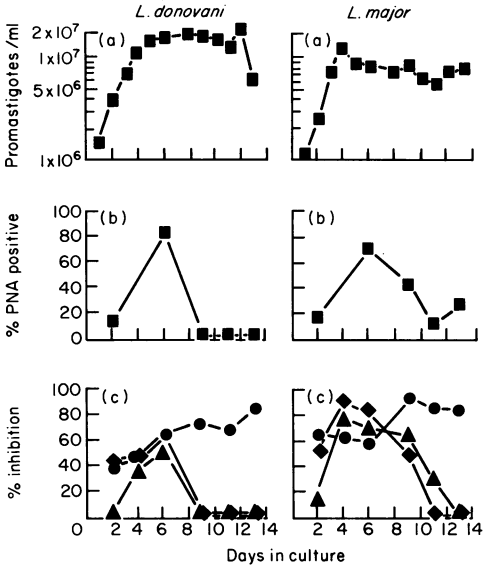

Figure 1. Parasites per ml (a), percentage PNA-positive parasites (b), and inhibition of parasite binding to murine resident peritoneal macrophages (c) for parasites harvested on different days after seeding *in vitro* cultures with 1×10^6/ml freshly transformed *Leishmania donovani* LV9 (left panel) or *L. major* LV39 (right panel). Results for (a) and (b) show one representative culture maintained throughout the experiment. Parasites from replicate cultures were used in the macrophage-binding inhibition assays employing the anti-CR3 mAbs, M1/70 (●) and 5C6 (▲), and the nucleophile Saha (♦). Results show mean percentage inhibition for each inhibitor for the four possible comparisons between duplicate control and duplicate treated coverslips examined at each time point. Duplicates fell within ±10% of each other. Parallel results were obtained for *L. major* NIH173 as those shown for LV39. Similar results have been obtained in two repeats of the complete experiment.

RESULTS

Growth curves, PNA-binding capacity and macrophage-binding inhibition

Figure 1 shows the different growth curves obtained for *L. donovani* versus *L. major* promastigotes over 13 days of culture, their ability to bind the lectin PNA, and the percentage inhibition of parasite binding to macrophages obtained with the two anti-CR3 mAbs and Saha. PNA positivity (Fig. 1) peaked (70–80%) for both parasite species at Day 6 of culture *in vitro*, parasites remaining >75% viable through 11 days of culture. For *L. donovani* only, viability dropped to <60% on Day 13. Patterns of macrophage binding inhibition were similar for the two parasite species. Inhibition with M1/70 was high (>40%) throughout and increased to >90% after Day 6 when late stationary phase PNA-negative parasites appeared in the cultures. Inhibition with 5C6 followed closely the curve for PNA positive parasites in the cultures, the drop in 5C6 inhibition observed in late stationary phase cultures being paralleled by a decrease in Saha inhibition.

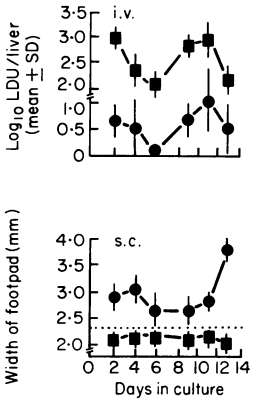

Figure 2. Infectivity *in vivo* for *L. donovani* LV9 (■) and *L. major* LV39 (●) promastigotes harvested at different times in the growth cycle (days in culture) and inoculated i.v. (2×10^7 parasites/mouse; three to four mice per group), or s.c. in the footpad (10^6 parasites/mouse; four to five mice per group). Parallel results were obtained for *L. major* NIH173 as those shown for LV39.

Promastigote infectivity *in vivo*

Figure 2 compares i.v. and s.c. infectivity for *L. donovani* and *L. major* promastigotes harvested at various times during their growth cycle. *L. donovani* promastigotes failed to produce any increase in footpad thickness over 6 weeks following s.c. inoculation. Similarly, infectivity of *L. major* promastigotes given i.v. was much reduced (approximately 100-fold) compared to numerically equivalent inocula of *L. donovani* promastigotes. Promastigotes of both species thus maintained their expected tissue tropism. However, as with previous studies (Da Silva & Sacks, 1987), peak PNA positivity in the promastigote cultures *in vitro* (Day 6, Fig. 1) coincided with the lowest infectivity *in vivo* (Day 6, Fig. 2) for both species of parasite inoculated i.v. or s.c.

DISCUSSION

Results presented here demonstrate dramatic differences in the ability of the two anti-CR3 mAbs, M1/70 and 5C6, and Saha to block macrophage binding and ingestion of *L. donovani* and *L. major* promastigotes harvested at different phases of the growth cycle. Most striking was (i) the absolute correlation between 5C6 inhibition and the expression of the PNA lectin binding site on the parasite surface, with a parallel drop in Saha inhibition as PNA-negative parasites appeared in the cultures; and (ii) a high level of M1/70 inhibition observed throughout which increased as the number of PNA-negative parasites increased in the cultures. In previous studies both M1/70 (Beller *et al.*, 1982) and 5C6 (Rosen & Gordon, 1987) have been shown to inhibit binding of iC3b-coated erythrocytes to CR3. In the case of 5C6, inhibition with whole IgG and its $F(ab')_2$ fragment but not Fab indicates (Rosen & Gordon, 1987) that this ability to block EiC3b rosetting relies on steric hindrance rather than direct binding to the iC3b binding site of CR3. The direct correlation between Saha and 5C6 inhibition observed here suggests that 5C6 IgG similarly blocked complement-dependent binding of PNA-positive *Leishmania* promastigotes to the iC3b binding site of CR3. The alternative hypothesis (Russell & Wright, 1988) that *Leishmania* promastigotes bind directly to the iC3b binding site of CR3 via an RGD sequence in the major surface glycoprotein GP63 is inconsistent with our functional data showing inhibition with Saha. The mAb M1/70 was also capable of blocking binding mediated by Saha-inhibitable covalently bound iC3b on PNA-positive parasites to a degree similar to the 5C6 inhibition. The increase in M1/70 inhibition observed for PNA-negative parasites together with a failure to observe Saha inhibition suggest, however, that M1/70 also blocks epitopes on CR3 other than the iC3b binding site. That the two anti-CR3 mAbs recognize distinct epitopes on CR3 was demonstrated clearly in the earlier studies of Rosen & Gordon (1987). 5C6 but not M1/70 was shown to block CR3-mediated adhesion to bacterial plastics with the epitope recognized by 5C6 being more resistant to proteolysis than the M1/70 epitope. Our data now demonstrate that M1/70, but not 5C6, is a potent inhibitor of direct complement-independent attachment of PNA-negative *Leishmania* promastigotes to murine CR3.

The precise correlation between 5C6 inhibition and the presence of PNA-positive parasites suggests that the acceptor site for the covalently bound iC3b mediating binding to CR3 may coincide with the PNA binding site on the parasite surface. Studies by Da Silva & Sacks (1987) suggest that this site resides in a subterminal galactose residue of a major surface lipophosphoglycan molecule (Turco *et al.*, 1987) shown by the earlier studies of Handman & Goding (1985) to be a ligand for parasite attachment to macrophages. More recently Puentes *et al.* (1988) have focused on the role of the lipophosphoglycan as a major C3 acceptor molecule. Their results show that PNA-positive parasites activate the alternative complement pathway and bind C3 by O-ester linkages inhibitable by nucleophiles like Saha, 10–15% of the total C3 bound being in the form iC3b. PNA-negative parasites, on the other hand, activate the classical pathway with most C3 bound non-covalently to the parasite surface. These results are consistent with our hypothesis that PNA-positive parasites bind to CR3 by Saha-inhibitable covalently bound iC3b, whereas PNA-negative parasites do not. Loss of the PNA binding site and transformation to metacyclic forms apparently results from further glycosylation of the lipophosphoglycan molecule (Sacks & Da Silva, 1987). Whether this form of the molecule mediates the direct lectin-like attachment of PNA-negative parasites to CR3 awaits further study. Such a role for the LPG molecule would, however, parallel observations of direct binding of bacteria via their lipopolysaccharide molecules to CR3 and other members of the LFA-1 family of adhesion promoting receptors (Wright & Jong, 1986), and has broader implications for how the parasite might influence T-cell independent priming/activation of macrophages (Blackwell *et al.*, 1988).

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