MAJOR ARTICLE

Antibodies Directed against *O*-Acetylated Sialoglycoconjugates Accelerate Complement Activation in *Leishmania donovani* Promastigotes

Sumi Bandyopadhyay,[1] Mitali Chatterjee,[2] Tanusree Das,[1] Suman Bandyopadhyay,[1] Shyam Sundar,[3] and Chitra Mandal[1]

[1]Immunobiology Division, Indian Institute of Chemical Biology, and [2]Department of Pharmacology, Institute of Postgraduate Medical Education and Research, Kolkata, and [3]Department of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India

Background. An enhanced presence of 9-*O*-acetylated sialoglycoconjugates *(9-O*-AcSGs) triggers the alternate pathway (AP) in Indian visceral leishmaniasis (VL). Antibodies directed against these epitopes are present in high titers. The biological relevance of these antibodies, with regard to activation of the classical pathway (CP), was investigated.

Methods. Complement activators were affinity purified, complement activation via the CP, AP, and lectin-mediated complement pathway was measured by use of an anti-C3 radio-binding assay, and the number of C3 molecules was quantitated by Scatchard analysis. Cell death induced via the complement pathways was measured by use of MTT (tetrazolium salt 3- [4, 5-dimethylthiazol-2-yl] -2, 5-diphenyltetrazolium bromide) assay, and uptake of propidium iodide (PI) was measured by flow cytometry.

Results. Anti–*O*-AcSGs from both healthy donors and patients with VL elicited C3 deposition as early as 3 min, which triggered parasite lysis, as demonstrated by use of MTT assay and corroborated by the high rate of uptake of PI. Analysis of complement activation by mannan-binding lectin and C-reactive protein demonstrated their negligible contribution during the 3-min time frame.

Conclusions. Anti–*O*-AcSGs were identified as an important source of CP activation under normal physiological conditions, suggesting that they play a role in conferring host protection against parasite infection.

Leishmaniasis is endemic in ~88 countries, resulting in a tenth of the world's population being at risk of infection [1]. In 2001, the disease caused a loss of 2.4 million disability-adjusted life-years and 59,000 deaths [2]. The visceral form of the disease is mainly caused by *Leishmania donovani, L. chagasi,* or *L. infantum,* and >500,000 new cases of visceral leishmaniasis (VL) occur each year [3]. Approximately 50% of the world's cases of VL occur in the Indian subcontinent, and ~90% of Indian patients with VL live in Bihar [4].

The disease process is initiated by promastigote inoculation into the host macrophage [5], whereby the host defense responds by activating its complement system, culminating in cleavage of the third complement component (C3) and followed, ultimately, by the lytic pathway [6, 7]. This promastigote-C3 opsonization is mediated mainly by 3 pathways—namely, the classical pathway (CP), the alternate pathway (AP), and the lectin-mediated pathway, leading to the formation of a cytolytic membrane attack complex (C5b-9). The involvement of anti-leishmanial IgM, a complement activator minimally present in normal human serum (NHS), causes parasite agglutination, CP activation, and parasite killing [8, 9]. Additionally, parasite-specific IgG induces lysis of *Leishmania* [10] and *Trypanosoma* organisms [11].

By use of Achatinin-H, a 9-*O*-acetylated sialic acid (9-*O*-AcSA)–binding lectin [12], an enhanced presence of 9-*O*-acetylated sialoglycans (9-*O*-AcSGs) in an $\alpha 2 \rightarrow 6$ linkage on hematopoietic cells from patients with VL has been demonstrated [13–15] concomitant with an enhanced presence of antibodies directed against them [16]. This enhanced presence of 9-*O*-AcSGs on eryth-

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Reprints or correspondence: Dr. Chitra Mandal, Immunobiology Div., Indian Institute of Chemical Biology, 4 Raja S.C. Mullick Rd., Jadavpur, Kolkata 700 032, India (cmandal@iicb.res.in or chitra_mandal@yahoo.com).

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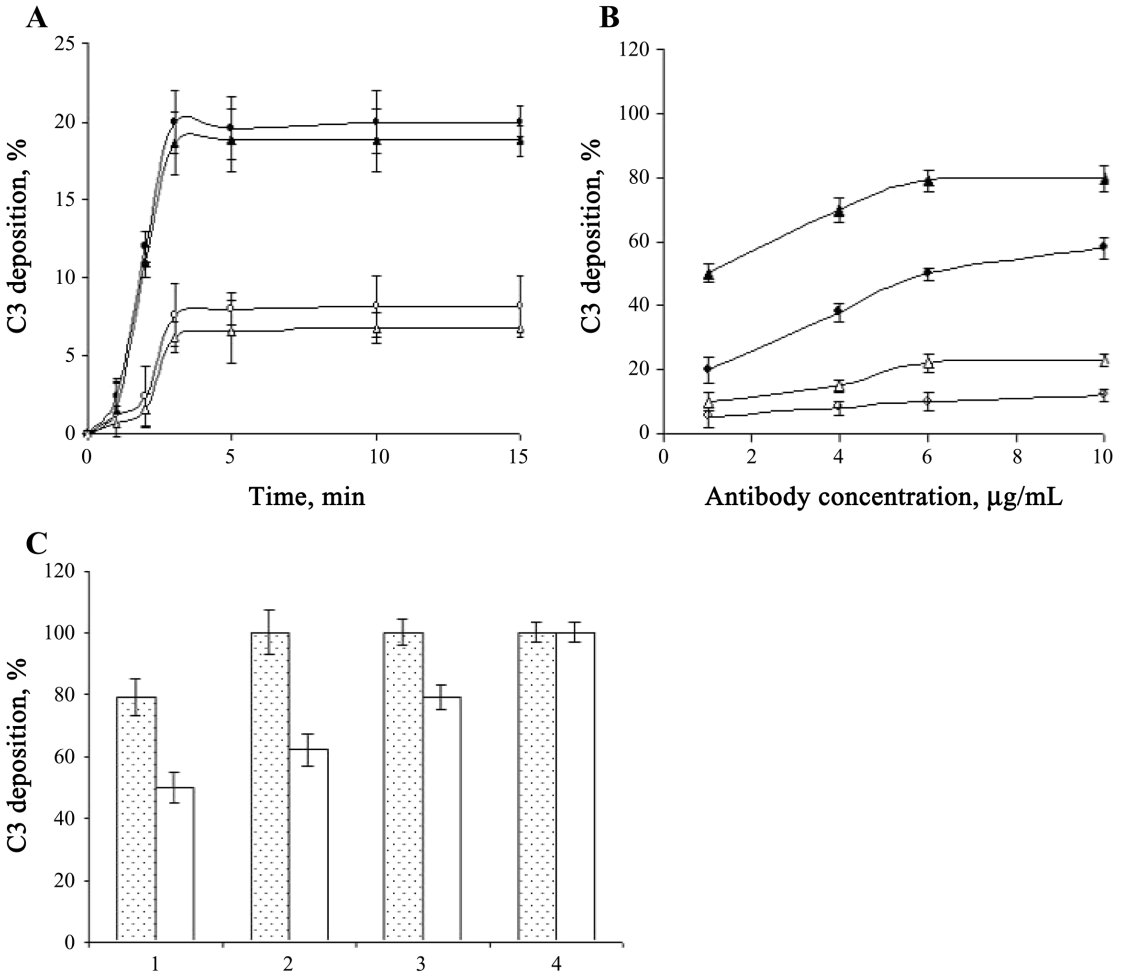

Figure 1. Classical complement pathway activation induced by anti–*O*-acetylated sialoglycoconjugate $(AcSG)_{\text{normal human serum (NHS)}}$ vs. that induced by total antibody_{NHS}. The parasites were incubated in the presence of adsorbed NHS (25%), as a source of complement, and several complement activators. C3 deposition was measured by use of ^{125}iodine–anti-C3 monoclonal antibody, as described in Materials and Methods. *A,* Fixed concentrations (6 μg/mL) of different antibodies—namely, anti–*O*-AcSG IgM_{NHS} (●), anti–*O*-AcSG IgG_{NHS} (▲), total IgM_{NHS} (○), and total IgG_{NHS} (△)—were selected to study C3 deposition induced by the antibodies, individually, at different periods. *B,* Comparison of C3 deposition within 3 min, triggered by anti–*O*-AcSG IgM (▲ and △) and anti–*O*-AcSG IgG (● and ○), purified from serum samples from patients with visceral leishmaniasis (VL) (▲ and ●), and by NHS (△ and ○), in different concentrations. *C,* Comparison of C3 deposition within 3 min, triggered by 6 μg/mL anti–*O*-AcSG IgM_{VL} *(bars with dots)* and anti–*O*-AcSG IgG_{VL} *(white bars),* in 4 strains—MHOM/IN/83/AG83 *(1),* MHOM/IN/90/GE1 *(2),* NS1 *(3),* and NS2 *(4)*—isolated from patients with VL.

rocytes was associated with an enhanced susceptibility to AP-mediated hemolysis [17], but their role both in health and in disease, in relation to CP activation, has not been studied. To the best of our knowledge, this is the first report demonstrating that, within a very short time frame (3 min), anti–*O*-AcSGs, even under normal physiological conditions, could trigger CP-mediated C3 (CP-C3) deposition on promastigotes, causing their lysis, whereas other complement pathways play a negligible role.

MATERIALS AND METHODS

Parasites and Culture

Four *L. donovani* strains—MHOM/IN/83/AG83, MHOM/IN/90/GE1, NS1, and NS2—isolated from patients with VL were cultured at 22°C in M-199 medium supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco-BRL) and gentamycin (200 μg/mL), until they reached the stationary phase. In parallel, promastigotes were also cultured in M-199 medium supplemented with completely defined serum-replacement TCH (10%) (ICN Biomedicals) [18]. To ensure that only virulent promastigotes were used, we routinely checked their capacity to infect the hamster. Informed consent was obtained from either the patient or the guardian, and the study received approval from the institutional human ethical committee.

Complement Activators

Purification of antibodies. Antibodies (IgM and IgG) directed against 9-*O*-AcSGs were affinity purified from both

Table 1. Comparison of classical, alternate, C-reactive protein (CRP), and mannan-binding lectin (MBL) complement pathways for triggering promastigote (GE1)–C3 opsonization via C3 deposition, within 3 min.

Incubation with (time)[a]		
Complement activator (30 s)[b]	Complement (120 s)	C3 deposition on promastigotes, %
Anti–*O*-AcSG IgM_{VL}	Ads-NHS	100
Anti–*O*-AcSG IgG_{VL}	Ads-NHS	62
Anti–*O*-AcSG IgM_{NHS}	Ads-NHS	23
Total $antibody_{NHS}$	Ads-NHS	6
Anti–*O*-AcSG IgG_{NHS}	Ads-NHS	10
Anti–*O*-AcSG IgM_{VL}[c]	Ads-NHS	25
Anti–*O*-AcSG IgG_{VL}[c]	Ads-NHS	20
PBS	…	8
PBS	…	8
PBS	Ads-NHS	7
PBS	HI-NHS	5
PBS	VLS + EGTA	28
CRP (− EDTA)	Ads-NHS	29
CRP (+ 10 mmol/L EDTA)	Ads-NHS	3
MBL	Ads-NHS	19
MBL (+ 10 mmol/L EDTA)	Ads-NHS	9
MBL (+ 30 mmol/L mannose)	Ads-NHS	8

NOTE. AcSG, acetylated sialoglycoconjugate; Ads-NHS, adsorbed normal human serum [NHS]; HI, heat-inactivated NHS for 30 min at 56°C; VL, visceral leishmaniasis; VLS, VL serum, used to investigate alternate pathway by blocking complement pathway with EGTA; …, absence of complement, but presence of anti–*O*-AcSG.

[a] Cells were incubated with complement activators for 30 s and subsequently incubated with complement (Ads-NHS; 25%) for 120 s.

[b] EDTA was added along with complement activators.

[c] Esterase-treated promastigotes.

pooled serum samples (12 mL) from 3 patients with VL (anti–*O*-$AcSG_{VL}$) and pooled serum samples (20 mL) from 3 healthy individuals (anti–*O*-$AcSG_{NHS}$) [16]. Total antibodies (IgM and IgG) were also similarly affinity purified. All these antibodies were used as complement activators for analysis of the CP.

Purification of mannan-binding lectin (MBL) and C-reactive proteins (CRPs). MBL [19] and CRP [20] were purified from pooled human plasma (40 mL) and serum (VLS) (~4 mL) samples from patients with VL, respectively.

Preparation of adsorbed NHS (Ads-NHS) as the Source of Complement

NHS (diluted 1:50 in PBS) was adsorbed by incubating it with promastigotes (1×10^9) for 30 min on ice, followed by centrifugation at 11,000 *g* for 3 min. The supernatant (Ads-NHS; 25%) was reabsorbed twice with fresh parasites and filtered, and the clear serum was used as the source of complement [8].

Tool for Measuring C3 Deposition

The antihuman C3α-chain monoclonal antibodies (MAbs) SIM 27–49 (anti-C3) were raised against the C3α domain of complement component purified from mouse ascites [8] and iodinated with Na^{125} iodine (I) [21].

Complement Activation, Judged by Analysis of C3 Deposition on Promastigotes

Triggering of CP was analyzed by use of Ads-NHS (25%), as the source of complement, and various types of antibodies, MBL, and CRP, as the source of complement activators. *L. donovani* promastigotes (5×10^6) were incubated with 6 μg/mL antibodies (complement activators) from healthy donors or patients with VL, for 30 s at 37°C, and were immediately washed twice with PBS (0.02 mol/L), by centrifugation (1157 *g* for 5 min). Cells were then resuspended in Ads-NHS (25%; 100 μL) and incubated further, for 2 min at 37°C, followed by 2 centrifugation washes. Finally, the promastigotes were resuspended in cold PBS (0.2 mL) containing FCS (2.5%) and NaN_3 (0.05%), designated as "PFS," to terminate the reaction. The parasites were then incubated with ^{125}I–anti-C3 MAb (3×10^5 cpm) for 1 h on ice. After 2 washes, the amount of C3 deposited by individual complement activators was determined by measuring the radioactivity incorporated in C3–anti-C3 complexes, by use of a gamma counter (IC4702A; Electronic Corporation of India). Binding of MBL and CRP to promastigotes was prevented by addition of EDTA (10 mmol/L). For confirmation of the direct involvement of 9-*O*-AcSA in triggering the CP, promastigotes were pretreated with *O*-acetyl esterase, an enzyme capable of cleaving the 9-*O*-acetyl group from sialic acid [22, 23], and C3 deposition was determined.

Quantitation of CP-C3 Molecules on Promastigotes, by Scatchard Analysis

To quantitate CP-C3 deposition on promastigotes, cells (5×10^6) were allowed to be opsonized by the individual complement activators in the presence of Ads-NHS. They were then washed, resuspended in PFS containing increasing concentrations of ^{125}I–anti-C3 MAb, and equilibrated on ice ($\geq$3 h), and bound C3 was determined as described above. The association constant was calculated by use of a direct linear plot and Scatchard analysis [24].

Detection of Complement-Mediated Cytolysis of Promastigotes

MTT (tetrazolium salt 3- [4, 5-dimethylthiazol-2-yl] -2, 5-diphenyltetrazolium bromide) assay. Promastigotes (5×10^6) were incubated with different complement activators for 30 s then incubated with Ads-NHS for 2 min (at 37°C in microtiter plates), were washed with RPMI 1640 medium (without phenol red) containing 10% FCS, and were incubated with MTT solution (100 μg/50 μL) for 3 h at 37°C. The crystals formed were dissolved in DMSO (450 μL), and optical density (OD) was measured at 570 nm in a spectrophotometer [25]. Cell

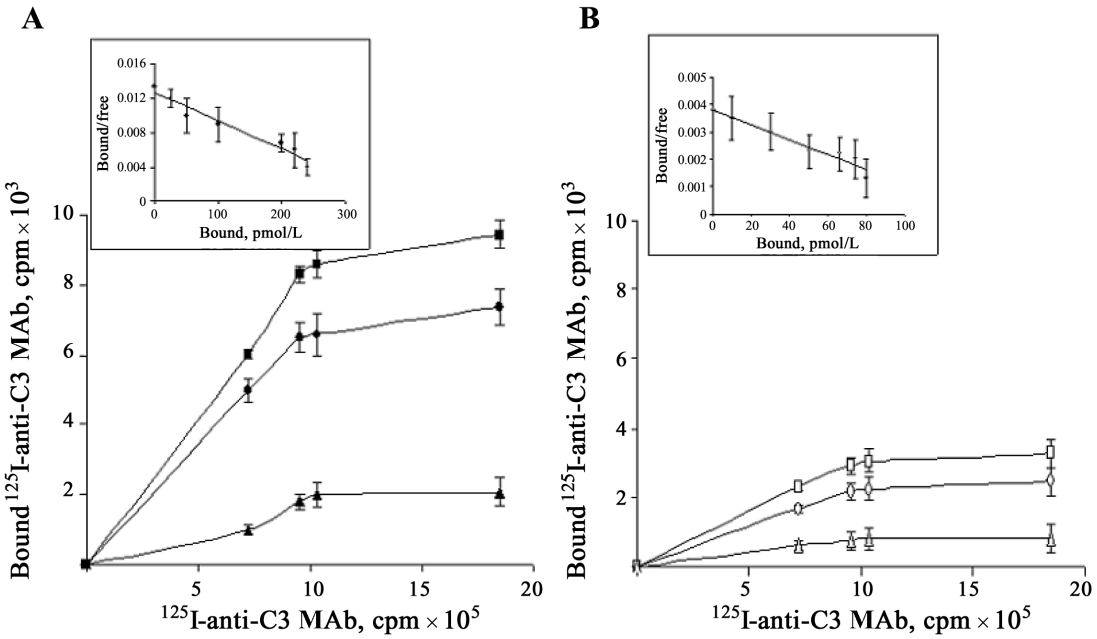

Figure 2. Estimation of bound C3 with anti-C3 monoclonal antibody (MAb) on promastigotes. Promastigotes (5×10^6) were incubated with equal amounts (6 μg/mL) of anti–*O*-acetylated sialoglycoconjugate (AcSG) $IgM_{visceral\ leishmaniasis\ (VL)}$ (●; *A*) or anti–*O*-AcSG IgG_{VL} (○; *B*), in the presence of adsorbed normal human serum (25%), and increasing amounts of ^{125}iodine (I)–anti-C3 MAb, as described in Materials and Methods. Specific binding (● and ○) was calculated by subtracting the difference between total binding (■ and □) and nonspecific binding (▲ and Δ) and was plotted against various amounts of ^{125}I–anti-C3 MAb. Results are expressed as mean ± SD from triplicate experiments. Insets are Scatchard plots of the binding of ^{125}I–anti-C3 MAb with deposited C3 molecules on parasites, induced by anti–*O*-AcSG IgM_{VL} (●) or anti–*O*-AcSG IgG_{VL} (○). cpm, counts per minute.

death (percentage) was calculated as follows: $100-[100 \times (OD_{sample}-OD_{100\%\ lysis}/OD_{0\%\ lysis}-OD_{100\%\ lysis})]$.

Flow-cytometric analysis of uptake of propidium iodide (PI). Parasites (5×10^6) were incubated with an optimum dose (6.0 μg/mL) of anti–*O*-AcSG IgM_{NHS} or anti–*O*-AcSG IgM_{VL} or with total purified IgM_{NHS}, in the presence of Ads-NHS, for 3 min at 37°C, as described above; promastigote lysis was detected by uptake of PI, by use of a FACSCalibur flow cytometer (BD Pharmingen), in accordance with the manufacturer's protocol. For determination of maximum lysis, promastigotes were treated with acetone: methanol (1:1) for 30 min. This served as a positive control, whereas cells without any treatment served as a negative control for PI.

To correlate the expression of 9-*O*-AcSG on promastigotes with its subsequent propensity toward anti–*O*-AcSG IgM_{VL}–mediated complement lysis, the expression of 9-*O*-AcSG on promastigotes was monitored by use of fluorescein isothiocyanate–Achatinin-H, a 9-*O*-AcSG binding probe [12]. The expression of 9-*O*-AcSG was detected on promastigotes grown in serum-replacement medium, as well as on promastigotes treated with *O*-acetyl esterase. Subsequently, the appearance of 9-*O*-AcSGs within 3 min of transfer to medium supplemented with 5% and 10% FCS was also monitored. After the acquisition of 9-*O*-AcSGs, anti–*O*-AcSG IgM_{VL}–mediated complement lysis was monitored on promastigotes at all of the above stated conditions, by measuring uptake of PI.

Complement Activation Triggered via the AP

For AP activation, promastigotes (5×10^6) were resuspended in VLS or NHS (25%; 100 μL) containing EGTA (7 mmol/L) and were incubated for different periods (2–15 min) at 37°C. Promastigotes were resuspended in PFS containing ^{125}I–anti-C3 MAb and incubated for 1 h on ice, and C3 deposition was determined as described above.

Complement Activation of Promastigotes by MBL and CRP

The extent of MBL- or CRP-mediated complement activation was determined initially by incubating promastigotes (5×10^6) with either MBL or CRP (0–60 μg/mL) in Tris-buffered saline (0.01 mol/L) buffer containing bovine serum albumin (1.0%), NaN_3 (0.1%), and $CaCl_2$ (5 mmol/L), for different periods (0–100 min) on ice, washing them to remove the unbound MBL or CRP, and subsequently incubating them with Ads-NHS at 37°C [26]. Similarly, an optimum dose of purified MBL (20 μg/mL) or CRP (50 μg/mL) was also incubated with promastigotes for 1 min, which were washed to remove the unbound MBL or CRP, and then incubated with Ads-NHS (25%) for 2 min at 37°C, and C3 deposition was measured.

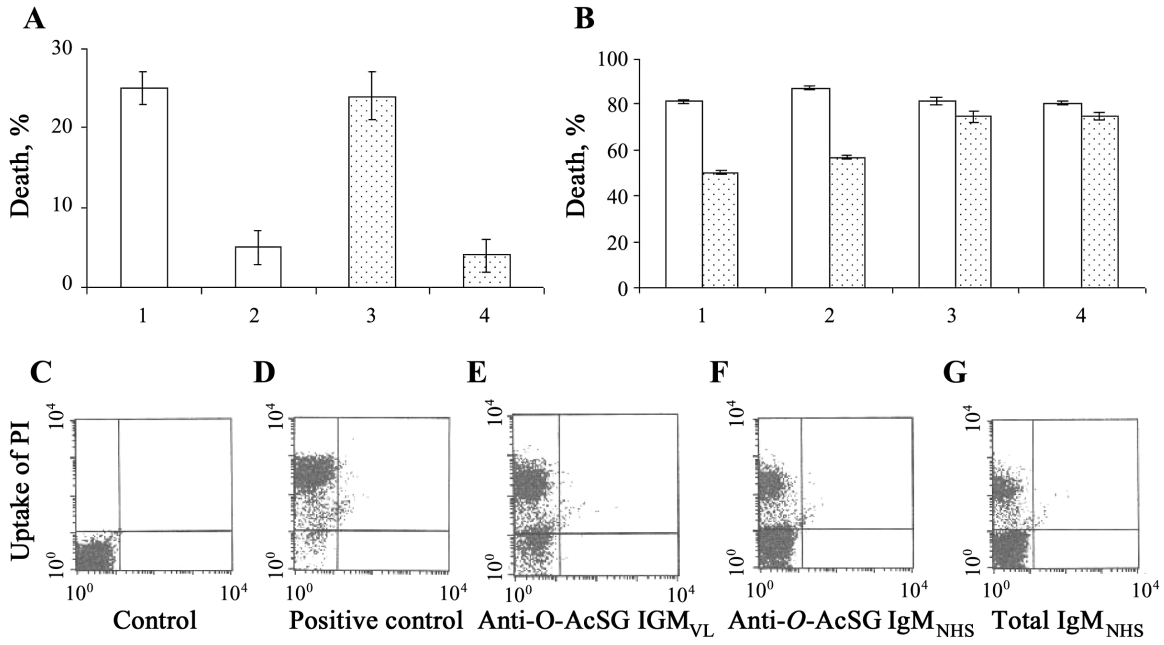

Figure 3. Induction of promastigote lysis due to C3 deposition induced by anti–*O*-acetylated sialoglycoconjugate (AcSG). *A,* Representative profile of cell death (%), detected by use of MTT (tetrazolium salt 3- [4, 5-dimethylthiazol-2-yl] -2, 5-diphenyltetrazolium bromide) assay, using 6 μg/mL purified anti–*O*-AcSG IgM$_{\text{normal human serum (NHS)}}$ *(1)* and anti–*O*-AcSG IgG$_{NHS}$ *(3)* vs. total antibodies$_{NHS}$ IgM *(2)* and total antibodies$_{NHS}$ IgG *(4),* in the presence of adsorbed NHS (Ads-NHS) (25%), within 3 min at 37°C. The cells were washed and incubated for another 3 h at 37°C with MTT (100 μg/50 μL) and were processed as described in Materials and Methods. *B,* Comparison of cell death in MHOM/IN/83/AG83 *(1),* MHOM/IN/90/GE1 *(2),* NS1 *(3),* and NS2 *(4)* isolated from patients with visceral leishmaniasis (VL), by use of a fixed concentration (6 μg/mL) of different antibodies: anti–*O*-AcSG IgM$_{VL}$ *(white bars)* and anti–*O*-AcSG IgG$_{VL}$ *(bars with dots). C–G,* Promastigote lysis analyzed by uptake of propidium iodide (PI) (flow-cytometric analysis) triggered by Ads-NHS (25%) along with 6 μg/mL purified anti–*O*-AcSG IgM$_{VL}$ *(E),* anti–*O*-AcSG IgM$_{NHS}$ *(F),* and total IgM$_{NHS}$ *(G),* compared with the absence of complement activators *(C)* and maximal uptake of PI (97%), after methanol:acetone treatment *(D).*

Statistical Analysis

For individual sets of experiments, results are expressed as mean ± SD and are representative of 2–3 experiments.

RESULTS

Purification of Complement Activators

Total IgM (1.8 mg) and IgG (1.2 mg) was purified from 20 mL of pooled NHS containing 800 mg of total serum proteins, of which anti–*O*-AcSG IgM and IgG were 0.05 and 0.04 mg, respectively. In 12 mL of pooled VLS containing 480 mg of total serum proteins, anti–*O*-AcSG IgG$_{VL}$ and anti–*O*-AcSG IgM$_{VL}$ were 0.13 and 0.11 mg, respectively. Therefore, the physiological concentration of total antibodies$_{NHS}$ and anti–*O*-AcSG antibodies were 60–90 and 11–13 μg/mL, respectively. MBL and CRP purified from VLS (40 and 4 mL, respectively) yielded 0.20 and 0.09 mg of protein, respectively (their levels in NHS were negligible).

Activation of CP by Anti–*O*-AcSG IgM$_{NHS}$ and Anti–*O*-AcSG IgG$_{NHS}$

To establish the optimum dose of anti–*O*-AcSG antibodies capable of inducing a maximum level of C3 deposition, a dose-response curve was established (figure 1*B*). At both 6 and 11 μg/mL (physiological concentration), equal levels of C3 deposition (percentage) were observed; accordingly, an optimal dose of 6 μg/mL may be considered to be near the physiological concentration.

Analysis of CP-C3 deposition on promastigotes, by use of 6 μg/mL anti–*O*-AcSG IgG$_{NHS}$ and anti–*O*-AcSG IgM$_{NHS}$ in a 2-stage incubation assay, revealed that C3 deposition occurred as early as 3 min, indicating direct involvement of 9-*O*-AcSG glycotopes (figure 1*A*). Their CP-C3 deposition was 3-fold higher than that of total antibodies$_{NHS}$, indicating that anti–*O*-AcSG IgG$_{NHS}$ and anti–*O*-AcSG IgM$_{NHS}$ are major contributors.

To confirm that the *O*-acetylated glycotope was critical, promastigotes were pretreated with *O*-acetylesterase; subsequently, C3 deposition induced by anti–*O*-AcSG IgG$_{NHS}$ and anti–*O*-AcSG IgM$_{NHS}$ decreased from 10% to 3% and from 23% to 7%, respectively, validating the idea that the 9-*O*-AcSA moieties are vital for eliciting complement activation.

Induction, within 3 Min, of a 5-Fold Higher CP-C3 Deposition by Anti–*O*-AcSG$_{VL}$ than by Anti–*O*-AcSG$_{NHS}$

The anti–*O*-AcSG$_{VL}$ produced in high titers during active VL [15] triggered C3 deposition that was 5-fold higher than that

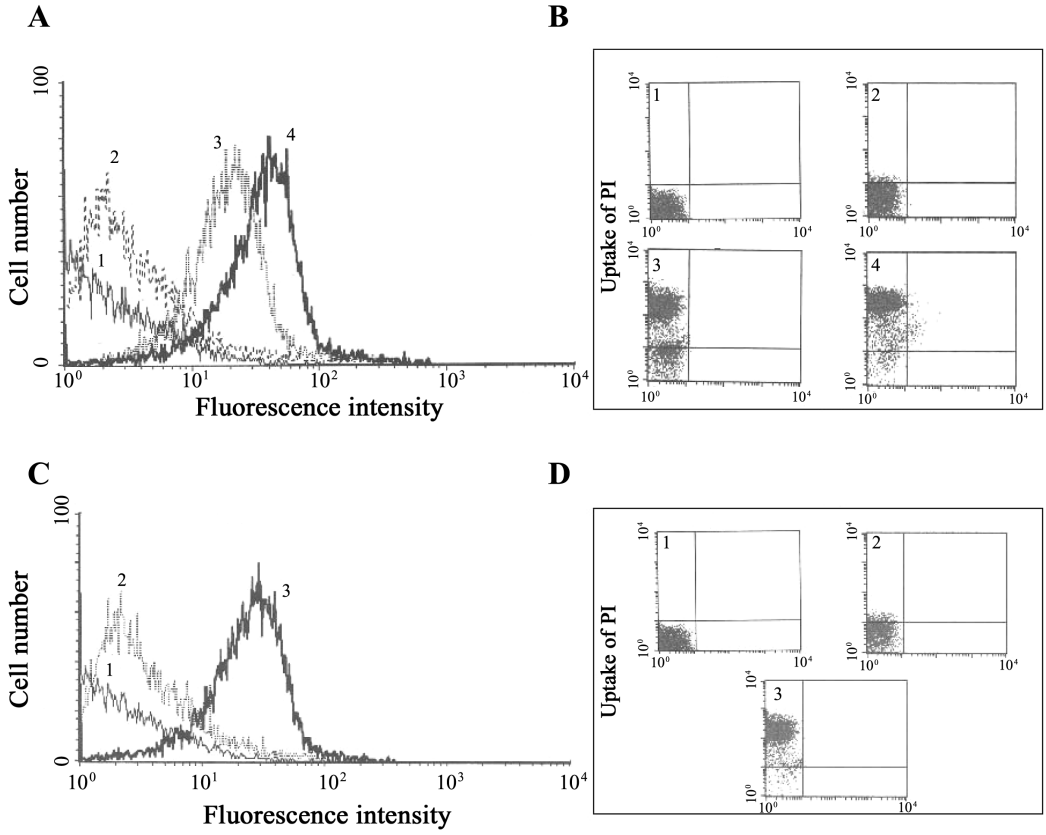

Figure 4. Correlation between 9-*O*-acetylated sialoglycoconjugate (AcSG) on promastigotes and its subsequent death, triggered by anti–*O*-AcSG $IgM_{visceral\ leishmaniasis\ (VL)}$ (6 μg/mL)–mediated complement lysis. *A,* Histogram analysis of expression of 9-*O*-AcSG on promastigotes grown in serum-replacement medium *(2)* and subsequent transfer to 5% fetal calf serum (FCS) *(3)* and 10% FCS *(4),* compared with controls *(1),* monitored by binding with fluorescein isothiocyanate (FITC)–Achatinin-H. *B,* Dot-plot analysis of anti–*O*-AcSG IgM_{VL}–mediated complement lysis, judged by uptake of propidium iodide (PI) by promastigotes grown in serum-replacement medium *(2)* and when transferred to 5% FCS *(3)* and 10% FCS *(4),* compared with controls *(1). C,* Histogram analysis of expression of 9-*O*-AcSG on esterase-treated promastigotes *(2)* and subsequent transfer to 10% FCS *(3),* compared with controls *(1),* by use of FITC–Achatinin-H. *D,* Dot-plot analysis of anti–*O*-AcSG IgM_{VL}–mediated complement lysis, judged by uptake of PI by esterase-treated promastigotes *(2)* and when transferred to 10% FCS *(3),* compared with controls *(1).*

triggered by anti–*O*-$AcSG_{NHS}$ (figure 1*B*). At a concentration of 6.0 μg/mL, both anti–*O*-AcSG IgM_{VL} and anti–*O*-AcSG IgG_{VL} showed significant C3 deposition on all 4 *L. donovani* strains (AG83, GE1, NS2, and NS1) (mean ± SEM, 94% ± 5% and 72% ± 10%, respectively) (figure 1*C*).

To confirm that C3 deposition was due to CP activation, cells were incubated with anti–*O*-AcSG IgM, anti–*O*-AcSG IgG, or Ads-NHS alone. All lacked the ability to trigger C3 deposition on promastigotes (C3 deposition, 8%, 8%, and 7%, respectively) (table 1). Although the addition of EDTA prevented MBL and CRP binding, the persistently high rate of C3 deposition indicated their minimal involvement.

Quantitation of C3 Molecules Deposited, within 3 Min, on Promastigotes by Anti–*O*-AcSG via CP Activation

To quantitate the C3 molecules deposited on promastigotes, 6.0 μg/mL anti–*O*-AcSG IgM_{VL} or anti–*O*-AcSG IgG_{VL} was added in the presence of complement. With anti–*O*-AcSG IgM_{VL}, the number of C3 molecules bound per cell was 3×10^5 (figure 2*A*), which was 2.5-fold higher than that with anti–*O*-AcSG IgG_{VL} (figure 2*B*). To evaluate the specific nature of binding, a 50-fold excess of anti-C3 was added; the apparent association constants (K_a) for C3 deposition triggered by anti–*O*-AcSG IgM_{VL} and anti–*O*-AcSG IgG_{VL} were 3.3×10^8 mol/L^{-1} and 2.7×10^8 mol/L^{-1}, respectively.

Increased Complement-Mediated Cytolysis of Promastigotes Induced by Anti–*O*-AcSG

MTT assay. Within the 3-min time frame, 6 μg/mL anti–*O*-AcSG IgG_{NHS} and anti–*O*-AcSG IgM_{NHS} induced a 5.6- and 6.0-fold higher level of cell death, respectively, compared with that induced by total $antibodies_{NHS}$ (28% vs. 5% with IgM and 24% vs. 4% with IgG, respectively) (figure 3*A*).

Disease-specific anti–*O*-AcSG IgM_{VL} and anti–*O*-AcSG IgG_{VL} induced an even higher level of C3 deposition on promastigotes, resulting in a greater level of cell lysis. Cell death (percentage)

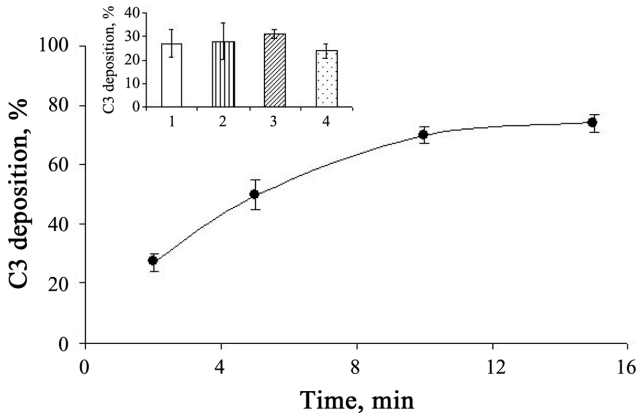

Figure 5. Kinetics of C3 deposition triggered via the alternate pathway. Promastigotes (AG83;●) were incubated at 37°C with EGTA (10 mmol/L)– and $MgCl_2$ (7 mmol/L)–treated serum samples (25%; 100 μL) from patients with visceral leishmaniasis (VL), at different time points (0–15 min), and C3 deposition was measured as described in Materials and Methods. *Inset,* Comparison of C3 deposition within 3 min, in MHOM/IN/83/AG83 *(1),* MHOM/IN/90/GE1 *(2),* NS1 *(3),* and NS2 *(4)* isolated from patients with VL.

triggered by anti–*O*-AcSG IgM_{VL}, compared with that triggered by anti–*O*-AcSG IgG_{VL}, was higher in 4 strains (AG83, GE1, NS1, and NS2; 81%, 87%, 81%, and 80% vs. 50%, 57%, 75%, and 75%, respectively) (figure 3*B*). However, MBL and CRP, when used as complement activators under similar conditions, caused negligible parasite lysis (2% and 3%, respectively; data not shown).

Flow-cytometric analysis of uptake of PI. Cell death triggered by anti–*O*-AcSG IgM via the CP, within the 3-min time frame, was corroborated by uptake of PI. The PI^+ promastigote population triggered by anti–*O*-AcSG IgM_{NHS} (figure 3*F*) and complement was 1.8-fold higher than that triggered by total IgM_{NHS} (42% vs. 23%) (figure 3*G*). The PI^+ promastigote population of anti–*O*-AcSG IgM_{VL} (figure 3*E*) triggered by complement was 2.0-fold higher than that triggered by anti–*O*-AcSG IgM_{NHS} (5% vs. 42%, respectively), indicating that cells were undergoing necrosis.

Interestingly, Achatinin-H binding increased from 8% to 81% or 93% when promastigotes grown in serum-replacement medium were transferred to medium supplemented with 5% or 10% FCS, respectively (figures 2–4). Similarly, enhanced Achatinin-H binding (85%) was also found when esterase-treated promastigotes were transferred to 10% FCS containing medium (figures 3 and 4*C*), demonstrating acquisition of 9-*O*-AcSGs from serum within 3 min. Subsequently, this enhanced presence of 9-*O*-AcSGs on promastigotes correlated well with their capacity to undergo lysis triggered by anti–9-*O*-AcSG IgM_{VL}, as confirmed by the high rates of uptake of PI (86%, 96%, and 97%, respectively) (figures 3 and 4).

Time Required for AP Activation versus That Required for CP Activation

Although C3 deposition on promastigotes triggered via the AP and CP followed a similar trend in all 4 parasite strains studied, a notable difference was the time needed for optimum deposition. In contrast to that of CP, maximal activation of AP occurred at 15 min, irrespective of the source of complement used (figure 5). At 3 min, the C3 deposition mediated via the AP was relatively lower in all 4 strains (AG83, GE1, NS1, and NS2; 27%, 28%, 31%, and 24%, respectively) (figure 5).

Negligible Role of MBL- and CRP-Mediated Complement Activation in *L. donovani* Promastigotes within the 3-Min Time Frame

Complement activation induced by MBL or CRP, under normal physiological conditions, was negligible within the 3-min time frame, possibly due to their presence in low concentrations. However, to obtain a measurable quantity of C3 deposition on promastigotes within 3 min, a 20-fold higher concentration of MBL (20 μg/mL) was needed, and it required 60 min (figure 6*A* and 6*B*). Within the initial 3 min, C3 deposition by MBL (20 μg/mL) on AG83 and GE1 was 32% and 19%, respectively (figure 6*C*). The specificity of this binding and its ability to trigger C3 deposition was confirmed by the near abolition of C3 deposition in the presence of mannose and EDTA (8% and 9%, respectively) (table 1).

Similarly, the optimal concentration for maximum C3 deposition by CRP was 50 μg/mL, which was 10-fold higher than the normal physiological concentration, and it required 30 min. Similar to MBL, CRP also showed a minimal contribution during the 3-min incubation (figure 6*A* and 6*B*). Within the initial 3 min, C3 deposition on AG83 and GE1 was 13% and 29%, respectively (figure 6*C*). As CRP-mediated complement activation requires Ca^{+2}, addition of EDTA caused abolition of CRP binding; C3 deposition was reduced to 3%.

Comparison of Different Complement Pathways Triggered in *L. donovani* Promastigotes within 3 Min

A comparative analysis of all 4 complement pathways of promastigotes revealed that maximum C3 deposition was triggered by anti–*O*-AcSG IgM_{VL} (mean ± SEM, 94% ± 5%), closely followed by anti–*O*-AcSG IgG_{VL} (mean ± SEM, 72% ± 10%). CRP, MBL, and AP played negligible roles (mean ± SEM, 22% ± 9%, 24% ± 5%, and 27% ± 1.4%, respectively) (figure 7).

DISCUSSION

CP-C3 deposition on promastigotes is an extremely rapid process; the reaction is completed within 2–3 min, during which >90% of C3 is activated via the CP by natural antibodies present in NHS [8]. A very specific anti–*O*-$AcSG_{NHS}$ is reported to be

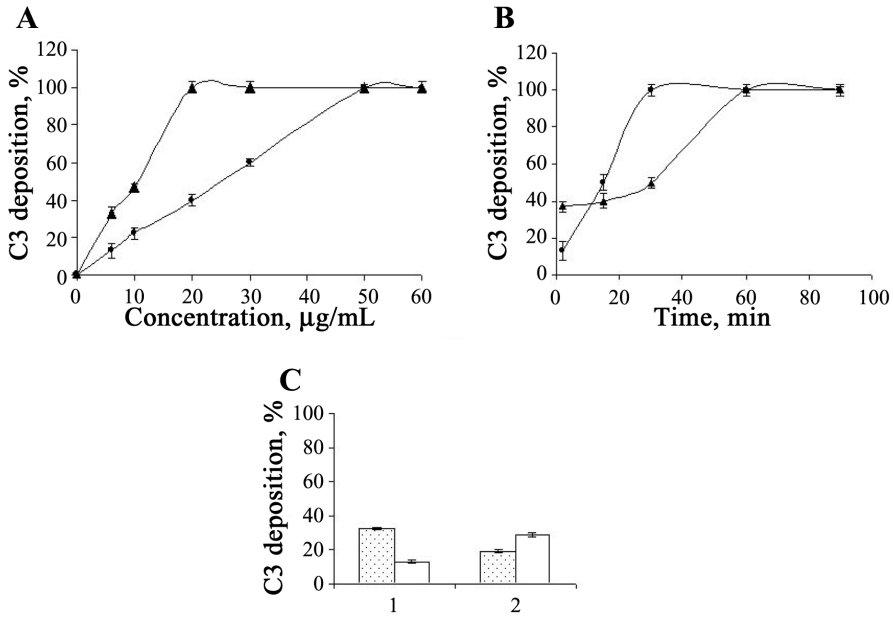

Figure 6. C3 deposition triggered by mannan-binding lectin (MBL) and C-reactive proteins (CRPs). *A,* C3 deposition triggered by different concentrations (0–60 μg/mL) of MBL (▲) or CRP (●) purified from serum samples from patients with visceral leishmaniasis (VL). *B,* MBL (20 μg/mL) or CRP (50 μg/mL) incubated for 0–60 min with promastigotes, in the presence of adsorbed normal human serum. C3 deposition was measured as described in Materials and Methods. *C,* Comparison of C3 deposition triggered by the optimum concentration of MBL (20 μg/mL) *(white bars)* or CRP (50 μg/mL) *(bars with dots)* within 3 min, in MHOM/IN/83/AG83 *(1)* and MHOM/IN/90/GE1 *(2)*.

present in low titers in response to 9-*O*-AcSGs minimally present on hematopoietic cells from healthy donors [15]. These antibodies are directed against 9-*O*-AcSAα2→6GalNAc glycotopes. The levels of anti–*O*-AcSG are drastically enhanced in patients with active VL [16]. In the present investigation, we aimed to study the contribution of anti–*O*-AcSG present naturally in NHS, as well as that induced in patients with VL, in relation to CP activation on promastigotes, within this narrow time frame. The C3 depositions induced by MBL and CRP were also compared. The major achievement of this investigation was the establishment of anti–*O*-$AcSG_{NHS}$ as a potent CP activator under normal physiological conditions. We therefore hypothesize that anti–*O*-$AcSG_{NHS}$ plays a significant role in conferring host protection against *Leishmania* infection.

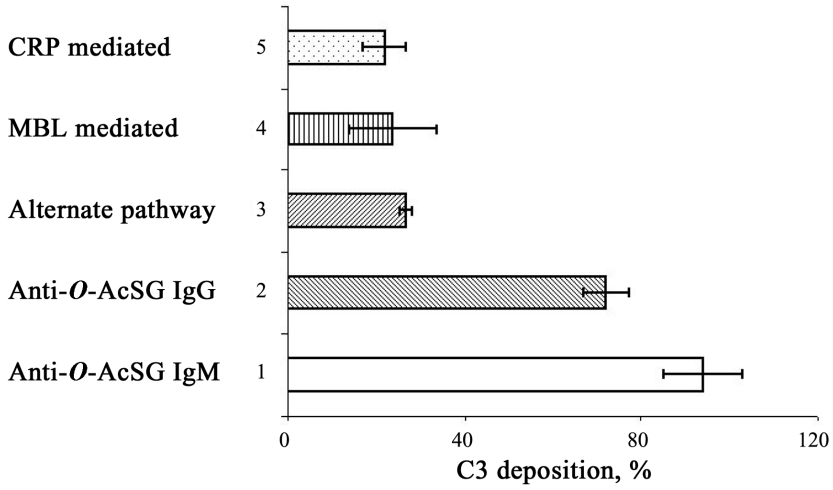

Figure 7. Comparative analysis of C3 deposition on promastigotes by all the complement pathways induced by the different complement activators—namely, C-reactive proteins (CRPs) (50 μg/mL), mannan-binding lectin (MBL) (20 μg/mL), alternate pathway, anti–*O*-acetylated sialoglycoconjugate $(AcSG)_{visceral\ leishmaniasis\ (VL)}$ IgM (6 μg/mL), or anti–*O*-AcSG IgM_{VL} IgG (6 μg/mL), within 3 min.

Previous reports have demonstrated that total IgM antibodies present naturally in NHS are a source of CP activation [8, 9]. However, we have reported that anti–*O*-AcSG$_{NHS}$ is 3-fold more potent than are total antibodies$_{NHS}$ (figure 1*A*), demonstrating, for the first time, that natural anti–*O*-AcSG is one of the major triggers of CP activation and promastigote opsonization.

Interestingly, in elicitation of C3 deposition on promastigotes, purified disease-specific anti–*O*-AcSG IgM$_{VL}$ and anti–*O*-AcSG IgG$_{VL}$ antibodies (6 μg/mL) were 5-fold more potent than anti–*O*-AcSG IgM$_{NHS}$ and anti–*O*-AcSG IgG$_{NHS}$ antibodies (figure 1*B*). The enhanced presence of 9-*O*-AcSG containing the 9-*O*-AcSAα2→6GalNAc glycotope, on the parasite surface, has been reported elsewhere [22, 27, 28] and has been corroborated here by the high rate of binding of anti–*O*-AcSG antibodies to promastigotes. Therefore, the enhanced presence of 9-*O*-AcSGs on parasites corroborates their increased susceptibility to complement lysis (figure 1*C*). Previous reports have shown that, irrespective of their linkage specificity, 9-*O*-AcSGs present on the surface of murine erythrocytes and murine erythroleukemia cells contribute significantly to their susceptibility to lysis by activation of the AP [29]. Further investigations from our group have shown that, in mammalian erythrocytes, the complement lysis induced via the AP correlates more significantly with linkage-specific 9-*O*-AcSAα2→6GalNAc [30]. This correlation has been extended to erythrocytes from patients with VL [17].

Sialic acids are critical determinants of parasite protection against attack by the host complement system [28]. The removal of sialic acid by treatment with neuraminidase is known to increase MBL binding, with a subsequent increase in MBL-mediated complement-dependant cell cytolysis, in *Neisseria meningiditis* [31]. Sialylation is also known to protect *N. gonorrhoeae* from MBL-activated complement killing [32] and confer protection to both epimastigote and trypomastigote forms by hindering the binding of lytic anti-galactose antibodies [33]. Thus, it may be envisaged that the host induces enhanced 9-*O*-acetylation on the parasite, thus generating anti–9-*O*-AcSG titers that, in turn, induce parasite lysis via CP activation. The role that 9-*O*-AcSG glycotopes on parasites play in mediating complement activation was further corroborated by *O*-acetylesterase–treated parasites, which resulted in the removal of *O*-acetyl moiety from sialic acid. These treated cells, when incubated with anti–*O*-AcSG IgM and anti–*O*-AcSG IgG, caused a significant reduction of C3 deposition. Quantitation of the number of C3-bound molecules per cell revealed that, in triggering the activation of C3 deposition, anti–*O*-AcSG IgM$_{VL}$ was 2.5-fold more potent than was anti–*O*-AcSG IgG$_{VL}$ (figure 2*A* and 2*B*). Interestingly, although total antibodies$_{NHS}$ are capable of inducing cell lysis, as confirmed by use of MTT assay, however, they were 3-fold less potent than anti–*O*-AcSG$_{NHS}$, establishing the critical role of these glycotope-specific antibodies (figure 3*A*). Furthermore, anti–*O*-AcSG–induced death, compared with that induced by total antibodies$_{NHS}$, revealed a much higher population of necrotic cells, as confirmed by the massive uptake of PI (figure 3*E–3G*).

The importance of 9-*O*-AcSGs was further demonstrated when promastigotes grown in serum-free medium were found to be incapable of undergoing anti–*O*-AcSG–mediated complement lysis; however, they became susceptible to lysis when they were transferred to medium supplemented with FCS, the source of this interesting 9-*O*-AcSG (figure 4). The susceptibility to lysis, even in medium supplemented with low FCS (5%), confirmed that these glycotopes play important in vivo roles in complement activation.

In contrast to CP, within the 3-min time frame, AP, CRP and MBL have a negligible effect (figures 5 and 6), and no cell death could be detected during this time, as confirmed by use of MTT assay. A comparative analysis distinctly revealed that the C3 deposition (mean ± SEM) triggered by anti–*O*-AcSG$_{VL}$ (94% ± 5% by IgM and 72% ± 10% by IgG) was maximal, compared with that triggered by CRP (22% ± 9%), MBL (24% ± 5%), or AP (27% ± 1.4%) (figure 7).

The overall importance of glycan-specific antibodies in protection against infection or in the pathological nature of the infection is still not clear, and only a relatively modest amount of information is available about specific structures or expression of relevant glycan antigens. The present study has demonstrated, for the first time, the biological role that these *O*-AcSG–specific antibodies play in host protection. Thus, a vivid analysis of the differential recognition of antigenic glycoconjugates and antibodies is required for comprehension of the immune response in VL.

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