C1q Enhancement of Antibody-dependent Granulocyte-mediated Killing of Nonphagocytosable Targets In Vitro

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Abstract

A possible role for C1q in antibody-dependent granulocyte-mediated killing of nonphagocytosable targets was investigated utilizing IgG-dependent granulocyte cytotoxicity directed against microfilariae of *Dirofilaria immitis*. Granulocyte-mediated killing of microfilariae is enhanced by addition of fresh serum. Lack of C4 did not significantly reduce the observed increase in cytotoxicity. The addition of highly purified monomeric human C1q (0.2 μg/ml) in the presence of immune IgG resulted in a two- to fivefold enhancement of killing ($P < 0.025$). C1q enhancement of killing occurred in the absence of fluid-phase IgG, but killing was significantly less than when both fluid-phase IgG and C1q were present. The effect of C1q was inhibited by the addition of solubilized type I collagen (44–92% inhibition of killing, $P < 0.05$). Significant ^{125}I-C1q binding to microfilariae occurred only in the presence of immune IgG. In addition, C1q in concentrations ranging from 0.5 to 2.0 μg/ml resulted in a dose-dependent increase in binding of ^{125}I-immune IgG to microfilariae. Finally, when purified C1q was added to preopsonized, washed microfilariae, granulocyte production of superoxide was increased from 0.25±0.07 to 0.68±0.07 nm/10^6 cells · 10 min ($P < 0.01$). These results describe a novel functional role for C1q in enhancement of antibody-dependent cellular cytotoxicity towards nonphagocytosable targets.

Introduction

During antibody-dependent activation of the classical pathway, C1q, a subcomponent of the first component of complement, serves as the recognition subunit that binds to the C2 domain of IgG or C4 domain of IgM. C1q has a molecular weight of 410,000, and consists of a collagenous stalk and six protruding strands which terminate in globular heads (1). The immunoglobulin attachment site resides in the globular heads, while binding of C1r and C1s occurs on the collagenous stalk. Within native C1, C1q is complexed to the proenzyme $C1r_2C1s_2$, which is converted into an activated form after binding of C1q to appropriate structures such as immune complexes. Activated $C1r_2C1s_2$ cleaves C4 and C2, which leads to C3 cleavage and subsequent activation of the remainder of the complement cascade. The pivotal role of C1q in classical pathway activation is apparent in individuals who lack this component and who have repeated episodes of bacterial infections and severe skin disease (2).

Recently, a number of reports have demonstrated that C1q binds to peripheral blood leukocytes and other mammalian cells by way of specific cell surface receptors (3–12). Binding to blood leukocytes was found to be through the collagenous stalk of the C1q molecule (8). Further studies have demonstrated that C1q bound to nonphagocytosable particles, when presented to granulocytes in suspension, results in stimulation of an oxidative burst. This indicates that the leukocyte C1q receptor may provide a means for activation of cytotoxic mechanisms (13). In addition, it has recently been demonstrated that adherent mononuclear phagocytes are stimulated by surface-bound C1q and manifest increased Fc receptor-mediated phagocytosis (14). The potential importance of cell surface receptors for C1q is underscored by the observation that after binding of C1 to an activating substance, C1r and C1s are actively disassociated (15, 16), thereby exposing monomeric C1q bound to the activating substance.

We have investigated the possibility that C1q may play a role in cellular killing of nonphagocytosable targets. We have previously found that C1q appears to enhance eosinophil-mediated killing of schistosomula of *Schistosoma mansoni* (17). The model system which we have employed here is killing of microfilariae of *Dirofilaria immitis* by dog granulocytes (18, 19). In this experimental system, microfilariae are killed by granulocytes only in the presence of specific immunoglobulin directed against the surface of the parasite. The results of the present studies show that C1q binds to IgG on the surface of the parasite and enhances antibody-dependent killing of these nonphagocytosable targets. The mechanism appears to involve both stimulation via the C1q receptor on granulocytes and enhancement of IgG binding, thus defining a new functional role for C1q.

Methods

Microfilariae and granulocytes. Venous blood was obtained from dogs infected with *Dirofilaria immitis* in the patent microfilaremic phase and microfilariae were isolated as previously described (18).

Granulocyte cell populations were obtained from normal dogs by Ficoll-diatrizoate (Pharmacia Fine Chemicals, Piscataway, NJ) sedimentation as previously described (18). Residual red blood cells were lysed by brief exposure to distilled water.

Adherence and killing assays. Microfilariae (30–50) in 0.1 ml minimal essential medium (MEM) were added to wells of flat-bottomed microtiter plates in quadruplicate as previously described (18), and were counted before the addition of cells. All sera used in experiments assessing killing were heat-inactivated (56°C for 30 min) and used in a final dilution of 1:8 unless indicated otherwise. Cells were added in a volume of 0.05 ml at a concentration of 400,000 per well to obtain a total volume of 0.2 ml. Plates were incubated in a CO_2 incubator at 37°C for 18 h. In comparing results of experiments in which maximal

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killing varied markedly from experiment to experiment, killing percentages were transformed to logarithms for statistical analysis using a paired *t* test. In experiments to test the effect of collagen, type I calf skin collagen (Calbiochem-Behring Corp., La Jolla, CA) was solubilized in 0.5 M acetic acid, dialyzed against solutions of gradually increasing pH to pH 7.4. Finally, the collagen was dissolved in Hanks' balanced salt solution (HBSS, K. C. Biologicals, Lenexa, KS) pH 7.4. Cells were preincubated in collagen in HBSS, 1.5 mg/ml, pH 7.4 for 30 min on ice, washed once in HBSS, and suspended in MEM (K. C. Biologicals) plus 375 μg/ml collagen. Control cells underwent the same preincubation steps in the absence of collagen. Monoclonal antibodies against neutrophil Fc receptors (3G8, a gift from Dr. M. Berger, and anti-human Leu-11b) were obtained from New England Nuclear (Boston, MA) and Becton, Dickinson & Co. (Mountain View, CA) respectively. They were used at ~ 1:100 final dilution. Binding of anti-Fc receptor antibody to canine granulocytes was confirmed by fluorescence microscopy using anti-human Leu-11a antibody and anti-Leu-11b antibody followed by fluorescein-labeled goat anti-mouse IgM antibody (Becton, Dickinson & Co.)

Serum fractionation for isolation of IgM and IgG. IgM purification was as previously described (18). For isolation of IgG, immune serum (IS),[1] obtained ~ 2 yr after documented spontaneous clearing of microfilaremia from a dog that had patent infection with high levels of circulating microfilariae (19), or normal dog serum (NDS) was dialyzed against buffer consisting of 0.02 M potassium phosphate, pH 8.0. This serum was then applied to an Affi-Gel Blue DEAE column (Bio-Rad Laboratories, Richmond, CA). Absorbance was read at 280 nm and those fractions comprising a peak were pooled and precipitated using 50% saturated ammonium sulfate. This concentrated sample was then dialyzed twice against HBSS. The resulting IgG fraction was assayed for purity by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). For use in the killing assay, IgG was added to each well in a final concentration of 300–360 μg/ml.

Immunofluorescent localization of C3. Microfilariae of *D. immitis* were incubated in 25% NDS, or IS in MEM for 1 h at 37°C, followed by washing three times. FITC-conjugated antisera to dog C3 (Cappel Laboratories, Cochranville, PA) was added at a final dilution of 1:8 and tubes were incubated at 4°C for 30 min. The microfilariae were then washed three times and were examined for fluorescence.

C4-deficient guinea pig serum. A breeding colony of C4-deficient guinea pigs was obtained through the courtesy of Drs. E. J. Brown and M. M. Frank, National Institute of Allergy and Infectious Diseases, Bethesda, MD.

C1q isolation. C1q was purified by the method described by Tenner et al. (20). In brief, fresh normal human serum was made 5 mM in EDTA, and was applied to a Bio-Rex 70 (Bio-Rad Laboratories) column. The column was then washed with a buffer consisting of 102 mM NaCl, 2 mM EDTA, 50 mM sodium phosphate, pH 7.3, and C1q was eluted by application of a linear gradient of increasing ionic strength using a final buffer consisting of 300 mM NaCl, 2 mM EDTA, 50 mM sodium phosphate, pH 7.3. The major protein peaks were precipitated with 33% saturated ammonium sulfate, then subjected to gel chromatography over a Bio-Gel A5M (Bio-Rad Laboratories) column. The initial protein peak was precipitated with 33% saturated ammonium sulfate. The presence of C1q was established by Ouchterlony gel diffusion using goat anti-human C1q (Atlantic Antibodies, Scarborough, ME). Purity was assessed by SDS-PAGE.

To test whether C1q remained monomeric under the experimental conditions utilized, ^{125}I-C1q was suspended in MEM plus 5% BSA and filtered over a Bio-Gel A5M column. Molecular weight standards (Pharmacia Fine Chemicals) included thyroglobulin (669,000), catalase (232,000), and aldolase (158,000). The radiolabeled C1q eluted in a symmetrical peak between thyroglobulin and catalase, and thus indicated nonaggregation of the C1q.

Binding of ^{125}I-C1q or ^{125}I-IgG to microfilariae. Radioiodination of purified human C1q and immune dog IgG was carried out by the lactoperoxidase-glucose oxidase method as described by Tenner et al. (20).

^{125}I-C1q (5 μg/ml) was mixed with 100 microfilariae in the presence or absence of 200 μg/ml purified IgG from IS or NDS in a total volume of 500 μl in MEM with 1% ovalbumin, and incubated for 1 h at 37°C. After the incubation, the mixture was pelleted by centrifugation, washed once gently, and the radioactivities of the pellets were counted. Nonspecific binding (background) was measured by incubation of ^{125}I-C1q without microfilariae and IgG. Experimental values were corrected by subtraction.

^{125}I-IgG (10 μg/ml) and 100 microfilariae were reacted in MEM with 1% ovalbumin with varying concentrations of C1q in the range 0.1–2.0 μg/ml or medium, total volume 500 μl. Incubation and counting were as described above.

Superoxide production. The production of superoxide by neutrophils was measured by reduction of cytochrome *c* (21). Neutrophils were obtained using the Ficoll-diatrizoate method using HBSS without phenol red, Ca^{2+}, Mg^{2+}. As a final step, cells were suspended in HBSS without phenol red, with Ca^{2+} and Mg^{2+}. Microfilariae were preincubated in 1:9 concentration of heat-inactivated IS (56°C for ½ h) at 37°C for ½ h, and then washed twice with HBSS. 1-ml spectrophotometer cuvettes were made to contain 1.0×10^6 neutrophils and 1,000 microfilariae and brought to a total volume of 990 μl in HBSS with cytochrome *c* (Sigma Chemical Co., St. Louis, MO) 0.6 mg/ml. 10 μl of the appropriate C1q solution was added to each cuvette. As a positive control, one cuvette received 10 μl of a 0.01 mg/ml solution of phorbol myristate acetate (Sigma) in HBSS. OD_{550} was measured every 2 min. Cuvettes were agitated immediately before each reading. In experiments designed to quantify superoxide production, the incubation was carried out in separate tubes containing the same materials, but aliquots of 0.267 ml were aspirated at 10-min intervals and spun at 400 *g* for 10 min, and the absorbance at 550 nm was read in the supernatant. ΔE_{mM} for cytochrome *c* was taken to be 18.5 (22). To test for an effect of superoxide dismutase, 20 μl of HBSS containing both 1 and 0.1 mg/ml superoxide dismutase (Sigma Chemical Co.) was added to the 2-ml reaction mixture.

Results

Effect of C1q on in vitro killing of microfilariae. Microfilariae incubated with granulocytes in the presence of immune serum demonstrated significant enhancement of killing with the addition of fresh guinea pig serum (GPS) to the system (Table I). However, the absence of C4 (in C4-deficient GPS) did not abolish the fresh serum enhancement of killing (Table I). In two separate experiments, examination of microfilariae by fluorescence microscopy after incubation with heat-inactivated immune serum or IgM (tested because IgM does not activate the alternative pathway) and whole GPS showed surface deposition of C3, while none was seen with either source of antibody when C4-deficient GPS was used.

To test the possibility that partial activation of the classical pathway leading to generation of monomeric C1q may account for some of the observed fresh serum effect, C1q was purified from human serum and added to the killing system. In these experiments, the effect of exogenous serum factors was minimized by the use of 5% albumin, in place of whole serum, to preserve cellular and microfilarial integrity. C1q was added in a final concentration of 0.2 μg/ml to wells containing purified IgG. In four separate experiments, there was a two- to fivefold augmentation of microfilarial killing with the addition of purified C1q to the system (Table II) ($P < 0.025$, paired *t* test). This degree of augmentation was comparable to that seen

1. *Abbreviations used in this paper:* GPS, guinea pig serum; IS, immune serum; NDS, normal dog serum.

Table I. Enhancement of Granulocyte-mediated Antibody-dependent Killing of Microfilariae Incubated with IS (1:8) and to Which Fresh GPS or Fresh GPS Deficient in C4 Was Added

GPS	Experiment no. 1	2	3	4	5	Significance*
			%			
Heat-inactivated	65±6‡	80±2	23±7	75±7	47±14	—
Fresh	90±4	96±5	60±13	98±2	67±15	<0.005
C4-deficient	92±2	84±7	88±9	94±5	95±6	<0.05

* Probability of no significant difference when compared to the results with heat-inactivated GPS, paired *t* test.
‡ Mean±SEM in quadruplicate wells.

with the addition of whole fresh serum. There was no enhancement of background killing when C1q was added in the absence of immunoglobulin. In two experiments, heating to 56°C for 30 min completely abrogated the observed C1q effect, while heating to 47°C had no effect (data not shown).

In experiments designed to test whether C1q binding to IgG on the surface of microfilariae, in the absence of fluid-phase IgG, is associated with enhancement of killing, microfilariae were first preopsonized with IgG and washed. In three experiments, the addition of C1q consistently resulted in slight, but significant, increases in killing of preopsonized microfilariae (10.9±5.0% vs. 6.3±3.6% killing, $P < 0.01$). However, the presence of fluid-phase IgG is required for optimal killing when C1q is added, as evidenced by two separate experiments which showed that the killing in the presence of C1q and IgG (16.8±6.6%) was significantly higher than with preopsonized microfilariae which had been washed prior to addition of C1q (4.7±1.8%) ($P < 0.001$).

Binding of ^{125}I-C1q to IgG-coated microfilariae. ^{125}I-C1q (5 μg/ml) was incubated with microfilariae in the presence of normal dog or immune dog IgG. In the presence of immune dog IgG, binding of radiolabeled C1q was significantly increased (mean percent ^{125}I-C1q binding 36±11 with IS vs. 2±1 with normal dog IgG, $P < 0.025$). In the presence of immune IgG, the average number of molecules of C1q bound per microfilaria was 1.3×10^{10}. Binding of ^{125}I-C1q in the presence of normal dog IgG was not significantly increased over background.

Table II. Enhancement of Antibody-dependent Granulocyte-mediated Killing of Microfilariae by Monomeric C1q

Experiment no.	Components added to the microfilariae-cell mixture: C1q	IgG	IgG + C1q*
		% killing	
1	5.0±3.5‡	3.0±2.7	13.0±4.5
2	—	13.2±8.9	70.3±6.8
3	4.4±2.4	15.6±6.5	35.2±5.1
4	—	17.2±7.0	31.7±9.5

* Killing with added IgG plus C1q is significantly greater than with IgG alone, $p < 0.025$, paired t test.
‡ Mean±SEM in quadruplicate wells.

The effect of washing after preopsonization on C1q binding was tested in four experiments. There was a 1.6–1.8-fold increase in ^{125}I-C1q binding ($P < 0.001$) when fluid-phase IgG was present as compared with microfilariae that had been washed and kept free of fluid-phase IgG.

Collagen effect on C1q-enhanced killing. To test the possibility that C1q attached to microfilariae might bind to granulocytes via the collagenous portion of the molecule and thereby enhance killing, the effect of type I collagen on C1q enhancement of killing was tested. When cells were preincubated in type I collagen, there was a 44–92% inhibition of killing in three separate experiments ($P < 0.05$). Collagen had no significant effect on baseline killing in the absence of added C1q (data not shown).

Effect of antibody to Fc receptor on killing. The possibility that C1q is acting independently of the granulocyte Fc receptor was examined using monoclonal antibodies specific for Fc receptors. When monoclonal antibody to the neutrophil Fc receptor was added to the killing system, there was a significant reduction in IgG-mediated killing (Table III). With the addition of C1q, killing was significantly enhanced over IgG alone despite the presence of anti-Fc receptor antibodies (Table III).

Binding of ^{125}I-immune IgG to microfilariae. A second possible mechanism for the observed C1q effect, enhancement of IgG binding to the target, was examined using ^{125}I-labeled IgG. Microfilariae were incubated in medium containing 10 μg/ml ^{125}I-IgG and -C1q in concentrations in a range of 0.1–2.0 μg/ml. Binding of radiolabeled immune IgG to microfilariae was increased in a dose-dependent fashion by the addition of C1q (Fig. 1). In each experiment, there was a pronounced increase in ^{125}I-IgG binding to microfilariae in the concentration range of 0.1–0.2 μg/ml C1q, with a slight further increase at a concentration of 2.0 μg/ml. The increase in ^{125}I-IgG binding was significant at each C1q concentration tested ($P < 0.025$).

C1q stimulation of superoxide production. To examine a possible stimulatory effect of C1q attached to IgG opsonized targets, monomeric C1q was added to granulocytes and preopsonized, washed microfilariae. Superoxide production by exposed granulocytes was measured by reduction of cytochrome *c*. In three separate experiments to define superoxide

Table III. Effect of Monoclonal Antibody against Neutrophil Fc Receptor (FcAb) on IgG-mediated Killing in the Presence and Absence of Added C1q

Components added to microfilariae plus granulocytes in killing assays*	Experiment no. 1	2
	%	
IgG + C1q	21±6	100±0
IgG + C1q + FcAb‡	13±0	90±4
IgG	6±8	97±4
IgG + FcAb	3±4	15±15
C1q	6±4	5±4

* Microfilariae and cells plus 5% BSA were incubated with the components listed as described in Methods. Experiment 1 was performed using 3G8 and experiment 2 with anti-human Leu-llb antibody.
‡ Killing with IgG + C1q + FcAb is significantly greater than with IgG + FcAb (no C1q), $P < 0.05$.

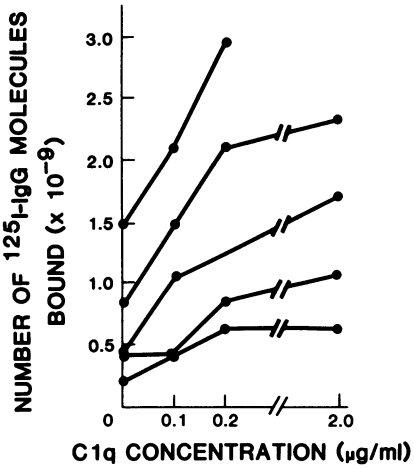

Figure 1. Enhancement of binding of ^{125}I-immune IgG to microfilariae by the addition of monomeric C1q. Radiolabeled immune IgG was added to microfilariae with C1q in concentrations ranging from 0.1 to 2.0 μg/ml and incubated at 37°C, and the microfilariae were washed and counted. Data are means of triplicate determinations in each experiment. Binding of ^{125}I-IgG was significantly increased ($P < 0.025$) at each concentration of added C1q.

production kinetics, the addition of C1q in concentrations of 0.002–20 μg/ml resulted in enhanced reduction of cytochrome *c*, with the onset of the change in absorbance consistently showing a lag of 14–20 min from the time of addition of C1q. In three further experiments focusing on the time interval 10–20 min after start of incubation, the amount of superoxide produced by cells exposed to opsonized microfilariae alone was 0.25±0.07 nm/10^6 cells · 10 min as compared with 0.68±0.07 nm/10^6 cells · 10 min in the presence of 0.2 μg/ml C1q ($P < 0.01$). Reduction of cytochrome *c* was inhibited by the addition of superoxide dismutase, but not boiled superoxide dismutase (data not shown).

Discussion

The present report suggests a novel functional role for C1q in the augmentation of granulocyte-mediated antibody-dependent killing of a multicellular, nonphagocytosable target. Fresh serum enhancement of killing even in the absence of C4 raised the possibility of partial activation of the classical pathway. Further, the disparity between the lack of surface C3 deposition and preservation of the fresh serum effect when C4-deficient serum was used suggested an unusual mechanism. By adding highly purified C1q to the killing system in the absence of whole serum, it was found that C1q does enhance antibody-dependent killing of microfilariae. The C1q enhancement of killing is seen only in the presence of antibody against the target, which, as demonstrated in this report, results in binding of the C1q molecule to the organism. This then appears to augment activation of granulocytes, which presumably accounts for the increased cytotoxicity.

The molecule, which was subsequently termed C1q, was first identified as a component of fresh serum which caused precipitation of soluble aggregated immunoglobulin (23, 24). This was later shown to be associated with an 11S sedimenting component (25, 26). Disassociation of native C1 by EDTA chelation and chromatographic separation of its three activities led to designation of the high molecular weight constituent C1q, and the other two C1r and C1s (27). It is now clear that C1q initiates classical pathway activation by attaching to IgG, IgM, or other activating substances, which under appropriate conditions leads to stabilization of the $C1r_2C1s_2$ complex and triggering of C1r and C1s esterase activity and classical pathway activation (1). Initiation of the activating process is dependent on the abundance and physicochemical state of the potential activating substance, and activation is further regulated by C1 inhibitor (1). Other major immunologic functions for C1q remain to be defined.

Previous results in the microfilarial killing system utilized here indicated that the neutrophil is the predominant effector cell (18), and that oxidative products of neutrophils possess potent cytotoxic properties toward microfilariae (El Sadr et al., manuscript submitted for publication). The present results indicate that C1q causes an increase in granulocyte-mediated killing and suggest that two mechanisms may be involved. Of possible relevance in this regard is the report by Tenner and Cooper (13) that monomeric C1q bound to nonphagocytosable latex beads stimulates hexose-monophosphate shunt activity and chemiluminescence by granulocytes. They postulated that particle-bound C1q interacts with the C1q receptor on the surface of granulocytes, and that this results in stimulation of metabolic and secretory activity. In the present system, the immunoglobulin requirement implicates initial binding of C1q to antibody. Inhibition of the C1q effect by solubilized type I collagen and the enhancement of killing even in the presence of anti-Fc receptor antibody suggest that C1q may be acting in part through its specific receptor on granulocytes, resulting in stimulation and perhaps enhanced binding of cell to target. The fact that microfilariae that had been preopsonized, washed, and then exposed to C1q stimulated increased superoxide production by granulocytes supports this hypothesis. However, enhancement of specific IgG binding to the target organism, as demonstrated in this report, or cross-linking of immunoglobulin molecules by C1q binding, also may be of major importance. The finding that killing and C1q enhancement of killing were less when microfilariae were preopsonized and washed before addition of C1q (as opposed to simultaneous incubation) suggests that a C1q effect on antibody binding is partially responsible for the effect. In addition, more C1q is bound to the microfilariae after simultaneous incubation with IgG and C1q. Of possible relevance in this regard is the previous report that partially purified C1q increases binding of cold reacting antibodies to human red blood cells (28). Further, it has been suggested that membrane-bound C1q may function as a cellular receptor for immunoglobulin Fc regions (12, 29). The present results suggest that the C1q effect noted here may result from both stimulation of granulocytes via the C1q receptor and enhancement of IgG binding to the target.

The previous demonstration of a granulocyte stimulatory role for C1q was also in a system employing nonphagocytosable targets, C1q-coated latex beads ~ 15 μm in diameter (13). Furthermore, we have demonstrated that C1q appears to enhance killing of schistosomula of *S. mansoni* (18). It is possible that C1q serves a particularly important function when the target for granulocyte-mediated killing is too large to be ingested (e.g., multicellular parasites, tumor cells, antibody-coated host cells), and killing is therefore dependent on a

mechanism for stimulating granulocytes to secrete cytotoxic products onto the surface of the target.

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