

C4b-binding Protein and Factor H Compensate for the Loss of Membrane-bound Complement Inhibitors to Protect Apoptotic Cells against Excessive Complement Attack*

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Apoptotic cells have been reported to down-regulate membrane-bound complement regulatory proteins (m-C-Reg) and to activate complement. Nonetheless, most apoptotic cells do not undergo complement-mediated lysis. Therefore, we hypothesized that fluid phase complement inhibitors would bind to apoptotic cells and compensate functionally for the loss of m-C-Reg. We observed that m-C-Reg are down-regulated rapidly upon apoptosis but that complement activation follows only after a gap of several hours. Coinciding with, but independent from, complement activation, fluid phase complement inhibitors C4b-binding protein (C4BP) and factor H (fH) bind to the cells. C4BP and fH do not entirely prevent complement activation but strongly limit C3 and C9 deposition. Late apoptotic cells, present in blood of healthy controls and systemic lupus erythematosus patients, are also positive for C4BP and fH. Upon culture, the percentage of late apoptotic cells increases, paralleled by increased C4BP binding. C4BP binds to dead cells mainly via phosphatidylserine, whereas fH binds via multiple interactions with CRP playing no major role for binding of C4BP or fH. In conclusion, during late apoptosis, cells acquire fluid phase complement inhibitors that compensate for the down-regulation of m-C-Reg and protect against excessive complement activation and lysis.

Cell death via apoptosis is an essential process for development, maintenance of tissue integrity, and resolution of inflammation (1). Several active processes ensure that the cell dies in a way that results in fast removal, to prevent release of its potential pro-inflammatory content as is the case in primary or secondary necrosis (2–4).

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Although billions of cells die via apoptosis every day, only a few apoptotic cells are present at any given point in time under healthy conditions (5) as the result of a very efficient clearance process. Only when clearance mechanisms are defective or overwhelmed by excessive apoptosis do apoptotic cells accumulate, which is thought to underlie autoimmune diseases such as systemic lupus erythematosus (SLE)³ (6).

Apoptotic cells express several ligands that enhance phagocytosis such as phosphatidylserine and calreticulin, and they down-regulate molecules that may work as "do not eat me" signals (7). In addition, apoptotic cells have been reported to bind complement-initiating molecules such as mannose-binding lectin and C1q that enhance uptake by phagocytes (8–10). Some discrepancies exist in reports of the stage of cell death necessary for binding of such recognition molecules, which can partly be explained by differences in semantics, experimental design, and readout (9, 11–14).

The complement system is an integral part of the innate immune defense and is also involved in the instruction of adaptive immunity and clearance of waste such as dead cells and immune complexes (15, 16). This potent enzyme cascade system has both membrane-bound and fluid phase inhibitors that protect host surfaces and prevent systemic depletion of complement activity caused by auto-activation (15, 16). The classical pathway and lectin pathway are initiated following binding of their recognition molecules, C1q and mannose-binding lectin, respectively, to their specific targets. The alternative pathway is activated spontaneously but is kept under control mainly by fluid phase inhibitor factor H (fH) (17). Importantly, the alternative pathway is also used as amplification for classical pathway and lectin pathway activity. The fluid phase inhibitor C4b-binding protein (C4BP) mainly regulates the classical pathway and lectin pathway but also has impact on alternative pathway activity (18).

The most common form of C4BP, the C4BP·protein S complex (C4BP·PS) consists of seven α -chains and one β -chain, to which anti-coagulant protein S is bound. Both C4BP and fH are built up from complement control protein domains (CCP)

³ The abbreviations used are: SLE, systemic lupus erythematosus; AV, annexin-V; C4BP, C4b-binding protein; C4BP·PS, C4b-binding protein-protein S complex; CCP, complement control protein (domain); CRP, C-reactive protein; mAb, monoclonal antibody; m-C-Reg, membrane-bound complement regulatory proteins; NHS, normal human serum; VP, via-probe; fH, factor H; FACS, fluorescence-activated cell sorter.

domains. The α -chains of C4BP have eight CCPs, the β -chain has three CCPs, and fH consists of a single chain of 20 CCPs (18).

During apoptosis cells down-regulate several surface molecules including their membrane-bound complement inhibitory molecules (m-C-Reg) (19–21). This would leave dying cells vulnerable to complement-mediated lysis if they did not acquire other means of protection. Lysis of apoptotic cells would result in release of potentially inflammatory intracellular content into the extracellular environment, which is not compatible with the anti-inflammatory property of apoptosis (2–4). Therefore, we investigated whether apoptotic cells acquire fluid phase complement inhibitors to compensate functionally for the loss of m-C-Reg as a way to remain protected against excessive complement attack.

In this study we observed that during apoptosis cells down-regulate m-C-Reg rapidly but do not yet activate complement. Only after a lag phase is complement activation initiated whereupon it is kept under control by simultaneous binding of fluid phase complement inhibitors C4BP and fH. In this way excessive complement attack and lysis is prevented. This process is also operating *in vivo* as shown here for healthy individuals and SLE patients.

EXPERIMENTAL PROCEDURES

Cells and Induction of Cell Death—Jurkat T cells and THP-1 cells (ATCC) were grown in RPMI containing glutamine, penicillin, streptomycin, and 10% heat-inactivated fetal calf serum (all from Invitrogen). Apoptosis was induced using 1 μ M staurosporine (Sigma) for 2, 4, or 6 h in RPMI without fetal calf serum at 37 °C. Necrosis was induced by heat as before (22).

Proteins, Antibodies, and Sera—The C4BP-PS complex (23), FH (24), C1q (25), and protein S (26) were purified from human plasma as described. Recombinant CRP was obtained from Calbiochem and heparin from ICN Biomedicals. All of the proteins were at least 95% pure, as judged by Coomassie staining of proteins separated by SDS-PAGE. CRP was confirmed to be mostly in pentameric form.

Antibodies against CD35, CD46, CD55, and CD59 were from Immunotools. Anti-protein S mAb HPS-54, HPS-21 (blocking), and anti-C4BP β -chain mAb 2B were generated in house. Function blocking mAb MH10 against CCP-20 of fH was a kind gift from Prof. J. D. Lambris (University of Pennsylvania). Anti-factor I mAb MRCOX-21 was a kind gift of Prof. R. Sim (Oxford University, Oxford, UK). Rabbit anti-C4BP antibodies were generated in house, goat anti-fH antibodies were from Quidel, rabbit anti-C1q and rabbit anti-C3c were from DAKO, and goat-anti-C9 was from Advanced Research Technologies. Normal human serum (NHS) and C4BP-deficient serum were prepared as before (26).

Complement Activation Assays—The cells were washed with DGVB²⁺ (2.5 mM veronal buffer, pH 7.3, containing 70 mM NaCl, 140 mM glucose, 0.1% gelatin, 1 mM MgCl₂, and 0.15 mM CaCl₂) and incubated at 10⁵ cells in a 50- μ l volume containing DGVB²⁺ and NHS, C4BP-deficient NHS, or C4BP-deficient reconstituted with physiological concentrations of C4BP-PS. We also analyzed the effect of a function-blocking antibody against fH, MH10. The mAb was preincubated with 100% NHS

for 15 min on ice at 1 mg/ml. Dilutions of treated NHS were made in DGVB²⁺. The samples were incubated on a shaker for 30 min at 37 °C, stained and analyzed by FACS.

For the lysis assay, Jurkat T cells were loaded with Calcein-AM (MoBiTech) following the instructions of the manufacturer. Following washing, the cells were used at 0.2 10^6 cells/well and incubated with sera as for the FACS assays for 1 h. at 37 °C. The percentage of specific lysis was calculated by dividing the experimental value by the maximum release, both corrected for spontaneous release, $\times 100$. The percentage of complement-mediated lysis was determined by subtracting the background value of heat-inactivated serum.

Flow Cytometry—To discriminate live, apoptotic, and necrotic populations, cells were stained with annexin-V-PE (AV) (BD Biosciences) and Via-probe (VP), (BD Biosciences). Expression of CD35, CD46, CD55, and CD59 and binding of C4BP, fH, and protein S were analyzed with specific antibodies as above, followed by fluorescein isothiocyanate-labeled matched secondary antibodies (DAKO). For the blood assays double stainings were performed using matched secondary antibodies conjugated with Alexa 647 (Invitrogen) and AV.

Binding Studies—C4BP and fH binding was analyzed by incubating cells with increasing concentrations of purified C4BP-PS or fH in binding buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂) or with NHS in DGVB²⁺, shaking for 30 min at 37 °C. For the free protein S versus C4BP-PS complex experiments, the cells were incubated with 400 nM C4BP-PS complex or 400 nM free protein S. For the blocking studies the cells were treated with buffer or with CRP (50 μ g/ml) for 30 min at 37 °C. Following washing, the cells were incubated with C4BP (5 μ g/ml), fH (25 μ g/ml), or C1q (5 μ g/ml) with or without heparin (1.5 mg/ml) or blocking mAb against protein S (mAb 21) or against fH (MH10) using anti-fI (MRCOX-21) as isotype control mAb, all at 2 molar excess. The mAb were preincubated for 10 min on ice with the proteins and incubated with the cells for 30 min at room temperature. The contribution of CRP to complement activation on dead cells was analyzed by incubating the cells with 20% NHS or 20% NHS containing 50 μ g/ml CRP followed by FACS analysis as above.

Blood Culture—Blood samples were obtained from SLE patients with active and inactive disease as defined by a SLE-DAI-2K value higher than 4 (27), and age and sex matched healthy controls. Ethical permission was obtained from the Lund University ethical committee. Characteristics for the groups are depicted in Fig. 3C. From both patients and controls we obtained two sets of tubes, one with citrate blood and one with clotted blood. The citrate blood tubes were mixed thoroughly and directly placed on ice, and the tube for serum was allowed to clot for 30 min at room temperature and then placed on ice for 1 h. Serum was prepared by centrifugation for 5 min at 1800 rpm. For each individual a total of 1 ml of citrate blood was washed once with 50 ml of phosphate-buffered saline to remove citrate and plasma, after spinning for 5 min at 1800 rpm the pellet was resuspended in 9 ml of RPMI and 1 ml of autologous serum. The samples were cultured, and 150 μ l of cultured blood was analyzed after 30 min and 48 h. When possible samples were analyzed pair-wise.

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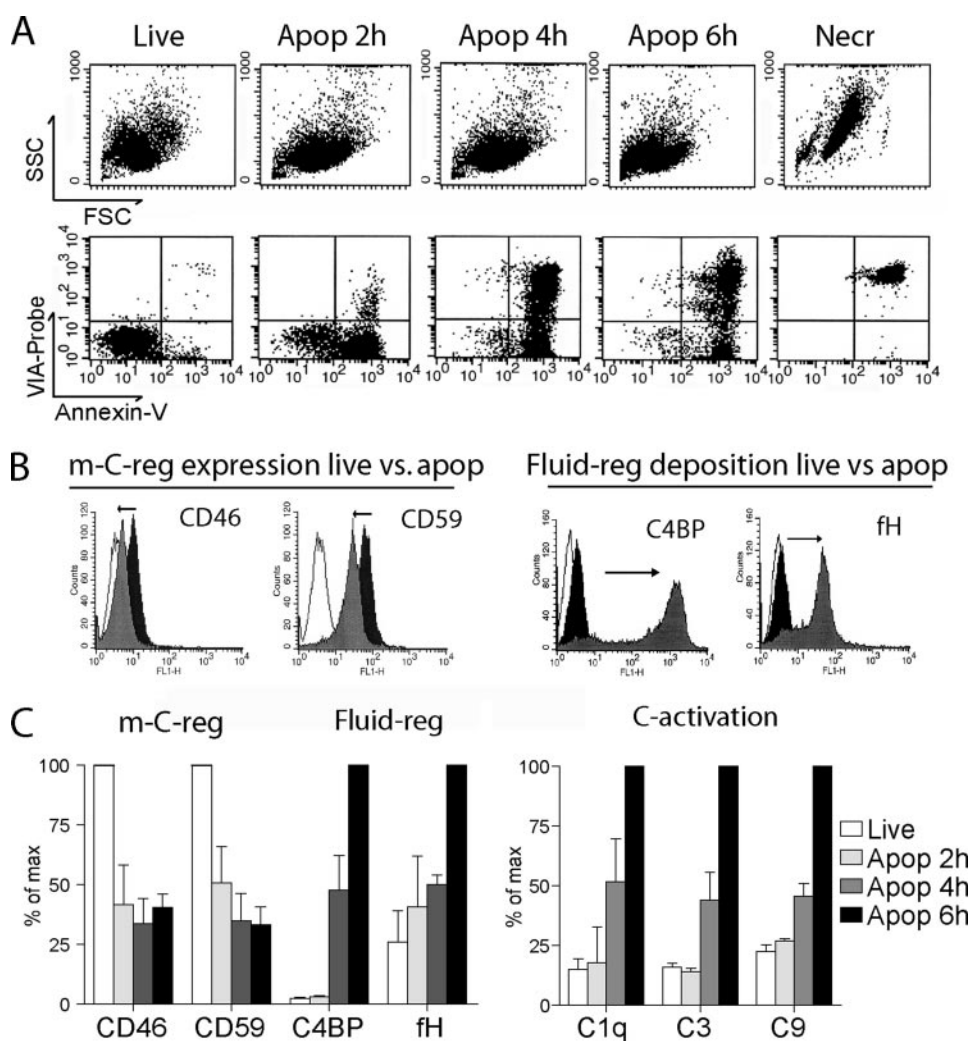


FIGURE 1. Membrane-bound and fluid phase complement regulators are differentially present on apoptotic cells. Jurkat T cells were rendered apoptotic (*Apop*) or necrotic (*Necr*) and analyzed for the membrane expression of complement regulators (m-C-Reg), binding of fluid phase inhibitors, and the capacity to activate complement over time. *A*, analysis of live cells, progressive apoptosis induced by staurosporine over time, and necrosis using scatter profiles and double staining with AV and Via-Probe. *B*, changes in the expression of CD46 and CD59 or binding of C4BP and fH from 20% serum on live (*black*) or apoptotic 6-h (*gray*) histograms. *Open histograms* represent negative controls with secondary antibody only. *C*, relative change of membrane expression of CD46 and CD59 and binding of C4BP and fH as well as the capacity to activate complement from 20% serum over time, expressed as percentages of maximum.

Statistical Analysis—We have used Mann-Whitney U, Student's *t* tests, and analysis of variance with Tukey post hoc test to test for significant differences between groups (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

RESULTS

Differential Expression of m-C-Reg and Binding of C4BP and fH to Dying Cells—Following induction of apoptosis in Jurkat T cells by staurosporine we observed progressive cell death, with after 2 h mainly early apoptotic annexin-V-positive (AV+) Via-Probe negative (VP-) cells; at 4 h a mix of AV+VP- and AV+VP+ cells; and at 6 h mainly double positive late apoptotic cells as is the case for primary necrosis (Fig. 1*A*). Jurkat T cells express significant amounts of m-C-Reg CD46 and CD59 that are rapidly down-regulated during early apoptosis (Fig. 1, *B* and *C*). By incubating these cells in serum, we observed that as these

cells go through the process of apoptosis, they start to bind C4BP and fH (Fig. 1*B*). Quantification of this binding revealed that they only bound to apoptotic cells in the latest stages of apoptosis (Fig. 1*C*). Binding of C1q and complement activation as measured by deposition of C3 and C9 only take place at 4 h and especially 6 h, indicating that complement activation is not the result of m-C-Reg down-regulation. A similar pattern of rapid m-C-Reg down-regulation and binding of fluid phase complement regulators was also observed for THP-1 cells and for Jurkat T cells using etoposide as an apoptosis-inducing agent (data not shown).

C4BP and fH Limit Complement Activation on Dead Cells—To analyze whether the binding of C4BP and fH has functional consequences regarding complement activation and/or lysis, we performed experiments using C4BP-deficient serum and a function-blocking mAb against fH.

Incubating apoptotic cells with C4BP-deficient NHS resulted in strongly enhanced C3 and C9 deposition as compared with NHS (Fig. 2, *A* and *B*), indicating that C4BP inhibits complement activation on the surface of apoptotic cells. To prove that it was really the absence of C4BP only that was responsible for this effect, we reconstituted C4BP-deficient serum with purified C4BP-PS, which resulted in levels of complement activation similar to those seen with NHS, indicating that the observed effects were C4BP-specific. In the presence of the fH blocking mAb MH10, we observed more C3 and C9 deposition (Fig. 2, *A* and *B*) compared with the isotype control. Blocking the function of fH in C4BP-deficient serum resulted in an additive increase in C3 and C9 deposition as compared with blocking fH in NHS.

Using apoptotic cells (6 h) and incubation with the same sera as above, we observed that C4BP and fH together provide protection against lysis of late apoptotic cells (Fig. 2*C*). Collectively these data show that C4BP and fH do not completely prevent complement activation but significantly limit complement activation on dead cells and by doing so prevent lysis.

C4BP and fH Bind Dead Cells in Blood—To confirm that C4BP and fH also bind dead cells *in vivo* and to human primary cells *ex vivo*, we analyzed blood either directly or after *ex vivo* culture. We used blood from healthy controls and from SLE patients

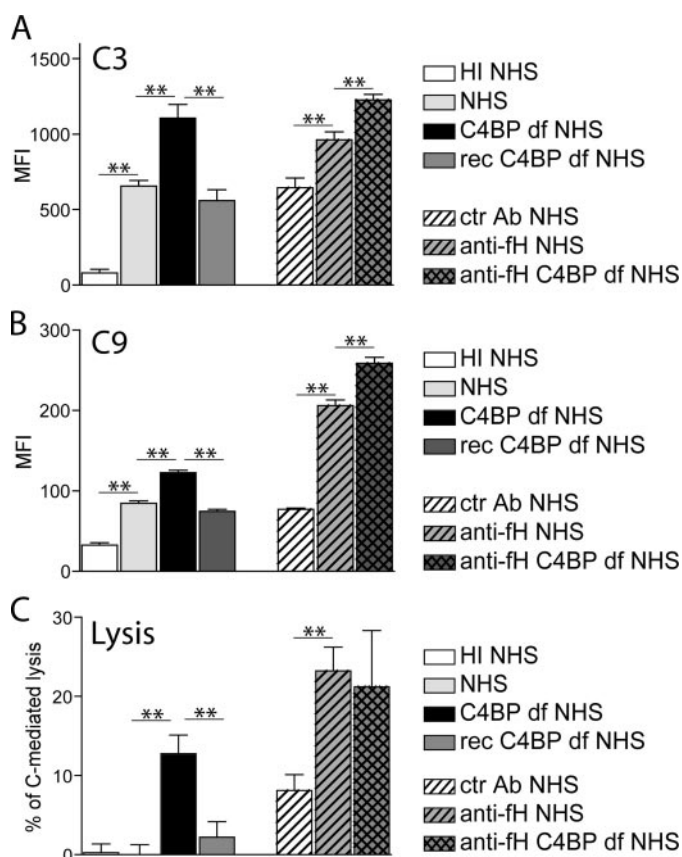


FIGURE 2. Both C4BP and fH inhibit complement activation and lysis of apoptotic cells. Apoptotic cells (6 h) were incubated in 10% serum treated in several ways to analyze the functional effects of binding of C4BP and fH to dead cells regarding complement inhibition. Apoptotic cells were incubated with, heat-inactivated serum (HI NHS), normal serum (NHS), C4BP-deficient serum (C4BP df NHS), or C4BP-deficient NHS that was reconstituted with physiological concentrations of purified C4BP (rec C4BP df NHS). Alternatively, apoptotic cells were incubated with serum that was pretreated with either with a function-blocking antibody against fH or with control antibody. Also C4BP-deficient serum was pretreated with the anti-fH mAb. All of the samples were analyzed by FACS for deposition of C3 (A) and C9 (B). C, in a lysis assay calcein AM loaded live and apoptotic (6 h) Jurkat T cells were incubated with the above-described sera. The release of calcein was measured in the supernatant after 1 h of incubation. Lysis was calculated and is expressed as a percentage of complement-mediated specific lysis (C-specific lysis). MFI, mean fluorescence intensity.

during active and inactive disease because it was reported that SLE samples may contain more apoptotic cells (28). We analyzed the presence of C1q, C3, C4BP, and fH and the percentage of apoptotic and necrotic cells both directly after blood sampling and after 2 days of culture *in vitro*. Whole blood contains few early apoptotic (AV+ only) and even fewer late apoptotic cells (AV+VP+), but upon culture these numbers increase (Fig. 3, A and B). We did not observe major differences in the percentage of early or late apoptotic cells comparing healthy individuals to SLE patients (Fig. 3B). Both groups had a similar increase in the percentage of late apoptotic cells following 48 h of culture (Fig. 3B).

Double staining of whole blood for either C4BP or fH with AV revealed that in freshly obtained blood there are cells positive for C4BP and for fH and that all of these cells are AV-positive. Not all cells positive for AV are also positive for C4BP and fH, which agrees with the observation that only cells in late apoptosis bind these proteins. Upon culture for 48 h, the num-

bers for C4BP and fH positive cells increase proportionally to the increase in late apoptotic cells (Fig. 3, B and D). As a positive control we have added necrotic Jurkat cells to whole blood, which showed a similar binding of C4BP, fH, and AV (data not shown). We observed no significant increase in C1q deposition upon culture, mostly because of large variation between the patients. The enhanced C1q binding in some of the patients with active disease was secondary to (auto)-antibody binding (data not shown). C3 deposition increased significantly over the 48-h culture in the healthy control samples but not significantly in patient samples. This lower C3 deposition is correlated to hypocomplementaemia in the SLE patients (Fig. 3C) (C3 on cells at 48 h versus C3 serum concentration, $p = 0.035$). FH deposition, in contrast to what we observed *in vitro*, did not increase significantly over time (Fig. 3D). C4BP deposition was strongly increased in all groups in line with our *in vitro* observations (Fig. 3D). These data indicate that there are only a few late apoptotic cells present in blood and that these cells are positive for C4BP and fH.

Complement Activation Is Not Required for Binding of C4BP or fH—To understand how these fluid phase regulators bind to dead cells, we analyzed whether C4BP and fH from serum bind to dead cells directly using a cellular ligand or as a consequence of prior deposition of their ligands, C4b and C3b, by complement activation. Comparing binding of purified C4BP-PS and fH with binding of C4BP-PS and fH from serum revealed that their binding patterns are virtually identical (Fig. 4, A and B). Cell death has been induced in the absence of serum or fetal calf serum, and these cells do not contain C3 or C4 fragments that could potentially serve as ligands for the purified C4BP and fH preparations (data not shown). Therefore, we conclude that the binding of C4BP and fH to apoptotic cells coincides with, but is independent from, complement activation.

Classical Early versus Late Apoptosis Does Not Explain Binding Restrictions of C4BP and fH—To get more insight into the binding specificities of C4BP and fH, we analyzed in detail which populations of dying cells actually bind the fluid phase inhibitors. We rendered cells apoptotic for 2 h, gated on individual cell populations (Fig. 4C), and analyzed the binding of C4BP and fH (Fig. 4D). At 2 h the population classically called early apoptotic (AV+/VP-) does not bind C4BP or fH, and even the population classically called late apoptotic (AV+/VP+) only binds very limited amounts of C4BP compared with similar (AV+/VP+) populations in apoptotic cells at, for example, 6 h (Fig. 4, A and B) or necrotic cells. Levels of C4BP binding to late 6 h apoptotic cells is similar as to necrotic cells, whereas for fH the binding is always strongest to necrotic cells.

Taken together, C4BP and fH do not yet bind to AV+VP+ cells at 2 h, but only bind to AV+VP+ cells after an extended period of time. This indicates that other as yet unknown changes need to take place on the cell surface in addition to membrane flip-flop (AV+) or cell permeability (VP+).

The above data show that the C4BP-protein S complex does not bind to early apoptotic cells despite the fact that they expose phosphatidylserine (AV+). Protein S binds to phosphatidylserine and is present in plasma in free form and as part of the C4BP-PS complex (18). We analyzed whether there is differential binding of free protein S and the C4BP-PS complex and

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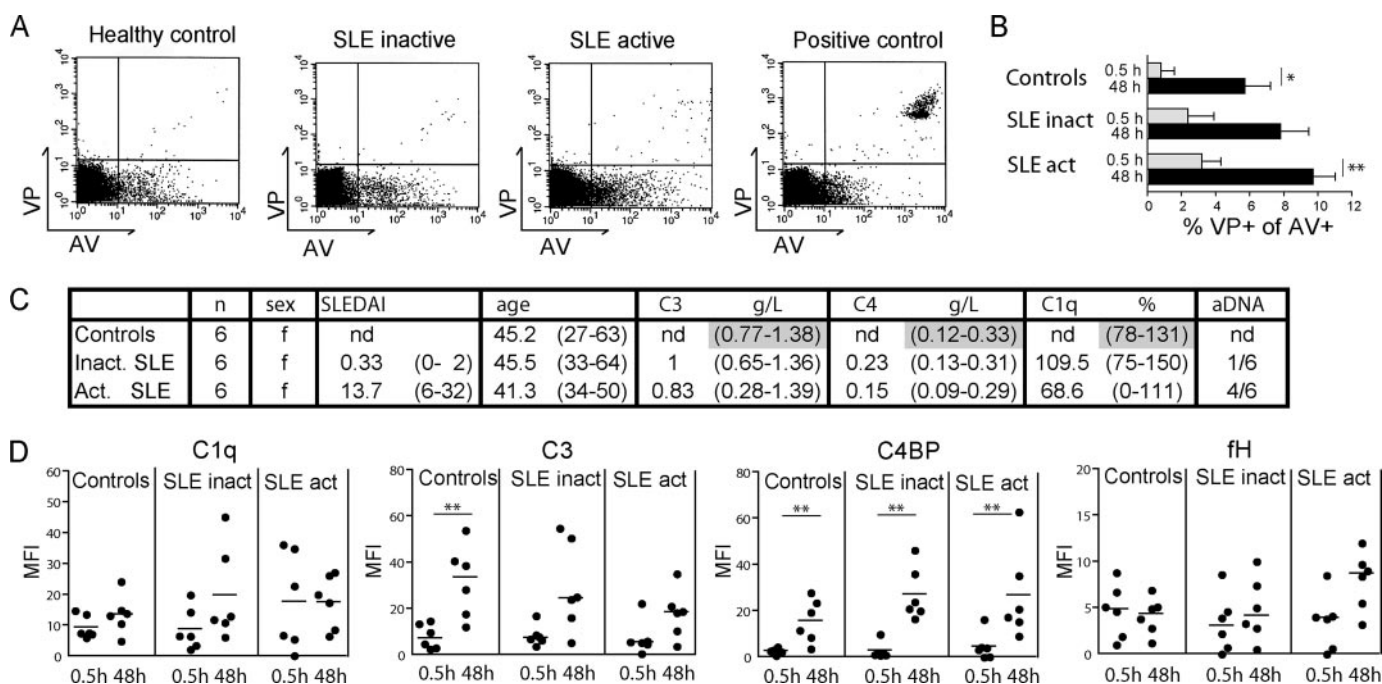


FIGURE 3. Apoptotic cells bind complement *in vivo*. Fresh whole blood and serum was obtained from healthy controls or SLE patients with active or inactive disease. The cells obtained from citrate blood were cultured with 10% autologous serum and analyzed either directly (0.5 h) or after 48 h of culture. Cell populations that bound AV or VP were analyzed by FACS. *A*, representative examples of samples analyzed directly after processing. *B*, quantification of the changes in the percentage of VP+ cells of all AV+ cells for all groups. *C*, description of patient characteristics. *D*, quantification of the mean fluorescence intensity (MFI) of binding of C1q, C3, C4BP, and fH on the AV+ cells for all groups on both time points.

observed that free protein S binds both early and late apoptotic cells, whereas the C4BP-PS complex only binds to cells in later stages of apoptosis (Fig. 4E). The pattern of free protein S binding over time resembles binding of AV, another small molecule that binds to phosphatidylserine and that of C4BP-PS resembles more that of Via-Probe (Fig. 4F) as a marker of the later stages of apoptosis. This indicates that membrane flip-flop alone is sufficient to allow binding of free protein S, whereas for binding of the larger C4BP-PS complex more changes on the apoptotic cell surface need to take place.

CRP Does Not Influence the Binding of C4BP and fH to Dead Cells—We have previously shown that C4BP binds to dead cells via the protein S part of the C4BP-PS complex (22, 29, 30). For fH the mode of binding is still unknown and is likely to be complex because fH has multiple binding sites for several ligands (17).

It was reported previously that both fH and C4BP bind to C-reactive protein (CRP) (31, 32) and that CRP binds to dead cells (33, 34). Therefore, we investigated to what extent CRP would influence the binding of C4BP and fH to dead cells and what the effect would be on overall complement activation. CRP binds dead cells in a way similar to fH, *i.e.* it does not bind to live cells, binds weakly to apoptotic cells, but binds strongly to necrotic cells both as purified protein or from NHS containing CRP (data not shown). Next we analyzed whether preincubation with CRP would change complement binding to dead cells by using purified C1q, as a well known binder of CRP (35, 36) as a positive control. Pretreating the cells with 50 $\mu\text{g/ml}$ CRP resulted in binding of CRP to dead cells and enhanced binding of purified C1q as expected (Fig. 5A). In contrast to this strong effect on C1q, we did not observe enhanced binding of

purified C4BP or fH following CRP pretreatment (Fig. 5B), suggesting that CRP is not a major ligand for C4BP and fH on dead cells.

In addition to testing the effect CRP preincubation on binding of purified C4BP and fH, we also tested the effect of this treatment on their binding from serum and on complement activation. CRP was reported to bind to apoptotic cells and enhance complement activation while inhibiting membrane attack complex formation by binding fH (33). Using 20% NHS with increasing concentrations of CRP, we observed that CRP bound to dead cells and increased the deposition of C1q and complement activation as measured by C3 and C9 on both apoptotic and necrotic cells (Fig. 5, C and D). However, CRP binding did not enhance the binding of C4BP and fH from serum (Fig. 5, C and D).

The Effect of Heparin and Blocking Antibodies on Binding of C4BP and fH to Dead Cells—Both C4BP and fH contain multiple heparin-binding sites that could mediate binding to cell surface-associated heparan sulfate (17, 18, 37). However, incubation of C4BP and fH with dead cells in the presence of heparin, which blocks protein interactions with heparan sulfate (37), did not affect the C4BP binding (Fig. 6A). Heparin did not inhibit the binding of fH to apoptotic cells, whereas it did inhibit its binding to necrotic cells (Fig. 6B). This observation together with the fact that on necrotic cells there is more fH binding than on late apoptotic cells indicates that different ligands for fH are expressed on these two populations of dead cells. Using blocking antibodies against protein S, we confirm that binding of C4BP-PS is mainly mediated via protein S (Fig. 6A). Using a blocking antibody against fH, we observed a significant reduction in fH binding to dead cells (Fig. 6B). This indi-

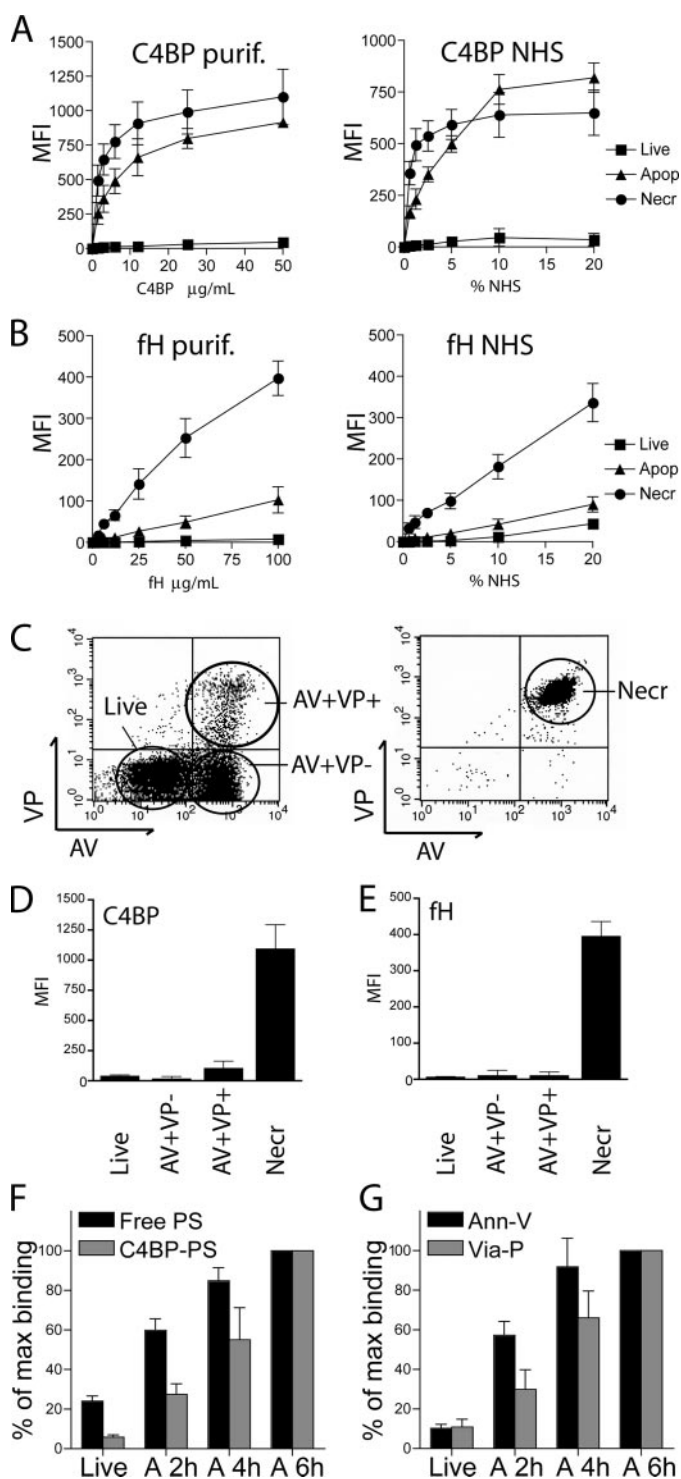


FIGURE 4. Binding of C4BP and fH is independent of complement activation. Live, apoptotic (6 h), or necrotic (Necr) cells were incubated with increasing concentrations of either NHS or with purified (Purif) C4BP and fH in concentration similar to those present in serum. Binding of C4BP (A) and fH (B) was analyzed by FACS and expressed as mean fluorescence intensity (MFI). Binding of purified C4BP and fH was analyzed on the different gated cell populations of AV and Via-P stained apoptotic (2 h) and necrotic cells as indicated (C). Binding of C4BP at 50 $\mu\text{g/mL}$ (D) and fH at 100 $\mu\text{g/mL}$ (E) to the gated cell populations. F, both free protein S and C4BP-PS were added to live, apoptotic or necrotic cells in equimolar concentrations and analyzed for their binding by FACS. Binding of free protein S or C4BP-PS is expressed as the percentage of maximum binding. G, binding of AV and Via-P to the different live and dead populations also expressed as a percentage of maximum binding.

ates that at least part of the binding of fH to dead cells is mediated via sites on CCP-20 of fH, which contains the epitope for the blocking antibody. However, the residual binding indicates that multiple interactions, potentially with multiple ligands, are involved in fH binding (Fig. 6B).

DISCUSSION

The complement system is a very powerful inflammatory cascade that among other functions can lyse target cells. Under normal conditions m-C-Reg protect host cells from complement attack. Deficiency of m-C-Reg give rise to pathology, e.g. paroxysmal nocturnal hemoglobinuria, but is not strongly associated with autoimmunity (38). Host cells also bind fH to a certain extent, and fH deficiency results in spontaneous alternative pathway activation and renal disease (39), but not often in autoimmunity, likely because the ensuing secondary C3 deficiency prevents inflammation and lysis of dead cells. So far no full genetic deficiency of C4BP has been reported, whereas it has been analyzed in large cohorts in relation to anti-coagulant protein S (40, 41). Combining these observations with our current data makes it tempting to speculate that deficiency of C4BP would lead to embryonic death because of complement-mediated lysis of apoptotic cells.

What initiates complement activation on late apoptotic cells is not clear. Down-regulation of m-C-Reg expression is not sufficient, because we noticed a gap in time between down-regulation and complement activation (Fig. 7). Exposing phosphatidylserine as such was not sufficient for C1q binding or complement activation, and also membrane permeability is not sufficient for this. It is likely that a combination of direct binding of C1q (9) and binding of C1q ligands such as IgM antibodies (14, 42) to an as yet unknown ligand that becomes exposed late during the apoptosis process is responsible for the initiation of complement activation.

Although C4BP binds dead cells mainly via protein S (22, 29, 30), which is known to bind strongly to phosphatidylserine, we did not observe significant binding of C4BP to early apoptotic cells despite the fact that they are AV+/VP- or even AV+/VP+ at 2 h. In contrast we show that free protein S does bind to such early apoptotic cells. This difference in binding could be explained by the presence of bulky macromolecules on the cell surface that sterically hinder binding of the large (500 kDa) C4BP-PS complex. Apparently during late apoptosis the cell surface is significantly altered to the extent that the membrane is accessible to large proteins. This differential binding fits with the observations that free protein S bound to apoptotic cells enhances phagocytosis (43), whereas binding of the C4BP-PS complex inhibits phagocytosis (26). We argue that free protein S enhances clearance by binding to early apoptotic cells and that the C4BP-PS complex only binds during the late stages of apoptosis when it is necessary to protect against excessive complement activation. The molecular interaction of fH is more difficult to understand; most of the binding involves CCP-20, because the blocking antibody had a significant impact on fH binding to both apoptotic and necrotic cells. Heparin-binding sites were also involved but mainly on necrotic cells. Overall it seems that several ligands are involved in binding to different sites on the fH molecule.

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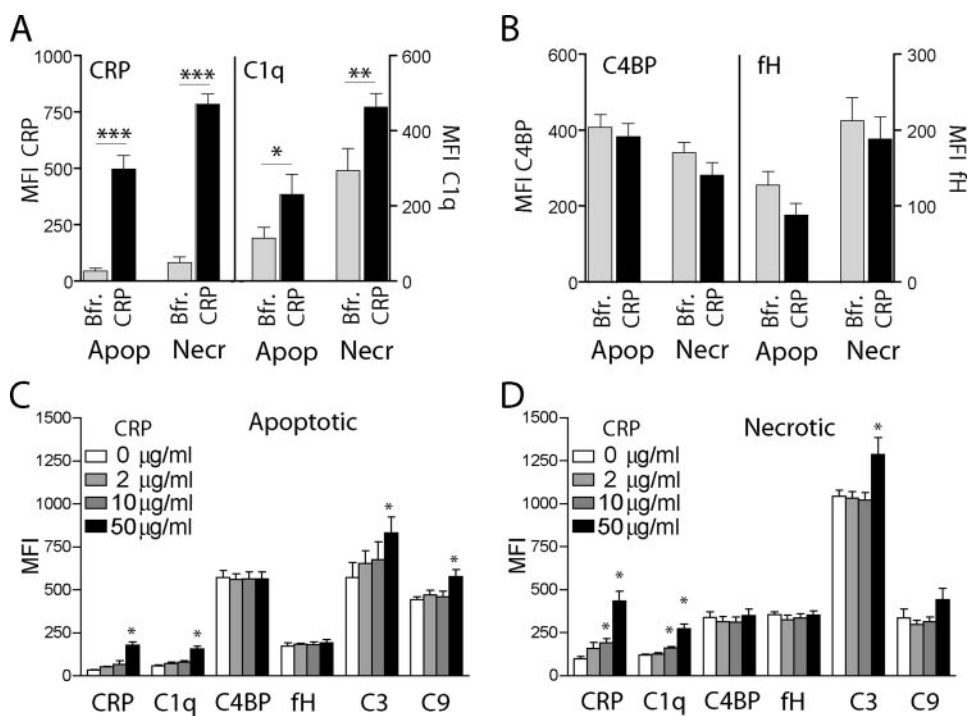


FIGURE 5. **CRP is not major ligand for C4BP or fH on dead cells.** *A*, effect of preincubation with CRP on the binding of C1q to apoptotic (*Apop*) and necrotic (*Necr*) cells. *B*, analysis of the effect of CRP preincubation on C4BP and fH binding. Effect of adding increasing concentrations of CRP to NHS on complement activation on apoptotic (*C*) and necrotic cells (*D*). *MFI*, mean fluorescence intensity. *Bfr.*, buffer only.

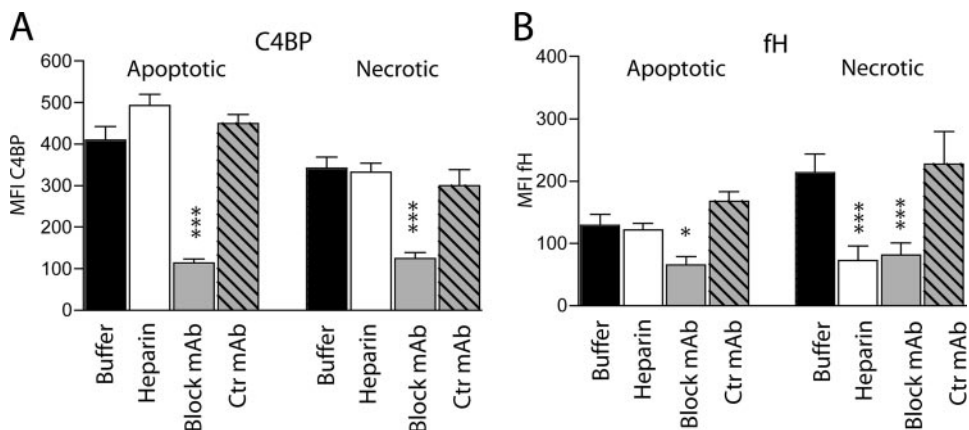


FIGURE 6. **Effect of heparin and blocking antibodies on the binding of C4BP and fH to dead cells.** Apoptotic (6 h) and necrotic cells were incubated with C4BP-PS (*A*) or fH (*B*) in buffer or in the presence of heparin, blocking antibodies against Protein S (mAb 21) or blocking antibodies against CCP-20 of fH (MH-10) or matched control antibodies. Binding of C4BP and fH was analyzed by FACS, and the data are expressed as mean fluorescence intensity (*MFI*). Statistical differences were calculated compared with samples incubated in buffer only.

The role of CRP in the process of recognition of apoptotic cells and complement activation has been a matter of debate (33, 34, 44, 45). It was reported that CRP binds to apoptotic cells, increases C1q deposition, but limits later stages of complement activation by binding fH (33). Now we and others, (34) provide evidence that CRP only binds to late apoptotic or necrotic cells. We show that it enhances C1q binding and as a consequence gives more C3 and C9 deposition. However, we do not observe enhanced fH binding or any protective effect of CRP regarding C9 deposition. Why these observations differ from some other reports may relate to the quality of the proteins analyzed and the semantics regarding stages of cell death.

Although fH (31, 46) and C4BP (32) have been reported to interact with CRP, we did not observe enhanced binding of fH or C4BP in the presence of CRP. This does not mean that these molecules do not interact with CRP but shows that they do not need CRP for binding to dead cells. This agrees with the fact that the CRP-fH binding is of relatively low affinity (31, 32) and that CRP binds C4BP via the center portion of C4BP (32), a part that is not readily available in the common form of the C4BP-PS complex because of steric hindrance by protein S. The CRP-C4BP interaction may therefore be most important during an acute phase response when the form of C4BP lacking the β -chain and protein S is up-regulated (47).

Testing blood and tissue sections of healthy controls for the presence of noningested apoptotic cells revealed that there are only a few free apoptotic cells (5, 48) and hardly any apoptotic cells that are positive for complement components. This provides indirect evidence for the speed of phagocytosis in the healthy host. This early recognition and uptake of apoptotic cells is also observed *in vitro* (49) and is thought to stimulate an anti-inflammatory response (50).

It seems that by far most cells are cleared before the stage when they acquire complement. However, this does not mean that a proper interaction of complement with dead cells is not relevant. Although C1q binding to apoptotic cells is a relatively late event, C1q deficiency leads to SLE in 90% of the cases, which according to the waste disposal theory is because of defective

clearance of late apoptotic cells (51). This indicates that there are moments when apoptotic cells are not cleared before the stage when complement comes into play. This is when we rely on enhanced recognition of dead cells for uptake by binding of C1q followed by limited complement activation, which is kept under control by C4BP and fH. Possibly this is during moments of reduced phagocytic capacity or excessive apoptosis, *e.g.* during infections, sunlight exposure, or pregnancy. Several groups have indeed reported that subgroups of SLE patients display defective clearance (48, 52, 53) or enhanced apoptosis that may overwhelm the uptake machinery (54, 55).

C4BP and fH do not block complement activation but rather

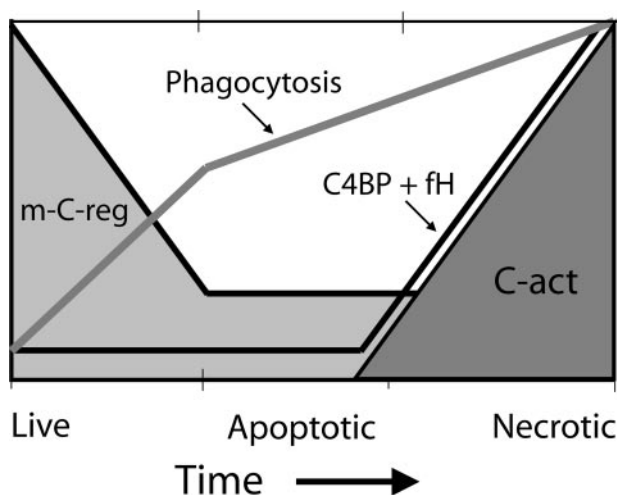


FIGURE 7. Schematic representation of the presence of complement inhibitors on apoptotic cells. Membrane-bound complement regulators are down-regulated rapidly after induction of apoptosis, and phagocytosis is initiated. Following a gap phase complement activation is initiated and phagocytosis is enhanced. Simultaneous binding of fluid phase complement inhibitors C4BP and fH prevent excessive complement activation and lysis.

form an inhibitory environment that will prevent excessive complement activation and lysis. In the healthy controls we observed C3 deposition on the apoptotic cells, whereas in the patients there was significant variation and no significant increase in C3 deposition upon culture. C3 bound to antigen is involved in the instruction of the adaptive immune system, and a lack of C3 on apoptotic particles may alter their retention in the immune system and influence the self-reactive repertoire of immune cells. This scenario reconciles the waste disposal hypothesis (56), regarding the enhanced clearance of apoptotic cells in the presence of complement with the altered immune activation hypothesis (57) regarding the presence of C3 on apoptotic cells. For the SLE samples we did not observe any differences regarding the binding of C4BP and fH, whereas we observed clear abnormalities regarding complement opsonization by C1q and C3 compared with healthy controls.

In conclusion (Fig. 7), when cells become apoptotic they down-regulate m-C-Reg, and phagocytosis is initiated. After a lag phase they acquire the capacity to bind C1q and activate complement. At the same moment, but independent from complement activation, fluid phase complement inhibitors C4BP and fH bind and provide protection against excessive complement activation on the apoptotic cell. This process may allow enhanced recognition by the phagocyte via complement receptors while preventing lysis of the dying cell and an exaggerated inflammatory response.

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