



Complement binding is an early feature of necrotic and a rather late event during apoptotic cell death

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Abstract

The phagocytosis of dying cells is an integral feature of apoptosis and necrosis. There are many receptors involved in recognition of dying cells, however, the molecular mechanisms of the scavenging process remain elusive. The activation by necrotic cells of complement is well established, however, the importance of complement in the scavenging process of apoptotic cells was just recently described. Here we report that the complement components C3 and C4 immediately bound to necrotic cells. The binding of complement was much higher for lymphocytes compared to granulocytes. In case of apoptotic cell death complement binding was a rather late event, which in lymphocytes was preceded by secondary necrosis. Taken together complement binding is an immediate early feature of necrosis and a rather late event during apoptotic cell death. We conclude that complement may serve as an opsonin for fragments of apoptotic cells that have escaped regular scavenging mechanisms. *Cell Death and Differentiation* (2001) 8, 327–334.

Keywords: Complement binding; apoptosis; necrosis

Abbreviations: AS, fresh autologous serum; AxV, annexin V; DiO₆C(3), fluorescent dye 3,3'-dihexyloxycarbocyanine iodide; FITC, fluorescein isothiocyanate; FSC, forward scatter; IS, heat inactivated autologous serum; NGH, nuclear granularity and hypochromicity; PBL, peripheral blood lymphocytes; PI, propidium iodide; PMN, polymorphonuclear cells; PS, phosphatidylserine; SSC, side scatter; $\Delta\psi_m$, mitochondrial membrane potential; UVB, ultraviolet B light

Introduction

Physiological cell death usually proceeds by the process of apoptosis. After an initial phase of commitment to cell death the execution phase is characterised by distinct structural changes.¹ Recognition and removal by phagocytes is the final common event of the death program. Genetic studies of *C. elegans* revealed that at least six out of 14 genes regulating apoptosis are necessary for engulfment of apoptotic cells,

highlighting the importance of their clearance.² The fast and efficient removal of apoptotic cells results in a low steady state of dying cells even in tissues of mass apoptosis like the thymus.³ Both macrophages and neighbouring semi-professional phagocytes^{4–8} usually remove cells undergoing apoptosis before membrane integrity is lost.⁹ Thereby, fast recognition, uptake, and nonphlogistic degradation promote the resolution of inflammation.^{10,11} If phagocytosis is insufficient to remove apoptotic cells in the early phases of cell death, noxious cell contents may leak and cause severe tissue damage and autoimmunity.^{9,12} This is of major importance for the removal of aged granulocytes containing high amounts of toxic compounds.^{13,14} Lectin-like molecules, the receptors for thrombospondin (CD36) and vitronectin ($\alpha_V\beta_3$ and $\alpha_V\beta_5$),^{15,16} class A and B scavenger receptors,¹⁷ the ATP-binding cassette transporter ABC1,¹⁸ as well as CD14¹⁹ have all been described to recognise surface changes on apoptotic cells. Since phagocytosis of apoptotic lymphocytes by macrophages is stereospecifically inhibited by phosphatidyl-L-serine liposomes, a specific receptor is supposed to be involved in phosphatidylserine (PS) recognition.²⁰ In addition, β_2 -glycoprotein, annexins and gas-6 may serve as adaptor proteins.²¹ Antibodies recognising either phospholipid of β_2 -glycoprotein-I were able to opsonise apoptotic cells and substantially enhanced their immunogenicity.^{22,23}

Surface blebs of apoptotic cells have been shown to contain high amounts of autoantigen that had been modified in the execution phase of apoptotic cell death.^{24–28} Furthermore, keratinocytes undergoing apoptosis develop the capacity to bind C1q in the absence of antibodies.²⁹ This complement factor was supposed to influence the immune response to self-antigens comprised within surface blebs of apoptotic cells.³⁰ In animals with targeted deletion of C1q, impaired clearance of apoptotic cells and a mild form of nuclear autoimmunity were observed.³¹ Furthermore, it was shown that complement was able to augment the *in vitro* uptake of apoptotic cells by monocyte-derived macrophages.³²

Here, we investigated the complement binding of necrotic and apoptotic cells in different states of cell death. To induce necrosis of polymorphonuclear cells (PMN) and of peripheral blood lymphocytes (PBL) cells were treated with heat shock. PBL apoptosis had to be induced by ultraviolet B light (UVB) irradiation whereas in PMN apoptosis occurred spontaneously during some hours of *in vitro* culture (ageing process). We demonstrated that the binding of the complement factors C1q, C3 and C4 to aged PMN and apoptotic PBL is a very late event in apoptosis. In contrast, induction of necrosis by heat shock resulted in an immediate binding of complement, more pronounced in PBL than in PMN. Analysis of C5 binding revealed that the membrane attack complex was not activated by apoptotic or necrotic cells, respectively.

Results

Complement does not change the morphology of the cultured cells

Untreated PMN (Figure 1a) as well as irradiated PBL (Figure 1b) underwent apoptotic cell death during culture. While apoptosis was proceeding, PMN as well as PBL underwent morphological changes resulting in a typical decrease of the forward scatter (FSC) concomitant with an increase in side scatter (SSC) as detected by flow cytometry (viable cells: population 1, apoptotic cells: population 2, subcellular fragments: population 3) (Table 1). In ageing PMN these changes occur faster than in UV-B irradiated PBL and the population 2 is much less pronounced (Figure 1). In the late phases of apoptosis, cells disintegrated resulting in numerous subcellular fragments with reduced FSC/SSC (marked with 3 in Figure 1a,b). A different behaviour could be seen in necrotic PMN compared to necrotic PBL. Compared to viable PMN, necrotic PMN showed a slightly decreased SSC and a markedly reduced FSC (Table 1a). In contrast, necrosis of PBL resulted in a moderate increase in SSC and a decrease in FSC (Table 1b). In comparison to cultures in medium containing 10% heat inactivated fetal calf serum (R10) neither

active nor heat inactivated autologous serum exerted relevant influences on the FSC and SSC properties of all populations (Figure 1c,d).

Time course of apoptosis and necrosis

Table 2 summarises the properties of the three distinct populations defined in Figure 1 and Table 1. Most PBL in population 1 are viable (annexin-V^{neg} (AxV)^{neg}, propidium iodide^{neg} (PI)^{neg}, mitochondrial membrane potential^{high} ($\Delta\psi_m$)^{high}), whereas populations 2 and 3 mainly consist of apoptotic (AxV^{pos}, PI^{neg}, $\Delta\psi_m$ ^{low}) or secondary necrotic (AxV^{pos}, PI^{low}, $\Delta\psi_m$ ^{low}) cells. Since primary necrotic cells (AxV^{pos}, PI^{pos}) can clearly be distinguished from populations 2 and 3 by the $\Delta\psi_m$ (Table 2a) and the FSC/SSC properties (Figure 1 and Table 1), PI^{low} cells of both populations are referred to as secondary necrotic cells.

The major differences between UVB irradiated PBL and *in vitro* aged PMN are the low amounts of secondary necrotic cells and the relative stability of the $\Delta\psi_m$ in PMN. Even incubation for 30 min at 56°C yielded only 64% of primary necrotic PMN, defined by permeability for PI (Table 2b). Importantly, neither addition of freshly isolated nor

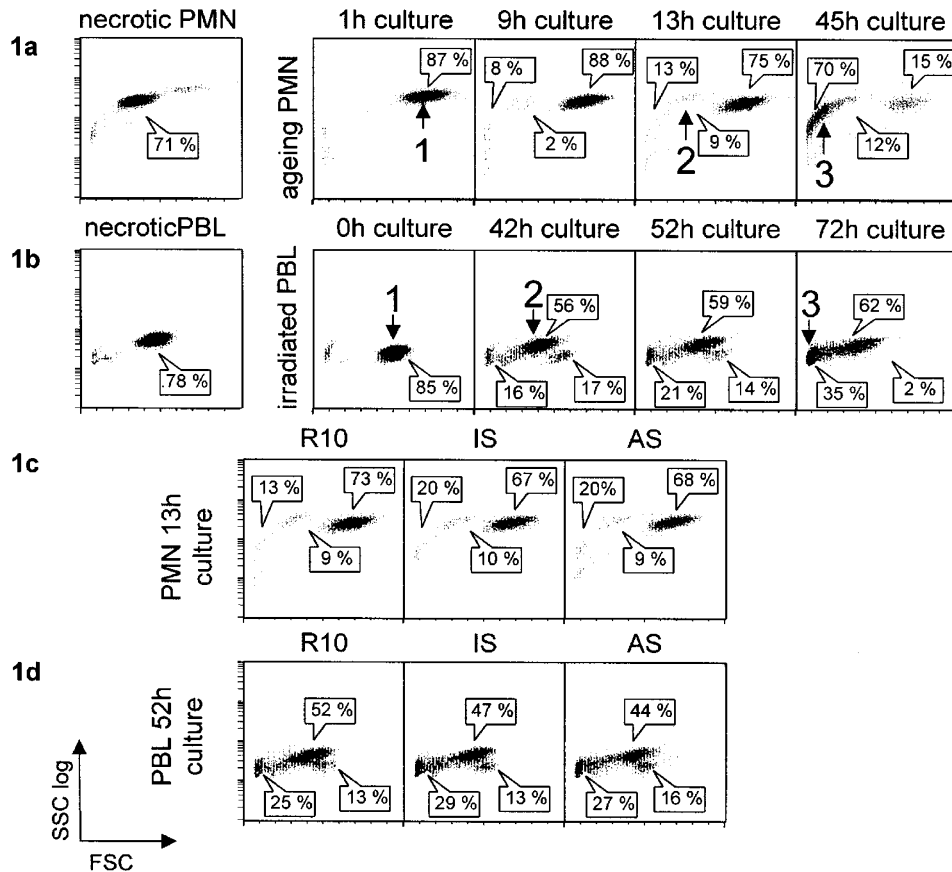


Figure 1 Flow cytometric analysis of forward and side scatter of PMN and PBL undergoing apoptosis or necrosis. Time course of morphological changes in ageing PMN (a) and UVB irradiated PBL (b) was analyzed by FSC/SSC dot blots. During execution of apoptosis three distinct populations could consecutively be observed (marked with 1, 2, and 3). The addition of heat inactivated (IS) or fresh serum (AS) to the culture medium (RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum (R10)) did not further change the cellular morphology of PMN (c) or PBL (d), respectively. Primary necrotic PMN and PBL (incubated for 30 min at 56°C) are shown in the left dot blots of a and b

Table 1 Properties of the cellular subpopulations defined in Figure 1 analyzed by cytofluorometry

1a									
PMN	1 h culture		9 h culture		13 h culture		45 h culture		
	Mn FSC	Mn SSC	Mn FSC	Mn SSC	Mn FSC	Mn SSC	Mn FSC	Mn SSC	
Population 1	179.1	29.1	179.2	28.5	168.9	23.9	168.2	26.2	
Population 2			101.1	31.5	98.7	33.4	96.1	34.4	
Population 3			35.2	8.24	45.8	12.4	42.4	13.7	
Necrotic	86.6	23.1							

1b									
PBL	0 h culture		42 h culture		52 h culture		72 h culture		
	Mn FSC	Mn SSC	Mn FSC	Mn SSC	Mn FSC	Mn SSC	Mn FSC	Mn SSC	
Population 1	152.1	2.38	141.2	2.28	142.9	2.38	134.5	3.17	
Population 2			104.5	4.11	105.3	4.28	102.7	4.21	
Population 3			27.6	2.52	27.7	2.47	27.9	2.44	
Necrotic	123.5	5.77							

The median of the forward scatter (FSC) and side scatter (SSC) of PMN (1a) and PBL (1b) were calculated at the times indicated

Table 2 Overview: Properties of viable, apoptotic, primary and secondary necrotic PBL and PMN as defined by forward and side scatter in Figure 1 and Table 1

2a									
PBL	AxV ⁻ PI ⁻ /% (viable)	AxV ⁺ PI ⁻ /% (apoptotic)	AxV ⁺ PI ^{low} /% (2nd necrotic)	AxV ⁺ PI ⁺ /% (necrotic)	$\Delta\psi_m$	C1q	C3	C4	C5
Population 1	97	3	0	0	80	0.00	0.64	0.00	0.00
Population 2	2	39	46	14	8	0.05	2.50	0.49	0.00
Population 3	5	25	51	19	1	2.92	11.6	3.49	0.49
1st necrosis	0	1	6	94	65	4.98	127	47.3	1.45

2b									
PMN	AxV ⁻ PI ⁻ /% (viable)	AxV ⁺ PI ⁻ /% (apoptotic)	AxV ⁺ PI ^{low} /% (2nd necrotic)	AxV ⁺ PI ⁺ /% (necrotic)	$\Delta\psi_m$	C1q	C3	C4	C5
Population 1	89	11	0	0	90	0.50	4.40	3.12	0.00
Population 2	2	80	7	11	66	3.90	7.30	13.4	0.00
Population 3	3	97	1	0	51	11.5	18.3	20.8	0.58
1st necrosis	0	23	13	64	85	12.5	17.8	33.2	0.00

AxV⁻PI⁻: no AxV binding, PI impermeable cells (viable). AxV⁺PI⁻: AxV binding, PI impermeable cells (apoptotic). AxV⁺PI⁺: AxV binding, PI permeable cells with G1 DNA content (primary necrosis). AxV⁺PI^{low}: AxV binding, PI permeable cells with sub-G1 content (secondary necrosis). The mitochondrial membrane potential ($\Delta\psi_m$) and the mean values of the specific binding of the complement components C1q, C3, C4 and C5 are displayed as well

inactivated serum to the culture medium (RPMI-1640 supplemented with 10% heat inactivated fetal calf serum (R10)) markedly influenced annexin V binding, PI permeability, $\Delta\psi_m$ (data not shown), or FSC/SSC properties (Figure 1 and Table 1).

We used loss of $\Delta\psi_m$ (staining with fluorescent dye 3,3'-dihexyloxycarbocyanine iodide (DiO₆C(3))), exposure of PS (staining with AxV-FITC (fluorescein isothiocyanate)), dye exclusion (staining with PI in the absence of detergent), nuclear granularity and hypochromicity (NGH) (staining with PI in the presence of detergent) as markers for apoptotic and necrotic cell death. As shown in Figure 2a, 42 h after UVB irradiation with 120 mJ/cm² more than 70% of the PBL displayed reduction of $\Delta\psi_m$, exposure of PS and nuclear changes. Less than 20% of the cells displayed a primary necrotic phenotype. In late phases (100 h after UV-B irradiation) PI binding in absence of detergent increased.

In cultured PMN apoptosis proceeds faster than in UVB irradiated PBL. After 29 h of culture more than 70% of the

PMN exposed PS and more than 90% showed nuclear changes. Only 44% of PMN had reduced $\Delta\psi_m$. The membrane integrity was preserved in most PMN at this time point since more than 92% of the cells excluded PI (Figure 2b).

Complement binds subcellular fragments of PBL generated during the very late phases of apoptotic cell death

To detect complement binding, cells were stained with affinity purified, FITC labelled antibodies against C1q, C3c, C4c and C5 (Figure 3). Heat treated primary necrotic lymphocytes showed a strong binding of C3 and C4, whereas there was very little binding of C1q and C5. In contrast, irradiated lymphocytes displayed only a weak binding of complement for the first 52 h of culture. During this period most PBL had undergone apoptosis and many of them already displayed a primary (PI^{pos}) or secondary necrotic (PI^{low}) phenotype (Table

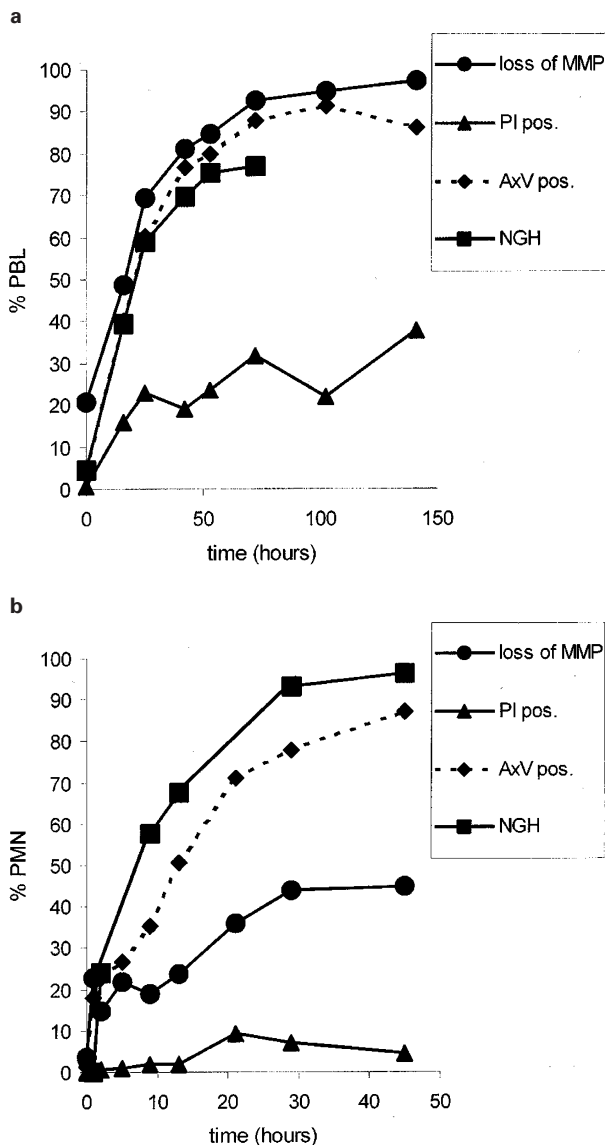


Figure 2 (a) Time course of apoptosis in PBL. Changes in AxV-FITC binding, PI binding to DNA in absence of detergent, PI binding to DNA in presence of detergent (detection of NGH) and loss of mitochondria membrane potential (MMP) of UV-B irradiated PBL were analyzed at different time points of culture. (b) Time course of apoptosis in PMN. Changes in AxV-FITC binding, PI binding to DNA in absence of detergent, PI binding to DNA in presence of detergent (detection NGH) and loss of mitochondria membrane potential (MMP) of ageing PMN were analyzed at different time points of culture

2). However, between 52 and 72 h many subcellular fragments were formed (population 3), which bound C1q, C3 and C4 (Figure 3 and Table 2). C5 was not detected on UVB irradiated PBL. PBL of population 2 showed weak binding of C1q and those of population 1 never showed any complement binding (Figure 3 and Table 2a). Therefore, morphological changes, PS exposure, nuclear hypochromicity and loss of mitochondrial membrane potential precede the complement binding of irradiated lymphocytes. The latter appeared to be focused on small subcellular fragments that are generated during the very late phases of apoptotic cell death.

In addition to UVB induced apoptosis we analyzed the complement binding of PBL undergoing either spontaneous apoptosis or apoptosis induced by staurosporine. Although the kinetics of apoptosis differed considerably between all three protocols, the complement binding was focused on subcellular fragments in all cases (data not shown). When we performed the experiments with up to 30% human serum in R10, we also observed a strong specific complement binding only for subcellular fragments (data not shown).

Complement mainly binds subcellular fragments generated during *in vitro* culture of PMN

Ageing PMN retained their membrane integrity much longer than apoptotic PBL (Table 2). Concerning complement binding apoptotic PMN showed some striking differences to UVB irradiated PBL (Figure 4). Interestingly, necrotic PMN bound much less C3 and C4 compared to necrotic PBL. A low binding of C3 and C4 was observed as early as 1 h and a low C1q binding was detected after 13 h of *in vitro* culture of PMN that had remained in the viable cell gate (population 1). However, during prolonged culture subcellular fragments were generated that showed a very high C3 and a high C4 binding activity. In addition, these fragments moderately bound C1q and C5 (Figure 4 and Table 2b). Although a weak C1q, C3, and C4 binding was observed in the early phases of apoptotic cell death, high complement binding to PMN was a late event during apoptosis preceded by PS exposure and nuclear hypochromicity. In contrast to PBL most of the PMN binding complement still excluded PI.

Discussion

In vivo cells undergoing apoptosis are timely recognised and swiftly ingested while still intact, thereby protecting tissues from the potentially harmful exposure to the contents of the dying cell.¹⁵ The clearance of dying cells is mainly performed by professional phagocytes like macrophages. In addition, apoptotic corpses are also taken up by neighbouring cells acting as semi-professional phagocytes.⁹ Many receptors and adaptor molecules have been reported to be involved in the fast and efficient removal of apoptotic cells in various tissues (reviewed in³³). Recently, the receptor involved in PS recognition on apoptotic cells was identified.²⁰ The clearance of apoptotic cells is usually non-phlogistic.^{34,35} In contrast, cells with disintegrated membranes have been shown to release proinflammatory substances capable to activate dendritic cells and thereby function as natural adjuvants.³⁶ Therefore, the non-inflammatory clearance of apoptotic cells has to be completed before the loss of membrane integrity. Granulocytes contain huge amounts of toxic compounds and are constitutively programmed to undergo apoptosis. Therefore, the removal of apoptotic neutrophils and eosinophils is a prerequisite for the resolution of inflammatory responses.¹⁰

Mice with targeted disruption of the C1qa gene provided evidence that C1q is necessary for proper clearance of apoptotic cells, since apoptotic corpse accumulated in the kidneys of these animals.³¹ Furthermore, it was shown that C1q binds to surface blebs of apoptotic keratinocytes.²⁹

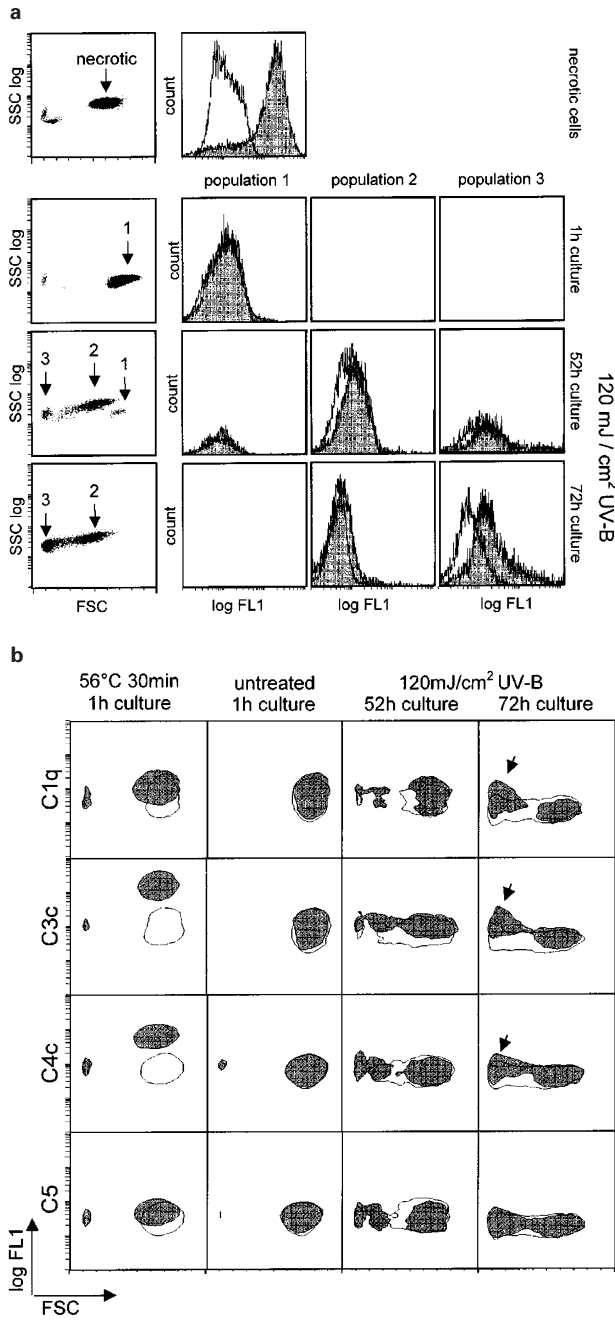


Figure 3 Analysis of complement binding to PBL by cytofluorometry. Thirty minutes at 56°C incubated (necrotic, **b**, col. 1), freshly isolated (viable, **b**, col. 2), and UV-B irradiated (apoptotic, **b**, col. 3 and 4) PBL were cultured for the time indicated in medium either containing 10% heat inactivated FCS only (complement inactivated control, white) or 10% heat inactivated FCS plus 7% fresh autologous human serum (containing active complement, grey). The cells were stained with affinity purified, FITC-labelled antibodies against C1q, C3c, C4c, or C5. (a) shows the dot blots of FSC/SSC log and histograms for the C3c staining. (b) displays the FSC/FL1 log dot blots for C1q, C3c, C4c, and C5, respectively. Binding of the complement components C3 and C4 was much higher in necrotic than in apoptotic cells. The latter showed a binding of C1q, C3, and C4 focused on small cellular fragments observed in late phases of apoptosis (arrow)

The authors concluded that C1q may mediate antibody-independent clearance of apoptotic keratinocytes, and

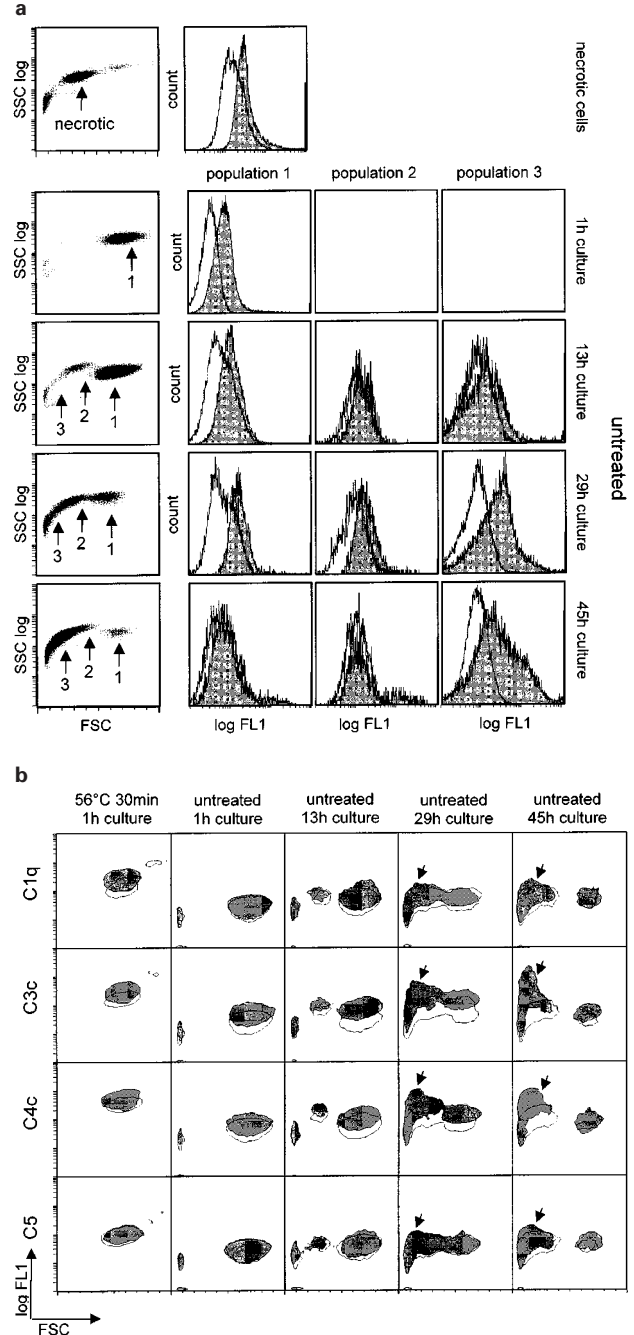


Figure 4 Analysis of complement binding to PMN by cytofluorometry. Thirty minutes at 56°C incubated (necrotic, **b**, col. 1), freshly isolated (viable, **b**, col. 2), and *in vitro* aged (apoptotic, **b**, col. 3–5) PMN were cultured for the time indicated in medium either containing 10% heat inactivated FCS only (complement inactivated control, white) or 10% heat inactivated FCS plus 7% fresh autologous human serum (containing active complement, grey). The cells were stained with affinity purified, FITC-labelled antibodies against C1q, C3c, C4c, or C5. (a) shows the dot blots of FSC/SSC log and histograms for the C3c staining. (b) displays the FSC/FL1 log dot blots for C1q, C3c, C4c, and C5, respectively. Necrotic PMN showed an increased binding of C1q, C3, and C4. Binding of complement was to be observed after ageing in culture mainly focused on small cellular fragments (arrow). Freshly isolated PMN showed a low binding of C3 and C4 which steadily increased during *in vitro* culture

prevents immunisation with autoantigens of cutaneous origin. In addition, experiments with *in vitro* cultured

macrophages showed that the uptake of apoptotic cells was significantly increased in the presence of active serum complement.³² Therefore, under certain conditions complement may contribute to the removal of dying cells. For this reason we investigated the binding of complement components to apoptotic, as well as primary and secondary necrotic cells. Whereas necrotic PBL efficiently bind C3 and C4 immediately after induction of necrosis by heat shock, UVB irradiated PBL executed the early stages of apoptosis without detectable C3 and C4 binding. Similar results had also been observed with induction of apoptosis by treatment with staurosporine or by cultured cells undergoing spontaneous apoptosis (data not shown). We used various methods to analyze cell death. All these methods identified cells as apoptotic before detectable amounts of C1q, C3 and C4 bound to the cells. In the early phases of apoptotic cell death the apoptotic PBL expose PS and become therefore targets for phagocytes expressing the PS receptor.²⁰ Also many other receptors recognising surface changes of apoptotic cells act in the early phases of cell death.^{18,21,37–39} Normally, the removal of dying cells is so efficient that the steady state of apoptotic cells is extremely low even in tissues with very high rates of apoptosis such as thymus or germinal centres of lymph nodes.³ These findings argue for a clearance of most apoptotic cells in the very early phases of apoptosis *in vivo*. Our *in vitro* data showed that a marked complement binding could be observed in the late phase of apoptosis, only. This activity is usually preceded by secondary necrosis and is mainly focused on small cells and subcellular fragments. Therefore, complement-mediated uptake of apoptotic cells may primarily represent a back-up mechanism for the clearance of late apoptotic cell material that had escaped first line clearance mechanisms. High local rates of apoptosis and impaired phagocytic capabilities of macrophages represent situations where complement may be necessary for the proper clearance of apoptotic cell material. Interestingly, high rates of apoptosis,⁴⁰ impaired phagocytosis⁴¹ and defects in the components of the classical complement pathway⁴² are discussed to contribute to the aetiology of human systemic lupus erythematosus. The phagocytic capability for cells in the peripheral blood is relatively low. Dead cells are usually removed from the peripheral blood during the passage of liver or spleen by macrophages of the reticuloendothelial system. Therefore, complement may be involved in the opsonisation and clearance of apoptotic debris that has been trapped in the blood vessels, e.g. in the glomeruli of the kidneys.³¹

The target structure on apoptotic cells which binds complement is not defined yet. Since secondary necrosis often precedes complement binding, cell surface changes as well as internal structures are candidates for complement activation by late apoptotic cells. Regarding complement binding there were two major differences between PBL and PMN. (I) Heat shocked, primary necrotic PMN bound C3 and C4 much less efficiently than necrotic PBL. (II) In contrast to PBL, in PMN complement binding to subcellular fragments was not preceded by loss of membrane integrity. This observation makes it unlikely

that complement binds to internal structures of aged PMN. The prolonged membrane integrity of PMN prevents leakage of toxic compounds from PMN and may be advantageous for the resolution of inflammation. The complement binding of both PMN and PBL takes place in the very late phases of apoptotic cell death and is focused on small cells or subcellular fragments. In contrast to the substantial binding of the complement components C1q, C3 and C4 to small cells or subcellular fragments, the binding of C5 was only marginal. Therefore, it can be concluded that the membrane attack complex is usually not activated by apoptotic or necrotic cells, respectively.

Clearance of apoptotic cells is a very important process which has implications for inflammation, autoimmunity, infectious diseases and tumour vaccination. Defective clearance or reduced anti-inflammatory properties of apoptotic cells may represent important susceptibility factors for autoimmunity.⁴³ Specific plasma proteins like serum amyloid P play an important role in preventing autoimmune diseases. Serum amyloid P binds to apoptotic cells and to nuclear debris released by necrotic cells and regulates the degradation of chromatin.⁴⁴

Complement is an important humoral defence system, which can initiate and amplify inflammation, even in the preimmune phase when specific antibodies are still lacking and specific lymphocytes are scarce. We suggest that opsonisation of dying cells and subcellular fragments is an important additional feature of complement. Future investigations are necessary to define the molecular targets on dying cells which are recognised and bound by the components of the classical complement pathway.

Materials and Methods

Cells and reagents

Venous blood was drawn from normal healthy volunteers according to institutional guidelines. The blood was anti-coagulated by the addition of 20 U/ml heparin. Peripheral blood mononuclear cells were isolated by Ficoll density centrifugation (Lymphoprep[®], Gibco-BRL, Eggenstein, Germany). Cells were resuspended in phosphate buffered saline (Gibco-BRL, Eggenstein, Germany) and remaining platelets were removed by centrifugation through fetal calf serum (Gibco-BRL, Eggenstein, Germany). To obtain non-adherent PBMC (PBL) monocytes were removed by adherence to plastic in Dulbecco's modified eagle medium (Gibco-BRL, Eggenstein, Germany). PMN were isolated by density centrifugation using discontinuous gradients of 70% and 62% percoll (Seromed, Berlin, Germany), respectively. Remaining erythrocytes were removed by hypotonic lysis. All cells were cultured in RPMI-1640 medium (Gibco BRL, Eggenstein, Germany) supplemented with 10% heat inactivated fetal calf serum (R10) in a humidified atmosphere containing 5% CO₂. As a source of complement or inactivated complement, respectively, the following additions were used: (1) 5% freshly isolated autologous human serum (active complement); and (2) 5% heat inactivated (30 min, 56°C) autologous human serum. This procedure was shown to inactivate the components C1 and C2 of the classical⁴⁵ and factor B of the alternative complement activation pathway.⁴⁶

Induction and detection of apoptosis and necrosis

PMN undergo spontaneous progressive apoptosis during the ageing process. Apoptosis of PBL was induced by irradiation with UV-B (120 mJ/cm²). Necrosis of PBL and PMN was induced by incubation of the cells at 56°C for 30 min. Monitoring of apoptosis and necrosis was performed by (1) microscopic examination,⁴⁷ (2) analysis of morphological changes by flow cytometry (FSC/SSC),^{48,49} (3) staining with PI in the presence of Triton X-100,^{50,51} (4) staining with FITC labelled AxV/PI⁵² and (5) staining with the fluorescent dye 3,3'-dihexyloxycarbocyanine iodide DiO₆C(3).⁵³

FITC-labelled annexin-V was purchased from Boehringer Mannheim, Germany. PI, Triton X-100, and DiO₆C(3) were obtained from Sigma, Munich, Germany.

Complement binding assay

All cells were cultured in RPMI-1640 medium (Gibco-BRL, Eggenstein, Germany) supplemented with 10% heat inactivated fetal calf serum (R10) in a humidified atmosphere containing 5% CO₂. As source of complement the following additives to the culture medium were used: (1) 5% freshly isolated autologous human serum, (2) 5% heat inactivated (30 min, 56°C) autologous human serum. We added an additional 2% of the serum to the cultures 1 h before harvesting the cells to make sure that sufficient active complement is present in the long term cultures. For the detection of bound complement components the cells were harvested, washed with phosphate buffered saline containing 1% heat inactivated fetal calf serum and stained for 30 min on ice with affinity purified, FITC-labelled polyclonal antibodies recognising either C1q, C3c, C4c, or C5 (DAKO, Glostrup, Denmark). For all experiments cells cultured in RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum in the absence of complement served as negative controls.

Flow cytometric analysis

Analysis of immunofluorescence, FSC/SSC, hypodiploid nuclei, PS exposure, and mitochondrial transmembrane potential ($\Delta\psi_m$) was performed employing an EPICS XL[™] cytometer (Coulter, Hialeah, USA), typically collecting at least 20 000 events.

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