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PROGRAMMED CELL DEATH OF ERYTHROCYTE, AN APOPTOTIC PHENOMENON. IMPACT ON BLOOD TRANSFUSION

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Human red blood cells are terminally differentiated cells of the erythroid lineage that are devoid of organelles and have a definite life span of 120 days that is ended by a process of senescence leading to their clearance from the circulation (Bratosin D. *et al*.,1998).

Every day, 360 billions of RBCs are phagocytized, *ie 5* millions per second. This fascinating phenomenon of programmed cell death (PCD) raises the following questions: i) what signals the death sentence of RBCs; ii) what are the physiological mechanisms for sequestration of the effete RBDs from the blood stream with such precision? and iii) by what specific membrane signal(s) do the reticulo-endothelial cells distinguish between the truly senescent RBCs and others? (Figure 1)

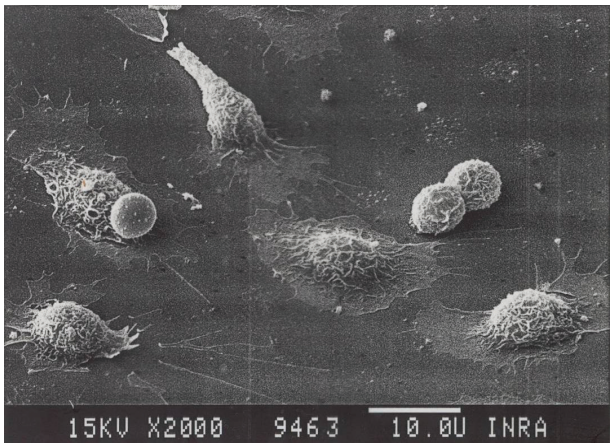

Fig.1. Scanning electron microscopy showing the preferred capture of a senescent erythrocyte after incubation of total unseparated population of human erythrocytes with murine peritoneal macrophages. Only a spheroechinocyte (arrow) has been captured. White bar, 10 µm

Numerous, and often conflicting, hypotheses have been proposed. Each investigator focusing on but one of the many modifications that afflict the cell surface of the ageing erythrocyte.

In this regard, how to define and discriminate aged and senescent erythrocytes ? According to Bessis (Bessis M.,1972): "The senescence is not the deed to become older but the fact to exhibit dramatic disorders which lead to a decrease in the ability to survive and to an increase of susceptibility towards hostile conditions". In the particular case of RBCs according to Aminoff (Aminoff D., 1988): "The choice of the term senescent was made because, by most criteria, the senescent RBCs do show the greatest number of time-dependent changes". In other words, senescent RBCs represent the erythrocyte population which has been condemned to death and which thus has one foot in the grave.

TIME-DEPENDANT CHANGES OF ERYTHROCYTES *IN VIVO*

The aim of the research concerning the age-related alterations of physical and chemical properties of the RBC membrane is to answer two fascinating questions concerning erythrocyte ageing. The first one consists, at the molecular level, to evidence the processes responsible for cellular ageing. The second one concerns at the cellular level the elucidation of mechanisms of recognition and specific removal of senescent RBCs. One of the earliest parameters that appeared to correlate with age of RBCs was an increase in cell density, resulting from the shedding of vesicles with minimal loss of hemoglobin. This resulted in extensive studies to compare the changes in young and old RBCs by simply separating the lightest and densest fractions obtained by ultracentrifugation, in continuous or discontinuous density gradients.

With a view to verifying this hypothesis, we have undertaken experiments mostly founded on flow cytometry and using two types of senescent

erythrocytes: the ones isolated from heparinized blood by ultracentrifugation in a self-forming Percoll gradient (physiological senescence) shown in figure 2, the others obtained by incubation with Ca^{2+} alone or in presence of ionophore A 23187(induced PCD).

Morphological changes and cell injury were assessed by flow cytometry using forward (cell size) and side-angle (cell density) scatters (FSC/SSC). This is a simple, rapid and sensitive method we previously described (Bratosin D. *et al.,* 1995).

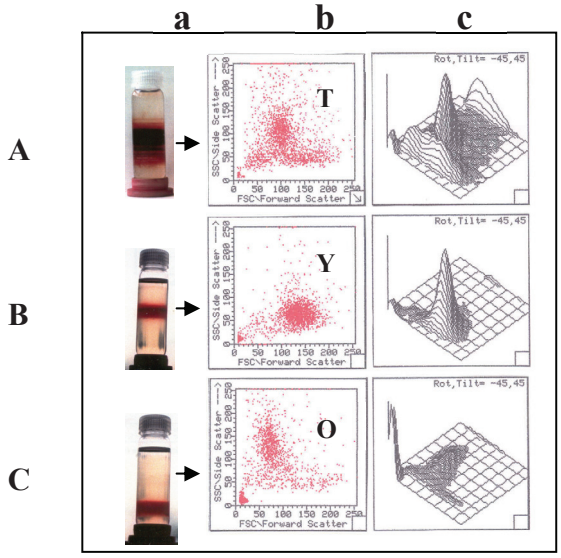

Fig. 2. Separation of young and old erythrocytes by ultracentrifugation in a self-forming Percoll gradient according to Lutz et al. [4]
(a) A: Total RBCs; B: Young RBCs; C: Old RBCs;
(b) Dot-plot analysis of total (T), old (O) and young (Y) erythrocyte populations. Abscissa, forward scatter; ordinates, side scatter.
(c) Three-dimensional representation of subpopulations.

SIALIC ACIDS RESIDUES ARE ANTIRECOGNITION SIGNALS BY MASKING THE SIGNALS FOR ERYTHROPHAGOCYTOSIS

Desialylation of RBCs was measured by flow cytometric analysis of the binding of FITC-labeled lectins specific for sialic acids (WGA, SNA and MAA) and β-galactose (RCA^{120}) residues (Table I) (Bratosin D., *et al.,*1995).

Analysis of the binding of FITC-labelled lectins specific for sialic acids shows that the aged erythrocytes bind less WGA, LPA, SNA and MAA than the young ones. The binding of DSA and LCA is not modified. On the contrary, the number of binding sites of UEA-I and AAA decreases significantly due to the release of membrane vesicles, according to Sharon and Fibach (Sharon R. and Fibach E.,1991; Fibach E. and Sharon R., 1994). RCA^{120} as well as E. cristagalli and E. corallodendron agglutinins specific for terminal β-galactosyl residues gave unexpected and inexplicable results with a decrease in the number of lectin binding sites despite the increased desialylation associated with the smallest cells. Similar result were reported by Shinozuka *et al.* (Shinozuka T. *et al.,* 1998). To date this result remains to be explained.

Table I. Characteristics of FITC-lectin binding to the membrane of total, young , old and neuraminidase-treated erythrocyte population determined by flow cytofluorimetriy and expressed as the number of binding sites per cell

	ERYTHROCYTE POPULATIONS			
	Total	*Young*	*Old*	*Neuraminidase-treated*
WGA	40 000	60 000	37 000	9 000
LPA	8 800	10 200	8 500	1 500
MAA	9500	11 000	6 300	4 500
SNA	11 000	11 000	9 200	7 000
RCA_{120}	16 000	24 000	16 000	11 000
Erythrina crystagalli	3 900	3 300	2 200	1 800
Erythrina corallodendron	3 000	2.600	2 100	1 900
AAA	22 700	29 400	20 400	20 000
LCA	3 700	3 500	3 400	1 900
UEA-I	36 000	40 000	33 000	nd
DSA	5 700	6 000	5 400	4 400
PNA	No binding			
GNA	No binding			
Nd.not determined				

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CHARACTERIZATION OF THE SENESCENT RBCS THAT ARE PHAGOCYTIZED BY MACROPHAGES

Regarding the ageing of erythrocytes and their removal from circulation, the study of the extracellular exposure of phosphatidylserine was of the first importance. In fact, as early as 1985, Schroit *et al.* (Schroit A.J. *et al.*,1985) and Mc Evoy *et al*. in 1986 (Mc Evoy L. *et al.,*1986) observed that erythrocytes with an abnormal, symmetric distribution of their membrane phospholipids were phagocytised four times more readily than normal, asymmetric intact erythrocytes. They postulated that the presence of external phosphatidylserine in the outer leaflet of RBCs might serve as a signal for triggering their recognition by macrophages. Later, Schroit and collaborators (Connor J. *et al.,*1994) and Allen *et al.* (Diaz C. *et al.,*1996) demonstrated unambiguously that the presence of phosphatidylserine on the cell surface is directly correlated with the propensity of the RBCs to be bound *in vitro* by autologous monocytes and to be rapidly cleared *in vivo* by the spleen.

On the assumption that there is a direct relationship between exposure of phosphatidylserine on the outleaft of RBC membrane and erythrophagocytosis, we have undertaken a series of researches, founded on the solid ground of the flow cytometry, with the aim to characterize the senescent RBCs. We have demonstrated that it is the densest population of RBCs obtained by ultracentrifugation on self-forming gradient of Percoll (a fine nebulous cloud of RBCs between Lutz's densest fraction and the bottom of the centrifuge tube) that: i) is the most desialylated; ii) presents the greatest reactivity with annexin-V; iii) shows the greatest susceptibility to phagocytosis. In addition, scanning electron microscopy shows that this population contains spheroechinocytes with filopodia and smooth sphere forms (Figure 3) (Bratosin D. *et al.,* 1997 (1)).

The in vitro phagocytosis of RBCs was measured by using a rapid, sensitive and reproducible flow cytofluorimetric procedure we previously described (Bratosin D. *et al*., 1997(2)) and which is based on the use of RBCs labeled with the fluorescent probe PKH-26. The procedure involves the following steps: i) incubation of PKH-26-labeled erythrocytes with murine macrophages, ii) removal of unbound red blood cells, iii) lysis of membrane-bound RBCs and iv) measurement of extent of phagocytosis by direct flow-cytometric analysis of intact macrophages. Dot-plot analysis of red PKH-26-labeled RBC cytofluorescence (ordinate) against the green macrophage autofluorescence (abscissa) clearly defines two regions: those of non-phagocytic and of phagocytic macrophages. Use of fluorescent, instead of radio-labeled RBCs, makes the methods more sensitive, rapid, and avoids radioactive hazards. Furthermore, this approach is multi-parametric and can distinguish different populations of macrophages with reference to their erythrophagocytic potential.

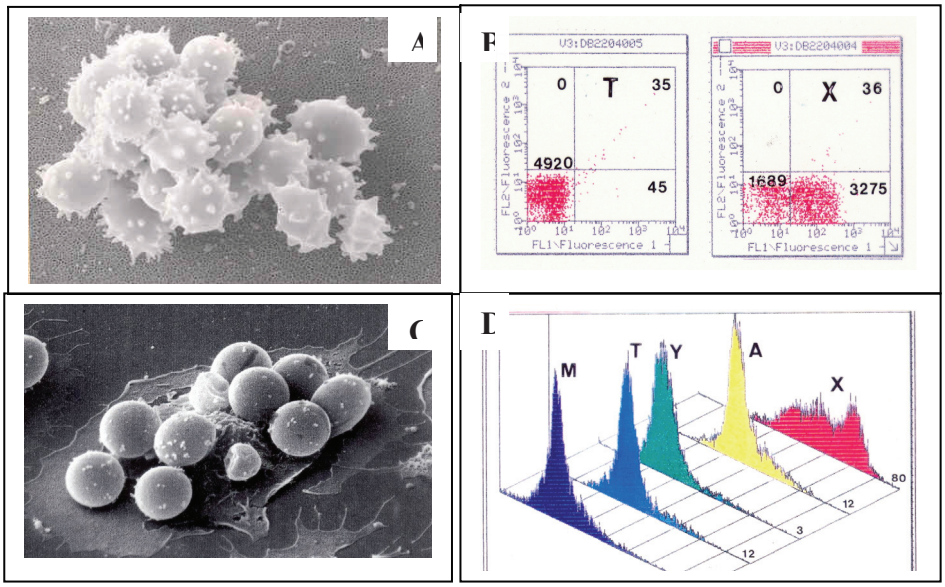

Fig. 3. Characterization of the senescent erythrocytes (1% of total RBCs)
A-Scanning electron microscopy; B-Dot-plot (FL1/FL2) of annexin-V- FITC binding to total (T) and senescente erythrocyte populations (X). Lower left quadrant (LL), region of annexin negative young, mature and old erythrocytes; lower right quadrant (LR), region of annexin positive senescent erythrocytes. Events acquired in each quadrant are indicated in the upper right corners. C-Scanning electron microscopy of preferred capture of senescent RBC after incubation with murine peritoneal macrophages; D-Comparative three-dimensional representation of phagocytosis of different PKH-26-labeled erythrocyte populations. M-macrophage autofluorescence; T-total unseparated erythrocyte population; Y, A, X,: young, old and senescent erythrocyte populations, respectively. Numbers represent the fluorescence mean values after deduction of macrophage autofluorescence (mean: 75) on a logarithmic scale. X, fluorescence intensity; y, cell numbers of engorged macrophages.

According to numerous authors, the phagocytosis of senescent RBCs appears to be a multifactorial process which is at least a three-step mechanism involving initially the β-galectin of macrophage membrane ensuring the capture of desialylated RBCs, secondly the recognition by a macrophagic receptor of phosphatidylserine residues exposed in the RBC outer leaflet which then triggers the phagocytosis induced by natural antibodies.

DO MATURE RED BLOOD CELLS DIE BY APOPTOSIS ?

On the basis of the following series of well-known characteristics of senescent RBCs which are specific of apoptosis: i) modifications of erythrocyte membrane such as progressive release of microvesicles leading to a decrease of cell size and an increase of density; ii) alteration of erythrocyte cytoskeleton, of spectrin in particular, leading to membrane budding and to the so-called echinocyte forms (crenated forms) and then to the spheroechinocyte form with spicules and to the smooth sphere form which are both specifically phagocytized; iii) enzymatic desialylation demasking terminal β-galactose residues and inducing the capture of erythrocytes by a β-galactolectin of macrophagic membrane; iv) progressive appearance in the cell outer leaflet of phosphatidylserine that serves as a signal for triggering the recognition and phagocytosis of senescent erythrocytes by macrophages, and, despite the similarities between this senescent phenotype and some cytoplasmic features of apoptosis, mature erythrocytes have been considered as the sole mammalian cell lacking the machinery required to undergo PCD in all human nucleated cells studied so far, i.e.treatment with the protein kinase inhibitory drug staurosporine, and culture in the absence of serum or other potential survival-promoting factors (Weil M. et al.,1996). We reasoned that the absence of response of erythrocytes to staurosporine and to serum deprivation was not sufficient to exclude the existence of a death machinery allowing erythrocytes to self-destruct in response to environmental changes, and that erythrocyte senescence may represent a particular, time-dependent outcome of the induction of such a self-destruction program. Indeed, the normal life span and senescence of several terminally differentiated nucleated cells is controlled by a time-dependent process of PCD induction that can be either accelerated or delayed by environmental signals.

Since erythrocyte senescence has been reported to be associated with a progressive increase in intracellular calcium (Ca^{2+}) (Aiken N.C. et al.,1992; Romero P.J. et al.,1999), we decided to investigate whether Ca^{2+} entry into freshly isolated human erythrocytes may suffice to induce premature erythrocyte death, by incubation in vitro with a physiological concentration (2.5mM) of Ca^{2+} .

A rapid calcium-dependent erythrocyte death process with several features of apoptosis

Ca^{2+} treatment led to a rapid and complete disappearance of the cells in 48 h and death were dose-dependent in presence of ionophore A 23187. A 23187 did not induce cell death in the absence of extracellular Ca^{2+}, nor in the presence of both Ca^{2+} and the Ca^{2+} chelator EDTA, indicating that erythrocyte death was an active process requiring Ca^{2+} entry into the cells. As also show in Figure 4A, incubation of erythrocytes with Ca^{2+} alone, in the absence of A 23187, induced a much slower death process, that only began to induce erythrocyte disintegration after 48 h and required 6 days to lead to the disappearance of more that 80% of the erythrocytes.

Morphological changes

Scanning electron microscopy showed that Ca^{2+}-induced erythrocyte death was preceded by dramatic morphological changes (Figure 4B). Indeed, 3.5 h after A 23187 treatment in the presence of Ca^{2+}, or 2 days after incubation with Ca^{2+} alone, the shape of erythrocytes changed from normal discocytes to echinocytes or spheroechinocytes, with plasma membrane microvesiculation. These rapid morphological changes induced by Ca^{2+} in vitro were identical to the features expressed by the very small subpopulation of in vivo senescent erythrocytes (less than 1% of the erythrocytes) that we purified from peripheral blood of healty donors.

Flow cytometry analysis indicated that the morphological changes induced by Ca^{2+} were associated with cell shrinkage (decreased forward scatter and increased side scatter), one of the characteristic features of apoptosis that distinguishes this active and regulated self-destruction process from the passive and chaotic process of necrosis induced by plasma membrane damage (Figure 4C).

Phosphatidylserine externalization

Flow-cytometry analysis using labeled Annexin-V indicated that incubation of human mature erythrocytes with A 23187 in the presence of Ca^{2+}, or with Ca^{2+} alone, induced a rapid phosphatidylserine externalization (Figure 4C), a feature characteristic of both apoptosis in nucleated cells and of senescence in erythrocytes (Raff M.C.,1992; Clark M.R.,1998; Boas F.E. et al.,1998].

Erythrocyte capture and ingestion by macrophages

Phosphatidylserine exposure on the outer leaflet of the plasma membrane is one of the signals that allow macrophages to bind and ingest apoptotic cells as well as senescent erythrocytes (Zwaal R.F.A. and Schroit A.J., 1997; Bratosin D. et al.,1997 (1)).

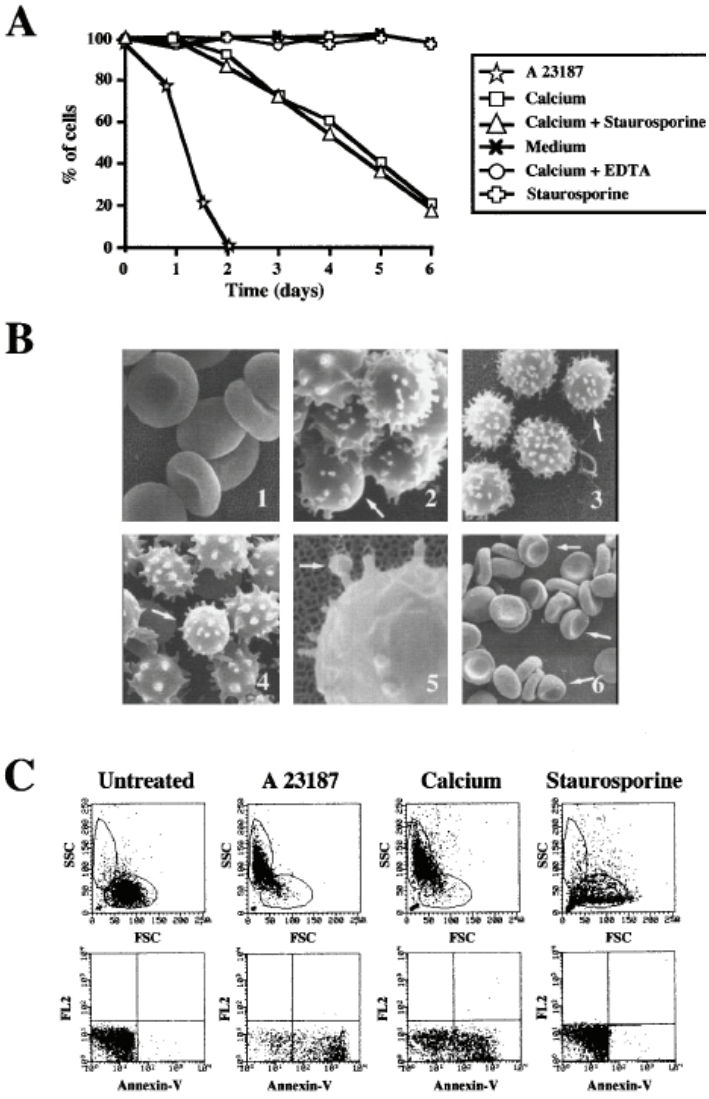

Fig. 4. Morphological changes, phosphatidylserine exposure and cell death induced by calcium ionophore and calcium in human mature erythrocytes.

(A) Cell death. Freshly isolated and purified mature human erythrocytes were incubated either with the calcium ionophore A 23187 (0.5 mM) in the presence of Ca^{2+} (2.5 mM); or with Ca2+ (2.5mM) in the absence or presence of staurosporine (10 mM); or with medium alone, in the absence of Ca^{2+}; or with Ca^{2+} (2.5mM) and the calcium chelator EDTA (5 mM); or with staurosporine (10 mM) in the absence of Ca^{2+}. Per cent of cells indicate the percentages of erythrocytes remaining in the culture at the indicated time points.

(B) Scanning electron microscopy analysis of morphological changes. Freshly isolated and purified human erythrocytes after 48 h incubation in medium alone, in the absence of Ca^{2+} (1); or after 3 h incubation with A 23187 and Ca^{2+} (3); or after 48 h incubation with Ca^{2+} alone (4); or after 24 h incubation with staurosporine in the absence of Ca^{2+} (6). (2) A small subpopulation (51%) of senescent erythrocytes, purified from freshly isolated human mature erythrocytes by a self-forming Percoll gradient, shows the same morphological features as A 23187 and Ca^{2+}-treated (3) or Ca^{2+}-treated (4) erythrocytes. (5) Close-up from (4) showing a microvesicle in formation (arrow). In (2- 4) the arrows show a typical spheroechinocyte. In (6), arrows show cup-shaped erythrocytes (stomatocytes).

(C) Flow cytometry analysis of shape changes and phosphatidylserine exposure. Freshly isolated and purified human mature erythrocytes were analyzed after either 48 h incubation in medium alone, in the absence of Ca^{2+} (Untreated); or 3 h incubation with A 23187 and Ca^{2+} (A 23187); or 48 h incubation with Ca^{2+} alone (calcium); or 24 h incubation with staurosporine, in the absence of Ca^{2+} (staurosporine).

During apoptosis, ingestion of the dying cells by macrophages is an early event that precedes the disintegration of these dying cells that will occur in the absence of macrophages (Savill J. and Fadok V., 2000).

In the absence of macrophages, ionophore A 23187-treated erythrocytes disintegrated in 48 h (Figure 4A). In the presence of macrophages, however, A 23187 pre-treated erythrocytes were rapidly captured and ingested by macrophages 3 h after having been incubated with A 23187, as shown by scanning electron microscopy analysis of erythrocyte binding to macrophages (Figure 6A), and by flow cytometry analysis of macrophages that had been incubated with erythrocytes pre-labeled with the lipophilic cationic agent PKH-26 (Figure 6B).

Cysteine proteinase inhibitors prevent Ca^{2+}-dependent erythrocyte death

Caspases are a family of aspartate-directed cysteine proteinases that are crucial effectors of apoptosis, (Hengartner M.,2000; Thornberry N. A. and Lazebnik Y.,1998; Earnshaw W.C. et al.,1999) and calpain is another Ca^{2+}-dependent cysteine proteinase that has been reported to also participate in apoptosis (Squier M.K.T. and Cohen J.J., 1996; Wang K.K.W., 2000) and to be involved in a Ca^{2+}-induced apoptosis-like death process of blood platelets. For these reasons, we investigated whether Ca^{2+}-induced erythrocyte death may depend on the activation of cysteine proteinases, by exploring the effect of Ac-DEVD-CHO, an inhibitor of caspase 3, one of the main effector caspases involved in the execution of apoptosis,and of leupeptin, a broad inhibitor of cysteine proteinases, including calpain.

Prevention of morphological changes and phosphatidylserine externalization.

Treatment with the cysteine-proteinase inhibitors also prevented erythrocyte shrinkage induced by A 23187 and Ca^{2+}, or by Ca^{2+} alone, as assessed by flow cytometry (Figure 5B), and allowed erythrocytes to maintain their discoid shape, as assessed by scanning electron microscopy (Figure 5A). A typical picture of discoid erythrocytes without plasma membrane microvesicles 48 h after incubation with Ca^{2+} and Ac-DEVD-CHO is shown in Figure 5, while in the absence of the inhibitor, erythrocytes became spheroechinocytes with plasma membrane microvesicles, or smooth spherocytes.

Incubation of erythrocytes with Ca^{2+} alone, in the absence of A 23187 also led to their capture (data not shown) and ingestion (Figure 6B) by macrophages at a time point (48 h) that preceded by several days the disintegration of erythrocytes that occurred in response to Ca^{2+} in the absence of macrophages (Figure 6A).

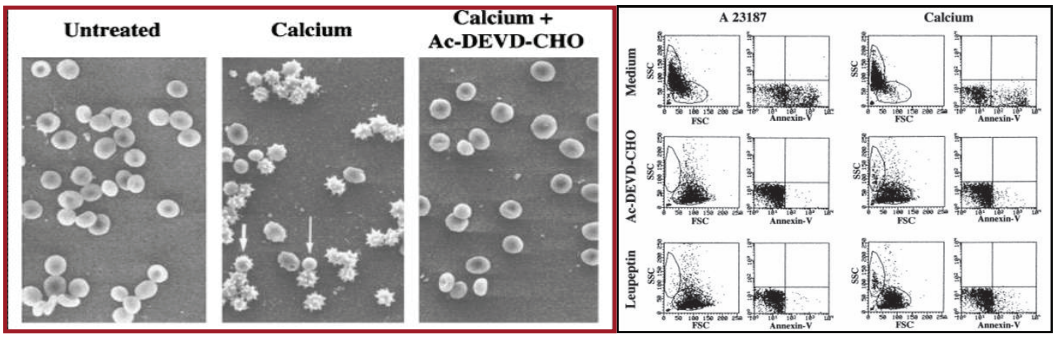

Fig. 5. (A) - Scanning electron microscopy analysis of the preventive effect of cystein proteinase inhibitors on calcium-induced human mature erythrocyte morphological changes. Freshly isolated and purified human mature erythrocytes were incubated for 48 h either in medium alone, in the absence of Ca^{2+} (Untreated), or with Ca^{2+} in the absence (Calcium) or presence (Calcium+Ac-DEVD-CHO) of pretreatment for 30min with Ac-DEVD-CHO. The short arrow shows a spheroechinocyte with spicules, and the long arrow a smooth spherocyte B) - Flow cytometry analysis of the preventive effect of cystein proteinase inhibitors on calcium ionophore- and calcium-induced human mature erythrocyte shape changes and phosphatidylserine exposure. Freshly isolated and purified human mature erythrocytes were analyzed after incubation either for 48 h in medium alone, in the absence of Ca^{2+} (Untreated), or for 3 h with A 23187 and Ca^{2+} (A 23187), or for 48 h with Ca^{2+} (Calcium) in the absence (Medium) or presence of pretreatment for 30min with Ac-DEVD-CHO or Leupeptin. In each condition, erythrocyte shape (left box) and Annexin V-labeling (right box) were analyzed. At least 5000 erythrocytes were analyzed in each experimental condition.

In contrast, erythrocytes that had been incubated for 48 h in the absence of Ca^{2+} were neither captured (Figure 6A) nor ingested (Figure 6B) by macrophages. Together, these data indicated that human mature erythrocytes can undergo a rapid and orderly Ca^{2+}-dependent death process sharing several features with apoptosis and leading either to an early elimination of the dying cells through ingestion by neighbouring macrophages, or, in the absence of macrophages, to a more progressive elimination of the dying cells through self-disintegration.

Treatment of erythrocytes with A 23187, Ca^{2+}, and cysteine proteinase inhibitors exerted an inhibitory effect on their further capture (Figure 6A) and ingestion (Figure 6B) by macrophages.

In vitro treatment with cysteine proteinase inhibitors allows in vivo survival of Ca^{2+}-activated murine erythrocytes

Murine mature erythrocytes freshly purified from BALB/c mice were incubated in vitro for 4 days with Ca^{2+}, in the absence or presence of Ac-DEVD-CHO, leupeptin, or a mixture of both. As we observed with human erythrocytes, incubation with each inhibitor alone, or with a mixture of both, very effectively prevented PS externalization by murine erythrocytes. In order to be able to subsequently follow the in vivo survival in the blood circulation of the treated erythrocytes, we labeled them with PKH-26 prior to injection in recipient mice. As a control, untreated freshly isolated murine erythrocytes were also labeled with PKH-26.

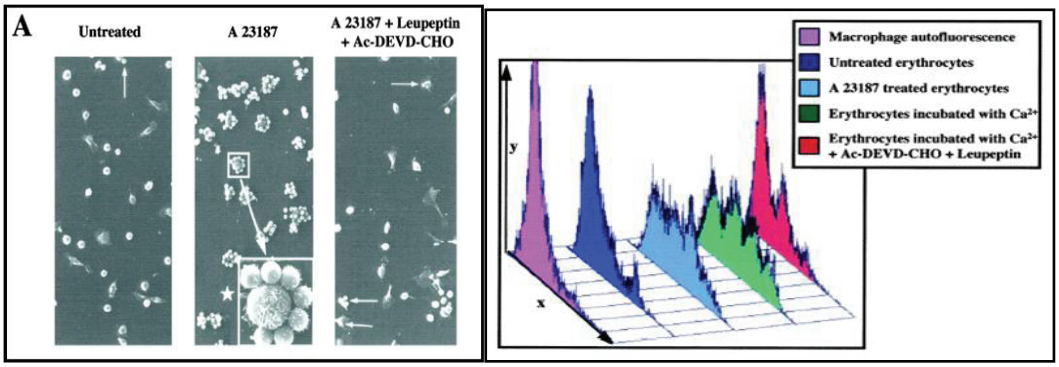

Fig. 6. Preventive effect of cystein proteinase inhibitors on the capture and ingestion of human mature erythrocytes by macrophages.
(A) - Scanning electron microscopy analysis of erythrocyte capture by macrophages. Murine peritoneal macrophages were incubated for 20 min with freshly isolated and purified human mature erythrocytes that had been preincubated either for 48 h with medium alone, in the absence of Ca^{2+} (Untreated); or for 3 h with A 23187 and Ca^{2+} in the absence (A 23187) or presence (A 23187+Leupeptin+Ac-DEVD-CHO) of pretreatment for 30 min with a mixture of Ac-DEVD-CHO and leupeptin. Arrows in untreated and in A 23187+Leupeptin+Ac-DEVD-CHO designate macrophages having captured one or two erythrocytes. The star in A 23187 designates a close-up picture of one of the erythrocyte-rosetted macrophages.
(B) - Flow cytometry analysis of the ingestion of erythrocytes by macrophages. Murine peritoneal macrophages were incubated for 2 h with either medium alone (Macrophage autofluorescence), or with freshly isolated and purified human mature erythrocytes that had been labeled with PKH-26 after preincubation either for 48 h with medium alone, in the absence of Ca^{2+} (Untreated erythrocytes); for 3 h with A 23187 and Ca^{2+} (A 23187 treated erythrocytes), or for 48 h with Ca^{2+} in the absence (Erythrocytes incubated with Ca^{2+}) or presence (Erythrocytes incubated with Ca^{2+}Ac-DEVD-CHO+Leupeptin) of pretreatment for 30min with a mixture of Ac-DEVD-CHO and leupeptin. The x axes indicate the mean PKH-26 fluorescence intensity, on a logarithmic scale; the y axes indicate the number of macrophages

The labeled treated and untreated erythrocytes (10^9 erythrocytes per recipient mouse) were then injected intravenously in syngeneic BALB/c recipient mice. Erythrocytes that had been incubated in vitro with Ca^{2+} in the absence of cysteine proteinase inhibitor were almost completely cleared from the circulation 48h afterinjection. On the contrary, erythrocytes that had been incubated in vitro with Ca^{2+} in the presence of Ac-DEVD-CHO, leupeptin or a mixture of both, remained in the circulation for at least 48 h at the same level as the freshly isolated untreated control erythrocytes. Results from two different representative experiments are shown in Figure 7. In both experiments, a small and progressive decrease in the number of labelled untreated control erythrocytes in the circulation was observed, that may be due both to the physiological clearance of the murine erythrocytes, whose normal life span in vivo is 40 days, and to the in vitro procedures used to purify and to label the cells.

As shown in Figure 7 and Table II, 94 and 86%, respectively, of the labelled untreated control erythrocytes detected in the circulation 1 h after injection were still present 48 h later. The numbers of erythrocytes that had been treated in vitro with Ca^{2+} and a mixture of both cysteine proteinase inhibitors and that remained in the circulation 48 h after injection was identical (Figure 7) or almost identical to the numbers of untreated control erythrocytes. In contrast, when compared to untreated control erythrocytes, only around 20% (Figure 7) and 10% of erythrocytes treated with Ca^{2+} in the absence

of inhibitor were detected in the circulation 1 h after injection, and less than 3% 48 h after injection.

Together, our data indicated that a form of Ca^{2+}-dependent apoptosis like cell death process can be induced in mature erythrocytes, and that cysteine proteinase inhibitors are able to prevent this death process and to allow subsequent erythrocyte survival both in vitro and in vivo.

Table II. Preventive effect of cysteine proteinase inhibitors on phosphatidylserine (PS) exposure and subsequent in vivo clearance of murine erythrocytes induced by in vitro treatment with calcium

Murine erythrocytes	Percentage of erythrocytes expressing PS *in vitro**	Number of PKH-26-labeled erythrocytes remaining in the circulation at different time points after injection**			
		+1 h	+6 h	+20 h	+48 h
Untreated	1	364	343	317	305
Incubated with Ca^{2+} for 4 days					
In the absence of inhibitor	84	34	11	11	10
In the presence of inhibitor					
Ac-DEVD-CHO	9	276	260	220	210
Leupeptin	8	219	230	250	230
Leupeptin+Ac-DEVD-CHO	4	293	247	284	292

*Flow cytometry analysis of annexin-V labeling was performed as in Figures 1 and 3, prior to erythrocyte PKH-26 labeling and injection in recipient mice. **Erythrocyte PKH-26 labeling, injection in recipient mice, and subsequent flow cytometry analysis were performed as in Figure 5, in three additional recipient mice. Results are from one representative recipient mouse

Proforms of caspase 3 are present in human mature erythrocytes, but are not activated during Ca^{2+}-dependent death

In mature erythrocytes, the presence of the calpain cysteine proteinase has been documented (Michetti M. et al., 1997) but the existence of caspases has never been investigated. Caspases are synthesized as inactive proforms that are activated after proteolytic cleavage.

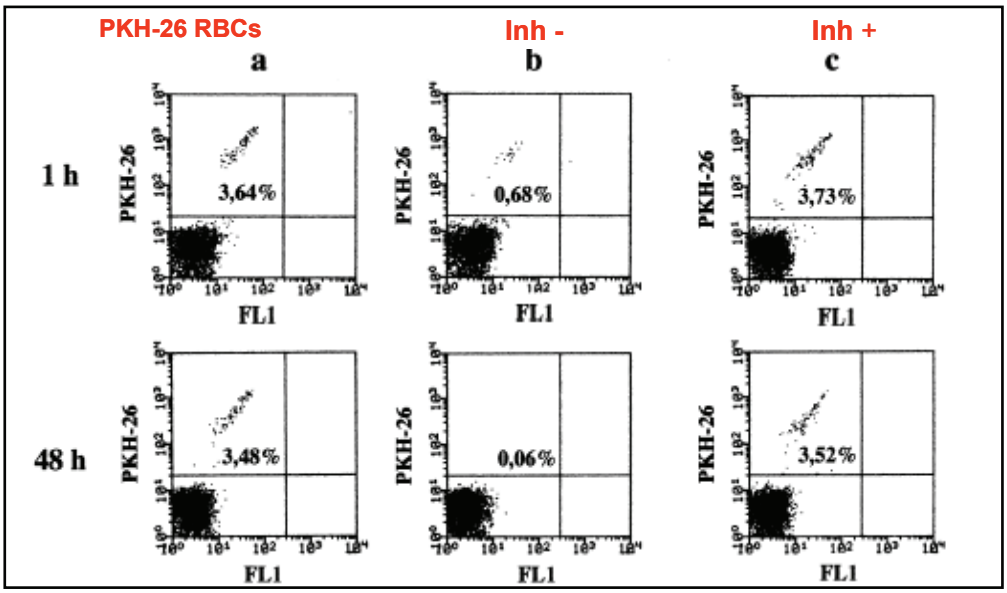

Fig. 7. In vitro treatment of murine mature erythrocytes with calcium and cystein proteinase inhibitors prevents their subsequent in vivo clearance from the circulation. Freshly isolated and purified murine erythrocytes were labeled with PKH-26 and injected intravenously in syngeneic recipient mice either immediately after their isolation (a) or after incubation for 4 days at 20˚C with Ca^{2+} in the absence (b) or presence (c) of pretreatment for 30 min with a mixture of Ac-DEVD- CHO and leupeptin. Flow cytometry analysis of the peripheral blood of the recipient mice was then performed 1 h or 48 h after injection. Upper left quadrant: PKH-26 labeled erythrocytes retrieved from the circulation (numbers indicate their percentages). At least 10 000 erythrocytes were analyzed in each experimental condition. Results shown are from one representative recipient mouse out of the three used in this experiment

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In nucleated cells, most, if not all, of the pro-apoptotic stimuli cause the mitochondrial release of cytochrome c in the cytosol, required for the formation of the apoptosome, that induces caspase 9 activation, and leads to the activation of the effector caspase 3 (Hengartner M.,2000; Green D.R.,2000; Martinou J.C. and Green D.,2001]. We investigated whether erythrocytes contain caspase 3 and cytochrome c (Figure 8).

Erythrocyte extracts were subjected to Western blotting with antibodies directed against these proteins. As a positive control, equivalent amounts of cytoplasmic extracts from the nucleated human Jurkat T-cell line were explored. We detected no cytochrome c in extracts from freshly purified erythrocytes, strongly suggesting that erythrocytes do not have the ability to form a functional apoptosome. The absence of detectable cytochrome c also confirmed the lack of significant contamination of the purified erythrocyte preparations by other cells that contain mitochondria and cytochrome c, such as nucleated cells or blood platelets.

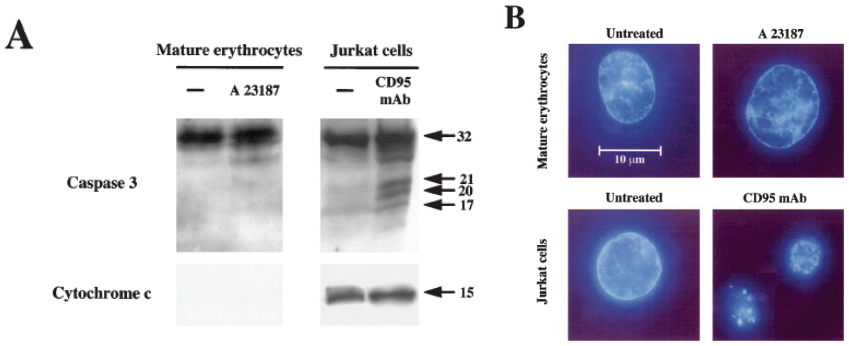

Fig. 8. Lack of cytochrome c in human mature erythrocytes, and of both detectable caspase 3 activation and cytoplasmic effectors of nuclear apoptosis during calcium ionophore-induced erythrocyte death.

(A) Western blotting. Extracts from freshly isolated and purified human mature erythrocytes that had been incubated for 24 h either in medium alone, in the absence of Ca^{2+} or with A 23187 and Ca^{2+} (A 23187), were subjected to Western blotting with antibodies specific for human caspase 3 or human cytochrome c. As a positive control,Western blotting was performed on cytoplasmic extracts from Jurkat T cells that had been incubated for 6 h in medium alone or with an agonistic mAb specific for the CD95 death receptor (CD95 mAb). (B) Cell free assays of nuclear apoptosis. Isolated human nuclei were incubated for 2 h with cytoplasmic extracts from human mature erythrocytes (Mature erythrocytes) that had been incubated for 24 h with either medium alone, in the absence of Ca^{2+} (Untreated), or with A 23187 and Ca^{2+} (A 23187). As a positive control, isolated human nuclei were incubated for 2 h with cytoplasmic extracts from human Jurkat T cells (Jurkat cells) that had been incubated for 6 h with either medium alone (Untreated) or with the agonistic anti-CD95 antibody (CD95 mAb). Nuclei were visualized by fluorescent microscopy after staining with Hoechst 33342. Results shown in A and B are representative of three independent experiments.

As shown in Figure 8A, we identified the uncleaved proform of caspase 3 (32 kD) in extracts from freshly purified erythrocytes. However, the caspase 3 proform remained unprocessed in extracts of dying erythrocytes that had been treated for 24 h with A 23187 and Ca^{2+}. As a positive control, we analyzed Jurkat cells either untreated or after apoptosis induction by antibody-mediated engagement of the CD95 death receptor, using an agonistic anti- CD95 antibody (1 mg/ml). As shown in Figure 8A, caspase 3 was present as an uncleaved proform of 32 kD in cytoplasmic extracts from untreated Jurkat cells, and was processed into the active 20, 21 and 17 kD forms in cytoplasmic extracts from apoptotic Jurkat cells. During apoptosis of nucleated cells, the various pathways of caspase activation always converge on caspase 3 processing and activation. Therefore, our finding of a lack of caspase 3 processing strongly suggested that erythrocyte death proceeded in the absence of caspase activation. It also indicated that Ac-DEVD-CHO did not prevent erythrocyte death by inhibiting caspase 3, and implied that its preventive effect was due to the inhibition of another proteinase. In nucleated cells, apoptosis is associated with the caspase-mediated activation of effectors that induce nuclear chromatin damage (Hengartner M., 2000; Thornberry N. A. and Lazebnik Y.,1998; Green D.R.,1997). Accordingly, cytoplasmic extracts from apoptotic cells induce nuclear features of apoptosis when they are incubated with intact nuclei in cell free assays (Earnshaw W.C. et al.,1999; Sahara S., et al. 1999; Nagata S., 2000). Using a cell free assay, we incubated cytoplasmic extracts from untreated- or CD95 antibody-treated Jurkat cells, and extracts from untreated- or A 23187 and Ca^{2+}-treated erythrocytes with isolated nuclei. While cytoplasmic extracts from

CD95 antibody-treated Jurkat cells induced typical nuclear chromatin condensation and fragmentation in the isolated nuclei, extracts from A 23187 and Ca^{2+}-treated erythrocytes induced no detectable damage in isolated nuclei (Figure 8B).

Finally, we wondered whether the absence of detectable nuclear effector activity in dying erythrocytes may be due to the presence, in erythrocyte extracts, of putative inhibitors of nuclear degradation. When extracts from A 23187 and Ca^{2+}-treated erythrocytes were mixed (1:1) with cytoplasmic extracts from CD95 antibody-treated Jurkat cells, no inhibition of nuclear degradation was observed (data not shown). Together, these data suggested that erythrocyte death proceeds in the absence of effectors allowing the induction of the nuclear features of apoptosis, a finding consistent with the absence of detectable caspase 3 activation in dying erythrocytes.

Mitochondria have been proposed to play a central role in programmed cell death (PCD), (Martinou J.C. and Green D., 2001; Desagher S. and Martinou J.C., 2000 - Kroemer G. and Reed J., 2000) through the release of pro-apoptotic factors that induce either caspase-dependent or caspase-independent executionary pathways. Until now, however, PCD has only been investigated in cells that contain mitochondria.

Mature erythrocytes represent the end stage of the erythroid lineage emerging from erythroid progenitors through a complex process of differentiation that involves the physiological loss of their nucleus, mitochondria and other organelles. Recent findings indicate that terminal differentiation into mature erythrocytes is preceded by a form of abortive apoptosis induction that involves transient caspase activation (Zermati Y. et al., 2001). Is the apparently peculiar death program operating in mature erythrocytes a consequence of their particular process of terminal differentiation? Or is it a legacy of cryptic effectors of cytoplasmic apoptosis that are already present in their erythroid progenitors? Identification of the molecular mechanisms of self-destruction in mature erythrocytes should allow one to assess to what extent the death effectors involved may be components of the various independent death programs that have been recently suggested to exist in parallel in nucleated mammalian cells (Sperandio S. et al., 2000; Wyllie A.H. and Golstein P. et al., 2001), and whose multiplicity may be related to the ancient evolutionary origins of PCD (Wyllie A.H. and Golstein P., 2001 - Ameisen J.C.,1998).

While we did not identify the death effectors operating in mature erythrocytes, our findings that inhibitors of cysteine proteinases are able to prevent Ca^{2+}-induced erythrocyte death both in vitro and in vivo has implications for therapeutic modulation of erythrocyte survival. Firstly, blood transfusion may benefit from treatments able to inhibit premature erythrocyte death in vitro. Secondly, shortened survival and accelerated clearance of erythrocytes from the blood circulation occur in several diseases (Boas F.E., 1998; Forman L. and Beutler E.,1998). Provided that accelerated erythrocyte clearance in such diseases depends on a process of Ca^{2+}-dependent PCD similar to that we have evidenced, our findings suggest the possibility that cysteine proteinase inhibitors might allow the in vivo prevention of premature erythrocyte death. Thirdly, senescence is the physiological process that ends the normal erythrocyte life span of 120 days. Erythrocyte senescence is associated with progressive Ca^{2+} influx (Aiken N.C. et al.,1992, Romero P.J. and Romero E.A.,1999) and with most, if not all, of the apoptosis-like features that characterize the rapid Ca^{2+}-induced premature erythrocyte death process that we identified here. If senescence represents the time-dependent induction of the same self-destruction process, it is possible that the normal life span of erythrocytes might be extended through therapeutic intervention.

Finally, our findings suggest the interesting possibility that there are physiological survival factors, present in vivo, that act by preventing or delaying Ca^{2+}-induced death of mature erythrocytes. Indeed, while mature erythrocyte life span is 120 days in vivo, incubation in vitro with a physiological concentration (2.5 mM) of Ca^{2+}, identical to that present in the peripheral blood, was sufficient to induce premature erythrocyte death within 6 days. It has been previously proposed that the survival of all nucleated mammalian cells depends on the constant repression of their self-destruction program by signals provided by other cells (Weil M. et al., 1996; Raff M.C., 1992; Meier P. et al.,2000, Jacobson M.D. et al.,1997). Our findings suggest that this may be also the case for mature erythrocytes, but that signals provided by other cells would only be required for erythrocyte survival when erythrocytes are in the presence of Ca^{2+}.

In summary, the findings presented here indicate that mature erythrocytes share, with all other mammalian cell types, the capacity to self-destruct in response to environmental changes. They suggest that erythrocytes may represent a useful model for the identification of effectors of PCD and senescence that are able to operate in a minimal cell devoid of nucleus, mitochondria and other organelles. They also indicate that death and survival of mature erythrocytes, as death and survival of all other mammalian cells, may be modulated by physiological regulation, pathological dysregulation, and therapeutic intervention (Bratosin D. et al., 2001).

To end, we would like to mention the two following conclusions. The first one was the last sentence of a poster abstract we presented in 1999 at the 26th FEBS-Meeting in Nice and we wrote: "Taking all together, these results strongly support the hypothesis that phagocytosis of senescent RBC occurs at the end of an apoptotic phenomenon" (Bratosin D. et al., 1999). The second one is an extract from the Editorial written by Daugas, Cande and Kroemer (Daugas E. et al., 2001) in the issue of Cell Death and Differentiation containing the papers of Bratosin et al.: "A semantic and hence delicate problem arises from the use of erythrocytes for

the study of cell death or apoptosis. If we consider Ca^{2+} ionophore-induced RBC senescence as "apoptotic", then the results reported here may be interpreted to mean that "apoptosis" can occur in the absence of mitochondria, nuclei, and caspase activation. That apoptosis may occur in the absence of a nucleus (that is in freshly enucleated cells, so-called cytoplasts) or in the absence of caspase activation (that is in the presence of caspase-inhibitors or in Apaf-1 or caspase knock-out cells) has been reported in the past, and the true originality of the present articles would be to show death in the absence of mitochondria. However, in a way the erythrocyte must be transient, presumably mitochondrion-triggered caspase activation has lead to the formation of an organelle-free mummy (just as ancient Egyptians removed organs from the corpse before mummification). Seen from this angle, it appears logical that the final implosion of RBC occurs via a non-apoptotic process which we might call erythroptosis"

In conclusions, RBCs have not been a popular model for the study of gerontological mechanisms. The argument was that they are dead cells destined for oxygen transport only, non-dividing and non-nucleated. But the absence of a nucleus is a great advantage in that the cell cannot correct any damage of our ageing.

In 1985, Aminoff write that " One of the most extensively studied cells, the red blood cell with a definite life span, seems to me to be the ideal model to study senescence". At present, on the basis of the results we described, we are tempted to conclude that the red blood cell is the ideal model to study programmed cell death (Bratosin D. et al., 2001).

Active caspases - 8 and -3 in circulating human erythrocytes purified on immobilized annexin-V

In 1999, Bratosin et al. (Bratosin D. et al., 1999) reported that the programmed cell death (PCD) of RBCs induced by Ca^{2+} influx is prevented by protease inhibitors and hypothesized for first time that phagocytosis of senescent RBCs occured at the end of an apoptotic phenomenon. This view was confirmed two years later by Bratosin et al. (Bratosin D. et al.,2001) and Berg et al. (Berg C.P. et al., 2001) who reported simultaneously that the programmed cell death of RBCs could be induced by Ca^{2+} influx and prevented by caspase and calpain inhibitors. However, whereas proforms of caspases-3 and -8 were present in RBCs, they were not activated during RBC death-mediated by Ca^{2+}. Moreover cytochrome c that is a critical component of the apoptosome, was lacking. More recently, it has been shown by Basu's group (Mandal D. et al., 2002) that procaspase-3 present in mature human RBCs can be activated under oxidative stress mediated by t-butylhydroperoxide leading to phosphatidylserine externalization and erythrophagocytosis. Moreover, these authors, using staurosporine and erythroid cells, showed that caspase-3 favors the proteolysis of N-terminal cytoplasmic domain of the anion exchanger 1 (Band 3) that may explain why the loss of membrane integrity is a feature of RBC senescence (Mandal D. et al., 2003). In a similar manner, they reported that Fas-ligation induces caspase-8 activation in RBCs (Mandal D. et al., 2005). Activation of caspases-8 and -3 as well as the clustering of Band 3 was also observed by Pietraforte et al. (Pietraforte D. et al., 2007) in peroxynitrite-induced apoptosis of human RBCs. Caspase and cleavage of Band 3 were also reported in stored RBCs by Kriebardis et al. (Kriebardis A.G. et al., 2007). Interestingly, Makherjee et al. (Mukherjee K. et al., 2007) reported recently that 9-O-acetylated ganglioside GD3 induces a cell death program in mature RBCs including activation of caspase-3. Altogether, these results suggest that caspase activation in RBC may depend at least in part on the apoptotic stimuli.

Proteolysis by caspases plays a central role in the execution of apoptosis in different cell types. Based on the premise that anucleated red cells undergo senescence, recognition by macrophages and removal from the circulation over the course of a 120-day life span, we sought to investigate whether in vivo caspases could be activated in senescent erythrocytes.

In previous studies we and others (Bratosin D. et al., 2001; Berg C.P. et al., 2001) have demonstrated that red cells contain caspases-8 and -3 in inactive proforms, even after Ca^{2+} influx-mediated cytoskeleton cleavage (Berg C.P. et al.,2001) leading to cell shrinkage, phosphatidylserine exposure and death. These authors concluded that the results they obtained i) could explain the programmed cell death of circulating RBCs since it was well known that senescence of these cells is associated with progressive Ca^{2+} influx (Shiga T. et al.,1985; Romero P.J. and Romero E.A.,1999) and ii) indicate that calpains but not caspases are involved in calcium-mediated senescence of RBCs.

In a comment concerning these conclusions, Daugas et al. (Zermati Y. et al., 2001) wrote: "That apoptosis may occur in the absence of a nucleus or in the absence of caspase activation has been reported in the past and the true originality of the present articles would be to show death in the absence of mitochondria. However, in a way the erythrocyte must be transient, presumably mitochondrion-triggered caspase activation has lead to the formation of an organelle-free mummy (just as ancient Egyptians removed organs from the corpse before mummification). Seen from this angle, it appears logical that the final implosion of RBC occurs via a non-apoptotic process which we might call erythroptosis". However, in the mean time other groups have shown caspase-3 activity in RBCs treated in vitro with various drugs (Mandal D. et al.,2002 - Mukherjee K. et al.,2007) leading to the conclusion that RBCs could die by "true" apoptosis.

We demonstrated by isolating RBCs from blood circulation using immobilized annexin-V that those cells are senescent cells based on esterase activity and morphology, but also display caspase activity in half of this population (Fig.9 and Fig.10).

Interestingly we found that both caspases-3 and -8 are activated in these RBCs. Mature erythrocytes represent the end stage of the erythroid lineage emerging from erythroid progenitors through a complex process of differentiation that involves the physiological loss of their nucleus, mitochondria and other organelles. Recent findings indicate that terminal differentiation into mature erythrocytes is preceded by a form of abortive apoptosis induction that involves transient caspase activation (Daugas E. et al., 2001). Therefore, in physiological circumstances, active caspases could persist in erythrocytes and induce the mechanism of erythrophagocytosis to eliminate abnormal cells.

We could also hypothesize that activation of procaspases could be induced by reactive oxygen species (ROS) since RBCs treated in vitro with various peroxides display caspase-3 activation (Mandal D. et al., 2002; Pietraforte D. et al., 2007).

Because caspase-3 is an effector whereas caspase-8 is an initiator able to cleave procaspase-3, we can speculate that in those RBCs caspase-8 should be the upstream signal. However, how caspase-8 can be activated? We and others (Bratosin D. et al., 2001; Berg C.P. et al., 2001) have shown that calpain may also participate in the "erythroptosis" of red blood cells.

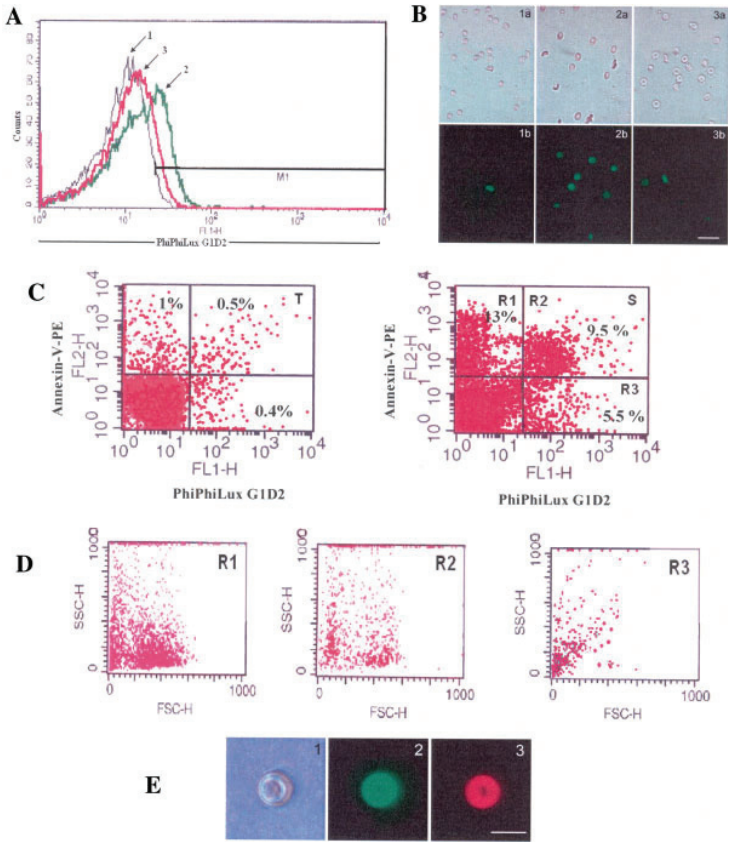

Fig. 9. Caspase-3 activity in human senescent RBCs using PhiPhiLux G1D2. A: Caspase-3 activity assessed by flow cytometric analysis. 1: Fluorescence of freshly isolated total human RBCs; 2 and 3: Fluorescence of senescent RBCs in the absence or in the presence of Ac-DEVD-cmk (200 µM), respectively. Abcissae: fluorescence (FL1). Ordinates: cell number. Number of counted cells: 10,000. B: Phase contrast (1a, 2a, 3a) and fluorescence (1b, 2b, 3b) microscopic analysis. Caspase-3 positive cells are green coloured. C: Flow cytometric quadrant analysis of caspase-3 activity (abscissae) and annexin-V-PE (ordinates) double-stained. T: freshly isolated human RBCs; S: affinity isolated senescent RBC population. Upper left quadrant (R1): caspase-3 negative and annexin-V-positive cells; upper right quadrant (R2): caspase-3 and annexin-V-positive cells; lower left quadrant (R3): viable cells (region of caspase-3 negative and annexin-V-negative cells); lower right quadrant: caspase-3 positive and annexin-V-negative cells. Numbering refers to the percentage of each cell population. Number of counted cells: 20,000. D: Dot-plot analysis FSC/SSC corresponding to the regions from Figure 4C-S. R1: caspase-3 negative and annexin-V-positive cells; R2: caspase-3 and annexin-V-positive cells; R3: caspase-3 positive and annexin-V-negative cells. Abcissae: forward scatter (cell size); ordinates: side scatter (cell density). Results shown are representative of ten independent experiments. E: Phase contrast (E1) and fluorescence (E2, E3) microscopy of a double stained discocyte using PhiPhiLux G1D2 (E2) and annexin-V-PE (E3) present in the population of RBCs obtained by centrifugation of heparinazed blood. Bar: 4B: 20 µm, 4E: 10 µm.

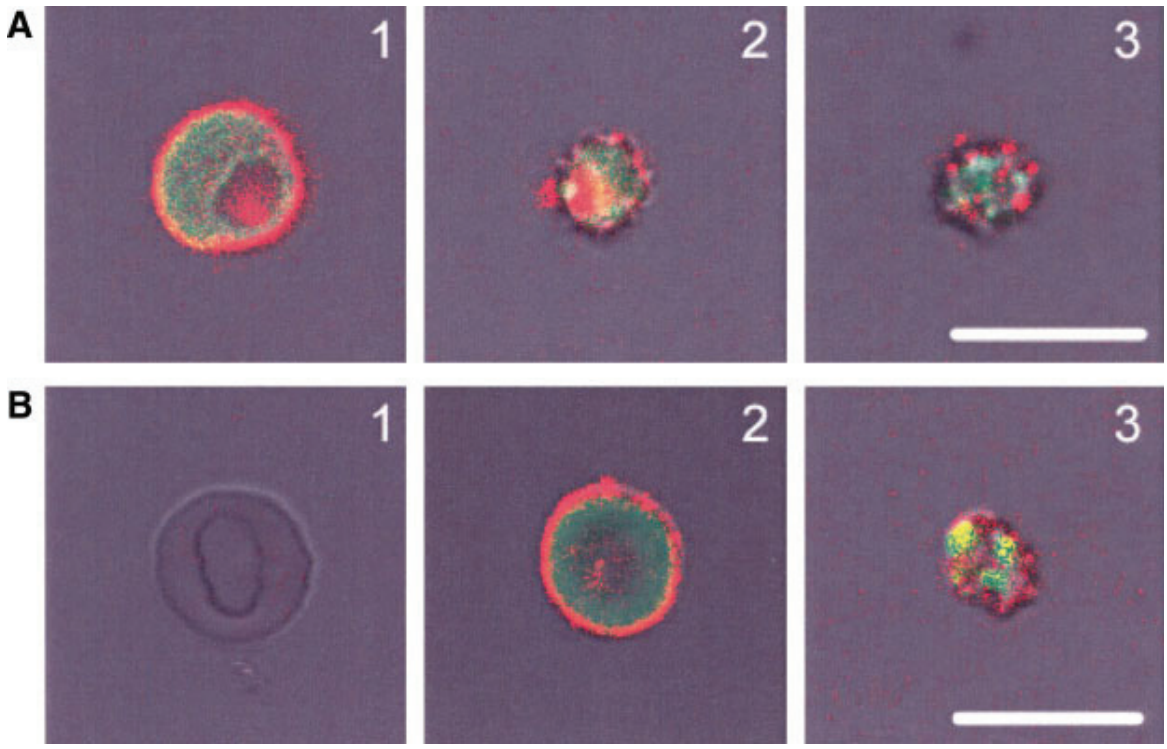

Fig. 10. Confocal microscopic analysis of human RBC senescent population double stained for caspase-8 (A) and -3 (B) activity using CaspGLOW™ (green colour) and for phophatidylserine externalization using annexin-V-PE (red colour). B1: Discocyte shape. A2, A3 and B3: Shrunken shapes. A1 and B2: pictures clearly showing the cytosolic location of caspases -8 and -3. Bar: 10 μm

However, Wolf et al. (Siegel R.M. et al., 1998) have demonstrated that calpain was unable to activate procaspases. Consequently we could hypothesize that erythrocyte apoptosis can be induced by cytoskeleton changes on the basis of the demonstration by White et al. (White S.R. et al. 2001). Another hypothesis is founded on the conception of death-effector domain (DED) that recruits caspases into complexes with members of TNF-receptor family causing cell apoptosis by forming cytoplasmic filaments that recruit and activate procaspase zymogen (Wolf B.B. and Green D.R., 1999). In others, loss of cell detachment from extracellular matrix mediated an apoptotic PCD termed "anoikis" (Grossmann J., 2002; Gilmore AP., 2005). Therefore, do senescent erythrocytes, annexin-V-positive displaying caspase-activity die through anoikis remains to be further explored?

In conclusion, we have clearly demonstrated for first time the presence of active caspases among the fraction of circulating erythrocytes displaying phosphatidylserine residues on line their surface (Bratosin D. et al.,2009). To end, the techniques we described and the findings we obtained could open the way to fruitful clinical investigations in the field of red blood cell pathology like haemolytic or parasitic diseases.

THE FUTURE IN RBC PRESERVATION IN BLOOD BANKS

The knowledege of the mechanism of the in vitro senescence of RBC is of the highest importance since it could lead to improvements of the storage conditions in blood banks by increasing the time of viability of stored RBC. In order to explain by which molecular and mechanism, 30 % and 70 % of transfused RBC disappear from the circulation after 1 and 3 days respectively, we have hypothetized that the in vitro senescence of RBC could be due to an identical mechanism of desialylation-phosphatidylserine exposure. In this regard, to ascertain whether stored RBCs would show similar, but accelerated, changes to those observed in the physiological aging of RBCs in circulation, we applied the same flow cytometric techniques to monitor the deterioration of the stored RBCs (Bratosin D. et al., 2001).

We focused our attention on the leukocyte population as the initiators of these accelerated changes for the following well known reasons. First, apoptosis occurs in white cells within the first 48-72 h of storage, leading to the cell lysis. Second, by this mechanism, active enzymes are released in the storage medium. These are mainly proteasesthat are responsable for hemolysis, because stored RBCs lack the protective effect of plasma which contains an array of antiproteases and sialidases

that have been identified in lytic products of leukocytes. Third, many authors have reported the advantages of leukodepletion prior to storage of RBCs in preventing the deleterious effects observed with nonleukodepleted RBC transfusion.

Experiments were comparatively carried out with nonleukodepleted and leukodepleted RBC concentrates prepared using blood bag set comprising an on-line leukocyte filter Leucoflex ® FQB 6250 (Maco Pharma, Tourcoing, France). In both cases, RBC concentrates were stored similarly at 4° C and analyzed every week for 6 weeks. On the basis of the following results we obtained it is clear that deleukocytation of blood before storage in blood banks protects the erythrocytes from desialylation and phosphatidylserine exposure in the outer leaflet of cell membrane and, consequently, from a rapid clearance of transfused erythrocytes : (i) After a 6-week storage of leukodepleted RBC, the three-dimensional representation of the dot-plot analysis in SSC / FSC remains unchanged compared with the pattern given by the " fresh " reference RBC. In contrast, storage of nonleukodepleted RBC induces dramatic changes of the pattern showing a progressive increase of senescent RBC versus viable ones ; (ii) Determination of RBC binding site number of lectins specific fo sialic acid and ß-galactosyl residues shows that an important desialylation of RBC membrane glycoconjugates occurs during the storage of nonleukodepleted RBC. In opposite, the number of ß-galactosyl residues, which are the signals for RBC capture, increases. RBC membrane desialylation was confirmed by applying the colorimetrc method of Warren. (ii) In parallel, residues of phosphatidylserine which are signals for RBC phagocytosis, are exposed in the outer leaftet of RBC membrane. (iii) Consequently, in vitro erythrophagocytosis progressively increases. All of these harmful effects are avoided by the leukodepletion of blood before storage in blood banks. In conclusion, blood destined to transfusion must be leukodepleted. In this regard, leukodepletion of blood before storage in blood banks is mandatory in Fance from the 1st of April 1998.

These results require consideration. In fact, up to now, the current storage parameters that affect RBC survival and function after transfusion involve the 1) determination of 2,3-diphosphoglycerate (2,3-DPG), necessary for oxygen transport by hemoglobin; 2) determination of adenosine triphosphate (ATP), which controls the erythrocyte shape, deformability, and in vivo survival; 3) changes in red cell morphology, from the typical discoid through echinocytes and finally into spherocytes; 4) quantitation of free hemoglobin, which signifies hemolysis if greater than 1 % of total hemoglobin; and 5) determination ot the percentage of RBCs remaining in circulation after 24 h of transfusion, 75 % of circulating red cells 24 h after transfusion being considered as an acceptable standart. However, according to Beutler (Beutler E.,2001), the ATP level is by no means the only limiting factor in determining whether or not a red cell will survive when reinfused, unless 2,3-DPG level regulates the oxygen affinity of RBCs. Therefore, allmedia, even those that do maintain ATP levels, require evaluation by in vivo viability testing of RBCs. In fact, authors found low in vivo recovery of stored nonleukodepleted RBCs in spite of excellent morphology and ATP level.

To conclude, we ground our present research strategy on the very interesting and relevant recent editorial by Beutler (Beutler E., 2001) who made the following salient comments: 1) the fundamental nature of the " storage lesion " remains unknown; 2) no good surrogate test has ever been found for the performance of viability studies in human volunteers; 3) it is unclear which, if any, of the components of the storage systems were effective in improving the viability of stored RBCs after transfusion; and 4) many of the improvements were achieved without the discovery of any new principles.

We propose that the flow cytometric criteria we have developed could provide that missing window that would permit the direct observation of the changes on the surface of RBCs as possible determinants to evaluate the viability of stored erythrocytes. In fact, most of the "classical" above-mentioned parameters that were used as criteria of stored RBC viability are cytosolic criteria whereas the cell membrane is truly responsible for erythrophagocytosis. In this regard, whatever the complex mechanism of RBC membrane damages due to leukocytes, desialylation and phosphatidylserine exposure remains fundamental criteria of RBC viability.

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