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# Granulocyte Apoptosis and the Control of Inflammation

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# Granulocyte apoptosis and the control of inflammation

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## SUMMARY

We have described a novel pathway available for the clearance of extravasated granulocytes from inflamed tissues whereby aging granulocytes undergo apoptosis, a process which leads to their phagocytosis by inflammatory macrophages. By contrast with necrosis, which may also be seen at inflamed sites, apoptosis represents a granulocyte fate which by a number of mechanisms would tend to limit inflammatory tissue injury and promote resolution rather than progression of inflammation: (i) apoptosis is responsible for macrophage recognition of senescent neutrophils with intact cell membranes which exclude vital dyes and retain their potentially histotoxic granule contents; (ii) the apoptotic neutrophil loses its ability to secrete granule enzymes on deliberate external stimulation; (iii) the macrophage possesses a huge phagocytic capacity for apoptotic neutrophils which it rapidly ingests and degrades without disgorging neutrophil contents; and (iv) the macrophage utilizes a novel phagocytic recognition mechanism which fails to trigger the release of pro-inflammatory macrophage mediators during the phagocytosis of apoptotic neutrophils. Preliminary characterization of the recognition mechanism implicates the integrin $\alpha$ v $\beta_3$ (vitronectin receptor) and CD36 (thrombospondin receptor) on the macrophage surface. Macrophage phagocytosis of apoptotic neutrophils is greatly influenced by the microenvironmental pH and by the presence of cationic molecules. Moreover, it can be specifically modulated by external cytokines and intracellular second messenger systems. By controlling the functional longevity of neutrophil and eosinophil granulocytes and their subsequent removal by macrophages, granulocyte apoptosis, with its potential for modulation by external mediators, is likely to play a key dynamic role in the control of the 'tissue load' of granulocytes at inflamed sites. Further elucidation of the mechanisms and control of apoptosis in granulocytes is likely to shed new light on the pathophysiology of inflammation and suggest new approaches to the therapy of inflammatory diseases.

## 1. INTRODUCTION: RESOLUTION VERSUS PERSISTENCE OF INFLAMMATION

It is now widely recognized that inflammation is central to the pathogenesis of a range of diseases which impose a heavy burden of mortality and untimely deaths in developed societies. In the lung these include chronic bronchitis and emphysema, asthma, and respiratory distress syndromes of the adult and neonate. Important inflammatory diseases in other organs include glomerulonephritis (the major cause of renal failure requiring dialysis), arthritides and inflammatory bowel disease. Most inflammatory diseases are characterized by the persistent accumulation of inflammatory cells which is associated with chronic tissue injury and scarring. In organs with delicate exchange membranes, such as the lung and kidney, these processes often result in catastrophic loss of function. However, it is also clear that, paradoxically, inflammation has evolved as a highly effective component of the body's defences against infection and injury. Indeed, until the latter half of this century, inflammation was perceived as an entirely beneficial host response. Neutrophil and eosinophil granulocytes play essential protective roles in bacterial infections, such as lobar Streptococcal pneumonia, and parasitic infections, such as schistosomiasis. The acute inflammatory response in lobar Streptococcal pneumonia exemplifies the effectiveness of a rapidly mounted inflammatory response. In the pre-antibiotic era, Streptococcal pneumonia was widely prevalent, being responsible for more than 90% of pneumonias, yet the inflammatory response was effective enough to save the lives of more than 70% of patients. Perhaps more remarkable, given what we now know of the destructive and pro-fibrotic potential of neutrophils and activated macrophages, there was clear evidence that in more than 95% of

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cases lobar Streptococcal pneumonia resolved completely, with less than 2.5% progressing to fibrosis (Robertson & Uhley 1938).

It is reasonable to suppose that research aimed at determining how inflammation may normally resolve will not only provide important insights into the circumstances leading to the persistent inflammatory states which characterize most inflammatory diseases but will also suggest novel therapeutic strategies directed at promoting those mechanisms which favour resolution. By contrast with the initiation and amplification mechanisms of inflammation, however, comparatively scant attention has been paid to the processes responsible for its termination. Hurley (1983) considered that the acute inflammatory response might terminate by the development of: chronic inflammation; suppuration (abscess); scarring; or by resolution. Clearly, all the alternatives to resolution are potentially detrimental to organ function, but until very recently little research effort had been focused on the cellular and molecular mechanisms underlying the normal resolution processes of inflammation.

The resolution of inflammation is likely to be as complex as the initiation phase, but one pre-requisite is that extravasated inflammatory cells and their contents must be removed from tissues. We have been particularly interested in elucidating the mechanisms whereby granulocytes are cleared from inflamed sites.

## 2. THE TISSUE CLEARANCE OF EXTRAVASATED GRANULOCYTES

The neutrophil granulocyte is the archetypal acute inflammatory cell. It is essential for host defence, but it is also implicated in the pathogenesis of a wide variety of inflammatory diseases (Malech & Gallin 1988). It is usually the first cell to migrate to the scene of tissue perturbation, and a number of subsequent inflammatory events including monocyte emigration (Doherty *et al.* 1988) and generation of inflammatory oedema (Wedmore & Williams 1981) may depend on the initial tissue accumulation of neutrophils. Neutrophils contain a large number of agents with the capacity not only to injure tissues (Weiss 1989), but also to cleave matrix proteins into chemotactic factors (Vartio *et al.* 1981) with the potential to amplify inflammation by attracting more cells. Eosinophils play an important part in host defence against worms and other parasites, but they are also implicated in the pathogenesis of allergic diseases such as asthma. Although we have for some years been aware of the histotoxic potential of neutrophil and eosinophil contents there has been little formal study of the tissue fate of these cells. It is generally agreed that most extravasated neutrophils meet their fate at the inflamed site, but it had been widely assumed that they inevitably underwent disintegration (necrosis) before the fragments were removed by local macrophages (Hurley 1983). However, if this was the rule, healthy tissues would inevitably be exposed to large quantities of potentially injurious neutrophil contents. Although a number of pathological descriptions have favoured neutrophil necrosis as a major mechanism operating in the inflammation, many of these examples have been taken from disease states rather than from 'beneficial' self-limited inflammation. Moreover, there has been evidence for over a century of an alternative fate for extravasated neutrophils, based on the original work of Metchnikoff, who, in vital preparations, was the first to describe the cellular events occurring during the evolution and resolution of the acute inflammatory response. Rather than neutrophil necrosis as the major mechanism during inflammatory resolution, he observed the ingestion of intact senescent neutrophils by macrophages (Metchnikoff 1891). Since then there have been several sporadic reports of macrophages phagocytosing neutrophils, and of particular relevance to the resolution of inflammation is the clinically described phenomenon of 'Reiter's cells': neutrophil-containing macrophages which have been described in synovial fluid from the inflamed joints of patients with Reiter's disease and other acute arthritides (Spriggs *et al.* 1978). In experimental peritonitis, macrophage ingestion of apparently intact neutrophils is clearly the dominant mode of neutrophil clearance (Chapes & Haskill 1983).

The mechanisms underlying these observations have only recently been addressed *in vitro*. Newman *et al.* (1982) showed that human neutrophils harvested from peripheral blood and 'aged' in culture were recognized and ingested by inflammatory macrophages (but not by monocytes) whereas freshly isolated neutrophils were not ingested. We have recently discovered that aging neutrophils and eosinophils (Savill *et al.* 1989*a*; Stern *et al.* 1992) constitutively undergo apoptosis and that this process is responsible for the recognition and ingestion of intact senescent granulocytes by macrophages.

## 3. APOPTOSIS IN AGING GRANULOCYTES LEADS TO THEIR PHAGOCYTOSIS BY MACROPHAGES

Neutrophils harvested from blood or from acutely inflamed human joints remain intact, retain their granule enzyme contents, and continue to exclude vital dyes for up to 24 h in culture. However, over this period there occurs a progressive increase in the proportion of cells exhibiting the classical light microscopical and ultrastructural (figure 1) features of apoptosis together with the 'ladder' pattern of chromatin cleavage which is indicative of endogenous endonuclease activation (Savill *et al.* 1989*a*). Only the apoptotic subpopulation of aged neutrophils is recognized and ingested by macrophages. Apoptotic neutrophils are not indestructible, and beyond 24 h in culture there is a progressive increase in the proportion of cells that fail to exclude vital dyes and spontaneous release of granule enzyme contents is observed. However, when neutrophils are cultured beyond 24 h in the presence of macrophages, the removal of apoptotic cells is so rapid and effective that no trypan blue positive cells are seen and there is no




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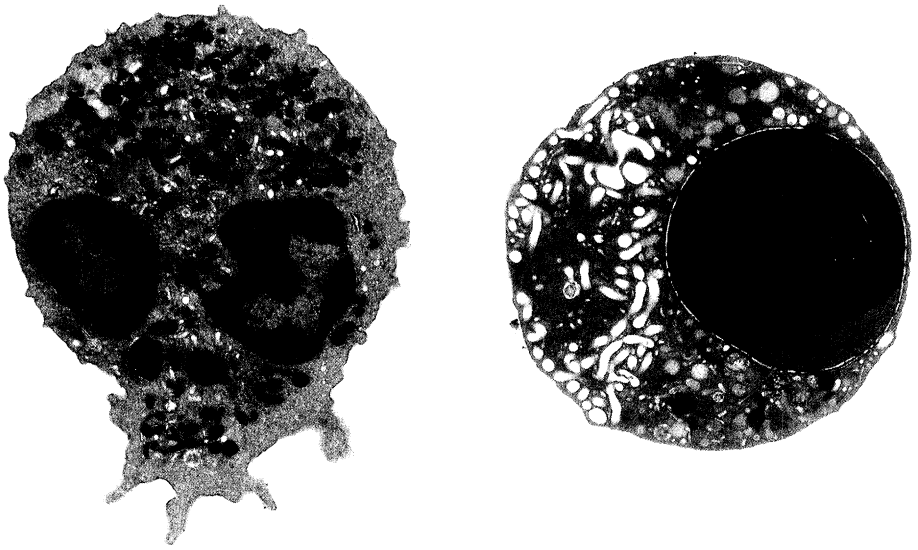

Figure 1. Electron micrograph of an apoptotic human neutrophil granulocyte (×*ca.* 11 000) showing classical nuclear chromatin changes and dilatation of the endoplasmic reticulum (seen on the right) as compared with a non-apoptotic neutrophil (left).

release of granule enzyme markers into the surrounding medium (Kar *et al.* 1993). Macrophages *in vitro* can ingest and destroy several neutrophils with remarkable speed, such that in ultrastructural studies it is necessary to fix macrophages within minutes of the initial interaction between apoptotic cells and the macrophages in order to demonstrate recognizable neutrophils within phagosomes; thereafter ingested cells are no longer recognizable. This may represent part of the explanation why the dynamic contribution of this process to inflammatory tissue kinetics has not been fully appreciated until recently. Nevertheless, there are now several clear histological demonstrations of a role for apoptosis in the *in vivo* removal of granulocytes in acute inflammation. These include acute arthritis (Savill *et al.* 1989*a*), neonatal lung injury (Grigg *et al.* 1991) and experimental acute Streptococcal pneumonia during its resolution phase (figure 2).

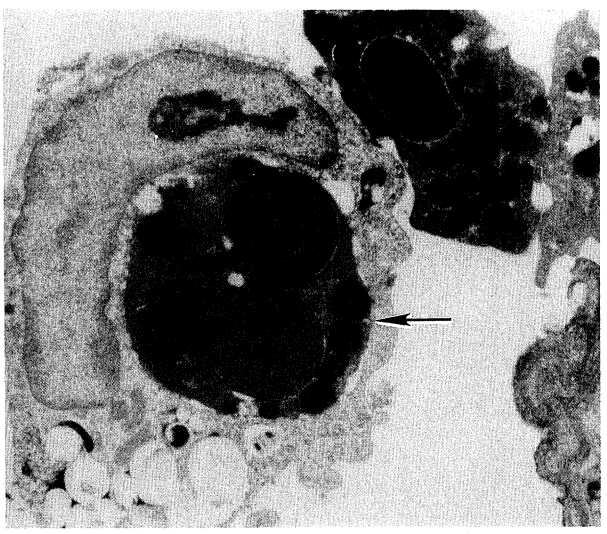

Figure 2. Electron microscopy of resolving streptococcal pneumonia showing a macrophage that has ingested an apoptotic neutrophil (arrow).

Several lines of *in vitro* experimental evidence have emerged in support of the hypothesis that apoptosis provides a granulocyte clearance mechanism in tissue which would tend to limit inflammatory tissue injury and promote resolution rather than persistence of inflammation.

1. During apoptosis, the cell membrane remains functionally intact, as assessed by vital dye exclusion, and continues to retain cytosolic enzymic contents, but there is marked loss of a number of neutrophil functions, including secretion of granule enzymes after deliberate external neutrophil stimulation (figure 3). This suggests that the apoptotic neutrophil becomes 'functionally isolated' from external stimuli which would otherwise trigger responses with the potential to damage tissue (Whyte *et al.* 1993). We have recently shown that during neutro-

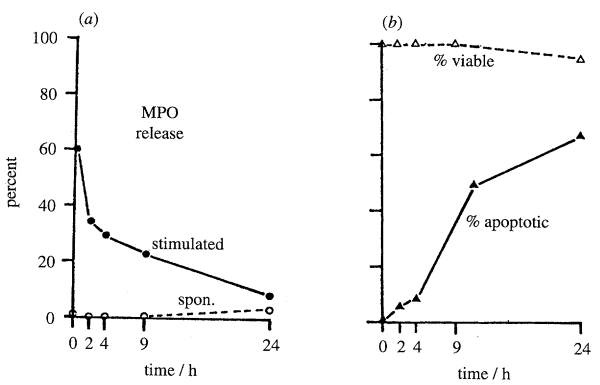


Figure 3. Release of the granule enzyme marker myeloperoxidase (MPO) during the *ex-vivo* culture of human neutrophils. Over 24 h there is a progressive increase in the proportion of apoptotic cells in the population but no significant necrosis (assessed by trypan blue exclusion) or spontaneous release of MPO. Moreover, with time the neutrophil population increasingly loses the ability to secrete MPO in response to deliberate external stimulation with the formylated peptide FMLP.

phil apoptosis there may be selective loss of surface receptors which exert important cellular functions (Dransfield *et al.* 1994). This down-regulation of neutrophil function would be expected to be particularly important if fully mature, competent phagocytes are not immediately available in the vicinity of neutrophils undergoing apoptosis.

2. Large numbers of apoptotic neutrophils can be cleared by macrophages without 'leakage' of potentially injurious neutrophil contents into the surrounding medium (Kar *et al.* 1993).

3. Although the usual response of macrophages to the ingestion of particles *in vitro* is to release mediators such as thromboxane, enzymes and pro-inflammatory cytokines, even maximal uptake of apoptotic neutrophils fails to stimulate the release of pro-inflammatory mediators (Meagher *et al.* 1992; figure 4). However, if apoptotic granulocytes are cultured beyond apoptosis to a point when they fail to exclude trypan blue, their ingestion by macrophages induces massive release of pro-inflammatory mediators (M. Stern, unpublished data). It was subsequently shown that this lack of a macrophage secretory response is not a function of the apoptotic body itself, but relates to the special mechanism by which the apoptotic cell is normally ingested (Meagher *et al.* 1992). These observations provided considerable impetus for our work on the molecular mechanisms responsible for macrophage recognition of apoptotic cells.

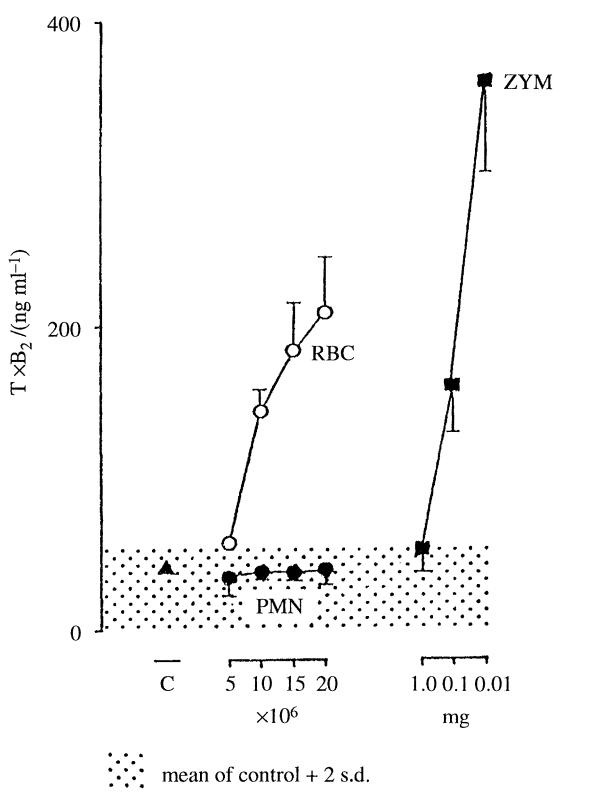


Figure 4. Release of Thromboxane $B_2$ from monolayers of human monocyte-derived macrophages after maximal phagocytosis of opsonized zymosan (ZYM), allogenic erythrocytes (RBC) or apoptotic human neutrophils (PMN).

## 4. MECHANISMS WHEREBY MACROPHAGES RECOGNIZE APOPTOTIC NEUTROPHILS

In their early studies of the effects of sugars on macrophage recognition of apoptotic thymocytes, Duvall & Wyllie (1985) suggested that phagocytes possess a lectin mechanism capable of recognizing sugar residues on the apoptotic thymocyte surface exposed by loss of sialic acid. This mechanism does not appear to be involved in macrophage recognition of apoptotic granulocytes, but these findings stimulated our early work showing that recognition of apoptotic neutrophils was inhibited by cationic molecules, e.g. amino sugars, and was directly influenced by reductions of pH in a fashion implicating negatively charged moieties on the apoptotic neutrophil surface (Savill *et al.* 1989*a*). These observations were of considerable interest as a number of neutrophil and eosinophil products, e.g. elastase and major basic protein, are highly cationic and have been detected in tissues in inflammatory disease. Moreover, in sites of chronic inflammation, or in abscesses, tissue pH may be very low (Menkin 1956). Thus, local micro-environmental conditions at chronically inflamed sites could greatly retard macrophage removal of apoptotic neutrophils.

The observed amino sugar inhibition pattern led to a detailed series of investigations which implicated macrophage surface molecules, including the integrin $\alpha$ v $\beta_3$ (the vitronectin receptor) (Savill *et al.* 1990) and CD36 (Savill *et al.* 1992) (a thrombospondin receptor), in the recognition and phagocytosis of apoptotic cells. These appear to link via thrombospondin, which acts as an intercellular bridging molecule, with an as yet uncharacterized recognition site on the apoptotic neutrophil surface. Recent studies by colleagues in Denver have been focused on changes in the apoptotic cell surface responsible for macrophage recognition. Their work suggests that macrophages may recognize phosphotidlyserine residues which become exposed on the surface of murine thymocytes induced by glucocorticoids to undergo apoptosis (Fadok *et al.* 1992). The *in vivo* significance of these observations is as yet uncertain, but it appears that the main difference between the two recognition systems relates to the utilization of alternative recognition mechanisms by different subpopulations of macrophages (Savill *et al.* 1993).

The definition of macrophage surface molecules responsible for apoptotic cell recognition suggests mechanisms by which neutrophil clearance may be regulated. Experiments so far have shown that a number of cytokines can promote macrophage uptake of apoptotic neutrophils (Ren & Savill 1993), and mediators which influence intracellular cyclic AMP may control the process through modulation of $\alpha$ v $\beta_3$ function (McCutcheon *et al.* 1994).

## 5. REGULATION OF GRANULOCYTE APOPTOSIS BY EXTERNAL MEDIATORS

Histological observations in models of experimental Streptococcal pneumonia suggested that neutrophils




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at inflamed sites underwent apoptosis at a much slower rate than those derived from peripheral blood (C. Haslett, unpublished observations). This implied that factors present at the inflamed site might have retarded the inherent rate of neutrophil apoptosis. It has now been shown that the rate of neutrophil apoptosis *in vitro* is inhibited by a variety of inflammatory mediators including endotoxic lipopolysaccharide, C5a and GMCSF. Furthermore, inhibition of neutrophil apoptosis by these agents not only increased the lifespan of cultured neutrophils, but also greatly prolonged their functional longevity assessed by a number of parameters including chemotaxis and stimulated secretion (Lee *et al.* 1993). It had been known for several years that fibroblast-conditioned medium and specific growth factors, including GMCSF, could prolong the life of neutrophils and eosinophils in culture as assessed by failure of the cell to exclude the vital dye trypan blue (necrosis). Because we have clearly shown in healthy cultured granulocytes that apoptosis precedes ultimate necrosis of the cell, it seems likely that these previous observations can be explained by growth factor-induced modulation of the process of apoptosis. By modulating the lifespan and functional activity of neutrophils, apoptosis may represent a pivotal mechanism controlling their functional longevity at sites of inflammation. Experiments with eosinophils *in vitro* show that GMCSF inhibits eosinophil apoptosis, but that interleukin-5 is also extremely potent in this regard, whereas it has no effect on neutrophil longevity (Stern *et al.* 1992). It is intriguing that the apoptotic 'programme' should be under different controls in two such closely related cells.

There has been a great deal of recent interest in the role of intracellular signalling pathways and proto-oncogene expression in the control of apoptosis in a variety of cell types (e.g. Vaux *et al.* 1988). However, there has been comparatively little work on intracellular mechanisms controlling granulocyte apoptosis, and there are indications that internal controls in granulocytes may differ from those in lymphoid cells. In thymocytes, elevation of intracellular calcium by calcium ionophores induces apoptosis, and apoptosis induced by other stimuli, such as glucocorticoids is associated with rises in intracellular calcium (e.g. McConkey *et al.* 1989). However, in neutrophils spontaneously undergoing apoptosis there were no such rises in $[Ca^{2+}]i$ and agents increasing $[Ca^{2+}]i$ caused dramatic slowing of neutrophil apoptosis without inducing necrosis (Whyte *et al.* 1993). Furthermore, treatment of aging neutrophils with intracellular calcium chelators was associated with an increase in the rate of neutrophil apoptosis. Again in contrast to lymphoid cells, culture of neutrophils in the presence of inhibitors of protein synthesis, e.g. cycloheximide,

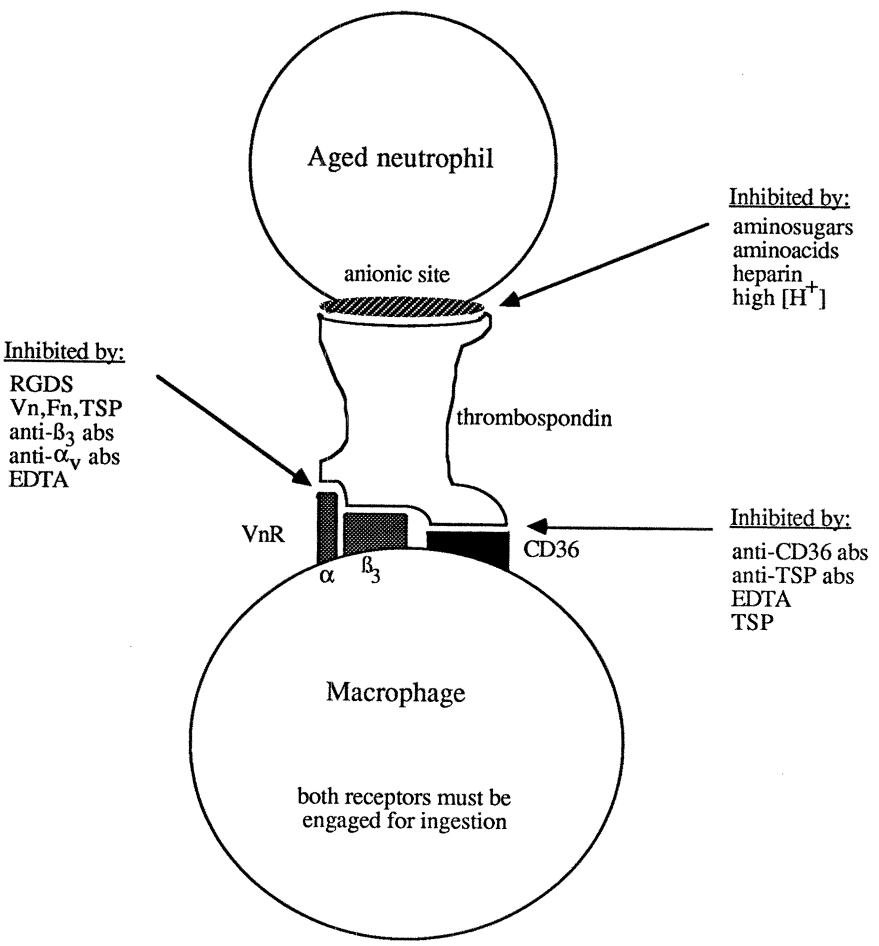


Figure 5. A model of the recognition mechanism whereby human monocyte-derived macrophages phagocytose apoptotic human neutrophils.




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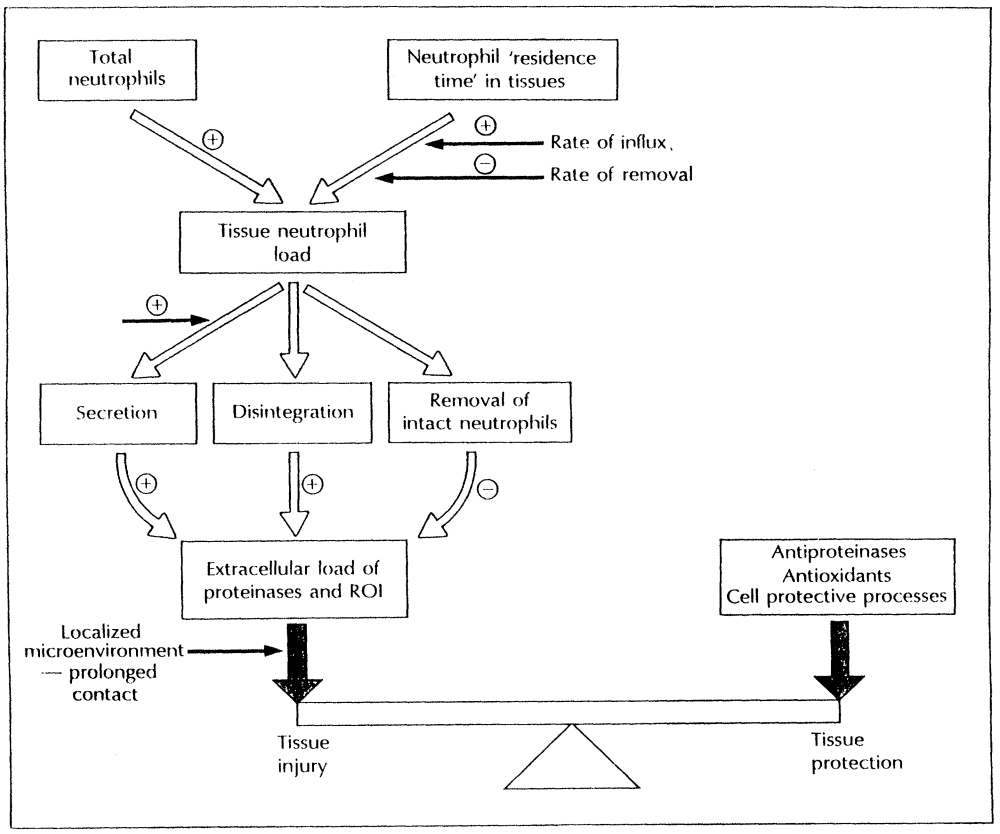


Figure 6. Some factors controlling the tissue 'load' of neutrophils at inflamed sites and the balance between injurious influences of inflammatory cells and tissue protective mechanisms.

caused an acceleration of the constitutative rate of apoptosis (Haslett *et al.* 1990). As yet there is little information on the role of proto-oncogenes in the control of neutrophil apoptosis.

## 6. HYPOTHESIS: A ROLE FOR GRANULOCYTE APOPTOSIS IN THE CONTROL OF INFLAMMATION?

The central role of apoptosis in the clearance of granulocytes from inflamed sites implies several levels of control but also the potential for disorders at various points of the clearance pathway which might promote inflammatory tissue injury and contribute to disease processes. Whether neutrophils meet their fate by disintegration, disgorgement of their contents, and phagocytosis by macrophages which respond by releasing inflammatory mediators (necrosis) or by removal of the intact senescent cell by macrophages which fail to release pro-inflammatory mediators (apoptosis) is likely to impinge on the precarious balance which normally exists between potentially damaging processes and tissue protective mechanisms in inflammation. While in all the spontaneously resolving examples of inflammation we have examined, the removal of whole granulocytes by apoptosis appears to be a major mechanism, examples of neutrophil necrosis are also seen. Therefore, it is possible that the balance between neutrophil apoptosis and necrosis at an inflamed site may represent a pivotal point in the control of tissue injury and in the propensity of an inflamed site to resolve or to progress.

By prolonging the functional longevity of neutrophils and eosinophils through inhibition of their constitutive rate of apoptosis, a variety of inflammatory mediators including growth factors and chemotactic cytokines exert important controls on the 'tissue load' of granulocytes (see figure 6). The removal of apoptotic granulocytes by macrophages is also under the control of inflammatory mediators including cytokines (Ren & Savill 1994) and agents which modulate macrophage cAMP levels (McCutcheon *et al.* 1994). Moreover, the phagocytic recognition mechanism is profoundly inhibited by physical conditions, including acidic pH and the presence of cationic molecules (Savill *et al.* 1989*b*) which may exist at chronically inflamed sites.

Finally, these observations may have some relevance for the development of novel approaches to anti-inflammatory therapy. With increasing knowledge of the internal mechanisms of apoptosis, it may be possible to specifically induce apoptosis in certain inflammatory cells at critical stages of the pathogenesis of inflammatory disease. The observation that apoptosis in such closely related cells as the neutrophil and eosinophil granulocyte appears to be controlled by different mechanisms lends some credence to this speculation.

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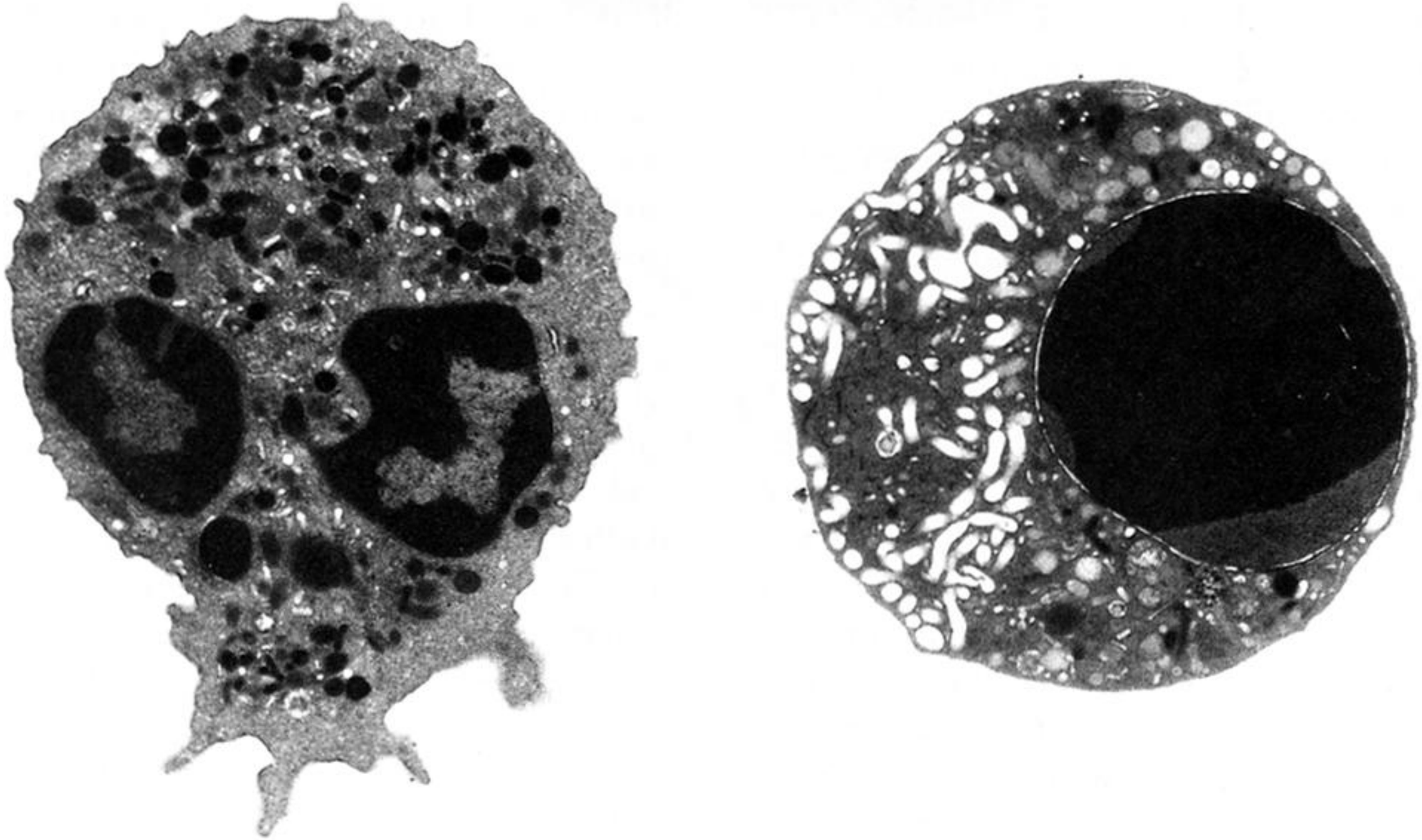

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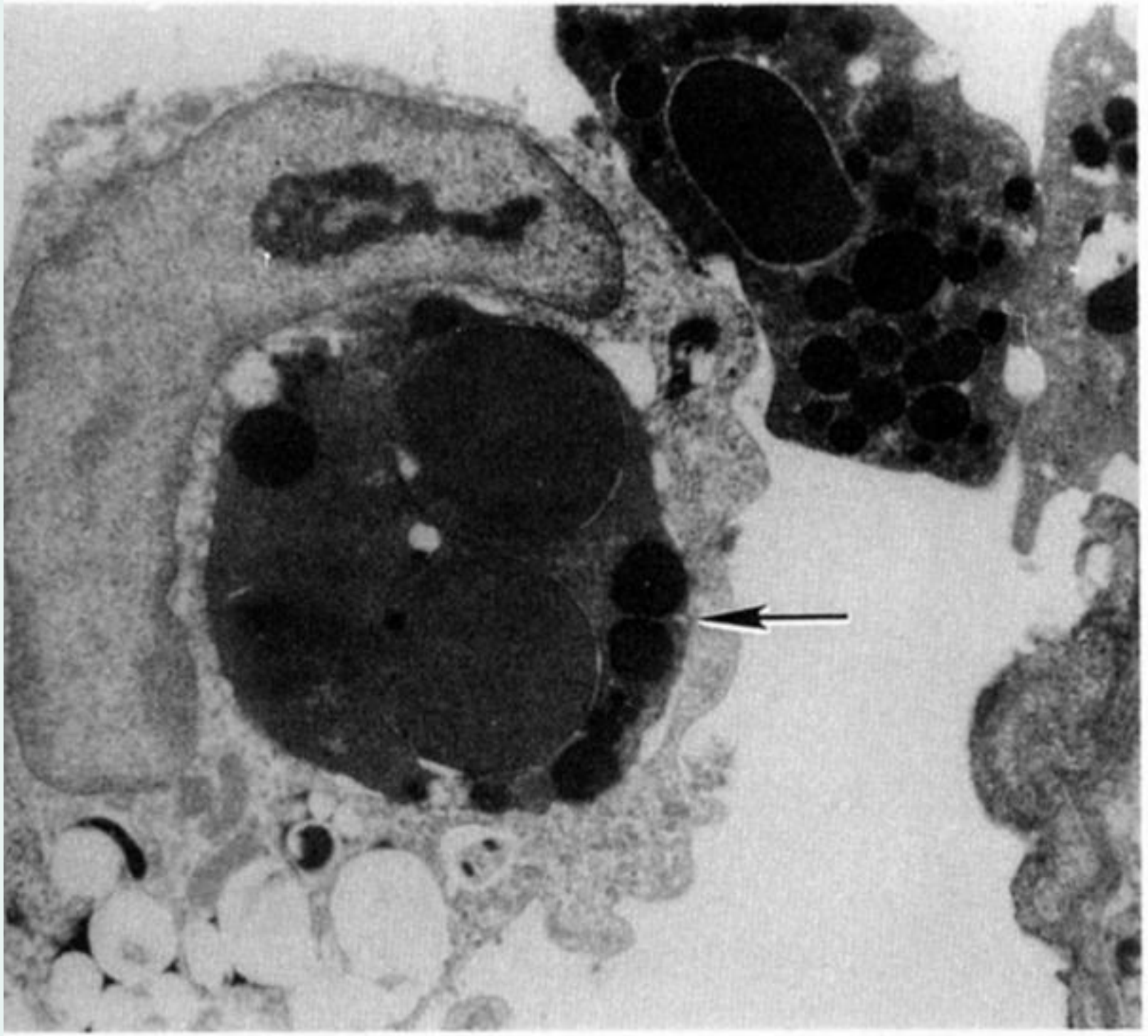

gure 2. Electron microscopy of resolving streptococcal neumonia showing a macrophage that has ingested an poptotic neutrophil (arrow).


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