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Development of Different Peroxidatic Activity Patterns in Peritoneal Macrophages *In Vivo* and *In Vitro*

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The peroxidatic activity (PA) pattern of rat peritoneal macrophages has been studied during both acute and chronic inflammation. The macrophages were divided into 4 types according to their different *in vivo* PA patterns: exudate macrophages, exudate-resident macrophages, resident macrophages and PA-negative macrophages. Cultured *in vitro*, peritoneal exudate macrophages of rat and mouse and rat blood monocytes acquired the characteristic PA pattern of resident macrophages via a transitional stage of cells with characteristics of exudate-resident macrophages. The *in vivo* and *in vitro* results indicate that the divergent PA patterns of macrophages represent differences in the stages of development or stages of activation of these cells. Thereby, it is suggested that PA-negative cells in fact may comprise different developmental stages of macrophages.

INTRODUCTION

The diaminobenzidine (DAB) technique of Graham and Karnovsky (15), developed in 1966 for the localization of horseradish peroxidase, has also been used to study the localization of endogenous peroxidatic activity (PA) in various types of cell. In 1970 Cotran and Litt (9) described endogenous PA in guinea pig peritoneal macrophages, while Robbins et al. (22) described it in rat peritoneal cells. Extensive studies by Daems *et al.* (10, 11) showed different PA of resident and exudate

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peritoneal macrophages in the guinea pig. Exudate macrophages show PA only in the lysosomal vesicles, while resident macrophages show PA only in the nuclear envelope and rough endoplasmic reticulum (RER).

Kinetic studies have shown unequivocally that inflammatory exudate macrophages are derived from blood monocytes that originate in the bone marrow (12, 13, 14). This was confirmed by studies which showed that the PA patterns of blood monocytes and exudate macrophages are identical (2, 21). On the basis of cell kinetics (12, 13, 14), resident macrophages have also been shown to originate from the bone marrow, but there are also kinetic studies which seem to refute this idea (20, 23, 26). The doubts of the origin of resident macrophages are, however, mainly based on the divergent PA patterns of blood monocytes and resident macrophages (10, 11, 16, 19). Recently we identified transitional exudate-resident macrophages, with cytochemical characteristics of both the exudate and the resident cells, *in vivo* during acute inflammation in the rat (2) and *in vitro* after culture of blood monocytes in man (3). During chronic inflammation in man, exudate-resident macrophages are not present *in vivo*; these cells did, however, appear *in vitro* in cultures of these exudates. In addition Bodel *et al.* (5, 6) have shown the appearance of PA in the RER *in vitro* after surface adherence in the rabbit, although this phenomenon was only of temporary nature. For these reasons we suggested that the divergent PA patterns of macrophages (2, 3, 18) represent differences in stages of development or stages of activation of these cells.

The present study is a further investigation of the possibility that the PA pattern is a function of development or functional activity. The PA pattern of peritoneal macrophages (PM) after both acute inflammation in mouse and rat and chronic inflammation in the rat was followed *in vivo*. The developments in PA pattern after culture of both peritoneal PM and blood monocytes were also studied *in vitro*, both in adherent and nonadherent cells.

MATERIALS AND METHODS

Animals. Male SPF-Wistar rats and SPF-Swiss mice were obtained from the central institute for the breeding of laboratory animals TNO (Zeist, the Netherlands).

Peritoneal Macrophages and Blood Monocytes. Peritoneal exudates were induced by i.p. administration of, respectively, 10 ml sterile newborn calf serum (NBCS, acute inflammation) or paraffin oil (chronic inflammation) in the rat or 1 ml NBCS in the mouse. The peritoneal cells were harvested as described previously (2). Blood monocytes were obtained according to Boyum (7, 8). The cells were fixed directly (*in vivo*) or processed further for culture (*in vitro*).

Cell Culture of Peritoneal Cells and Blood Monocytes. The cells were washed 3 times in an RPMI-1640 medium (Flow Laboratories, Glasgow, Scotland) with glutamine, 50 U/ml penicillin, 50 μ g/ml streptomycin (Mycofarm, Delft, the Netherlands) and 10% fetal calf serum (Gibco Bio-Cult, Irvine, Scotland). Subsequently, an aliquot was taken for fixation (0 time). For incubation, 6-ml aliquots, each containing 1 to 2 x 10⁶ cells/ml, were put in 60 x 15-mm Integrid culture dishes (Falcon Plastics, Oxnard, CA, USA). Cells were incubated at 37 C in 5% CO₂ in air.

After 2 hr of incubation, the nonadherent cells were washed off and the adherent cells (> 90% macrophages) were incubated for an additional 0-8 days.

The number of adhering cells was followed light microscopically; macrophages which no longer adhered at these times were also studied. Furthermore, cells were cultured on Teflon Film Dishes according to Van der Meer *et al.* (17) to prevent surface adherence from the start. As a control, cells obtained from nonstimulated peritoneal cavities were cultured simultaneously for 0 to 8 days.

Cytochemical Procedures. Cells were fixed for 10 min in 1.5% glutaraldehyde, washed 3 times in 0.1 M Na-cacodylate (pH 7.4), brought into an Eppendorf tube and reacted for PA in diaminobenzidine (DAB) as described previously (2, 3), i.e., pH 6.5; preincubation and incubation (0.01% H₂O₂) 1 hr at 20 C. The cells were then washed, postfixed in OsO₄, processed for electron microscopy as described previously and embedded in araldite. Since the oxidized DAB-polymer becomes electron-opaque with OsO₄ (25), staining with lead citrate was omitted. Staining with uranyl acetate was omitted because it could have extracted or masked a reaction product (6).

In vitro-incubated adherent cells were fixed, washed, reacted for PA and processed as above, while still in dishes, until dehydration in absolute alcohol was complete (6). The cells were then removed with propylene oxide as described previously (3, 6, 24), concentrated to small pellets and embedded in araldite. The nonadherent cells from the Teflon surface were processed as described previously (2).

Control preparations consisted of cells from incubations in which H₂O₂ was omitted or to which Aminotriazole (AT) was added.

All electron microscopic data were derived from 2 to 6 experiments in each of which 100 to 400 cells were studied.

RESULTS

Cytochemistry of Peritoneal Macrophages *In Vivo*. In the peritoneal cavity of the rat, resident macrophages are normally present and, in an acute inflammatory state induced with NBCS, exudate (exudate-resident) and resident macrophages, and low numbers of PA-negative cells are found (Table 1) (2). The mouse PM are

TABLE 1
The Various Types of Macrophages in the Peritoneal Cavity of the Rat in Normal Steady State and After Acute and Chronic Inflammation

Condition	Exudate macrophages (%)	Exudate-resident macrophages (%)	Resident macrophages (%)	PA-negative macrophages (%)
Normal steady state			95	5
Acute inflammation ^a	90		5	5
Chronic inflammation ^b	45	5	5	45

^a24 hr after NBCS.

^b2 to 8 days after paraffin oil.

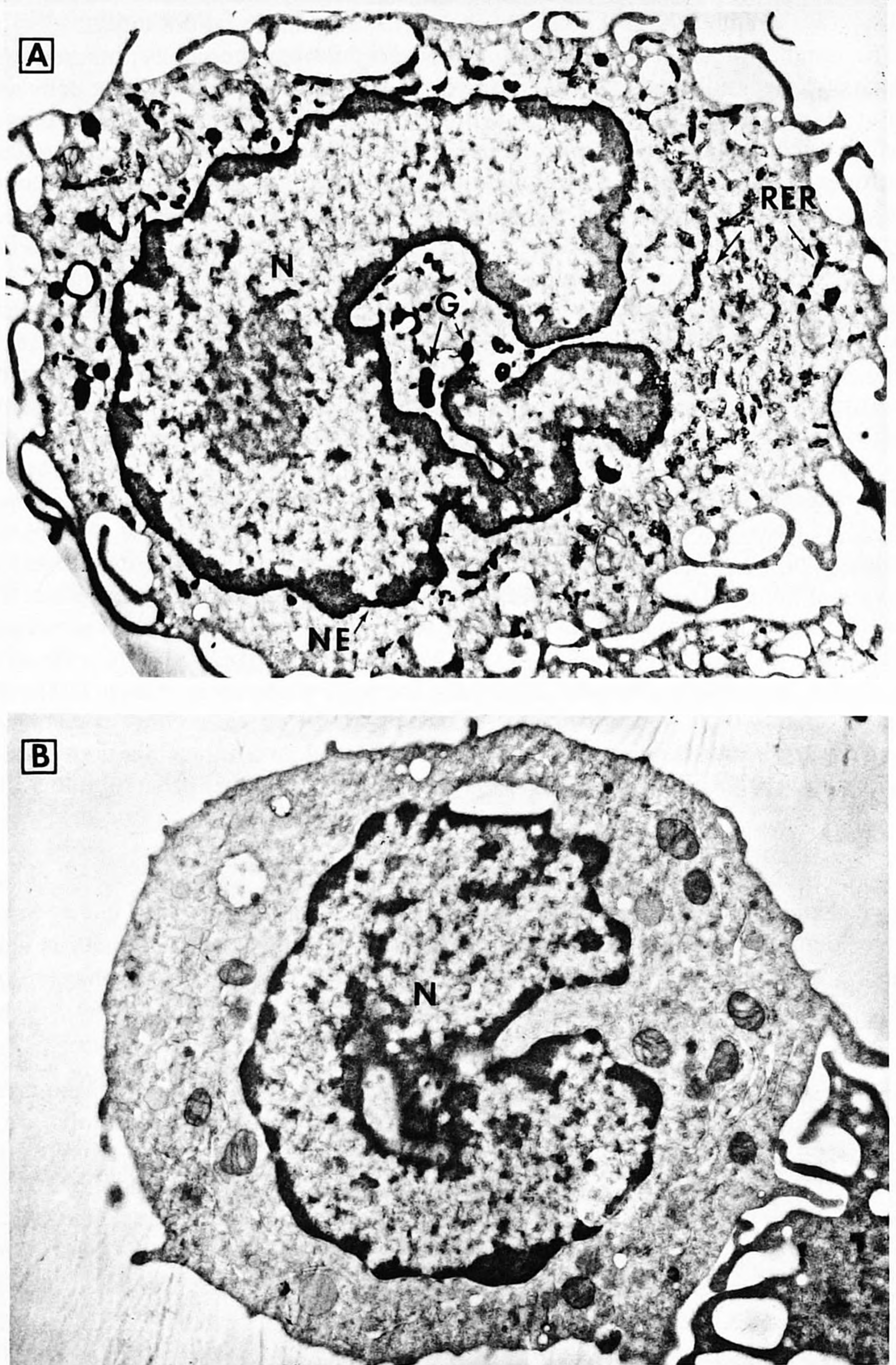


Fig. 1. Two patterns of PA observed *in vivo* in rat PM during chronic inflammation induced with paraffin oil. (A) Exudate-resident macrophages with PA in some of the lysosomes (G), in the nuclear envelope (NE) and in rough endoplasmic reticulum (RER). N, nucleus. X 10,200. (B) PA-negative macrophage. N, nucleus. X 10,000.

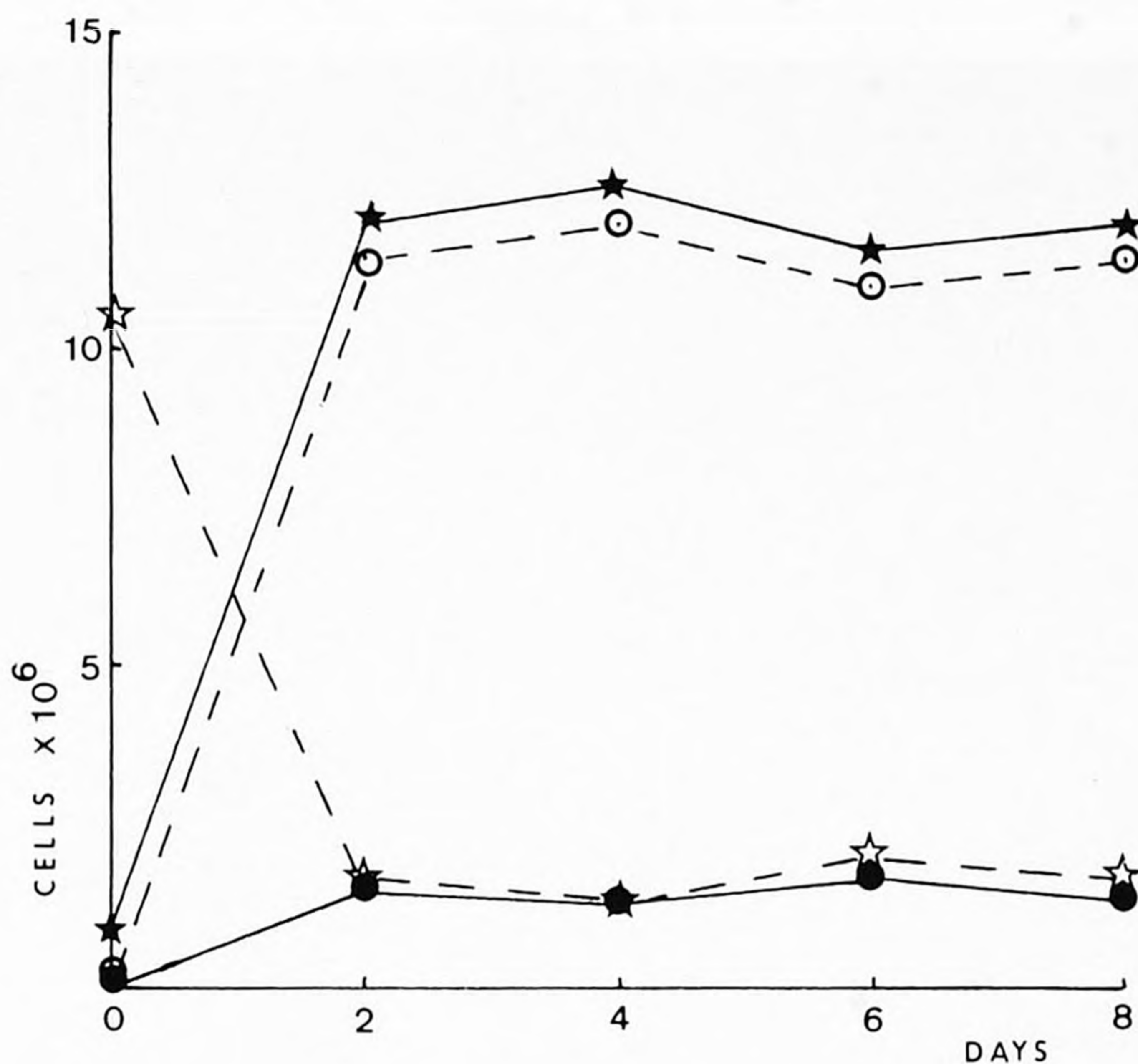


Fig. 2. The number of *in vivo* exudate (---○---), exudate-resident (—●—), resident (---☆---) and PA-negative macrophages (—★—) in the inflammatory peritoneal exudates in the rat 0 to 8 days after an injection with paraffin oil.

similar; however, in an acute inflammatory state more PA-negative cells are present.

During chronic inflammation provoked with paraffin oil, 4 types of macrophages were found (Table 1), viz., exudate macrophages with PA only in some lysosomal vesicles, exudate-resident macrophages with PA in both the lysosomal vesicles and in the RER and the nuclear envelope (Fig. 1A), resident macrophages with PA only in the RER and nuclear envelope and PA-negative macrophages which did not show any PA reaction product (Fig. 1B). The total number of both exudate macrophages and PA-negative cells rose sharply to approximately 1.2×10^7 , respectively, at 2 days (Fig. 2) and remained at that level. The total number of resident macrophages fell sharply from the onset of the inflammation, and their number remained very low throughout (Fig. 2). The total number of exudate-resident cells which first appeared during the inflammation also remained at a constant low level during chronic inflammation (Fig. 2).

Cytochemistry of Peritoneal Macrophages and Blood Monocytes *In Vitro*. After culture for 0 to 8 days, both rat blood monocytes and rat PM from an acute 24-hr exudate elicited by NBCS (2) displayed the same 4 different PA patterns *in vitro* as those found *in vivo* during chronic inflammation: macrophages with the cytochemical characteristics of blood monocytes and exudate macrophages showed PA in the lysosomes (Fig. 3A); macrophages with the cytochemical characteristics of

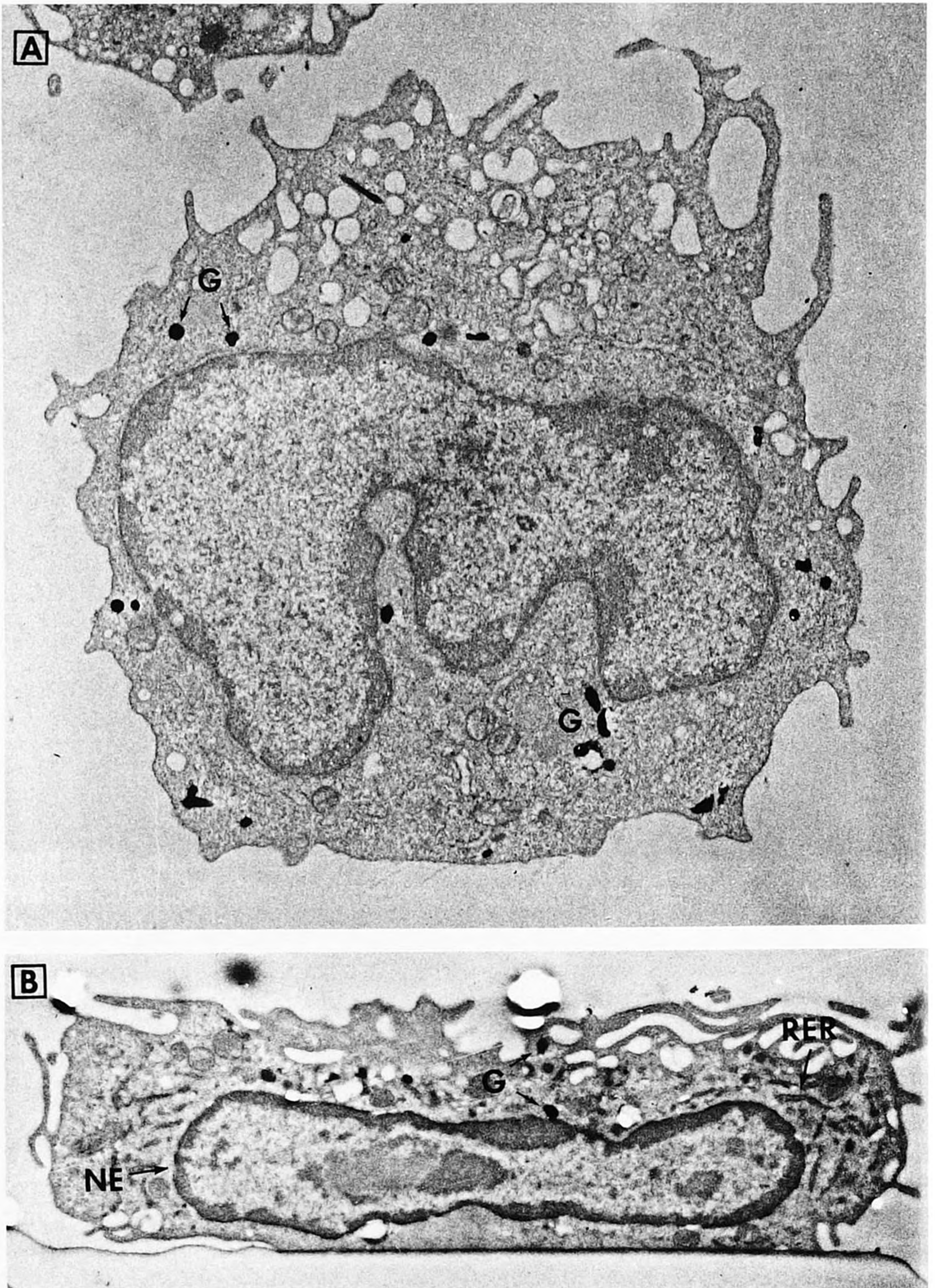


Fig. 3. Two patterns of PA observed *in vitro* after culturing 24 hr NBCS-induced rat PM. (A) Exudate macrophage at the start of culture (0 days). This cell shows PA only in the lysosomes (G). X 12,500. (B) Macrophage after 2 days of culture. This cell has the characteristics of an exudate-resident macrophage, i.e., it shows PA in the lysosomes (G) and in the nuclear envelope (NE) and RER (RER). X 8,000.

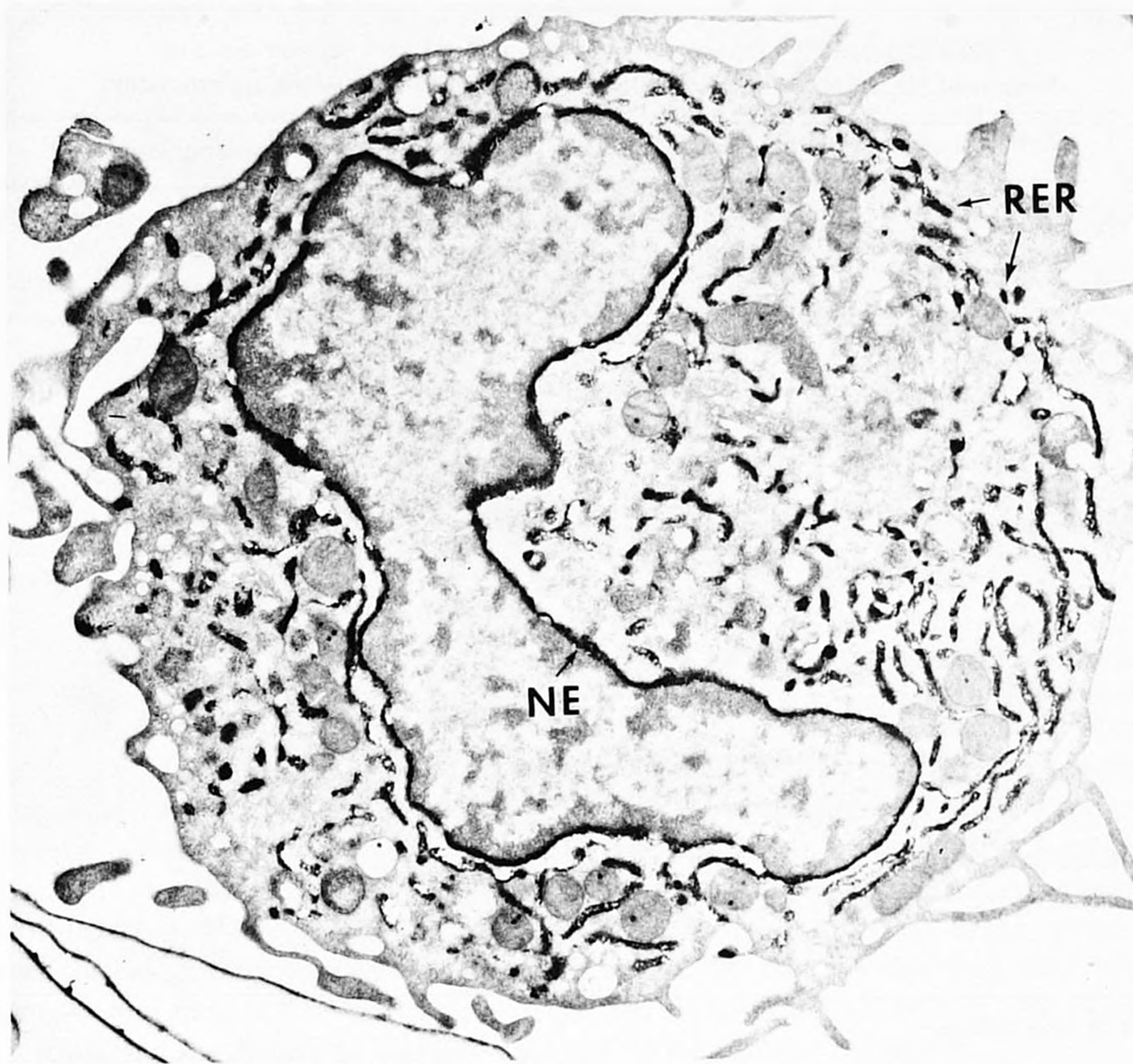


Fig. 4. The predominant PA pattern observed *in vitro* in rat blood monocytes after 8 days of culture. This cell has the cytochemical characteristics of a resident macrophage i.e., it shows PA in the nuclear envelope (NE) and RER (RER).

exudate-resident macrophages showed PA in the lysosomes and in the RER (Fig. 3B); macrophages with the cytochemical characteristics of resident macrophages showed PA only in the RER (Fig. 4); PA-negative macrophages did not show PA.

From Table 2 it can be seen that the relative number of exudate macrophages declined during incubation *in vitro*, while the relative number of resident macrophages rose, and that exudate-resident macrophages, which first appeared after *in vitro* incubation, were present in large numbers at days 2 and 4. At 8 days there was still a large percentage of cells with PA in the RER, a characteristic of resident macrophages. In cultured cells from an unstimulated peritoneal cavity the percentage of resident macrophages started high and remained so for at least 8 days (Table 2).

The percentages from the cultures in plastic dishes refer to the adherent cells. In fact, during the course of the experiments some cells became detached from the plastic so that the absolute number of cells counted fell slightly. These detached cells (~10%) were also investigated, however, and showed the same PA pattern as the adherent cells. In the Teflon culture dishes, in which surface adherence was

TABLE 2
Four Different PA Patterns in Cultures of Rat Blood Monocytes and
Peritoneal Macrophages in Normal Steady State and After Acute Inflammation

Cell type and condition	Days of culture	Localization of peroxidatic activity				
		Lysosomes only (%)	Lysosomes + RER (%)	RER only (%)	PA-negative (%)	
Blood monocytes	0	90	—	—	10	
	2	40	20	20	20	
	4	20	40	20	20	
	8	< 5	< 5	60	30	
Peritoneal macrophages in normal steady state	0	—	—	95	5	
	2	—	—	> 90		
	4	—	—	> 90		
	8	—	—	> 60		
Peritoneal macrophages in acute inflammation ^a	cultured on plastic	0	90	—	5	5
		2	40	20	20	20
		4	20	40	20	20
		8	< 5	< 5	60	30
	cultured on teflon	0	90	—	5	5
		2	40	20	20	20
		4	20	40	20	20
		8	< 5	< 5	60	30

^a24 hr after NBCS.

prevented, the cells developed the same PA patterns as those of the adherent and nonadherent cells in the plastic dishes (Table 2).

Cultures of rat and mouse PM gave fairly similar results. However, the appearance of cells with PA in the RER (a characteristic of resident macrophages) occurred earlier in the mouse. On the other hand, the percentage of PA-negative cells present 24 hr after induction of an acute inflammatory state in the mouse (about 30%) fell to approximately 5% during culture. Because the cell loss during the first 4 days of culture was less than 10%, a part of the PA-negative macrophages may acquire *in vitro* the cytochemical characteristics of resident macrophages.

DISCUSSION

In previous studies exudate-resident macrophages have been demonstrated *in vivo* in the rat after acute inflammation provoked by NBCS (2) and *in vitro* in human cell cultures (2). In the present study we have demonstrated the presence of such cells also after chronic inflammation provoked with paraffin oil in the rat (Table 3). Furthermore, in the present study it was shown that blood monocytes and exudate macrophages may develop the characteristic PA pattern of exudate-resident mac-

TABLE 3

Four Different PA Patterns in Cultures of Mouse Peritoneal Macrophages
in Normal Steady State and Acute Inflammation

Condition of peritoneal macrophages	Days of culture	Localization of peroxidatic activity			
		Lysosomes only (%)	Lysosomes + RER (%)	RER only (%)	PA-negative (%)
Normal steady state	0	—	—	98	2
	2	—	—	> 98	
	4	—	—	> 98	
Acute inflammation ^a	0	65	—	5	30
	2	—	20	75	5
	4	—	5	90	5

^a24 hr after NBCS.

rophages *in vitro* (Tables 2 and 3). Thus these results further confirm the existence of exudate-resident macrophages as a commonly occurring cell in inflammatory processes. Both the PA pattern and the kinetics of the appearance and disappearance of this cell type strongly suggest that it is a transitional form, intermediate to the exudate and the resident forms. Bodel *et al.* (5) could not demonstrate an exudate-resident form in the rabbit. This may be due to the fact that rabbit mononuclear phagocytes never have PA in their lysosomes; therefore, real exudate macrophages according to Daems' (10) definition cannot be found.

It has been suggested that the appearance of PA in the RER *in vitro* is due to surface adherence (5, 6). In this study, however, it was clearly shown that non-adherent cells, cultured on a Teflon surface, also develop PA in the RER *in vitro*. It is remarkable, however, that this PA in the RER develops so quickly *in vitro*. This is evident not only from the present studies with rat and mouse macrophages but also from previous studies in man (3) and rabbit (5). The possibility that this is due to the absence of inflammatory factors in the *in vitro* situation remains to be studied.

A remarkably high number of PA-negative cells was seen in the mouse after acute inflammation and in the rat after chronic inflammation. This was previously shown in man after chronic inflammation (3) and in the guinea pig after both acute and chronic inflammation (unpublished). The occurrence of this cell type *in vivo* in the present study (Table 1) and our previous study (3) suggests that exudate macrophages during inflammation also may develop into PA-negative cells. On the other hand, the *in vitro* kinetics of this cell type (Tables 2 and 3) indicate that a part of the PA-negative cells still may develop PA in the nuclear envelope and RER and, afterwards, may become PA-negative again as suggested in our earlier studies (3, 18). Therefore, PA-negative cells may comprise different developmental stages of macrophages and may be another transition between exudate and resident macrophages as well as an end-stage macrophage *in vitro*.

In conclusion, the results show that the divergent PA patterns *in vivo* and *in vitro* in PM and blood monocytes as well as in other mononuclear phagocytes (1, 4, 18) in

various species represent differences in stages of development or stages of activation of these cells.

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