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Non-immunological recognition and killing of xenogeneic cells by macrophages

II. MECHANISM OF KILLING

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Summary. Macrophages are cytotoxic to chicken embryonic fibroblasts without either previous activation or lymphocyte assistance. This cytotoxic activity (xenolysis) is expressed by non-activated macrophages from athymic mice as well as by pure macrophage populations. Neither macrophage lysate nor supernatants of macrophages cultivated with fibroblasts cause xenolysis. Unlike macrophage tumoricidal activity, killing of xenogeneic cells is not dependent on specific serum factors and is expressed by macrophages from a lipopolysaccharide (LPS) unresponsive strain (C3H/HeJ). Xenolysis is expressed also by trypsin-treated macrophages and by macrophages from 5-day-old cultures. Killing of chicken fibroblasts by macrophages is not affected by hydrocortisone (100 μg/ml) gold salt (1 mg/ml) and colchicine (100 μg/ml). On the other hand, cytochalasin B (10 μg/ml) completely abolishes the killing, probably by interfering with macrophage mobility and extension of filopodia toward the targets.

It is suggested that the xenolytic activity of macrophages represents a primitive trait of phagocytes which assists the body in defence against multicellular parasites.

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INTRODUCTION

It was demonstrated that *in vitro* activation and modulation of macrophage tumoricidal activity were triggered by administration of lymphokines (David, 1975; Ruco & Meltzer, 1977) lipopolysaccharide (LPS; Weinberg, Chapman & Hibbs, 1977) and foetal bovine serum factor(s) (Hibbs, Taintor, Chapman & Weinberg, 1977). In previous studies we have demonstrated that non-immune non-activated murine macrophages aggressively killed phylogenetically-distant avian target fibroblasts (xenolysis). In the present study we investigated the possible role of lymphocytes and their products in assisting macrophage-mediated xenolysis by determining the xenolytic activity of macrophages from athymic mice, pure populations of macrophages, and macrophages from long-term cultures. To compare macrophage tumoricidal to their xenolytic activity we studied the ability of serum from various sources to modulate the killing. Additionally, we examined the association between the LPS responsiveness gene which controls tumoricidal activity of C3H/HeJ macrophages (Ruco, Meltzer & Rosenstreich, 1978) and the xenolytic potential of macrophages. Various agents, known to affect lysosomal enzyme activity, were added to macrophage—fibroblast cultures to elucidate the role of lysosomal enzymes in xenolysis. The mode of interaction between macrophages and fibroblasts was studied further by scanning electron microscopy.

MATERIALS AND METHODS

Mice

Inbred female mice, 2–4 months old, of the following strains were used in this study: C57BL/6, C3H/CRGL (animal breeding farm, Hadassah Medical School, Jerusalem), C3H/HeJ, C3H/eb and C57BL/6 (*nu/nu*) from the breeding centre of the Weizmann Institute of Science, Rehovot, Israel.

Growth medium

As described in the accompanying paper (Cabilly & Gallily, 1981b).

Macrophages

Peritoneal macrophages were harvested from C3H and C57BL/6 mice 2 or 4 days, respectively, after intraperitoneal (i.p.) injection of 2 ml thioglycollate medium (TG, Difco). Peritoneal macrophages from nude mice were harvested without previous elicitation. Macrophage lysates were prepared by freezing and thawing five times.

Pure macrophage cell population

Ten million C57BL/6 TG-elicited peritoneal exudate cells (PEC) were cultured for 1 hr onto 97 mm Petri dishes (Nunc, Denmark) in serum-free Dulbecco's modified Eagles medium (DMEM). The cultures were rinsed by a jet of phosphate-buffered saline (PBS) and treated with a solution of versene-trypsin (0·02% and 0·25%, respectively) for 24 hr in a serum-free DMEM. The cultures were then rinsed well and the cells removed from the plates. By criteria of *Staphylococcus albus* phagocytosis and morphology, >99% of the cells were macrophages.

Removal of macrophages from plates

Macrophages that were cultured on plastic petri dishes (Nunc) for various periods of time were incubated in a solution of PBS containing 1% FBS and 10^{-4}M ethanol diamine acetic acid (EDTA) for 2 hr at 4°. Those cells which did not detach spontaneously were removed by gentle pipetting. The viability of cells obtained by this procedure is above 80%.

Fibroblasts

Chicken embryonic fibroblasts were prepared as previously described (Cabilly & Gallily, 1977) from chicken embryos (White Leghorn) obtained from a local flock.

Mixed macrophage-fibroblasts cultures

Preparation of the mixed cultures, and assessment of target cell lysis were described previously (Cabilly & Gallily, 1977) and in the accompanying paper (Cabilly & Gallily, 1981b).

Determination of relative adherence of ^{51}Cr labelled macrophages to fibroblasts was described in the accompanying paper (Cabilly & Gallily, 1981b).

Sera and reagents

The following sera were used in this study: rat (RS), guinea-pig (GPS), rabbit (RabS) and goat (GS) obtained by bleeding of the respective animals (the animal breeding farm, Hebrew University, Hadassah Medical School, Jerusalem). Horse serum (HS) was obtained from Biolab. Foetal bovine serum (FBS) and newborn bovine serum (NBBS) from Gibco. Human serum (HuS) was obtained from four normal donors. The reagents used in this study were: sodium aurothiomalate (Myocrisin, May & Baker Ltd), hydrocortisone succinate (N.V. Organon Oss, Holland), colchicine and cytochalasin-B (Sigma, stock solution ten times dissolved in dimethylsulphoxide 0·5% v/v).

Scanning electron microscopy

Preparation of specimens for scanning electron microscopy (SEM) was described in the preceding paper (Cabilly & Gallily, 1981b).

Experimental design

In each experiment, TG-elicited macrophages were pooled from three to five donor mice. Determination of macrophage activity was performed in triplicates. Each experiment was performed at least three times. Differences between experimental and control group values in individual experiments were analysed for significance by the one tail distribution-free Mann–Whitney U Test. Differences were considered significant where P was 0·05 or less.

RESULTS

Xenolysis by macrophages depleted of lymphocytes

To exclude the possible assistance of lymphocytes residing in macrophage adherent-cell populations during xenolysis, the following experiments were undertaken. Macrophages from C57BL/6 athymic mice were cultured with chicken fibroblasts for 24 hr. As seen in Table 1 experiment 2, these macrophages killed the target fibroblasts as efficiently as macro-

phages from normal C57BL/6 mice. In another experiment, cultures of macrophages were pretreated with a solution of versene-trypsin (0·02% and 0·25%, respectively for 40 min at 37°) and further incubated for 24 hr in the absence of serum. Following this procedure, the cultures consisted of more than 99% macrophages, as determined by morphological and phagocytic criteria. As seen in Table 1 experiment 3, killing of chicken fibroblasts by these cells was as efficient as untreated cells. Moreover, when non-adherent peritoneal cells, mostly lymphocytes, were added to chicken fibroblasts, they did not affect fibroblast viability even at an E:T ratio of 4:1 (experiment 1). These experiments indicate that xenolysis is not dependent on T or other lymphoid cells.

Xenolysis by *in vitro* cultivated macrophages

As seen in Table 2, macrophages retain their xenolytic potential after cultivation for 5 days in a serum-free medium, although a slight non-significant decrease in this activation was observed ($P > 0{\cdot}05$ tested by

Table 1. Xenolytic activity of C57BL/6 macrophages depleted of lymphocytes

Exp. no.	Chicken fibroblasts* incubated with	Fibroblast lysis† Residual label (c.p.m. ± SD)	(%)
1	—	5744 ± 136	
	PEC macrophages	2337 ± 587	59
	Peritoneal lymphocytes‡	5740 ± 882	0
2	—	1931 ± 343	
	Nude macrophages	668 ± 218	65
3	—	8966 ± 966	
	Macrophages deprived of lymphocytes§	3597 ± 1089	60

* Chicken fibroblasts/dish (3×10^5), macrophage fibroblast ratio (E:T) 10:1.
† After 24 hr.
‡ Lymphocytes ($1{\cdot}2 \times 10^6$).
§ See Materials and Methods.

Table 2. Xenolysis by long-term cultivated macrophages

Cells in culture*	Macrophages in culture (days)	Fibroblast lysis† Residual label (c.p.m. ± SD)	(%)
Fibroblasts	—	960 ± 45	
Fibroblasts + Macrophages	0	379 ± 54	61
Fibroblasts + Macrophages	1‡	548 ± 94	43§
Fibroblasts + Macrophages	3‡	541 ± 136	44§
Fibroblasts + Macrophages	5‡	502 ± 68	48§

*† See footnotes to Table 1.
‡ More than 85% of the macrophages were alive as determined by erythrosin B dye exclusion.
§ Insignificantly different from freshly obtained macrophages ($P > 0{\cdot}05$).

Mann–Whitney Test). This fact distinguishes the xenolytic potential of normal macrophages from the tumoricidal potential of activated macrophages which wanes by 24 hr of culturing (Chaffar & Cullen, 1976; Marino & Adams, 1979).

The effect of various sera on macrophage-mediated xenolysis

As seen in Fig. 1, C57BL/6 macrophages efficiently killed chicken fibroblasts in the presence of each of the following sera: FBS, NBBS, RS, GPS, RabS, GS, HS and HuS.

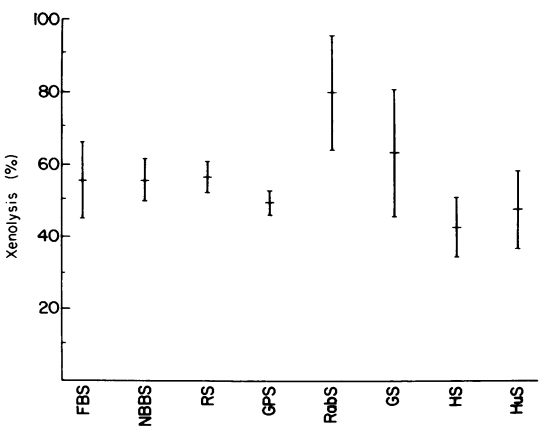

Figure 1. Destruction of chicken fibroblasts by murine macrophages in the presence of various types of serum (10%), lysis was assessed after 24 hr of incubation.

Xenolysis by macrophages derived from LPS non-responsive C3H/HeJ mice

As seen in Table 3, killing of chicken fibroblasts by macrophages from C3H/HeJ, a strain of mice lacking LPS responses, was similar to that demonstrated by macrophages of C3H/eb (an LPS responsive strain). Thus, the LPS responsiveness gene does not regulate macrophage xenolytic activity of C3H/HeJ macrophages, contrary to its effect on macrophage tumoricidal potential of this strain. However, macrophages from both strains of C3H tested demonstrated lower xenolytic activity when compared with that of C57BL/6 macrophages (43% and 44% compared with 73% lysis, see Table 3). This difference is correlated with the lower binding capacity of C3H macrophages to target fibroblasts compared to C57BL/6 macrophages (see column 3 Table 3 and Cabilly & Gallily, 1981b).

Role of macrophage lysosomal enzymes and cytoskeletal constituents in xenolysis

To determine whether macrophage lysosomal hydrolases take part in the killing event, agents affecting the activity or secretion of these enzymes were administrated before or during macrophage interaction with avian fibroblasts. As seen in Table 4, treatment of macrophages with up to 1 mg/ml gold salts, 2 hr before their interaction with the target cells did not affect macrophage xenolytic response. Similarly, macro-

Table 3. Lysis of chicken fibroblasts by C3H/HeJ macrophages

Strain of mice	Cells in culture*	Adherence of macrophages to fibroblasts† (%)	Fibroblast lysis‡ Residual label (c.p.m. ± SD)	(%)
	Fibroblasts		3418 ± 438	
C57BL/6	Fibroblasts + Macrophages	80	916 ± 177	73
C3H/eb	Fibroblasts + Macrophages	60	1941 ± 14	43§
C3H/HeJ	Fibroblasts + Macrophages	51	1931 ± 44	44§

* See footnote to Table 1.

† Expressed as percentage of ^{51}Cr labelled macrophages adhering to empty dish.

‡ After 24 hr.

§ Significantly different from C57BL/6 macrophages ($P < 0.05$).

Table 4. Effect of various chemicals on macrophage xenolytic activity

Agent	Treatment*	Concentration (μg/ml)	Relative fibroblast lysis† 24 hr	48 hr
None	—	—	100	100
Gold salt (sodium aurothiomalate)	Pre	100	78	ND§
Gold salt (sodium aurothiomalate)	Pre	1000	76	84
Hydrocortisone succinate	In	1	87	ND
Hydrocortisone succinate	In	10	86	92
Hydrocortisone succinate	In	100	92	96
Colchicine	In	100‡	92	127
Cytochalasin B	In	1	0	60
Cytochalasin B	In	10	0	0

* Pre, pretreatment of Mø for 2 hr at 37°: In, present during Mø fibroblast co-cultivation.

† Relative to lysis of chicken by untreated C57BL/6 Mø (E:T—10:1)

‡ $2{\cdot}5 \times 10^{-4}$M.

§ ND, not done.

phage-mediated lysis was not affected following administration of up to 100 μg/ml hydrocortisone succinate.

To find out whether agents affecting cell cytoskeleton constituents impair macrophage xenolytic potential, colchicine and cytochalasin B were added to macrophage–fibroblast cultures. It was observed (Table 4) that while colchicine ($2{\cdot}5 \times 10^{-4}$M) did not affect macrophage xenolytic response, cytochalasin B (10 μg/ml) totally inhibited macrophage-mediated lysis. In addition, administration of cytochalasin B (but none of the other tested agents) to the mixed cultures prevented the formation of typical clusters of macrophages around fibroblasts (observed by phase contrast microscopy).

Scanning electron microscopy

The first stage in the interaction of C57BL/6 macrophages with chicken fibroblasts was characterized by the extension of macrophages of undirectional filopodia toward the target fibroblasts (Fig. 3a, see also in Cabilly & Gallily 1981b Fig. 2b). Once contact between macrophage filopodia and target cells was firmly established they were not separable by treatment with cytochalasin B (10 μg/ml). Rather cytochalasin B caused breaks in the macrophage filopodia (Fig. 2). In the second stage of interaction, the area of membranal contact between the macrophage extensions and the target fibroblasts increased (Fig. 3b). After 24–48 hr of co-cultivation, macrophages were seen attached to sporadic fibroblast residues. (Fig. 3c).

DISCUSSION

To evaluate the role of lymphocytes or their mediators in assisting macrophage-mediated xenolysis, the xenolytic activity of non-activated nude (*nu/nu*) C57BL/6 macrophages was examined. It was observed that macrophages from athymic mice kill avian fibroblasts as efficiently as normal C57BL/6 macrophages. Thus, the presence of T lymphocytes is not required for macrophage xenolytic response. Furthermore, while pure populations of macrophages did not kill tumour cells without previous activation with MAF or LPS, pure macrophages efficiently killed avian target cells without any previous activation. Of course, one cannot rule out the possible effect of LPS traces which might contaminate the FBS added to the medium. The pure populations of macrophages, obtained by trypsin treatment of PEC followed by 24-hr incubation in

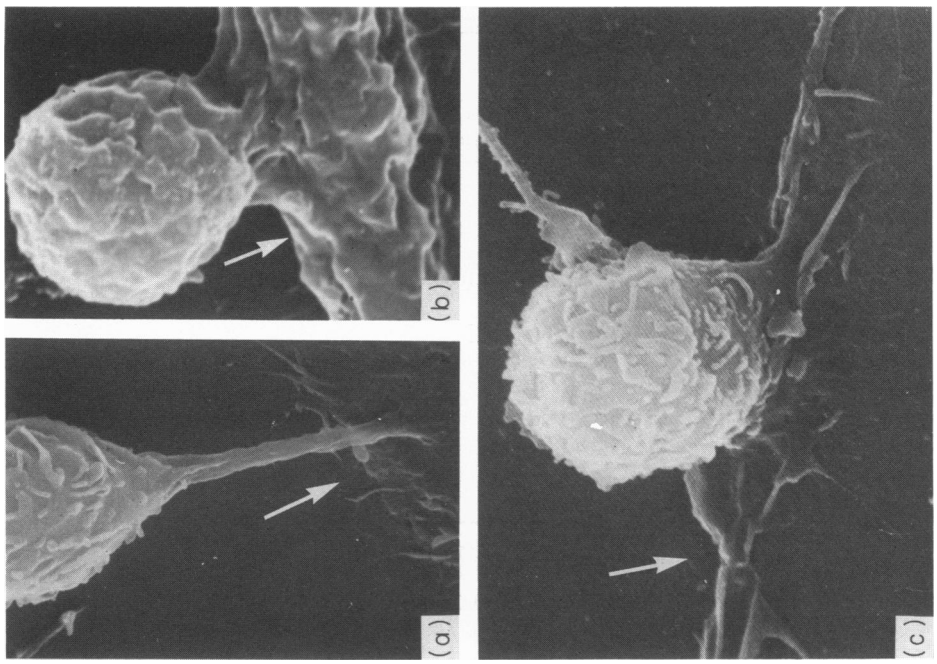

Figure 3. Interaction of murine macrophages with chicken fibroblasts leading to target cell destruction. (a) Interaction after 2–3 hr (magnification × 6650). (b) Interaction after 10–12 hr (magnification × 6200), note extensive membranal contact between the cells. (c) Fibroblasts lysis at 24 hr (magnification × 6300). Fibroblasts are indicated by arrow.

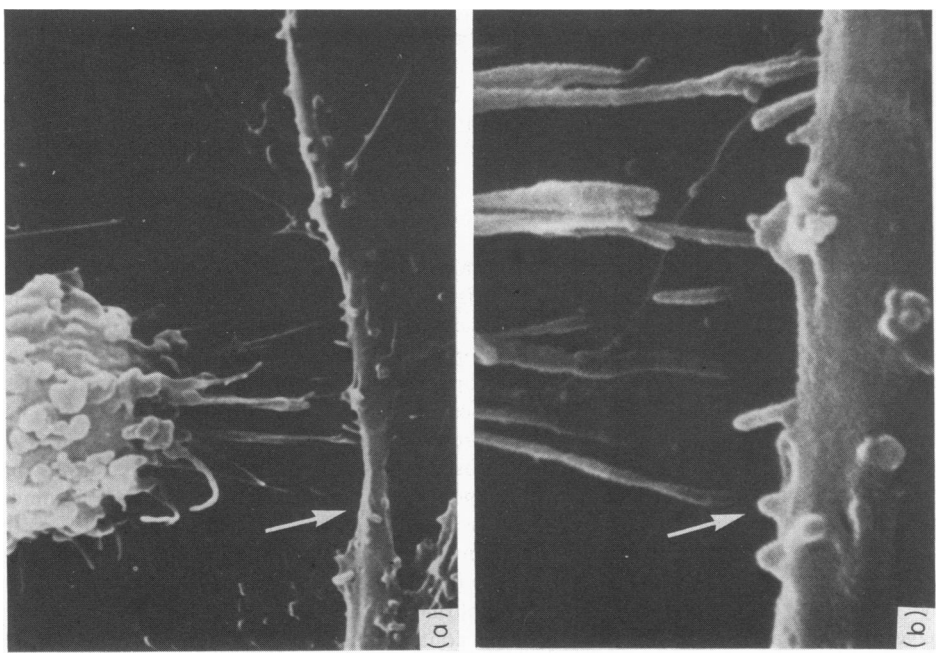

Figure 2. Breaks of macrophages filopodia attached to target fibroblasts by cytochalasin B (10 μg/ml), (a) magnification × 6600, (b) magnification × 25,600. Fibroblast is indicated by an arrow.

medium devoid of sera, killed avian fibroblasts as efficiently as untreated PEC-adherent cells which contain 2%–3% lymphocytes. This experiment also demonstrates that after trypsin treatment macrophages did not lose their xenolytic activity whereas similar treatment prevents the tumoricidal response of activated macrophages. Similarly, long-term cultivation of macrophages in serum-free medium for 1–5 days before their interaction with target cells did not affect their xenolytic response whereas their tumoricidal potential decayed after *in vitro* cultivation for 24–48 hr (Ghaffar & Cullen, 1976; Marino & Adams, 1979). These findings clearly demonstrate that macrophages exert their xenolytic activity independently of lymphoid cells, and this activity differs from the tumoricidal response of activated macrophages.

We also aimed at clarifying whether macrophage xenolytic activity is affected by any of the factors shown to abolish macrophage tumoricidal potential. To this end, macrophages were cultured in the presence of eight different sera (FBS, NBBS, RS, GPS, RabS, GS, HS and HuS) which included sera known to inhibit the tumoricidal potential of activated macrophages (Hibbs *et al.*, 1977). It was observed (Fig. 1) that macrophage xenolytic activity was unaffected by any of the sera added, including human and rat sera which contain LPS-inhibiting factors (Oroszlan, McFarland, Mora & Shear, 1966; Johnson, Ward, Goralnick & Osborn, 1977). Recent studies demonstrated that macrophages of C3H/HeJ, LPS unresponsive mouse strain, were not tumoricidal *in vitro*. The development of activated tumoricidal macrophages was shown to be controlled in C3H/HeJ strain by genes clearly linked, or identical, to endotoxin response gene (Ruco *et al.*, 1978). In our experiments the C3H/HeJ macrophages killed avian fibroblasts as efficiently as macrophages from C3H/eb—an LPS responsive strain. Thus C3H/HeJ macrophages devoid of tumoricidal response, possess xenolytic activity. All these findings support the notion that xenolytic activity of macrophages differs from their tumoricidal response.

The mechanism by which macrophages kill target cells is still unknown. Although several cytotoxic mechanisms were suggested as being operative in the tumoricidal response of activated macrophages, Hibbs (1974), and Bucana, Hoyer, Hobbs, Breesman, McDaniel & Hanna (1976) suggested that killing of tumour cells was accomplished by transfer of lysosomes from effector to target cells, whereas McIvor believed that actual death of target cells may be due to soluble specific macrophage cytotoxin (SMC) released by macrophages following contact with target cells (McIvor & Weiser, 1971). Nathan, Brukner, Silverstein & Cohn (1979) suggested that H_2O_2 released from pharmacologically-triggered macrophages participated in the extracellular cytolysis of target cells. In our assay system, no cytotoxicity was expressed by supernatant of macrophages cultivated with avian fibroblasts for 24 hr (see Table 5). Xenolysis was not inhibited by agents known to prevent activation and exocytosis of macrophage lysosomes. Hydrocortisone, a membrane stabilizer which inhibits lysosomal enzymes release (Weissmann & Dingle, 1961) and their transfer to tumour target cells did not affect the xenolytic activity of macrophages, whereas it has been shown to inhibit the tumoricidal response of activated macrophages (Hibbs, 1974). Gold salt, an inhibitor of lysosomal enzyme activity (Persellin & Ziff, 1966) has the capacity to abolish tumoricidal potentials of *Corynebacterium parvum* activated macrophages (Ghaffar, McBridge & Cullen, 1976). Nevertheless, it did not at all affect macrophage xenolytic potential. Xenolysis, in contrast with tumoricidal responses of activated macrophages was not inhibited by colchicine which interferes with microtubule polymerization (Sharma & Piessens, 1978). However, macrophage xenolytic potential was totally abolished by cytochalasin B. Cytochalasin B among its many other effects prevents polymerization of actin into microfilaments (Grumet, Flanagan, Lin & Lin, 1979), thus also interfering with cell locomotion. It is suggested that inhibition of macrophage xenolytic activity by this agent is due mainly to inhibition of both macrophage locomotion and extension of cytoplasmic filopodia

Table 5. Murine macrophage lysates and supernatants lack xenolytic activity

Treatment of chicken fibroblasts*	Fibroblast lysis (%)	
	24 hr	48 hr
None	0	0
Lysate from 3×10^6 Mø	11	13
Lysate from 6×10^6 Mø	13	40
Supernatant from Mø + fibroblasts†	0	ND
Supernatant from Mø†	0	ND

* Fibroblasts/dish (3×10^5).
† After 24-hr cultivation; 3×10^6 macrophages.

towards the target cells. Our morphological studies by SEM demonstrated that 2–3 hr after initial interaction of macrophages with the targets, macrophage filopodia were extended unidirectionally towards avian fibroblasts, establishing firm contact with the target cell membranes. This contact could not be detached by either trypsin or cytochalasin B. Moreover, the treatment of the mixed cultures with cytochalasin B, after the contact had been established, caused breakages along macrophage filopodia rather than at the point of contact with the targets (see Fig. 2). It seems that following filopodial contact, macrophages are pulled toward the fibroblasts forming aggregates of macrophages around and above the targets. When macrophages were cultivated with syngeneic fibroblasts only a few aggregates of macrophages around some target cells occurred. Our SEM studies also demonstrated that most of these macrophages (about 85%) did not send unidirectional pseudopods towards the targets (Fig. 3 in Cabilly & Gallily, 1981b). One might assume that the few aggregates of macrophages around syngeneic target cells demonstrate recognition of cells damaged or altered during *in vitro* cultivation. Thus, contact between macrophage and target cells, which presumably demonstrates a recognition stage, is a prerequisite for cytolysis. However, close contact between macrophages and targets is not sufficient for cytolysis, as our recent study demonstrated (Cabilly & Gallily, 1981a). When macrophages were bound by concanavalin A (Con A) or AMS to syngeneic fibroblasts, cytostasis but not cytolysis occurred. Thus a foreign recognition signal is required to induce cytolysis.

Our studies demonstrate an innate capacity of macrophages to recognize and kill a large panel of xenogeneic normal target cells (Cabilly & Gallily, 1981b) without any apparent assistance of lymphocytes or their products. This finding supports the notion emphasized by Mackaness (1976) that mononuclear phagocytes, in the absence of any immunological mechanism, might serve as effectors of a native form of immunity—an 'innate resistance'. We suggest that the xenolytic activity points toward a primitive phylogenetic origin of the macrophages. Indeed, wandering phagocytic cells analogous to mammalian macrophages are found throughout the invertebrates. These cells have an intrinsic ability to discriminate between indigenous and foreign materials, be they particulate or cellular in nature. The invertebrate phagocytes are also involved in destruction of allografts or xenografts in molluska (Hildemann, Dix & Collins, 1974), annelids (Velembois, 1974), arthropods (Jenkin & Hardy, 1975), and echinoderms (Hildemann, 1972). We assume that this primitive property of recognizing cells as foreign still resides in mammalian macrophages, although it is superimposed by the immunological amplifying mechanism(s). The invertebrate phagocytes are responsible for various homeostatic functions which might be adapted under the influence of selective evolutionary pressures, to various effector activities in mammalian macrophages. The macrophage non-immunogenic reactivity against xenogeneic cells might represent their ability to recognize and attack various unicellular and multicellular parasites. This capability probably plays a part in the defense of the mammalian organism.

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