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Ultrastructure of tumor cell interaction with alveolar macrophages stimulated by vitamin A

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Summary. F344 rats were given vitamin A for four consecutive days and then their alveolar macrophages (AMϕ) were obtained by bronchopulmonary lavage of the lung. Compared with unstimulated AMϕ, AMϕ from rats given vitamin A had more numerous and longer cytoplasmic projections, and these projections had many knobs on their sides and tip.

The AMϕ became attached to syngeneic mammary adenocarcinoma cells at many focal points and the tumor cells then lost surface microvilli around the contact zones. Detachment of the knobs from the projections on AMϕ was often observed in areas of close association between AMϕ and tumor cells. The detached knobs were 250 nm in diameter, gave a positive reaction for acid phosphatase, and frequently became attached to the surface of tumor cells. Then, many of the tumor cells in the vicinity of AMϕ exhibited cytolytic changes.

It is concluded that the cytotoxicity of stimulated AMϕ is due to their attachment to the surface of tumor cells and their release of particles with acid phosphatase activity into the narrow space between the cells, and then to uptake of these particles by susceptible tumor cells.

Key words: Alveolar macrophage – Cytoplasmic projection – Knobs

Introduction

Many immunologic parameters of macrophage-mediated cytotoxicity have been investigated extensively, but little attention has been focused on morphologic aspects of tumor cell lysis. Previously we reported that vitamin A at high levels can increase the immune response and tumoricidal activities of alveolar macrophages (AMϕ) in syngeneic rats (Tachibana et al. 1984).

In this work, we examined the process of cytolysis of tumor cells by AMϕ from vitamin A-treated rats by electron microscopy and a cytochemi-

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cal technique to obtain morphological evidence of the enhanced cytotoxicity of the AMϕ. In particular, we examined the properties of the surface of AMϕ and of their initial interaction with syngeneic mammary adenocarcinoma cells, which ultimately results in tumor destruction.

These observations may be useful in designing an effective method for prevention of lung cancer by interaction of cancer cells with AMϕ stimulated with vitamin A.

Material and methods

Animals and preparation of AMϕ

Male F344 rats of 7 weeks old (Shizuoka Animal Facility Center, Shizuoka, Japan) were given commercial stock diet (Oriental Yeast Co., Chiba, Japan). Experimental rats were given 500 IU/g body weight of retinyl palmitate (Wako Pure Chemical Co., Tokyo, Japan) suspended in soya bean oil by stomach tube once a day for 4 days. After this stimulation with vitamin A, the AMϕ destroyed 34% of the target tumor cells in tests *in vitro*, as reported previously (Tachibana et al. 1984). Control animals were given the same volume of soya bean oil for 4 days. AMϕ were obtained by broncho-pulmonary lavage of the animals. AMϕ collected by centrifugation of the lavage fluid were cultivated in multiwell plates (Falcon Plastics, Oxnard, CA, USA) containing 1 ml of RPMI-1640 medium and 5% fetal bovine serum. After 60 min, nonadhering cells were removed by washing the plates with the same medium.

Tumor cell line and its interaction with AMϕ

Cells of a syngeneic mammary adenocarcinoma (MADB-100) were used as target cells, as described in detail previously (Tachibana et al. 1984). AMϕ that had been cultivated for 60 min were incubated with 10^4 MADB-100 cells for 24 h or 48 h at 37° C.

Scanning electron microscopy (SEM)

Plates with attached cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h at 5° C and washed with the same buffer. They were then postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h and dehydrated in an ethanol series. Ethanol was removed from the samples with amylacetate, and then the samples were dried in a critical point apparatus (Hitachi HCP-2, Tokyo, Japan) under CO_2 and coated with gold. The specimens were examined in a Hitachi S-800 field emission scanning electron microscope at 25 kv.

Transmission electron microscopy (TEM)

Samples of cell monolayers were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), washed with the same buffer, and postfixed with 1% osmium tetroxide for 1 h. For cytochemical demonstration of acid phosphatase activity, preparations were fixed with 2.5% glutaraldehyde in buffer containing 8% sucrose for 15 min at 4° C, washed overnight in the buffer, and incubated for 20 min at room temperature in reaction mixture containing cytidine-5′-monophosphate (Sigma Chemical Co., St. Louis, Mo, USA) and lead nitrate (Novikoff et al. 1963). Samples were postfixed in 1% osmium tetroxide for 1 h. Control preparations were incubated in reaction medium without substrate. All samples were dehydrated by passage through a graded series of ethanol and then propylene oxide, and embedded in Epon 812. Thin sections were cut on an LKB microtome (LKB Instruments, Rockville, MD, USA), stained with uranyl acetate and lead citrate, and examined in a Hitachi HU-12 electron microscope (Hitachi Ltd, Tokyo, Japan).

Results

On SEM, most of unstimulated AMϕ appeared spherical with surface microvilli and occasionally ruffled membranes. AMϕ stimulated with vitamin A had more cytoplasmic projections than unstimulated AMϕ. Most cells had long, slend cytoplasmic projections, which often terminated in knobs. These knobs, ranging in size from 200 to 350 nm, were observed along the side of cytoplasmic projections as well as at their tip, giving the projections a rosary-like appearance, which was rarely seen in unstimulated AMϕ (Fig. 1).

On TEM, the cytoplasm of these AMϕ was seen to contain many phagocytic vacuoles and phagolysosomes of various sizes and densities. The many vacuoles in stimulated AMϕ gave a positive reaction for acid phosphatase (Fig. 2). The lead deposits indicating this positive reaction were frequently observed in the periphery of the vacuoles and also in lysosomes.

Interaction of AMϕ with tumor cells

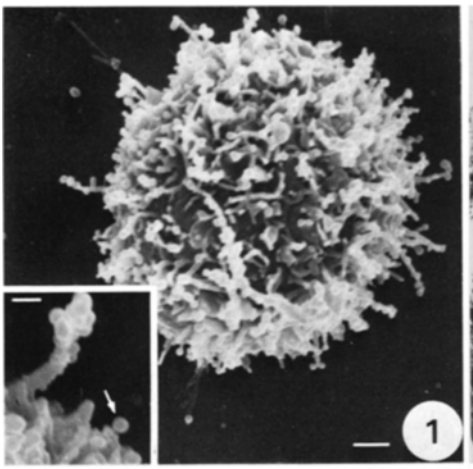
After incubation of AMϕ with tumor cells for 24 h, the AMϕ exhibited prominent cytoplasmic projections toward the tumor cells, as shown in figure 3A. The sites of contact of AMϕ with tumor cells were invariably at the edge of these projections (Fig. 3B), but no fusion of the two types of cells could be seen (Fig. 3C). Spherical knobs, generally of 250 nm diameter, were often detached from the projections of the AMϕ and were also observed on the surface of tumor cells in regions closely aposed to AMϕ (Inset of Fig. 4). During this phase, the affected tumor cells tended to lose their microvilli in regions near adherent AMϕ (Fig. 4). This loss of microvilli was the first sign of tumor cell lysis. Fine pits were seen on the surface of tumor cells, corresponding to pinocytotic vesicles (Inset of Fig. 4).

Particles of about the same size as the knobs on cytoplasmic projections were often observed in intercellular spaces between AMϕ and tumor cells (Fig. 5A), and on cytochemical staining for acid phosphatase, these particles between the AMϕ and tumor cell often gave a positive reaction. Lysosomes with acid phosphatase activity were also prominent adjacent to the cell surface in AMϕ (Fig. 5B–5D).

Extensive cytolysis (Fig. 6A, B) was seen in samples prepared after culture of stimulated AMϕ with tumor cells for 48 h, the time when tumor cell lysis was observed in previous work (Tachibana et al. 1984).

Discussion

The tumoricidal activity of rat AMϕ is increased by treating the animals with vitamin A for 4 days (Tachibana et al. 1984). In this work we found that after this stimulation the number and length of the cytoplasmic projections of AMϕ had increased. On interaction of AMϕ with tumor cells, these cytoplasmic projections elongated and adhered to the surface of the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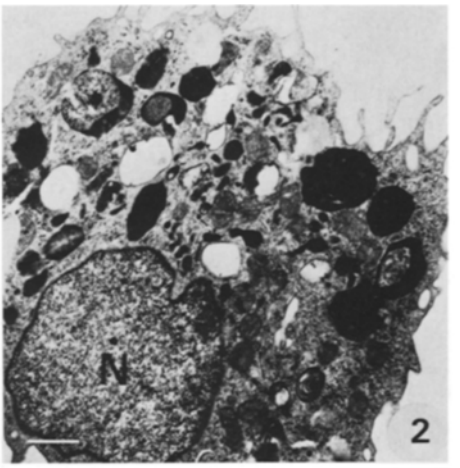
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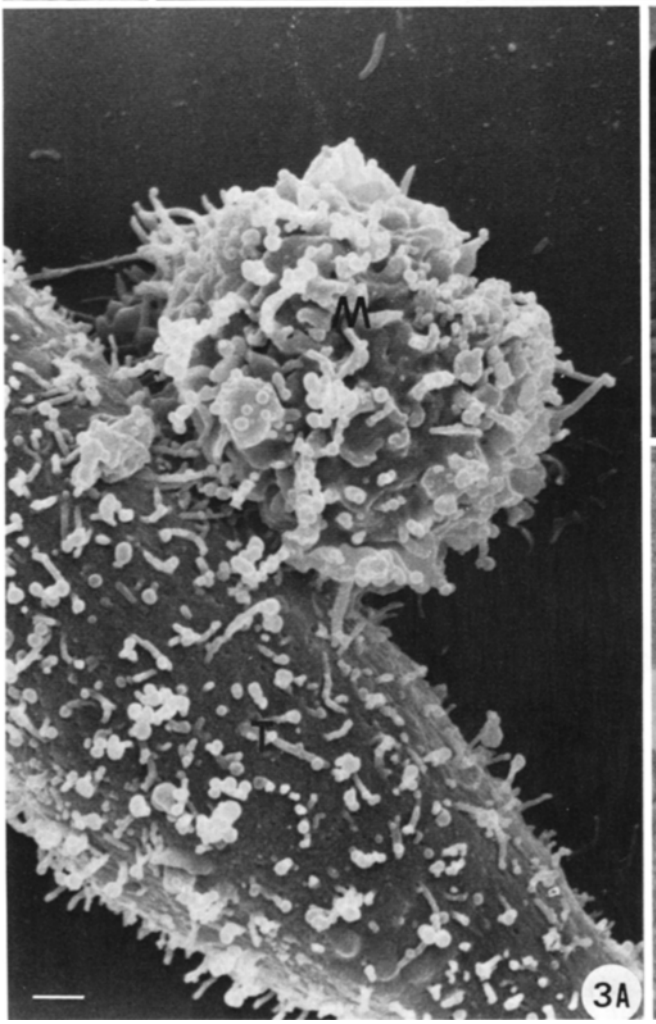
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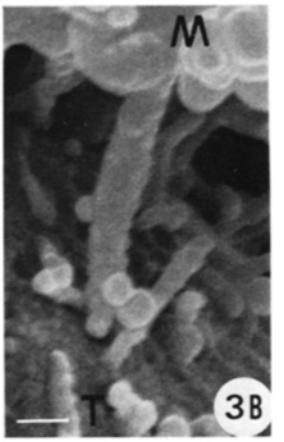
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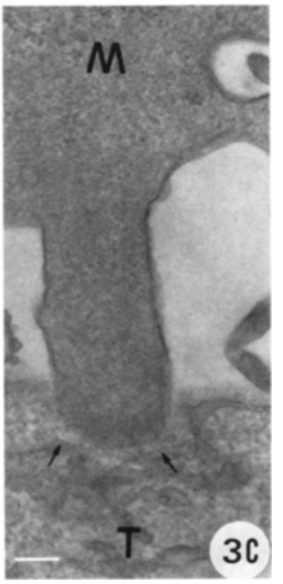
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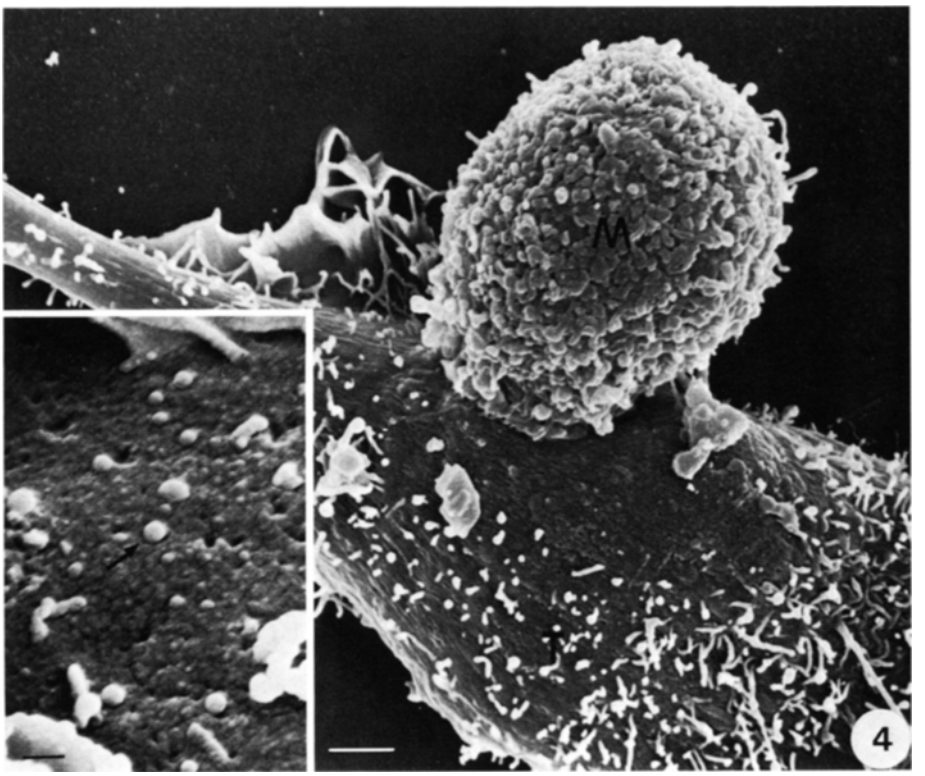

Fig. 4. SEM of the contact of an AMϕ (*M*) with a tumor cell (*T*) after incubation in medium for 24 h. Note the loss of microvilli on the tumor cell surface around the contact zone, probably representing an early stage of tumor cell damage. *Bar:* 2 μm. *Inset:* many particles of 250 nm in diameter (*arrow*), probably detached from AMϕ-projections, are seen on the tumor cells surface. *Bar:* 0.5 μm

Fig. 1. SEM of AMϕ stimulated by vitamin A showing numerous, long cytoplasmic projections with spherical knobs (250 nm in diameter). These knobs are seen on the sides and tip of the projections, and are frequently detached from the projections (*arrow in inset*). The presence of numerous knobs is in marked contrast to the ruffled surface and a few cytoplasmic projections of controls. *Bar*: 1 μm, *Bar in inset:* 0.5 μm

Fig. 2. Distribution of acid phosphatase activity in AMϕ of rats given vitamin A. AMϕ contain many lysosomes and vacuoles of various sizes. Lysosomes in the subplasmalemmal area give a positive reaction for acid phosphatase. The vacuoles appear to be essentially empty; namely, they contain no electron-dense material. N: Nucleus. Novikoff's method. *Bar:* 1 μm

Fig. 3. A SEM of a rat AMϕ (*M*) and a syngeneic tumor cell (*T*) after incubation in medium for 24 h. Close proximity between the two cells is seen. The long cytoplasmic projections of AMϕ are directed toward the tumor cell and are in contact with the tumor cell surface at their tip. *Bar:* 1 μm. **B** Enlarged SEM micrograph. Note point-like contact between the tip of projections of AMϕ and the tumor cell surface. Particles of similar size to the knobs of projections are seen in the narrow space between AMϕ (*M*) and tumor cell (*T*). *Bar:* 0.5 μm. **C** TEM of the contact zone between a tumor cell (*T*) and projection of an AMϕ (*M*). No fusion of the cells is detectable (*arrows*). *Bar:* 0.1 μm

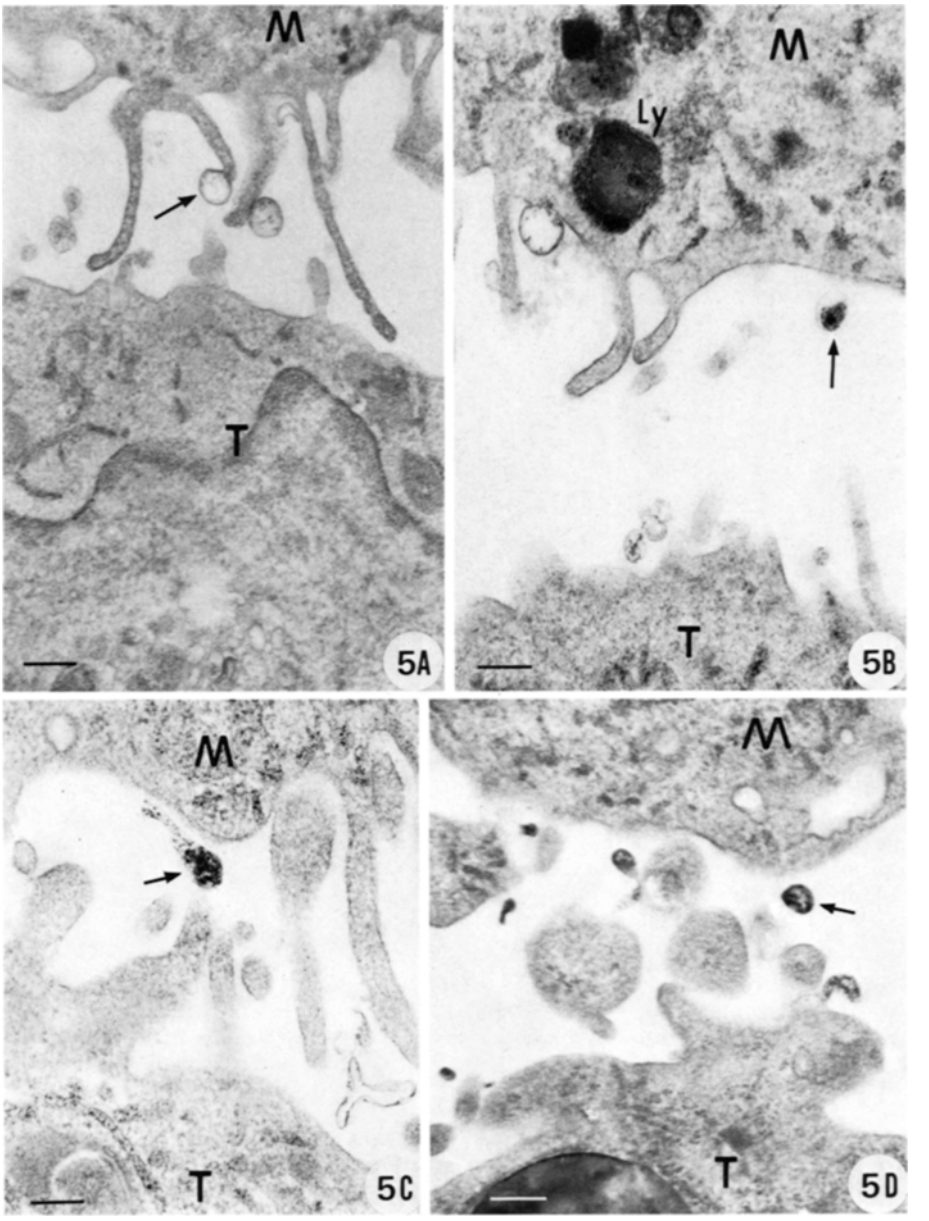

Fig. 5A–D. TEM of a rat AMϕ (*M*) and a syngeneic tumor cell (*T*) after incubation in medium for 24 h. **A** Less-dense round particles (*arrow*) are seen associated with AMϕ-projections interdigitating with the processes of the adjacent tumor cell. **B–D** Particles in the space between the two cells give a positive reaction for acid phosphatase (*arrows*). *Ly:* lysosomes stained for acid phosphatase. Novikoff's method. *Bar:* 0.5 μm

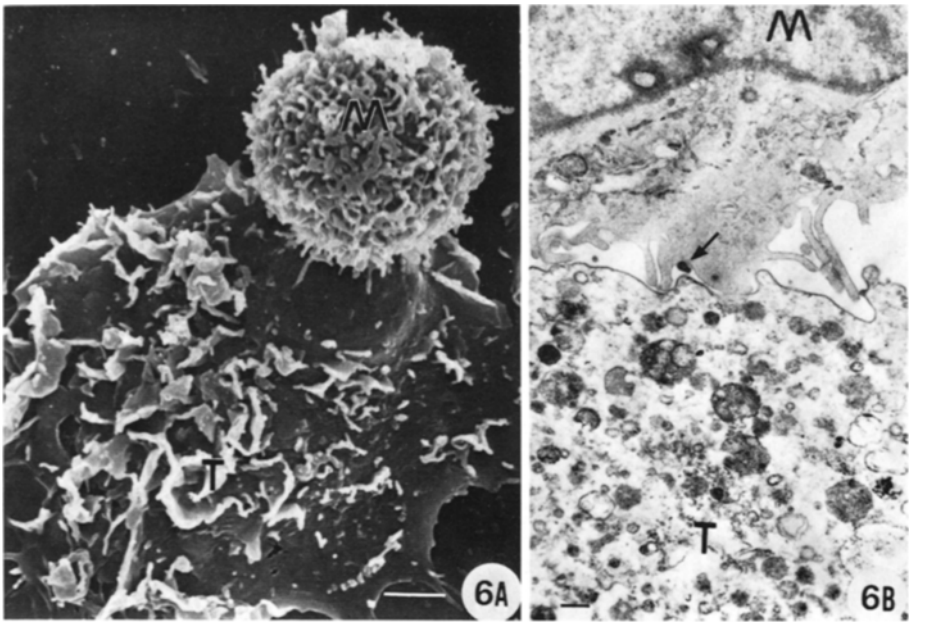

Fig. 6. A SEM of a rat AMϕ and a syngeneic tumor cell after incubation in medium for 48 h. Severely damaged tumor cell is seen attached to intact AMϕ (*M*). Damaged tumor cell (*T*) become spherical with plate-like debris associated with their flattened cell surface. *Bar:* 2 μm. **B** TEM of the zone of close contact between an AMϕ (*M*) and a damaged tumor cell (*T*). An electron-dense body is seen between the AMϕ and the damaged tumor cell (*arrow*). There is no obvious specialization in the AMϕ-cell membrane at the point of close proximity with the tumor cell. *Bar:* 0.5 μm

tumor cells. Morphologically, we found that the process of tumor cell lysis consisted of formation of knobs on the extended cytoplasmic projections of AMϕ, detachment of these knobs from the projections and then their attachment to the surface of the tumor cells. SEM showed that the microvilli of affected tumor cells were lost before the cells lyzed (Rosenau et al. 1981). The attachment of AMϕ to tumor cells is probably an important step in cytolysis mediated by stimulated macrophages (Bucana et al. 1976; Sone and Fidler 1980). In the present study, tumor cells were seen to come into contact with the tips of interdigitating spike-like projections of AMϕ, but there was no evidence of membrane fusion. The peculiar distribution of contact zones may be related to the localization of phagocytic receptors on the AMϕ membrane, but little is known about the distribution of these receptors (Papadimitriou 1973).

SEM and TEM preparations of tumor cells incubated with stimulated AMϕ showed the presence of spherical or oval bodies (250 nm) in the spaces between the AMϕ and tumor cells. These bodies appeared to be small vesicles and occasionally gave a strong positive reaction for acid phosphatase. Some of these bodies were also seen attached to the surface of tumor cells. They seemed to originate from AMϕ because they were the same

size as the knobs on the sides and tip of the cytoplasmic projections of AMϕ.

In previous studies Hibbs (1974) using macrophages activated by BCG (Bacillus Calmette-Guérin) observed direct secretion of macrophage lysosomes into susceptible tumor cells via temporary cytoplasmic bridges and suggested that lysosomal enzymes are the effectors of tumor cell destruction in this cytotoxicity system. Bucana et al. (1976) concluded that the intercellular electron-dense bodies were lysosomal organelles of macrophage origin, since like the latter they showed acid phosphatase activity. They also suggested that penetration of these particles into the tumor cells might occur by an active or passive transport process in the tumor cells. Nathan et al. (1979) reported that BCG-activated macrophages secrete hydrogen peroxide. The production and secretion of oxygen radicals and interleukin I by macrophages were found to be increase after activation of the cells with polysaccharides purified from the plant *Echinacea purpurea* (Stimpel et al. 1984). In general, mononuclear phagocytes have high activities of acid proteases, such as cathepsins, which play a role in intracellular digestion (Cohn and Benson 1965). Unkelss et al. (1974) reported that thioglycolate-stimulated macrophages produce and secrete large amounts of trypsin-like proteases *in vitro*, whereas unstimulated macrophages do not. Stimulated macrophages may also release a variety of proteases that are not formed by unstimulated cells. Currie (1978) and Currie and Basham (1975) observed that arginase was released from macrophages activated by zymosan and LPS, and lysed malignant cells, but not normal cells, in 24–48 h. Retinoids were also found to enhance production of arginase by macrophages (Rhodes and Oliver 1980). Increased release of arginase by macrophages may be one reason for the enhanced tumoricidal effect of vitamin A.

The present morphologic findings suggest that the knobs on cytoplasmic projections of AMϕ contain components that induce tumor cell lysis and that their release is probably another reason for the enhanced cytotoxicity of vitamin A-stimulated macrophages.

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