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Alteration of Physiological Activity of Activated Macrophages through L-Arginine Metabolism

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Our aim in this study was to define the effect of L-arginine on macrophages (Mϕ) in relation to the decay of tumoricidal activity of activated Mϕ. We found that the activated Mϕ retained their cytotoxicity when cultured in L-arginine-deficient medium but not in conventional medium. Such a decline of tumoricidal activity was associated with increase of glucose consumption and concomitant lactate production, resulting in Mϕ death. Addition of glucose to the culture medium of activated Mϕ appeared to cause only a slight delay of the decrease of tumoricidal activity and Mϕ death. These events were also coincident with a decrease of electron transport activity in mitochondria. Cytological observation by electron microscopy clearly showed the structural alteration or destruction of mitochondria, which preceded the changes of other physiological and functional activities. These results demonstrate that the L-arginine-dependent cytolytic activity against tumor target cells also impairs Mϕ functions and ultimately induces Mϕ death, which is primarily mediated by the inhibition of mitochondrial activity.

Key words: Macrophage — Cytotoxicity — L-Arginine — Regulatory mechanism

The reduction of physiological activity and lysis of tumor cells caused by activated macrophages (Mϕ)[5] is now considered to be related to L-arginine metabolism of Mϕ through a pathway producing NO_2^-/NO_3^- and citrulline and inhibiting aconitase and oxido-reductase.[1-4] This cytotoxic event of Mϕ has recently been reported to affect Mϕ themselves as well as tumor cells.[5-8] We have studied the decay of tumoricidal activity of activated Mϕ and found that activated Mϕ lost their cytotoxicity when cultured in conventional minimum essential medium (N-MEM) but not in L-arginine-deficient MEM (Arg^--MEM). In addition, it was observed that cytolysis of P815 target cells by activated Mϕ occurred in the presence of 0.1 m*M* or more of L-arginine, but that the activated Mϕ retained their cytotoxicity for over 3 days in the culture containing 0.15 m*M* or less of L-arginine (to be published elsewhere). These results suggest that L-arginine-dependent cytotoxicity also affects Mϕ themselves, though the sensitivity is lower in activated Mϕ than in tumor target cells. In the study presented here, we aimed to investigate the effect of L-arginine on the decay of Mϕ tumoricidal activity. The results showed that the activated Mϕ in N-MEM consumed more glucose and produced more lactate than those in Arg^--MEM. Addition of glucose resulted only in the delay, but not prevention, of the decline of tumoricidal activity. Furthermore, the loss of cytotoxicity occurred coincidentally not only with these events but also with the decrease of mitochondrial electron transport activity. Electron microscopic observation showed that the structural damage of mitochondria was concomitant with the reduction of mitochondrial electron transport activity and preceded the changes in other physiological activities.

MATERIALS AND METHODS

Mice Male and female C3H/HeSlc mice at 7 to 8 weeks of age (Japan SLC Inc., Shizuoka) were used to collect peritoneal Mϕ.

Reagents and culture media Recombinant murine interferon (IFN)-γ (2×10^7 JRU/mg protein) was produced by Shionogi Research Laboratories (Osaka). Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4, and Brewer's thioglycollate broth (TGC) were purchased from Difco Lab., Detroit, MI. Indomethacin (IM) was from Sigma Chemical Co. (St. Louis, MO). [Methyl-^{3}H]-thymidine (^{3}H-TdR) was obtained from ICN Radiochemicals, Irvine, CA.

Eagle's MEM (referred to as N-MEM in this paper) was prepared from a powdered mix (Nissui Seiyaku Co., Tokyo), which contained 0.6 m*M* L-arginine and 60 μg/

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[5] Abbreviations used are: Mϕ, macrophage(s); Arg^--MEM, MEM depleted of L-arginine; LPS, lipopolysaccharide; N-MEM, normal MEM; TGC, thioglycollate broth; LDH, lactate dehydrogenase.

ml of kanamycin, and was supplemented with 0.2 m*M* L-glutamine, 5 m*M* HEPES and 1.6 mg/ml of sodium bicarbonate. MEM depleted of L-arginine (Arg^--MEM) was prepared from powdered mix MEM-5 (Nissui), to which 0.4 m*M* L-leucine, 0.1 m*M* methionine, and 0.2 m*M* phenylalanine were added. In some experiments, additional glucose (8.5 m*M*) was supplemented to N-MEM or Arg^--MEM, which initially contains 5.6 m*M* glucose. Endotoxin-free fetal bovine serum (FBS) containing about 60 μM L-arginine and 4 m*M* glucose, was obtained from Bocknek Laboratories Inc. (Toronto). All tissue culture reagents were found to be negative for endotoxin contamination by a chromogenic endotoxin-specific assay (Endospacy: Seikagaku Kogyo Co. Ltd.).

Macrophage culture Peritoneal exudate cells were obtained from mice injected ip with 2 ml of TGC 4 days previously. Adherent Mϕ monolayers were prepared by depleting nonadherent cells after incubation of PEC in 96-well microculture plates at 2×10^5/well for 2 h at 37°C. For activation, Mϕ were cultured in fresh medium containing 20 JRU/ml of IFN-γ plus 10 or 100 ng/ml of LPS. Each group consisted of triplicate cultures. The same type of experiment was repeated at least 3 times. The duration of culture, the type of medium and supplements to the medium prior to or at the time of assay of Mϕ tumoricidal activity were recorded for each experiment.

Mϕ viability was determined by trypan blue dye exclusion or release of lactate dehydrogenase (LDH). For the dye exclusion method, Mϕ on cover slips (13-mm diameter) were activated as mentioned above, and cultured in 24-well multi-culture plates. At the end of culture, Mϕ were exposed to trypan blue, washed, and fixed with 2% glutaraldehyde, and the number of unstained cells was counted as viable cells before fixation. The LDH method was carried out according to Albina *et al.*[8] Briefly, Mϕ monolayers in 24-well multi-culture plates were activated and cultured for various periods in N-MEM or Arg^--MEM with or without extra glucose. At the end of culture, the LDH activity released into culture supernatant and that of cells lysed with Triton X-100 were determined by using a measurement kit for LDH (Lactate Dehydrogenase CII-Test, Wako Pure Chemicals, Osaka).

Assay of macrophage-mediated cytotoxicity Tumoricidal activity of Mϕ was assessed in terms of ^{3}H-TdR release from labeled P815 target cells according to a method described previously.[9] Values were calculated by using the following formula: % specific ^{3}H-TdR release = [(experimental cpm − spontaneous cpm)/(total cpm − spontaneous cpm)] × 100. Experimental cpm and spontaneous cpm were the radioactivities in wells containing target cells plus Mϕ and target cells alone, respectively. Total cpm was the radioactivity of the lysate of target cells after treatment with the final 1% (v/v) sodium dodecyl sulfate. Spontaneous cpm was less than 10% of the total cpm. The results are represented as the mean of triplicate cultures.

Determination of glucose consumption and lactate accumulation After preculture with IFN-γ and/or LPS for 8 h, Mϕ were washed, received fresh N-MEM or Arg^--MEM, and then were cultured for various periods. The cell-free supernatants of these Mϕ cultures were collected and the amount of glucose was determined enzymatically by using glucose oxidase and 4-aminoantipyrine (Glucose B-test; Wako Pure Chemicals).[10] The value was expressed as % glucose consumption by calculation using the formula: (initial concentration of glucose − resultant concentration of glucose/initial concentration of glucose) × 100. The concentration of glucose in the culture medium supplemented with 10% FBS was 6 m*M*. Lactate accumulation in the culture supernatant during post-activation culture was measured by means of a standard spectrophotometric assay in which NADH newly formed in the LDH reaction was determined.[11] Results are represented as the mean of triplicate cultures.

Measurement of mitochondrial activity Electron transport activity of mitochondria was measured by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrasolium bromide; Sigma Chemical Co.) method.[12] For this assay, 10 μl of 5 mg/ml MTT in phosphate-buffered saline was added to each well of Mϕ monolayers, and the plates were incubated for 4 h at 37°C. Then MTT-formazan produced was solubilized with 10% SDS and measured in a Multiscan MC (Flow Labs, Mclean, VA) at 570 nm.

Cytological study by electron microscopy After activation with 20 JRU/ml of IFN-γ plus 100 ng/ml of LPS in 96-well flat-bottomed culture plates, Mϕ were washed and cultured for a further 40 h in N-MEM or Arg^--MEM medium. Then Mϕ were washed with PBS and fixed with 3% glutaraldehyde in 0.1 *M* sodium cacodylate buffer at pH 7.4 overnight, followed by post-fixation with 2% osmium tetroxide. Each specimen was washed with PBS, dehydrated in graded ethanol solutions and embedded in Epon. The plate was immersed in liquid nitrogen to remove the specimens. Sections were stained with 0.25% uranyl acetate and lead citrate, and examined by transmission electron microscope (Model JEM-100X, Japan Electron Optics Lab., Tokyo).

RESULTS

L-Arginine-dependent loss of tumoricidal activity of activated Mϕ The experiment shown in Fig. 1 was conducted to determine the effect of L-arginine on the activation of Mϕ and the retention of their cytotoxicity. Mϕ were cultured with IFN-γ plus LPS in N-MEM or Arg^--MEM for various periods and then their tumor-

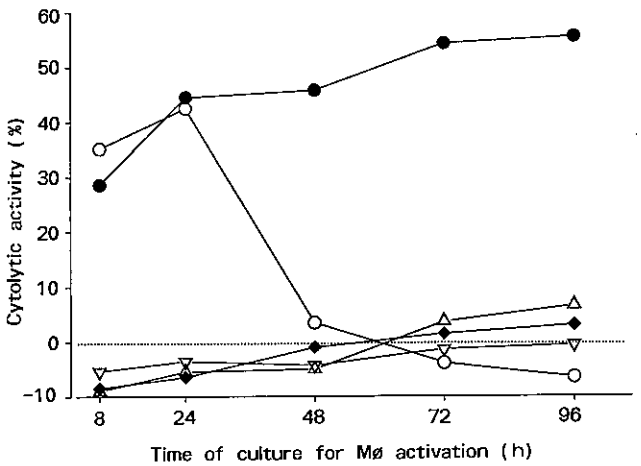

Fig. 1. Activation and retention of tumoricidal activity of Mϕ in N-MEM or Arg$^-$-MEM. Mϕ monolayers were cultured with 20 JRU/ml of IFN-γ plus 100 ng/ml of LPS in N-MEM (○) or Arg$^-$-MEM (●) for various periods as indicated on the abscissa, washed, and assessed for tumoricidal activity against ^{3}H-TdR-labeled P815 target cells in N-MEM. Mϕ cultured in the presence of either IFN-γ (△) or LPS (▽) or in their absence (◆) in N-MEM served as controls. Each point represents the mean of triplicate cultures.

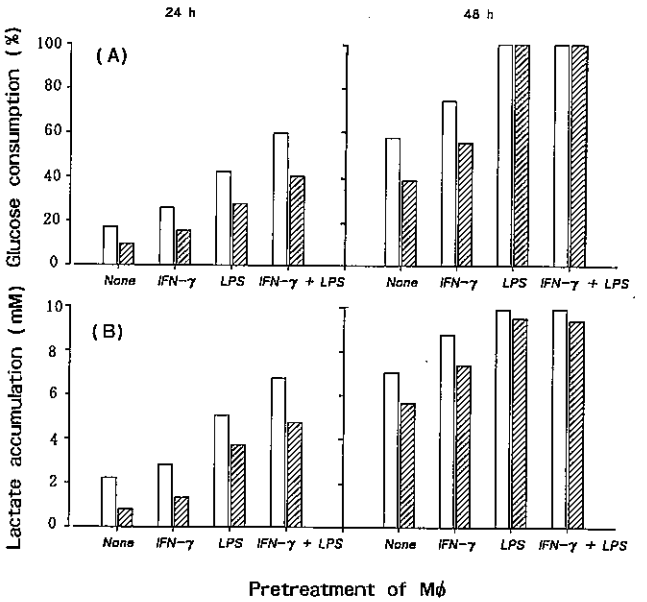

Fig. 2. Glucose consumption and lactate production by activated Mϕ during culture in N-MEM or Arg$^-$-MEM. Mϕ pretreated with 20 JRU/ml of IFN-γ and/or 100 ng/ml of LPS for 8 h were cultured in N-MEM (□) or Arg$^-$-MEM (▨) without further stimulation for a further 24 h or 48 h, and the concentration of glucose (A) and that of lactate (B) in media were determined. Mϕ precultured without any stimulating agent served as controls. Results in (A) represent the mean % decrease of glucose relative to the initial glucose content (5.9 m*M* in N-MEM with 10% FBS) of triplicate cultures, and those in (B) the mean concentration of lactate accumulated in the media of triplicate cultures.

icidal activity was evaluated against P815 target cells in N-MEM. Mϕ kept in N-MEM with IFN-γ plus LPS for 8 h showed substantial cytotoxic activity, which was higher than that of Mϕ in Arg$^-$-MEM. The tumoricidal activity appeared to increase with time of culture for a further 16 h but decreased drastically thereafter. In contrast, Mϕ maintained in Arg$^-$-MEM retained cytolytic activity.

Glucose consumption and lactate accumulation of activated Mϕ in N-MEM or Arg$^-$-MEM It is known that peritoneal Mϕ have high activities of glycolytic enzymes, and that the glucose consumption increases in activated Mϕ.[13-15] Such an enhanced rate of glucose consumption of activated Mϕ may result in a marked decrease of glucose in the tissue culture medium.[16] Therefore we measured the amount of glucose and that of lactate in the medium at the end of culture for 24 h or 48 h (Fig. 2). Mϕ preactivated with IFN-γ plus LPS consumed more than half of the glucose in the medium by 24 h of culture and depleted it by 48 h, when cultured in N-MEM. On the other hand, the rate of glucose consumption was lower in activated Mϕ cultured in Arg$^-$-MEM than in N-MEM. However, they ultimately used up the glucose in 48 h of culture (Fig. 2A). The accumulation of lactate in the medium was closely and inversely correlated with the consumption of glucose (Fig. 2B). These results suggest that the depletion of glucose may result in a shortage of cellular ATP. This may lead to the death of activated Mϕ.

Effect of glucose supplement on Mϕ viability and retention of tumoricidal activity To avoid inadequate energy supply, we added glucose to the culture medium. The Mϕ viability and the retention of tumoricidal activity were examined. All of the activated Mϕ were alive at 24 h in N-MEM, but their viability was decreased thereafter. About 80% of them were dead at 37 h and none was alive at 62 h. Prolongation of activated Mϕ viability by the addition of extra glucose, however, was evident only at 37 h of culture. In contrast, no activated Mϕ cultured in Arg$^-$-MEM were dead at 37 h, and only a slight reduction of the viability was observed even at 62 h after the start of culture, even without extra glucose (Fig. 3A). A similar trend was observed when the viability of Mϕ was determined in terms of the release of LDH into the culture medium (Fig. 3B).

In order to define the relation between the decline in tumoricidal activity of activated Mϕ and their viability,

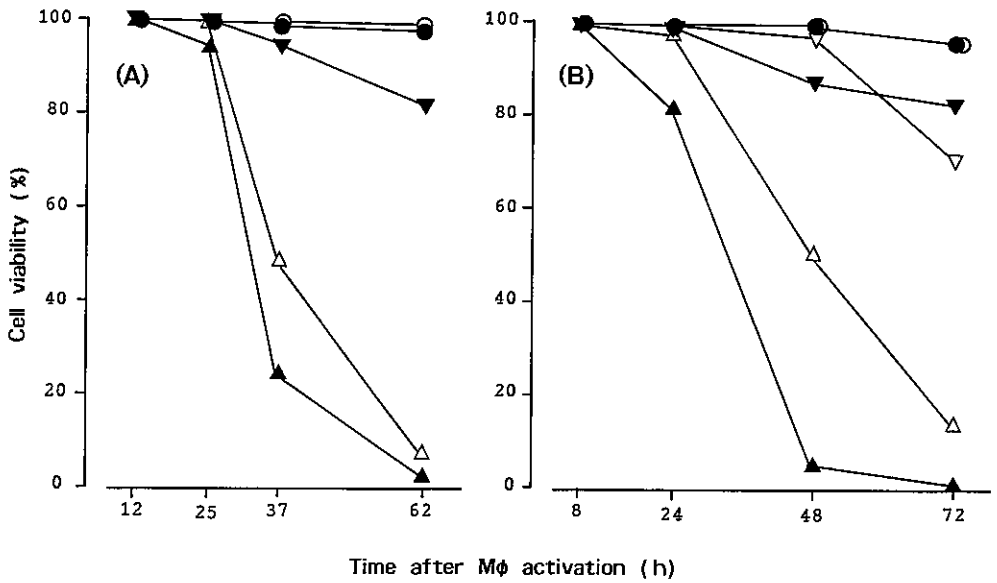

Fig. 3. Viability of activated Mφ in culture in the presence or absence of L-arginine. Mφ activated with 20 JRU/ml of IFN-γ plus 100 ng/ml of LPS for 8 h were cultured for various periods in N-MEM (△, ▲) or Arg^--MEM (▽, ▼) with (open symbols) or without (closed symbols) extra glucose. At the end of culture, viability was determined by the trypan blue dye exclusion test (A) and LDH method (B). Mφ precultured for 8 h without activation (○, ●) served as controls. Each point represents the mean of duplicate cultures.

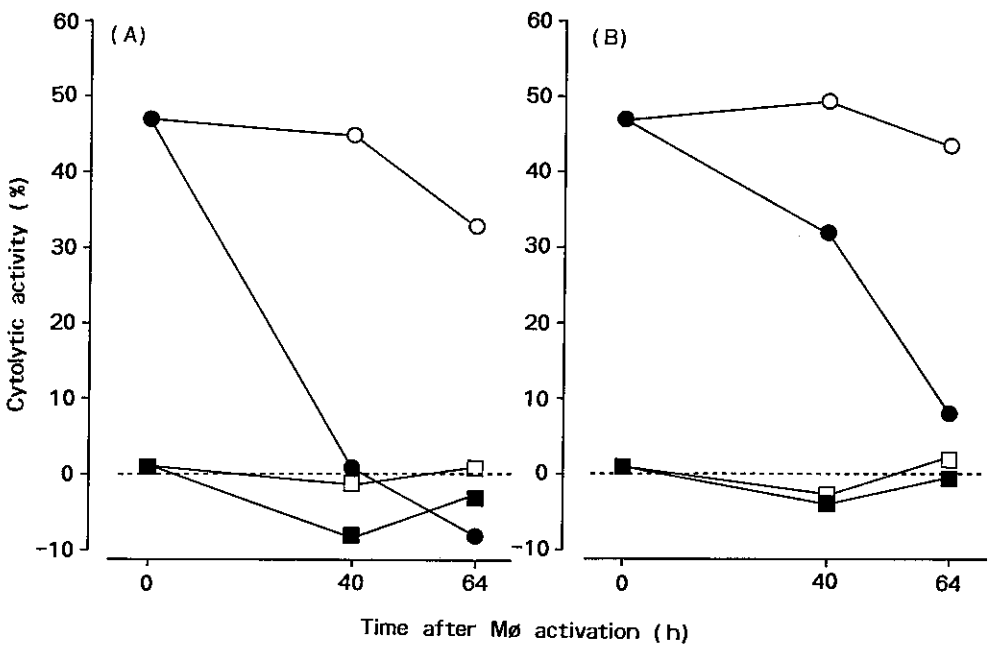

Fig. 4. Effect of glucose supplement to N-MEM or Arg^--MEM on the retention of Mφ cytotoxicity. After preactivation for 8 h with 20 JRU/ml of IFN-γ plus 100 ng/ml of LPS (○, ●), Mφ were cultured in N-MEM (closed symbols) or Arg^--MEM (open symbols) for 40 or 64 h (A). A half of the activated Mφ were cultured in medium supplemented with 8.5 m*M* extra glucose (B). Then tumoricidal activity was assessed. Mφ precultured without activation (□, ■) served as controls. Each point represents the mean of triplicate cultures.

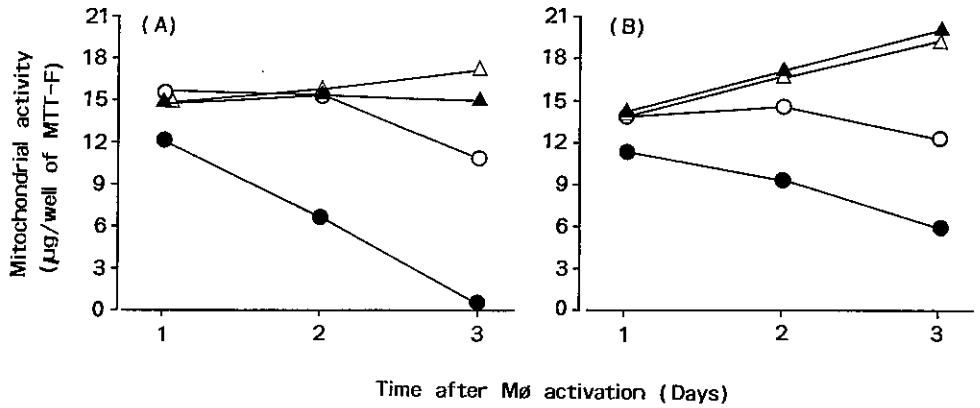

Fig. 5. Loss of mitochondrial activity in activated Mϕ during culture. After being washed, Mϕ precultured for 8 h in the presence (○, ●) or absence (△, ▲) of 20 JRU/ml of IFN-γ plus 100 ng/ml of LPS in N-MEM were further cultured for various periods in N-MEM (closed symbols) or Arg^--MEM (open symbols) supplemented with (B) or without (A) extra glucose. MTT was added to each well and culture was continued for a further 4 h, then the concentration of MTT-formazan produced was measured with a Multiscan MC at 570 nm.

the cytolytic activity of activated Mϕ was determined after culture for 40 h and 64 h in N-MEM with or without extra glucose (Fig. 4). At 40 h after activation, Mϕ in N-MEM without extra glucose lost the cytolytic activity, whereas Mϕ retained moderate activity in the presence of extra glucose. However, cytotoxicity was no longer detected at 64 h. On the other hand, regardless of the presence of additional glucose, Mϕ maintained in Arg^--MEM were still cytolytically active even at 64 h. These results show the correlation between the viability and the cytolytic activity of Mϕ.

Comparison of mitochondrial activity between activated Mϕ cultured in N-MEM and in Arg^--MEM Increased glucose consumption and concomitant increased lactate production of activated Mϕ in N-MEM may be due to the slump of mitochondrial electron transport activity. To test this possibility, we assessed the mitochondrial activity of activated Mϕ cultured for various periods in N-MEM or Arg^--MEM by the MTT method. As shown in Fig. 5, the activated Mϕ in N-MEM rapidly lost electron transport activity. In contrast, this activity of Mϕ in Arg^--MEM was retained for a fairly long time and only a slight reduction was detected even 3–4 days later. On the other hand, electron transport activity of Mϕ lasted longer in N-MEM supplemented with glucose than in that without extra glucose, but it eventually diminished.

Impairment of mitochondrial structure in the activated Mϕ cultured in N-MEM In order to examine the cell structure, especially mitochondria, we fixed the activated Mϕ cultured in N-MEM or Arg^--MEM in the presence of extra glucose after 40 h of activation and observed them under an electron microscope. The electron-dense structure of mitochondria remained intact in Arg^--MEM (Fig. 6B). In contrast, the mitochondria swelled, and the structure of inner membrane foldings, cristae, became irregular in shape and was often disrupted in Mϕ cultured in N-MEM, but plasma membrane of the cell surface and intracellular fine filaments appeared to be unaffected, although the cytoplasm was electron-lucent (Fig. 6A). The mitochondria were not changed in structure at 24 h but their electron density decreased (not shown). These results indicate that the functional impairment and damage of mitochondria precede Mϕ death, and are accompanied with the decay of the tumoricidal activity.

DISCUSSION

It has been reported that Mϕ tumoricidal activity is a transient event under both *in vivo* and *in vitro* conditions.[17–20] In the early studies, E-series prostaglandins produced by Mϕ themselves were considered to be one of the principal inhibitory mediators of the Mϕ cytolytic activity.[21–23] However, such a role of prostaglandins in the tumoricidal activity of Mϕ is not general.[24–26] Thus, the mechanisms by which the tumoricidal activity of activated Mϕ is depressed still remain unclear.

The metabolism of L-arginine through a pathway producing NO_2^-/NO_3^- and citrullin and causing inhibition of aconitase and certain oxido-reductases has been demonstrated to underlie the Mϕ-mediated tumoricidal

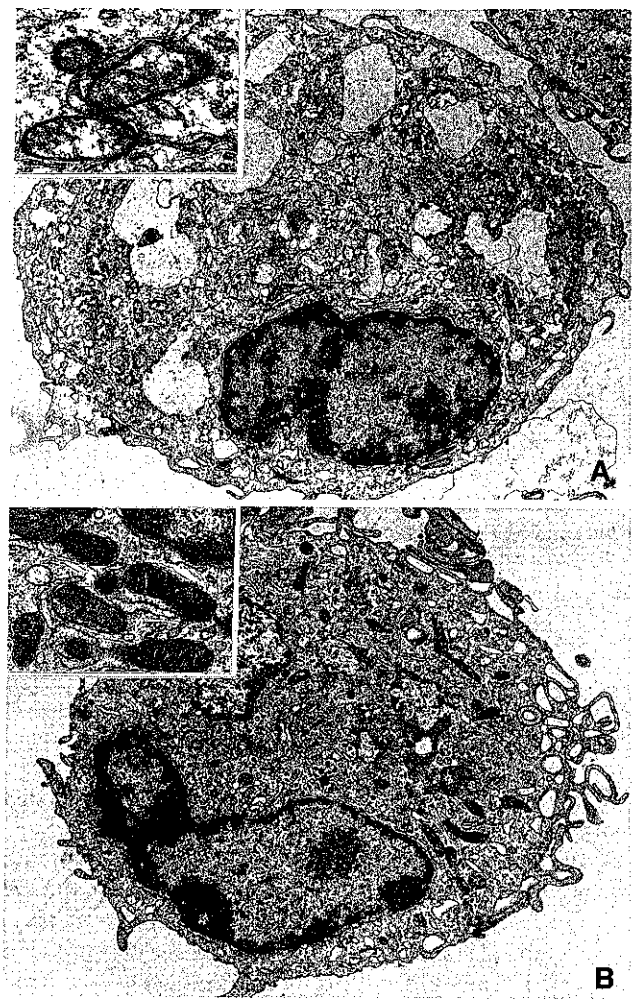

Fig. 6. Structural damage to mitochondria in activated Mϕ cultured in N-MEM but not in Arg^--MEM. Activated Mϕ were cultured in N-MEM (A) or Arg^--MEM (B) for 40 h and fixed prior to observation by electron microscopy. Insets represent typical figures of mitochondria. In (A), swelling of mitochondria and destruction of inner membrane foldings and cristae structure can be seen. ×6,000.

reaction.[1, 2, 27] Some workers have reported that this metabolic pathway may injure or depress various functions of Mϕ themselves.[6, 28] In those reports, depletion of L-arginine, or addition of N^G-monomethyl-L-arginine, a specific inhibitor of the oxidative L-arginine deiminase pathway, was demonstrated to result in prevention of the decrease of Mϕ viability. In the present study, therefore, we attempted to define the cellular mechanism of the decay of Mϕ cytotoxicity by L-arginine.

It has been well documented that peritoneal Mϕ from a relatively anaerobic environment have a high glycolytic activity, and that these Mϕ consume more glucose when cultured in the presence of immunomodulatory signals.[14] Here we showed that Mϕ precultured with IFN-γ plus LPS to make them cytolytic consumed glucose at a high rate and incidentally produced lactate rapidly in comparison with Mϕ precultured without any stimulating agent. This was the case with Mϕ either in N-MEM or Arg^--MEM (Fig. 2, A and B). However, glucose consumption and lactate accumulation were considerably lower in the activated Mϕ cultured in Arg^--MEM than in N-MEM. These profiles of metabolic activity appeared to correlate with the decline rate of tumoricidal activity (Fig. 1 vs. Fig. 2). Moreover, Mϕ death was observed concomitantly with the loss of cytotoxicity (Fig. 3). Drapier and Hibbs noted that depletion of glucose in the medium resulted in shortage of energy supply, since glucose is a substrate for ATP synthesis.[6] Supplementation of glucose caused a slight prolongation of the activated state of Mϕ (Fig. 4). In addition, glucose was used up even in the absence of L-arginine by 48 h after activation, when Mϕ retained the cytotoxic activity (Fig. 1). These results, therefore, suggest that Mϕ death is not primarily due to the depletion of glucose.

Albina *et al.* have shown that the lactate accumulation of rat peritoneal resident Mϕ cultured in conventional RPMI-1640 medium was greater than that of Mϕ in L-arginine-deficient RPMI medium, and the ATP content of these Mϕ was inversely correlated with lactate accumulation.[28] Furthermore, they demonstrated that the death of Mϕ was facilitated by L-arginine even in resident-type cells.[28] On the contrary, significant death of noncytotoxic Mϕ, such as those unstimulated or stimulated by either IFN-γ or LPS alone, was not observed in our present study during culture in N-MEM, although they consumed more glucose in N-MEM than in Arg^--MEM (Fig. 3 vs. Fig. 2). These discrepancies may be ascribed to the difference of metabolic activity between rat resident Mϕ and mouse elicited Mϕ. Vascular endothelial cells contain a constitutive nitric oxide synthase that is Ca^{2+}-dependent. In addition, these cells express, after activation with IFN-γ and LPS, an inducible Ca^{2+}-independent nitric oxide synthase.[29] In association with this, rat resident Mϕ may contain either or both of these enzymes, different from mouse Mϕ.

The mitochondrial activity of activated Mϕ in N-MEM rapidly decreases in comparison with that in Arg^--MEM. This is essentially consistent with the result of Albina et al. in the case of rat resident or *Corynebacterium*-elicited Mϕ.[28] The reduction of mitochondrial electron transport activity of activated Mϕ in N-MEM appeared to precede the decline of Mϕ tumoricidal activity (Fig. 1 vs. Fig. 5) and to be followed by the death of Mϕ themselves (Fig. 1 vs. Fig. 3). In addition, electron microscopic observation showed structural damage to

mitochondria in the culture with additional glucose at 40 h after activation (Fig. 6A), in which activated Mϕ still manifested moderate cytotoxicity (Fig. 4B). These results also demonstrate that Mϕ even under altered physiological conditions can still attack tumor cells.

It has been reported that the concentration of amino acids in the *in vivo* extracellular fluid is lower than in the medium used for cell culture, and that the concentration of L-arginine decreases at the site of lesions because of the elevated activity of arginase.[30, 31] Therefore, functional loss or death of activated Mϕ may be observed under only *in vitro* culture conditions with relatively high concentrations of L-arginine, and may not occur in *in vivo* Mϕ-infiltrated sites.

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