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Nitric oxide-mediated cytotoxic effects of alveolar macrophages on transformed lung epithelial cells are independent of the β_2 integrin-mediated intercellular adhesion

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SUMMARY

It is known that murine macrophages produce nitric oxide (NO) when stimulated with lipopolysaccharide (LPS) or interferon-γ (IFN-γ), and NO mediates the tumoricidal activity of activated macrophages. The present study was designed to investigate whether the intercellular adhesion is necessary for activated rat alveolar macrophages to exert the full cytotoxic effects. Rat alveolar macrophages produced NO dose dependently in response to either LPS or IFN-γ, and caused DNA fragmentation in rat type II pneumocytes transformed with SV40 (SV40T2). Chemically produced NO also caused the DNA fragmentation and viability loss in SV40T2, and both of them were inhibited by a NO radical scavenger. The cytotoxicity of activated macrophages was reduced by N^G-monomethyl-L-arginine, a competitive nitric synthase inhibitor, and neither superoxide dismutase nor catalase modulated the cytotoxicity. Although alveolar macrophages stimulated with either LPS or IFN-γ caused DNA fragmentation of SV40T2, only LPS increased the intercellular adherence between macrophages and SV40T2. The intercellular adhesion was reduced by both anti-CD18 and anti-CD11a. However, those antibodies did not affect the cytotoxicity of LPS-stimulated macrophages. These results clearly indicate that NO-mediated cytotoxicity is caused predominantly by diffusion of NO, and the β_2 integrin-mediated intercellular adhesion does not play an important role, if any, in activated macrophage-mediated cytotoxic effects on SV40T2.

INTRODUCTION

Nitric oxide (NO) has a variety of physiological and biological functions such as relaxation of vasculature,[1] neurotransmission,[2,3] bacteriostatic/bactericidal[4] and fungistasis activities,[5] and killing of parasites[6] and tumour cells.[7–11] Stuehr & Marletta[12] reported for the first time that activated macrophages produced NO, and one of the most effective mediators in the killing of target cells by activated macrophages has been reported to be NO.[7–10]

Alveolar macrophages play a pivotal role in the elimination of foreign substances from the alveolar surface,[13–15] the recruitment of circulating neutrophils into the alveolar space, and the subsequent lung inflammatory injury.[14,15] Through these processes macrophages produce reactive oxygen intermediates (ROI),[15] and ROI are implicated in cytotoxicity and tissue injury.[16–20] Peroxynitrite is a reaction product of NO and superoxide, and this molecule has been reported to be directly[16,18] and indirectly[17,20] responsible for the cytotoxicity. It has also been reported that NO production is not correlated with the killing of tumour cells in some activated macrophage/tumour cell combinations.[11] These findings suggest that although NO play an important role in the killing of tumour cells by activated macrophages, other molecules may be involved in the cytotoxic effects of macrophages.

On the other hand, it has been reported that effective molecules of T lymphocytes and natural killer (NK) cells for the lysis of target cells are granzymes, perfolin and Fas ligands, and the intercellular adhesion is a requisite for the cytotoxic effects of lymphocytes and NK cells.[21–23] It seems that a close cell–cell contact is required for guinea-pig macrophages to cause cytolysis of hepatoma cells..[24] However, very little is known about the involvement of intercellular adhesion in tumoricidal activity of activated macrophages compared to cytotoxic T lymphocytes and NK cells.

Recently the author reported that adherence of alveolar macrophages to rat lung type II cells transformed with SV40 (SV40T2) increased within 30 min following stimulation with lipopolysaccharide (LPS), and LPS-stimulated macrophages lysed SV40T2 by a NO-dependent mechanism.[25] The present study was designed to investigate whether the intercellular adhesion is a requisite for the maximum cytotoxic effects of activated macrophages on SV40T2, and whether ROI modulate NO-mediated cytotoxicity using the alveolar macrophage–SV40T2 co-culture system.

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MATERIALS AND METHODS

The following monoclonal antibodies and chemicals were used in the present experiment: anti-rat CD11a, CD11b and CD18 (Cedar Lane Lab., Hornby, Canada); murine interferon-γ (IFN-γ; Biosource Int., Camarillo, CA); LPS (*Salmonella minnesota*), superoxide dismutase (SOD; from bovine erythrocytes), catalase (from bovine liver, cell culture-tested) and calf thymus DNA (Sigma, St Louis, MO); N^G-monomethyl-L-arginine (NMMA; Wako Pure Chem., Osaka, Japan); S-nitroso-*N*-acetyl-DL-penicillamine (SNAP), (2-4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) and WST-1 assay kit (Dojindo, Kumamoto, Japan). Other chemicals of analytical grade were obtained from Wako Pure Chem.

Type II epithelial cells

The rat pulmonary type II cell line (SV40T2) was a kind gift from Dr A. Clement (Hospital Armand Trousseau, France), and the characteristics of SV40T2 have been reported elsewhere.[26] They were grown to confluence in Earl's minimum essential medium (EMEM) containing 4 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM HEPES, and 10% heat-inactivated fetal bovine serum in a 5% CO_2 atmosphere at 37°.

Collection of alveolar macrophages

Specific pathogen-free, male Sprague–Dawley rats, 7–9 weeks old (Clea Japan, Tokyo, Japan), were used. The rats were anaesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg body weight) and killed by exsanguination from the abdominal aorta. The lungs were exposed and lavaged eight times with saline and the lavaged fluid was centrifuged at 400 *g* for 5 min at 4°. The pellet was washed in EMEM and resuspended in fresh EMEM. The viability of the lavaged cells was more than 95% as determined by the exclusion ability of trypan blue. More than 97% of the lavaged cells were alveolar macrophages, as determined by differential cell counting using Diff-Quik® staining (Kokusai Shiyaku Co., Kobe, Japan).

Adhesion assay

SV40T2 cells were grown to confluence in an eight-well chamber slide (Nunc, Naperville, IL). The monolayer was washed with fresh EMEM and alveolar macrophages were placed into each well of the chamber slide ($8{\cdot}0 \times 10^4$ viable cells/well, $1{\cdot}0 \times 10^5$ viable cells/cm^2) and were precultured for 8 hr. In inhibition experiments using monoclonal antibodies, macrophages were incubated with 100 μg/ml anti-CD11a, -11b, or -18 on ice for 2 hr and precultured on the SV40T2 monolayer in the presence of each antibody (20 μg/ml) for 8 hr. LPS or IFN-γ was added to the co-culture system to final concentrations of 0·02–2 μg/ml and 10–1000 U/ml, respectively, and the co-culture was further incubated for 0·5 hr. Following 0·5 hr stimulation with LPS or IFN-γ, each well was filled with warmed (37°) Hanks' balanced salt solution (HBSS). A silicon gasket was layered between the top of the wells and the rim to seal the chamber slide assembly. The chamber slide was set vertically in a rotor of a cytocentrifuge (Autosmear CF-12D, Sakura, Tokyo, Japan) with the bottom of the chamber slide inward and centrifuged at 100 *g* for 10 min. The slide was air-dried and stained with Diff-Quik®. Alveolar macrophages that had remained on the SV40T2 monolayer of equally divided nine high power fields were differentially counted using a microscope. The adhesion assay was also performed without the SV40T2 monolayer to investigate effects of the antibodies on non-specific adhesion of alveolar macrophages (adhesion to the plastic substrate).

Measurement of NO_2^-

NO released in the culture medium from SNAP or activated macrophages was evaluated according to the method of Stuehr & Nathan[10] with minor modifications. Briefly, SNAP was added to EMEM in a 96-well culture dish (Costar, Cambridge, MA) to final concentrations of 0·2 and 0·5 mM. At 0, 1, 2, 4, 8, 12 and 24 hr of incubation, an aliquot of the supernatant was mixed with the same volume of Griess reagent (0·1% *N*-naphthylethylene diamine dihydrochloride, 1% sulophanilic acid and 5% H_3PO_4). Similarly, alveolar macrophages (1×10^6 viable cells/ml) were cultured in EMEM containing various concentrations of LPS or INF-γ for 24 hr, and the supernatant was mixed with Griess reagent. After 15 min of reaction at room temperature, an optical density (OD) at 560 nm was measured using a microtitre plate reader (CS-9300PC, Shimadzu, Kyoto, Japan). The NO_2^- production of LPS-stimulated (2 μg/ml) alveolar macrophages was also measured in the presence of anti-CD18 (20 μg/ml) to study effects of the β_2 integrin ligation on the NO production.

Viability assay

SV40T2 cells were grown to confluence in a 24-well culture dish. The monolayers were cultured in EMEM containing 0·2 and 0·5 mM SNAP in the presence or absence of carboxy-PTIO. The equimolar carboxy-PTIO was added twice at 0 and 12 hr of culture to a final concentration of 0·2 mM. The viability of SV40T2 was measured by WST-1 (a modified colorimetric tetrazolium assay) according to the manufacturer's instruction. Briefly, after gentle washing with phenol red-free HBSS, WST-1 solution was added to each well, and the dish was incubated at 37° for 1 hr. At the end of incubation, OD at 450 nm was measured with a reference of 650 nm using the microtitre plate reader.

DNA fragmentation of SV40T2

SV40T2 cells were grown to confluence in a 24-well culture dish (Costar). Alveolar macrophages ($1{\cdot}0 \times 10^5$ viable cells/cm^2) were cultured on the SV40T2 monolayer or in empty wells with or without 2 μg/ml of LPS or 1000 U/ml IFN-γ in the presence or absence of SOD (1000 U/ml), catalase (1000 U/ml) or NMMA (10 mM). After 24 hr of culture, the monolayer was washed twice with phosphate-buffered saline (PBS). Non-adherent cells were centrifuged and washed twice with PBS. Adherent and non-adherent cells obtained either from the co-culture or macrophages alone were combined and lysed together in the lysis buffer solution (20 mM Tris–HCl containing 0·5% dodecyl sulphate and 10 mM EDTA sodium, pH 7·4). The lysate was centrifuged at 12 000 *g* for 10 min at 4°. The sticky pellet was washed in the fresh lysis buffer and the sample was centrifuged again. Perchloric acid was added to both the supernatant (containing soluble DNA) and the pellet (containing insoluble DNA) to a final concentration of 4% to sediment DNA. DNA contents were measured by the diphenylamine reaction[27] using calf thymus DNA as a stan-

dard. The percentage of DNA fragmentation of SV40T2 was calculated by the following equation:

$$\text{DNA fragmentation of SV40T2 (\%)} = \frac{(\text{soluble DNA of co-culture} - \text{soluble DNA of macrophages})}{(\text{total DNA of co-culture} - \text{total DNA of macrophages})} \times 100$$

where total DNA = soluble DNA + insoluble DNA.

Antibodies (anti-CD11a, -CD11b, and -CD18) were added instead of enzymes or NMMA to investigate the effects of intercellular adhesion on DNA fragmentation of SV40T2. The DNA fragmentation of SV40T2 by chemically produced NO was also assayed in the presence or absence of SNAP and carboxy-PTIO.

Statistics

The measurement of NO_2^- production of activated alveolar macrophages was performed using four rats with duplicate replicates, and results are expressed as means ± SEM. The effects of anti-CD18 on NO_2^- production of LPS-stimulated alveolar macrophages were analysed statistically by a two-tailed *t*-test. Otherwise, each value represents the mean ± SEM of four to five replicates.

RESULTS

Figure 1 shows DNA fragmentation (Fig. 1a) and the viability change of SV40T2 (Fig. 1b) caused by chemically induced NO. SNAP spontaneously produced NO in the culture medium almost linearly with time up to 24 hr (Fig. 1a, inset). The killing of SV40T2 was predominantly mediated by NO, because carboxy-PTIO, a NO radical scavenger, abrogated the DNA fragmentation and viability loss of SV40T2.

Figure 2 shows the production of NO by LPS- (Fig. 2a) and IFN-γ-stimulated macrophages (Fig. 2b), as estimated by nitrite concentration in the medium, DNA fragmentation of SV40T2 caused by LPS- (Fig. 2c) and IFN-γ-stimulated macrophages (Fig. 2c), and the effects of enzyme inhibitors on DNA fragmentation of SV40T2 caused by LPS- (Fig. 2e) and IFN-γ-stimulated macrophages (Fig. 2f). Alveolar macrophages produced NO and fragmented DNA of SV40T2 in response to either LPS or IFN-γ dose-dependently. The DNA fragmentation was inhibited by NMMA, and neither SOD nor catalase affected the DNA fragmentation of SV40T2 caused by activated macrophage.

Figure 3 shows the effects of LPS and IFN-γ on the adherence of alveolar macrophages to the SV40T2 monolayer. The adherence of alveolar macrophages to the SV40T2 monolayer was increased dose-dependently by LPS, while IFN-γ did not change the adherence. Figure 4 shows that the adhesion of both control and LPS-stimulated macrophages to the SV40T2 monolayer was abrogated in the presence of anti-CD18 and partially inhibited by anti-CD11a (Fig. 4c, d). However, anti-CD11b did not inhibit the adherence of macrophages to SV40T2. It is of interest that anti-CD18 also inhibited non-specific (the plastic substrate) adhesion of control (Fig. 4a) and LPS-stimulated alveolar macrophages (Fig. 4b).

Figure 5 shows inversed photomicrographs of the alveolar macrophage–SV40T2 co-culture system in the presence (Fig. 5b–e) or absence of LPS (Fig. 5a) with (Fig. 5c, anti-CD11a; Fig. 5d, anti-CD11b; Fig. 5e, anti-CD18) or without antibodies (Fig. 5a, b). It appeared that those antibodies did not modulate the destruction of the SV40T2 monolayer, although anti-CD11b and -CD18 inhibited adhesion of LPS-stimulated macrophages to SV40T2 (Fig. 4). Table 1 shows the effects of antibodies on DNA fragmentation of SV40T2 caused by LPS-stimulated macrophages. These antibodies again did not reduce the activated macrophage-mediated DNA fragmentation of SV40T2.

To exclude the possibility that the ligation of β_2 integrin enhances NO production and the enhanced NO production may compensate for the loose contact or the reduced effective-

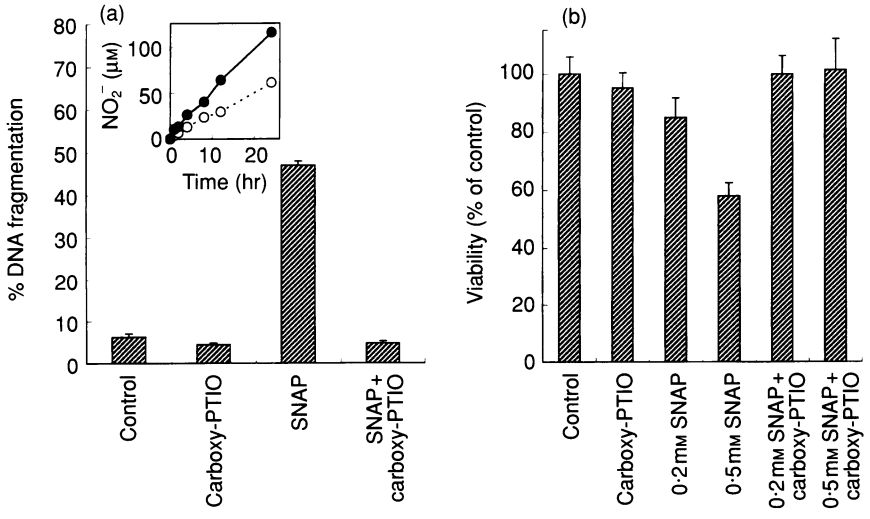

Figure 1. Changes in DNA fragmentation (a) and the viability of SV40T2 (b) in the presence or absence of SNAP with or without carboxy-PTIO. The spontaneous release of NO_2^- from SNAP in the culture medium is shown in the inset of Fig. 1(a) (○, 0·2 mM SNAP; ●, 0·5 mM SNAP). The SV40T2 monolayer was cultured in EMEM containing 0, 0·2 and 0·5 mM SNAP, for 24 hr. Carboxy-PTIO was added twice to a final concentration of 0·2 mM.

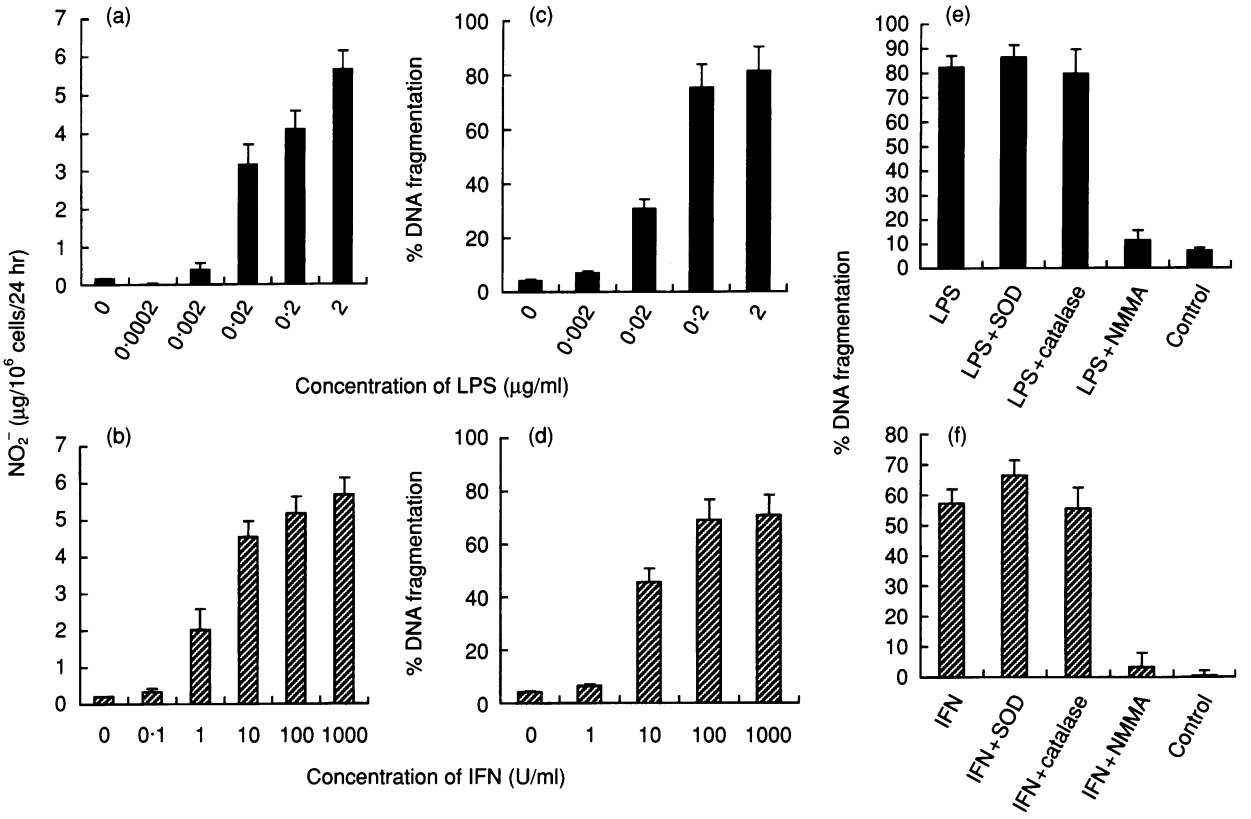

Figure 2. Dose-dependent changes in the production of NO_2^- by LPS- (a) and IFN-γ-stimulated alveolar macrophages (b), dose-dependent DNA fragmentation of SV40T2 caused by LPS- (c) and IFN-γ-stimulated macrophages (d), and effects of SOD, catalase and NMMA on the DNA fragmentation of SV40T2 caused by alveolar macrophages stimulated with 2 μg/ml LPS (e) and 1000 U/ml IFN-γ (f). Rat alveolar macrophages were cultured in the presence or absence of LPS and IFN-γ for 24 hr, and the NO_2^- concentration in the culture medium was measured by Griess reaction (a and b). The alveolar macrophage–SV40T2 co-culture was cultured for 24 hr in the presence of LPS (c and e) and IFN-γ (d and f). SOD, catalase and NMMA were added to final concentrations of 1000 U/ml, 1000 U/ml and 10 mM, respectively, to investigate effects of these inhibitors on DNA fragmentation of SV40T2 caused by activated alveolar macrophages (e and f).

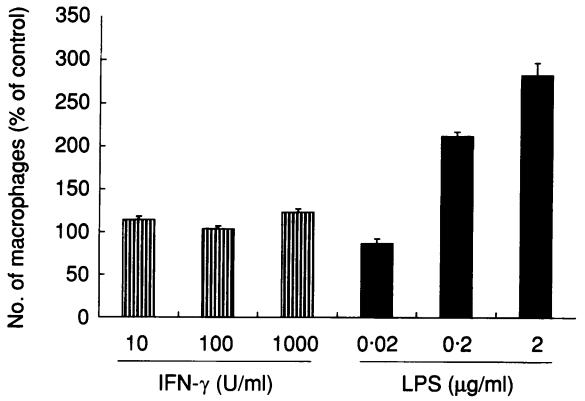

Figure 3. Effects of LPS and IFN-γ on the adherence of alveolar macrophages to the SV40T2 monolayer. Alveolar macrophages were cultured on the SV40T2 monolayer for 8 hr, and further cultured for 0·5 hr in the presence or absence of LPS (2 μg/ml) or IFN-γ (1000 U/ml). The adhesion assay was performed using the cytocentrifuge methods as described in the Materials and Methods. The number of alveolar macrophages that remained on the plastic surface or SV40T2 monolayer in nine high power fields was counted using a photomicrograph (×400).

ness of NO, the NO_2^- production of LPS-stimulated (2 μg/ml) alveolar macrophages was measured in the presence or absence of anti-CD18 (20 μg/ml). The presence of anti-CD18 in the medium slightly, but significantly ($P<0·01$), reduced NO_2^- production by LPS-stimulated alveolar macrophages (without anti-CD18, $5·90\pm0·099$ μg $NO_2^-/10^6$ cells/24 hr; with anti-CD18, $4·62\pm0·135$ μg $NO_2^-/10^6$ cells/24 hr).

DISCUSSION

It has been demonstrated that intercellular adhesion plays an important role in tumoricidal activity of cytotoxic T cells, lymphkine-activated lymphocytes, and NK cells.[21–23] However, very little is known about the role of intercellular adhesion in monocyte- or macrophage-mediated tumour-cell killing. On the other hand, although NO has been demonstrated to be an important mediator of the cytotoxic effects of macrophages, it is not clear whether other mediators, such as ROI and peroxynitrite, are involved in the cytotoxicity. The present study addressed both the requirement of intercellular adhesion and effective molecules in activated macrophage-mediated killing of SV40-transformed type II pneumocytes.

Figure 1 shows that SNAP caused DNA fragmentation of SV40T2, and decreased the viability of these cells. The DNA

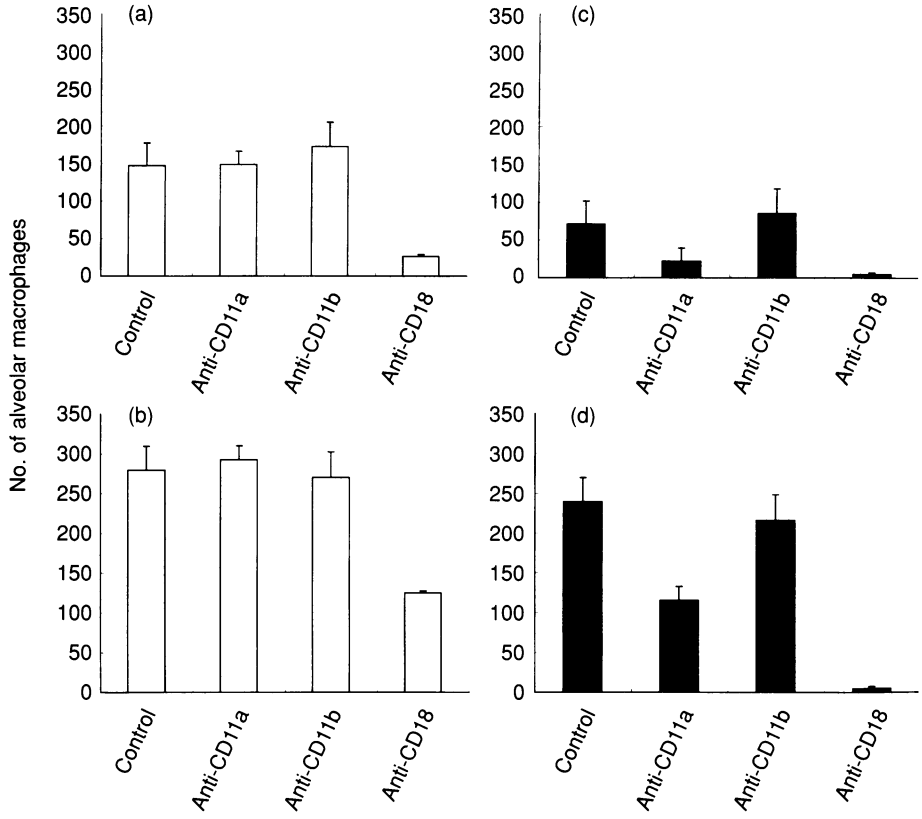

Figure 4. Effects of anti-integrin antibodies on the adherence of alveolar macrophages to the plastic substrate (a and b) or SV40T2 monolayer (c and d) in the presence (b and d) or absence (a and c) of LPS (2 μg/ml). Alveolar macrophages were incubated with 100 μg/ml of each antibody for 2 hr on ice, and cultuered on the SV40T2 monolayer or in the plastic dish for 8 hr. LPS was added to a final concentration of 2 μg/ml and the alveolar macrophage–SV40T2 co-culture was further cultured for an addtional 0·5 hr. The adhesion assay was performed using the cytocentrifuge method as described in the Materials and Methods. The number of alveolar macrophages that remained on the plastic surface or SV40T2 monolayer in nine high power fileds was counted using a photomicrograph (×400).

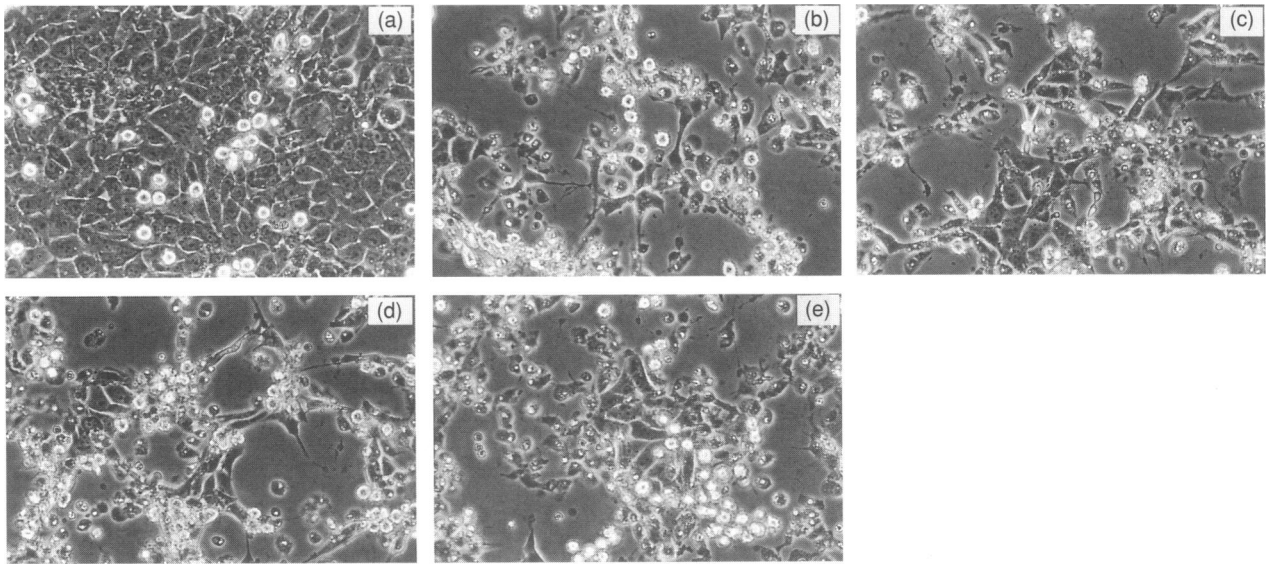

Figure 5. Inverted photomicrographs of the alveolar macrophage–SV40T2 co-culture system cultured with or without LPS (2 μg/ml) in the presence or absence of anti-integrin antibodies. Alveolar macrophages were incubated with 100 μg/ml of anti-integrin antibodies for 2 hr on ice, and cultuered on the SV40T2 monolayer with 20 μg/ml of each antibody for 8 hr. LPS was added to a final concentration of 2 μg/ml and the alveolar macrophage–SV40T2 co-culture system was incubated for an addtional 20 hr. (a) Control; (b) LPS; (c) LPS + anti-CD11a; (d) LPS + anti-CD11b; (e) LPS + anti-CD18.

Table 1. Effects of anti-integrin antibodies on DNA fragmentation of SV40T2 caused by LPS-stimulated rat alveolar macrophages

	Control	LPS	LPS + anti-CD11a	LPS + anti-CD11b	LPS + anti-CD18
DNA fragmentation mean ± SEM (%)	6·6 ± 0·74	80·6 ± 8·9	90·0 ± 9·9	72·7 ± 8·0	70·0 ± 7·7

fragmentation and viability loss were inhibited by carboxy-PTIO, a NO radical scavenger, suggesting that SV40T2 cells are susceptible to NO. LPS and IFN-γ are known to stimulate murine macrophages to produce NO.[4,6,12] Figure 2a, b also shows that rat alveolar macrophages produce NO in response to both LPS and IFN-γ in a dose-dependent manner. The production of NO was correlated with DNA fragmentation of SV40T2 (Fig. 2c, d). It has been reported that NO reacted with superoxide, yielding peroxynitrite,[28] which may be more toxic than NO for certain cells.[18] Figure 2e, f shows that the DNA fragmentation of SV40T2 caused by activated alveolar macrophages was abrogated in the presence of NMMA, while SOD and catalase did not reduce the DNA fragmentation (Fig. 2e, f). These results suggest that ROI does not play an important role, if any, in macrophage-mediated DNA fragmentation of SV40T2.

It has been reported that LPS and phorbol myristate acetate (PMA) up-regulated adhesion molecules of rat alveolar macrophages such as lymphocyte function-associated antigen (LFA-1) and intercellular adhesion molecule (ICAM-1),[29] and increased macrophage adherence to the plastic substrate[30] and laminin.[31] However, to the best of the author's knowledge, it has not been reported whether intercellular adhesion is required for activated macrophages to exert the maximum cytotoxicity. Figure 3 shows that LPS increased adhesion of macrophages to the SV40T2 monolayer, while the adherence was not changed by IFN-γ, although both LPS and IFN-γ promoted NO production of alveolar macrophages to the same extent (Fig. 2). The adhesion of control and LPS-stimulated alveolar macrophages to the SV40T2 monolayer was inhibited in the presence of anti-CD11a and anti-CD18 antibodies (Fig. 4c, d), suggesting that alveolar macrophages adhere to SV40T2 cells via LFA-1. However, the destruction of the SV40T2 monolayer and DNA fragmentation of SV40T2 were not reduced in the presence of those antibodies (Fig. 5 and Table 1). Moreover, the presence of anti-CD18 in the medium slightly reduced NO_2^- production of LPS-stimulated alveolar macrophages. These results indicate that NO-mediated killing of SV40T2 caused by activated alveolar macrophages is independent of the β_2 integrin-mediated intercellular adhesion. Probably the diffusion of NO from alveolar macrophages to the target cells is dominant in the killing of SV40T2 by activated macrophages, and the greater adherence is not necessary for the cytotoxic effects of activated macrophages.

An ancillary but interesting finding is that LPS increased the adherence of alveolar macrophages to the plastic substrate as well as to the SV40T2 monolayer, and anti-CD18 also inhibited this non-specific adhesion to the plastic. Rosen & Gordon[32] reported that the murine type 3 complement receptor inhibited adhesion of myelomonocytic cells to the plastic dish. Thus, it is conceivable that rat alveolar macrophages adhere to the plastic substrate, at least in part, using β_2 integrins.

In summary, LPS- and IFN-γ-stimulated rat alveolar macrophages produced NO. DNA fragmentation of SV40T2 cells by the activated macrophages appeared to be caused predominantly by NO. The β_2 integrin-mediated intercellular adhesion played a minimal role, if any, in alveolar macrophage-mediated DNA fragmentation of SV40T2.

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