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Nitric Oxide Production From a Macrophage Cell Line: Interaction With Autologous and Allogeneic Lymphocytes

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Abstract The indirect stimulation of macrophages to produce nitrite was examined by using the macrophage cell line J774. J774 spontaneously produced nitrite, when cultured at high concentration. J774 cultured in low concentration ($< 10^4$ cells in 100 μl) barely produced nitrite. J774 cultured in low concentration produced a large amount of nitrite by the co-culture of nonadherent spleen cells or nonadherent peritoneal exudate cells, which were stimulated with con A, anti-CD3, or staphylococcal enterotoxin A. J774 (BALB/c derived: H-2^d) cultured with either syngeneic (BALB/c) or allogeneic (B6; H-2^b B10BR; H-2^k) nonadherent lymphocytes, which were stimulated with conA or anti-CD3, produced nitric oxide. However, J774 produced nitric oxide by stimulation with SEA only when co-cultured with SEA-reactive T lymphocytes. Peritoneal exudate cells from mice, which did not proliferate by the stimulation of conA or anti-CD3, proliferated well by the addition of L-arginine homologue, N^G-monomethyl-L-arginine. The proliferation of nonadherent peritoneal exudate cells stimulated with conA or anti-CD3 was suppressed by the addition of peritoneal macrophages. This suppression was abolished by the addition of N^G-monomethyl-L-arginine.

Key words: nitric oxide, macrophage cell line, T lymphocyte stimulation, feedback suppression, inflamation site

Macrophages have been shown to regulate lymphocyte activity in contradictory ways. One is to support the T cell–mediated immune responses by antigen processing and presentation and by secretion of soluble mediators [Rosenthal and Shevack, 1973; Unanue and Allen, 1987]. Another is to suppress lymphocyte proliferation by releasing hydrogen peroxide, prostaglandins, and other suppressive mediators [Allison, 1978; Kung et al., 1977; Metzger et al., 1980]. It has been shown that activated macrophages produce reactive radical nitric oxide (NO), during metabolism of L-arginine to NO_2^-/NO_3^- [Hibbs et al., 1987; Stuehr et al., 1989], and its production is blocked by structural analogues such as N^G-monomethyl-L-arginine (N-MMA) [Hibbs et al., 1987]. Recently, nitrogen oxide biosynthesis has also been demonstrated in endothelial cells [Palmer et al., 1987], brain cells [Garthwaite et al., 1988; Knowles et al., 1989], and others [Nathan and Stuehr, 1990]. Nitric oxide synthases have been purified and cloned [Bredt et al., 1991; Bredt and Snyder, 1990] and been divided in two types from biological characteristics. One is produced constitutively from endothelial cells or cerebellar cells. Another is produced inducibly from macrophages [Nathan and Stuehr, 1990]. The reactive nitrogen intermediates from macrophages have potent cytostatic and anti-proliferative effects on tumors, parasites, bacteria, and lymphocytes [Albina et al., 1991; Liew and Cox, 1991; Stuehr and Nathan, 1989; Tarr, 1941].

Our recent observation [Isobe and Nakashima, 1992; Kawabe et al., 1992] and others [Albina et al., 1991; Hoffman et al., 1990] have provided evidences that the mode of the macrophage-mediated suppression of T cell proliferative responses to concanavalin A (conA), staphylococcal enterotoxin A (SEA), or staphylococcal enterotoxin B (SEB) involves NO release by activated macrophages. We showed that the spleen T lymphocytes, which were stimulated with SEA or SEB, activated macrophages. Then the activated macrophages in turn suppress T cell proliferation by releasing NO. We hypothesized that this feedback suppression might work at inflamation sites. In order to investigate the

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cell types more precisely which were engaged in the feedback suppression, we set up the co-culture experiments of macrophage cell lines and immune lymphocytes including nonadherent cells in peritoneal cavity.

MATERIALS AND METHODS

Macrophage Cell Lines

Murine macrophage cell line J774 was obtained from Onozaki, Faculty of Pharmacology, Nagoya City University, Nagoya, Japan.

Animals

BALB/c, B10BR, C57BL/6(B6) mice were obtained from the Shizuoka Agriculture Co. (Shizuoka, Japan) and maintained in the Institute for Laboratory Animal Research, Nagoya University School of Medicine. The animals were kept on a 12-h light-dark cycle in a temperature- and humidity-controlled environment.

Reagents

Staphylococcal enterotoxin A (SEA) (Serva, Heidelberg), N^G-monometyl-L-arginine (N-MMA) (Calbiochem-Behring Corp., La Jolla, CA), concanavalin A (conA) (Pharmacia), Lipopolysaccharides (LPS) (from *E. coli;* Sigma), and anti-CD3 (145-2C11) [Leo et al., 1987] were used in this experiment. Human recombinant IFN-γ was donated by Shionogi Corp. (Osaka, Japan).

Preparation of Spleen Cells and Peritoneal Exdate Cells

Single-cell suspensions of spleen cells were prepared by gently rubbing the spleen against a sterile stainless steel wire screen. The peritoneal exudate cells (PEC) were taken from mouse peritoneal cavity by infusion of MEM. The cells were washed and resuspended in complete medium [Kawabe et al., 1992]. These cell suspensions were incubated in plastic culture dishes for 12 h at 37°C in a humidified atmosphere in a 5% CO_2 incubator, and then nonadherent spleen cells or nonadrerent PEC were collected by pipetting. After taking nonadrerent PEC, residual cells were repeatedly washed, and peritoneal macrophages (Mø) were obtained by treating the plate with 0.05% EDTA and washed with MEM for use as a macrophage source.

Cell Proliferation Assay

Nonadherent spleen cells or nonadherent PEC (5×10^5/well) were dispensed in triplicate into 96 wells with or without J774 or peritoneal Mø (10^5 cells/well) in 200 μl complete medium with stimulating agents. These cell cultures were incubated for 2 days in a humidified atmosphere at 37°C in a 5% CO_2 incubator. Eight hours before the termination of the cell culture, 0.5 μCi [^{3}H]thymidine was added to all wells. Cultures were collected with a semiautomatic cell harvester and counted in a liquid scintillation counter.

Nitrite Concentration

Nitrite in culture supernatants was measured by the method described [Ding et al., 1988]. Briefly, 50 μl of supernatants were removed from the 96-well plates and incubated in triplicate with an equal volume of Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 550 nm was determined with a microplate reader. NO_2^- concentration was determined by using sodium nitrite as a standard.

RESULTS

Nitrite Production Was Dependent on J774 Cell Concentration

We analyzed the mode of the nitrite accumulation in the macrophage cell line. First, we cultured various numbers of J774 cells without stimulation or in the presence of low concentration of IFN-γ. As shown in Figure 1, nitrite accumulation was increased by the increase of J774 cell numbers per well. Cultures of 10^4 or fewer J774 cells per well barely accumulated nitrite at a level higher than that in medium alone. IFN-γ enhanced the production of nitrite from J774 cells.

Nonadherent Spleen Cells, Which Are Activated with ConA, Anti-CD3, or SEA, Stimulate J774 Cells to Produce Nitrite

J774 cells were co-cultured with or without BALB/c-, B6-, and B10BR-derived nonadherent spleen cells which were stimulated by conA, LPS, or anti-CD3. J774 cells cultured without nonadherent spleen cells produced nitrite only when stimulated with LPS. J774 cells with nonadherent spleen cells produced large amount of nitrite by *the* stimulation of anti-CD3 or conA. The production of nitrite in these cultures was abrogated by the addition of 0.5 mM N-MMA

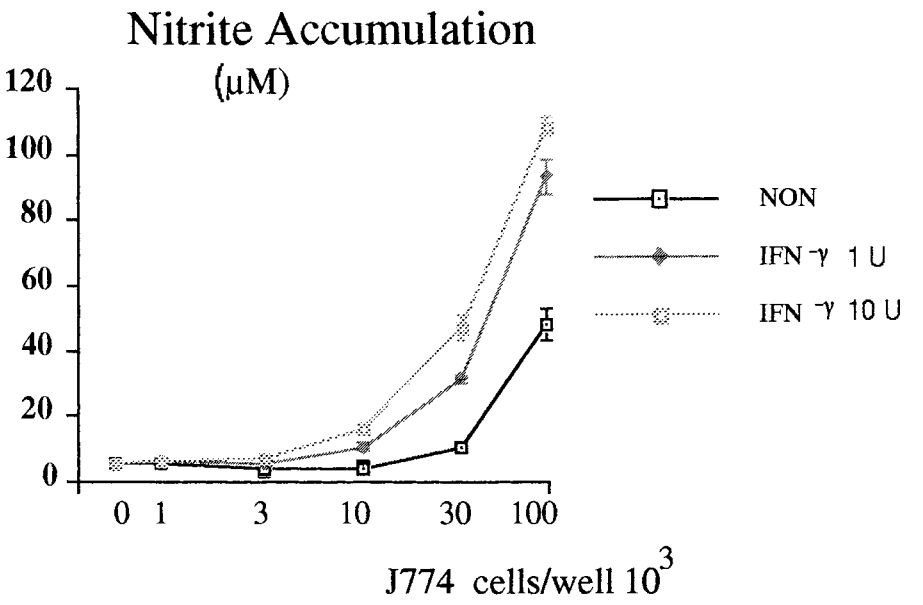

Fig. 1. High concentration of J774 cells spontaneously produced nitrite. Various concentration of J774 cells in 100 μl of RPMI-1640 plus 10% FCS were cultured in 96-well plates with or without recombinant IFN-γ. Nitrite accumulation was measured at 2 days culture as described in Materials and Methods. The values were the means from three replicated experiments, and error bars indicate standard deviations.

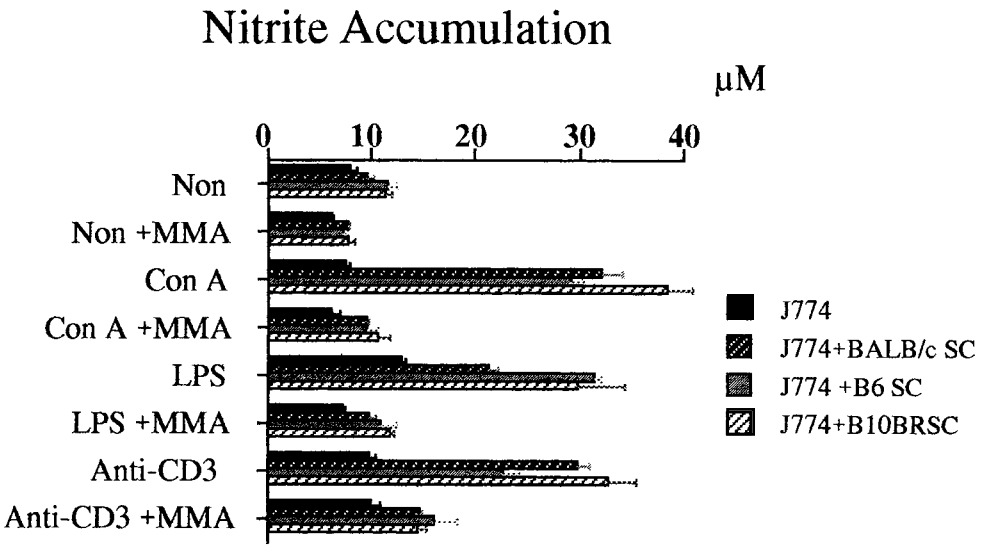

Fig. 2. Co-culture of J774 and nonadherent spleen cells, which were stimulated with conA, LPS, or anti-CD3, produced nitrite. J774 cells (10^4 cells/well) were cultured with or without BALB/c, B6, or B10BR nonadherent spleen cells (10^5 cells/well). They were stimulated with conA (5 μg/ml), LPS (5 μg/ml), or anti-CD3 (10 μl culture supernatant/well). N-MMA (0.5 mM) was added at the start of culture. Nitrite accumulation was measured at 2 days culture as described in Materials and Methods. The values were the means from three replicated experiments, and error bars indicate standard deviations.

(Fig. 2). Any concentration of nonadherent spleen cells cultured alone did not produce nitrite [Isobe and Nakashima, 1992]. Next, we stimulated the co-culture of J774 and nonadherent spleen cells *by* SEA. As shown in Figure 3, J774 cells produced a larger amount of nitrite by the stimulation of SEA, when co-cultured with B10 or B10BR nonadherent spleen cells. However, by the co-culture with BALB/c, J774 cells produced only small amount of nitrite. The production of nitrite in these cultures was also abrogated by the addition of 0.5 mM N-MMA.

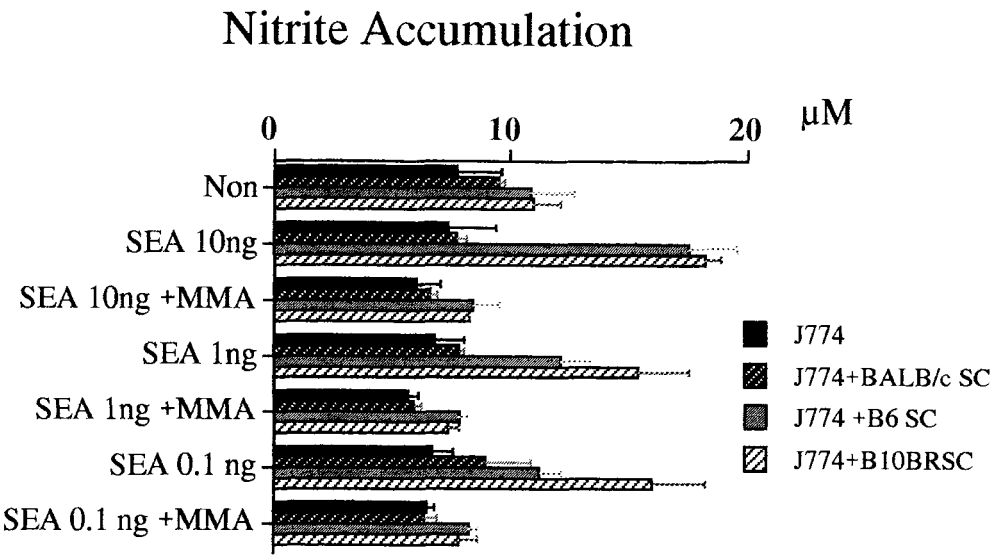

Fig. 3. Co-culture of J774 and nonadherent spleen cells, which were stimulated with SEA, produced nitrite. J774 cells (10^4 cells/well) were cultured with or without BALB/c, B6, or B10BR nonadherent spleen cells (10^5 cells/well). They were stimulated with SEA (0.1–10 ng/ml). Nitrite accumulation was measured at 2 days culture as described in Materials and Methods. The values were the means from three replicated experiments, and error bars indicate standard deviations.

Suppression of T Cell Proliferation in PEC by NO Produced From Murine PM

In order to examine the feedback suppression, which might well occur in a macrophage-rich peritoneal cavity, we tested the effect on the response of nonadherent PEC rather than nonadherent spleen cells. First, we stimulated PEC with conA or anti-CD3. T cell proliferation was not detected at any dose and duration of culture, when whole PEC were stimulated with conA or anti-CD3. However, by the addition of 0.5 mM N-MMA, a high level of T lymphocyte proliferation was observed. A large amount of NO was detected in the supernatant of the same whole PEC culture, and the addition of 0.5 mM of N-MMA reduced it (Fig. 4). Next, we separated nonadherent PEC by culturing whole PEC overnight. These nonadherent PEC proliferated well by the stimulation of conA or anti-CD3. On the other hand, nonadherent PEC cultured with PEC macrophages did not proliferate by the stimulation of conA or anti-CD3 and produced a large amount of NO. Addition of 0.5 mM N-MMA reversed the proliferation of nonadherent PEC and reduced the NO production (Fig. 5). In order to clarify that macrophages really produce NO by the co-culture with stimulated nonadherent PEC, we used the J774 macrophage cell line. J774 cells were co-cultured with or without BALB/c nonadherent PEC stimulated by anti-CD3 or conA. As shown in Figure 6, J774 cells cultured without nonadherent PEC did not produce nitrite. However, J774 cells cultured with nonadherent PEC produced a large amount of nitrite by stimulation of anti-CD3 or conA, and this production of nitrite was abrogated by the addition of 0.5 mM N-MMA. Any concentration of nonadherent PEC cultured alone did not produce nitrite (data not shown).

DISCUSSION

In previous reports, we showed that T cell proliferation of rat or mouse spleen cells stimulated with conA, SEA, or SEB was suppressed by macrophages via a nitric oxide synthase pathway [Isobe and Nakashima, 1992]. Our hypothesis was that the macrophage-mediated suppression was one of feedback suppression, which might occur in vivo. The macrophages, which we used in previous studies, might be contaminated with small amount of other cells, which were responsive for the phenomena observed. To rule out this possibility, here we used the macrophage cell line J774 for study of the mechanism of nitrite production. Results presented here support our previous conclusion.

We measured nitrite accumulation in this report, because NO was shown to be quickly converted to nitrite. Without stimulation, J774 cells produced NO in culture supernatant only when cultured at high concentration. Low concentration of J774 cells produced NO by direct stimula-

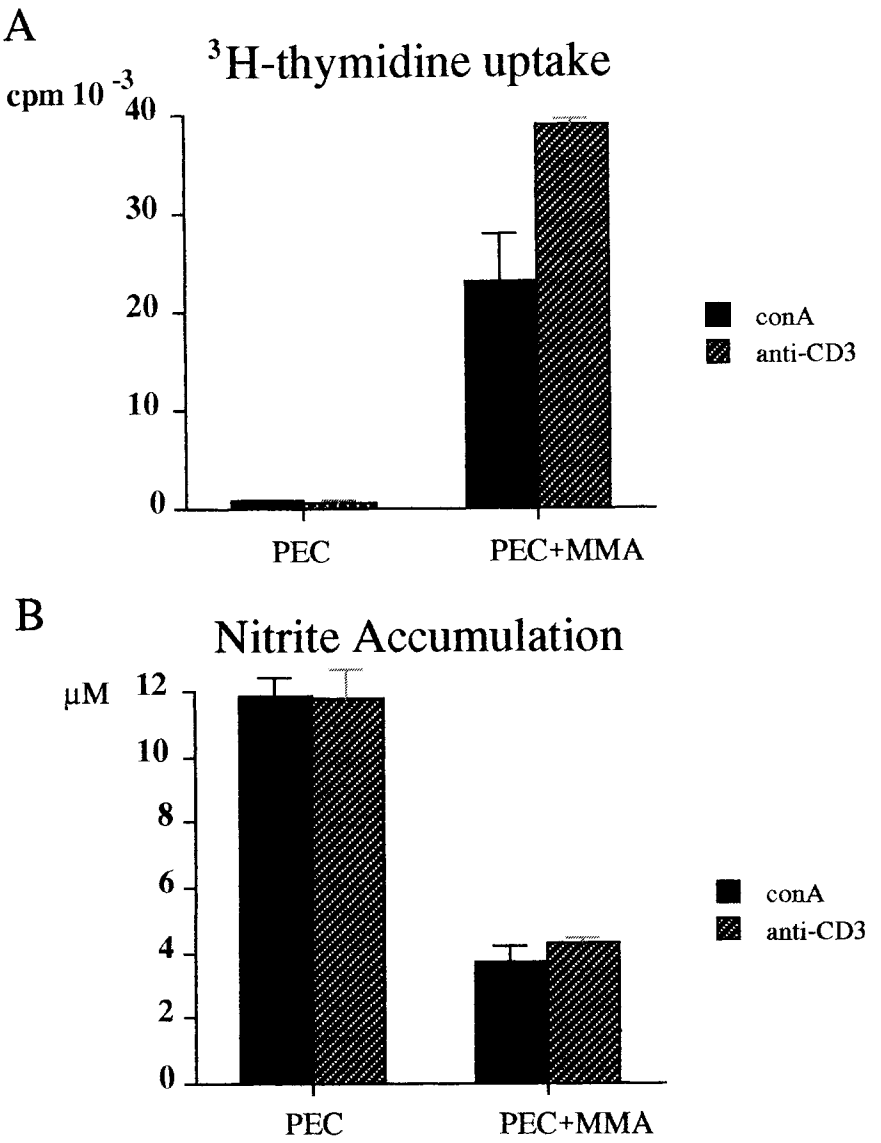

Fig. 4. Addition of N-MMA to PEC culture induced T cell proliferation. C57BL/6 PEC (2×10^5 cells/well) were stimulated with 5 μg/ml of conA or 10% anti-CD3, or at the initiation of culture 0.5 mM of N-MMA was added. The incorporation of ^{3}H-thymidine into DNA was determined in 48 h cultures (**A**). Nitrite accumulation was measured by the supernatant of the same culture as described in Materials and Methods (**B**). The values were the means from three replicated experiments, and error bars indicate standard deviation.

tion of IFN-γ or LPS as shown in many other reports [Albina et al., 1991; Hibbs et al., 1987; Stuehr and Nathan, 1989]. On the other hand, J774 cells at low concentration (10^4 cells/well) did not produce NO by direct stimulation of conA, anti-CD3, or SEA. However, they produced a large amount of NO, when co-cultured with nonadherent spleen cells stimulated with conA or anti-CD3. In the case of superantigen (SEA), NO production was variable among spleen cells from various strains of mice. BA1B/c strain-derived nonadherent spleen cells stimulated with SEA did not stimulate J774 cells to produce NO, although B6 or B10BR strain-derived nonadherent spleen cells, which were stimulated with SEA, stimulated J774 cells to produce large amount of NO. This variance might be due to the inability of SEA to stimulate BALB/c lymphocytes. Superantigen was shown to selectively stimulate T cells bearing specific Vβ TCR [Kappler et al., 1989; White et al., 1989].

The peritoneal cavity has a high number of macrophages, which should vary in inflamation. We found that whole PEC did not proliferate

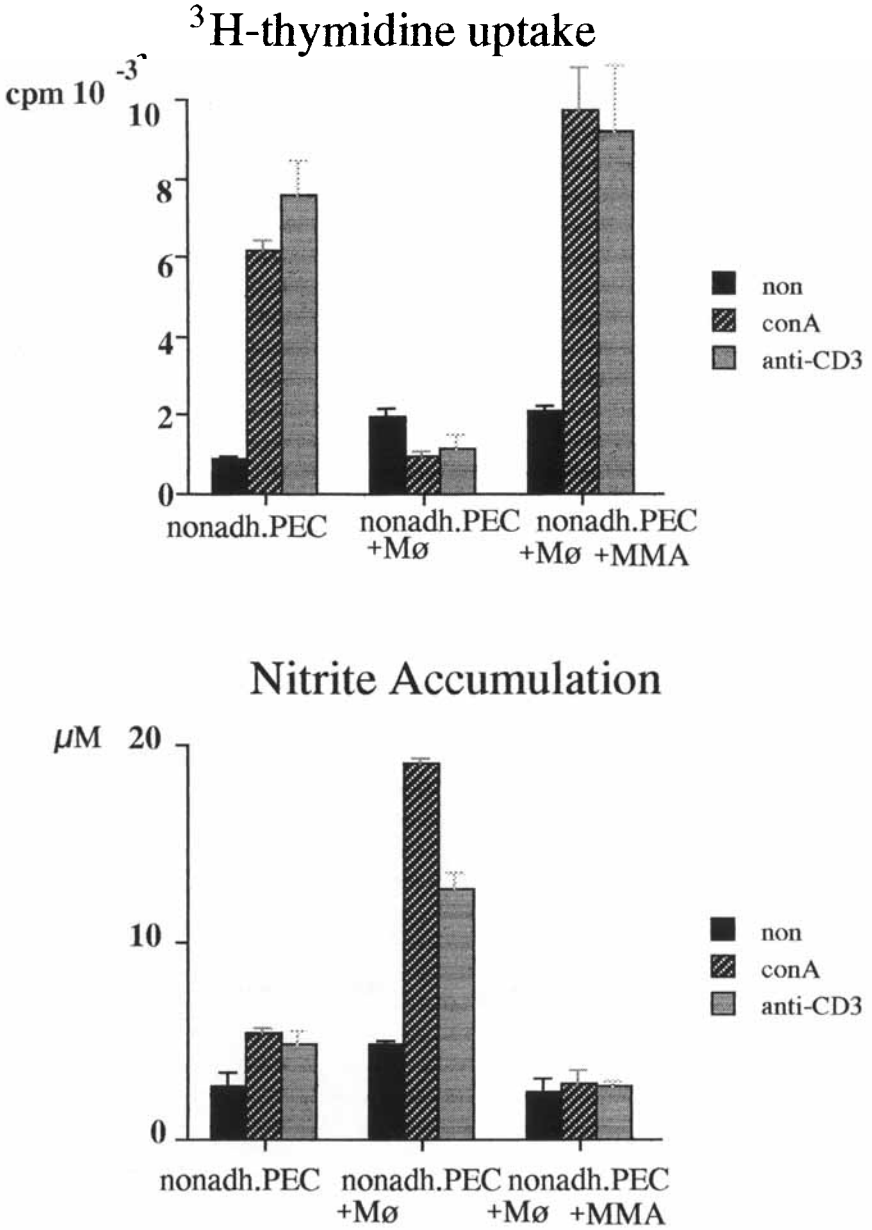

Fig. 5. Suppression of T cell proliferation and NO production in co-culture of nonadherent PEC and macrophages. C57BL/6 nonadherent PEC (2×10^5 cells/well) were co-cultured with peritoneal macrophages (Mø) (10^5 cells/well) stimulated with 5 μg/ml conA or 10% CD3. The incorporation of ^{3}H-thymidine into DNA was determined in 48 h cultures (**top**). Nitrite accumulation was measured by the supernatant of the same culture as described in Materials and Methods (**bottom**). The values were the means from three replicated experiments, and error bars indicate standard deviation.

detectably by the stimulation of conA or anti-CD3. Interestingly, when 0.5 mM N-MMA was added to the culture, PEC proliferated well by the same stimulation. These results partially correspond to those of Hoffman et al. [1990], who showed that rat spleen cells without removal of macrophages did not proliferate by the stimulation of PHA, but proliferated in the culture added with N-MMA. In the present study, we tried to identify more carefully the role of macrophages in the observed phenomena by testing PEC, which mainly consist of macrophages and lymphocytes. We collected the nonadherent PEC after 12 h of culture in RPMI-1640 plus 10% FCS. This was because the nonadherent PEC obtained by short duration of culture (2 h) were contaminated with PEC macrophages, which produced NO by stimulation of conA or

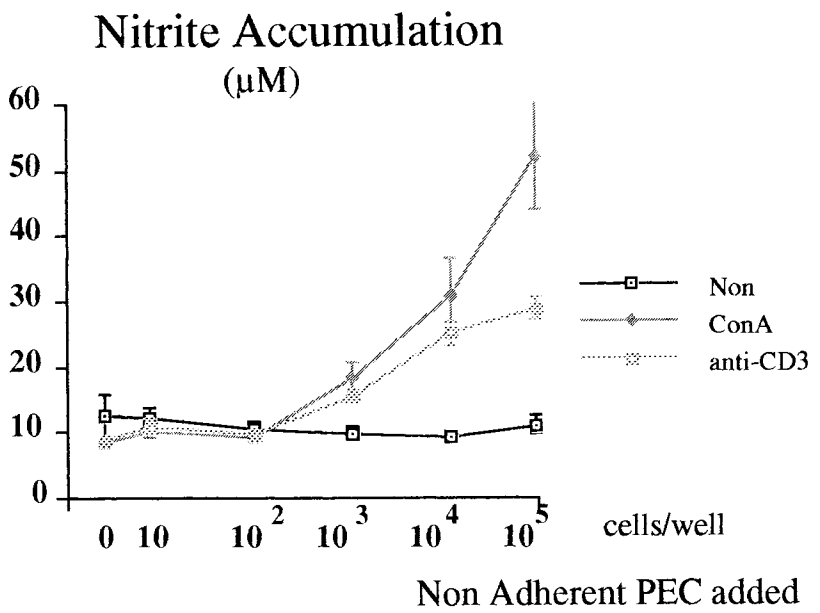

Fig. 6. Co-culture of J774 and nonadherent PEC, which were stimulated with conA or CD3, produce nitrite. J774 cells (10^4 cells/well) were cultured with or without BALB/c-derived nonadherent PEC (10^5 cells/well). They were stimulated with conA (5 μg/ml) or anti-CD3 (10 μl culture supernatant/well). Nitrite accumulation was measured at 2 days culture as described in Materials and Methods. The values were the means from three replicated experiments, and error bars indicate standard deviations.

anti-CD3 (data not shown). We further extended our study on analysis of the interaction of nonadherent PEC and J774 cells and finally confirmed the involvement of the macrophage cell type in this event. In contrast to the early events of T lymphocyte signal transduction, which start within a few minutes [Finkel et al., 1987], NO production from macrophages appears at a later time. Expression of nitric oxide synthase specific mRNA was first detected at 2 h and peaked at 6 h, when RAW 264 macrophage cell line was stimulated with LPS [Lowenstein et al., 1992]. NO accumulation in our system was initially detected at 24 h and reached maximum at 48 h, when nonadherent PEC and J774 cells were co-cultured and stimulated by anti-CD3 or conA (data not shown). Taken together, we hypothesize that T lymphocytes are activated by antigens or other biologically active products of microorganisms to proliferate and secrete factors, which in turn activate macrophages for feedback suppression of T cell proliferation at a later stage through delayed but progressive NO secreation.

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