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Interferon-γ-Dependent Expression of Inducible Nitric Oxide Synthase, Interleukin-12, and Interferon-γ-Inducing Factor in Macrophages Elicited by Allografted Tumor Cells

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We have examined the mechanisms of activation of macrophages (Møs) induced by *i.p.* allografted Meth A tumor cells (Meth A-Møs) during the rejection of the cells by C57BL/6 mice. Inducible nitric oxide (NO) synthase (iNOS), interleukin-12 (IL-12), and interferon-γ (IFN-γ)-inducing factor (IGIF) were transiently expressed in Meth A-Møs during the rejection. The expression was impaired in mice in which the gene encoding IFN-γ had been disrupted (IFN-γ $-/-$). *In vitro* studies showed that Meth A-Møs from IFN-γ $+/+$ mice induced an apoptotic type of cell death in P815 cells, without cell-to-cell contact, in an NO-dependent manner, whereas Meth A-Møs from IFN-γ $-/-$ mice could not lyse these cells. The iNOS, IL-12, and IGIF expression was also impaired in bacteria-activated Møs from IFN-γ $-/-$ mice, indicating that IFN-γ, but not IGIF, would be the initial signal that leads to the activation of Møs *in vivo.*

Macrophages (Møs) play a crucial role in the defense against tumors and parasites (1). Mø activation has been defined as a response of Møs to the stimulation by Mø-activating factors including IFN-γ; and the response includes the production of cytokines and cytotoxicity towards tumor cells and parasites. One of the main cytokines produced by activated Møs is IL-12, which is the determining factor in the development of the T cell response (2). L-Arginine-derived nitric oxide (NO), a soluble cytotoxic molecule, is also produced in activated Møs by inducible NO synthase (iNOS) (3, 4). Several reports have shown that *in vitro*-activated Møs kill some tumor cells (5-8) and bacteria (9,10) by an NO-dependent mechanism that can also work in the absence of cell-to-cell contact (5, 7). The antimicrobial activity of NO has been reaffirmed with the generation of iNOS mutant mice, which animals show altered responses to bacterial infection (11, 12). In addition, a new cytokine, IFN-γ-inducing factor (IGIF), which induces IFN-γ production in T cells, was recently reported to be expressed in activated Møs (13). IL-12 primes Møs for NO production *in vivo* (14), whereas the IL-12 p40 gene promoter is primed by IFN-γ in monocytic cells (15). These findings imply that the signals for Mø activation might be initiated by IGIF and followed by IFN-γ, IL-12, and iNOS expression.

Møs are the major population of cells infiltrating into the rejection site in skin or organ allografts, and may play a role in the process of allograft destruction (16). The obstacle to study of the mechanisms of allograft rejection seems to reside in the technical difficulty in

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Abbreviations: Mø, macrophage; Meth A-Møs, Møs induced by Meth A tumor cells; NO, nitric oxide; iNOS, inducible nitric oxide synthase; IL-12, interleukin-12; IFN-γ, interferon-γ; IGIF, interferon-γ-inducing factor; Meth A, 3-methylcholanthrene-induced ascites type fibrosarcoma; BCG, bacillus Calmett-Guérin; N-MMA, N^G-monomethyl-L-arginine; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction.

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harvesting host cells infiltrating into the grafts. Therefore, we have developed a model system in which 3-methylcholanthrene-induced ascites type fibrosarcoma (Meth A) was transplanted into the peritoneal cavity of allogeneic C57BL/6 mouse. Since Meth A cells are an ascites type, the advantage of this system is that we can obtain almost all of the cells infiltrating into the rejection site simply by lavage of the peritoneal cavity (17-21). In this system, we found that Meth A-Møs were the major cytotoxic cells against Meth A tumor cells *in vitro* (19) and *in vivo* (21). Here, to evaluate the level of Meth A-Mø activation, we have measured the time-dependent expression of iNOS, IL-12, and IGIF in Meth A-Møs in C57BL/6 mice during the rejection of the tumor cells using resident-Møs as a control. We also have examined the mechanism of activation, using bacillus Calmett-Guérin (BCG)-Møs as a control, by measuring the expression of iNOS, IL-12, and IGIF mRNAs in Meth A-Møs from mice in which the gene encoding IFN-γ had been disrupted (IFN-γ $-/-$). The results revealed the Meth A-Møs to be a type of highly activated Møs whose activation was IFN-γ dependent, and suggested that IFN-γ induction at the rejection site preceded the expression of IGIF mRNA in the *in vivo* -activated Møs (*e.g.*, Meth A-Møs and BCG-Møs).

MATERIALS AND METHODS

Preparation of Møs. Møs were isolated from male, 7-12-week-old, specific pathogen-free C57BL/6 mice (Japan SLC, Hamamatsu, Japan) or 9-week-old C57BL/6 IFN-γ $-/-$ mice (Jackson Lab., Bar Harbor, ME). For Meth A-Mø isolation each mouse was injected *i.p.* with 3×10^6 Meth A cells, which were kindly provided by Dr. S. Muramatsu, Dept. of Zoology, Faculty of Science, Kyoto University, Japan. At various time intervals after the transplantation Meth A-Møs were isolated as described (17-21) with some modifications. After the leukocyte-rich fraction had been obtained, Møs were separated from it by a discontinuous density gradient of Percoll (Pharmacia, Uppsala, Sweden). Resident-Møs and BCG-Møs were collected by *i.p.* lavage of untreated mice and mice that had received an *i.p.* injection of 10^7 CFU of *Mycobacterium bovis* BCG per mouse 5 days earlier, respectively. The Møs thus obtained were washed twice with RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) containing 10% fetal calf serum (Cell culture Labs., Cleveland, OH) and antibiotics, and plated. The Møs were further purified by removing the non-adherent cells by washings with warm phosphate-buffered saline after 2 h of incubation at 37 °C in 5% CO_2. Adherent cells were Møs with >90% purity as judged by Mac-1 staining and by morphological criteria. For Western blot analysis and RNA isolation, the cells were seeded at 2×10^6 cells/ml in 9 cm-diameter dishes (Falcon, Lincoln Park, NJ); and for NO_2^- accumulation, ^{51}Cr release, and DNA fragmentation assays, the cells were seeded at 2×10^6 cells/well in the lower chamber of a Transwell 24-well plate (Costar, Cambridge, MA).

SDS-PAGE and Western blot. SDS-PAGE was carried out following Laemmli's method (22). After finishing the SDS-PAGE, proteins were transferred onto 0.2 μm pore-size nitrocellulose membranes (Schleicher & Schuell, Tokyo, Japan) in 25 m*M* Trisbuffer (pH 8.3) containing 192 m*M* glycine and 20% methanol. The membrane was blocked for 30 min at room temperature with 1% BSA in TBST buffer [25 m*M* Tris buffer (pH 7.5) containing 150 m*M* NaCl, 0.05% NaN_3, and 0.2 % Tween 20] at room temperature for 30 min. The membrane was then incubated with rabbit anti-mouse iNOS antibody (Transduction Laboratories, Lexington, KY) at a 1:1000 dilution for 1 h at room temperature or overnight at 4 °C. After having been washed with TBST buffer, the membrane was incubated for 1 h at room temperature with a 1:5000 dilution of donkey anti-rabbit IgG antibody conjugated to alkaline phosphatase (Chemicon, Temecula, CA), washed again, and developed.

NO_2^- accumulation. NO_2^- accumulation was assayed by the Griess reaction (23). An equal volume of Griess reagent was added to 50 μl of each culture supernatant, and the amounts of NO_2^- were estimated by reading the OD_{540nm} against a standard curve of $NaNO_2$.

^{51}Cr release assay. After removal of the non-adherent cells from the lower chamber of the Transwell, 800 μl of RPMI 1640 medium was added to the chamber, and prewetted Transwell inserts were placed in the wells. An inhibitor of iNOS, N^G-monomethyl-L-arginine (N-MMA; Calbiochem, La Jolla, CA), was also included when indicated. P815 cells were collected during the logarithmic phase of growth and 10^7 cells/ml were labeled in medium with 1 μCi/ml of $Na_2[^{51}Cr]O_4$ (DuPont New England Nuclear, Boston, MA) for 2 h at 37 °C. The cells were then extensively washed and 6.6×10^4 cells in 200 μl of medium were added onto the insert. After an 18-h incubation at 37 °C in 5% CO_2, aliquots of the supernatants were collected and counted for ^{51}Cr or frozen at −70 °C for later measurements of NO_2^- accumulation as described above. The percent specific ^{51}Cr release was calculated as follows: % specific ^{51}Cr release= [(Experimental cpm-Spontaneous cpm)/(Total cpm-Spontaneous cpm)] × 100. Total release of ^{51}Cr was determined by lysis with 0.5% Triton X-100. Spontaneous release was determined in the absence of Møs and was ≤ 25%.

DNA fragmentation. DNA fragmentation was assayed as described (5) with some modifications. P815 cells in the logarithmic phase of growth were labeled in medium with 0.04 μCi/ml [*methyl* ^3H] thymidine (DuPont New England

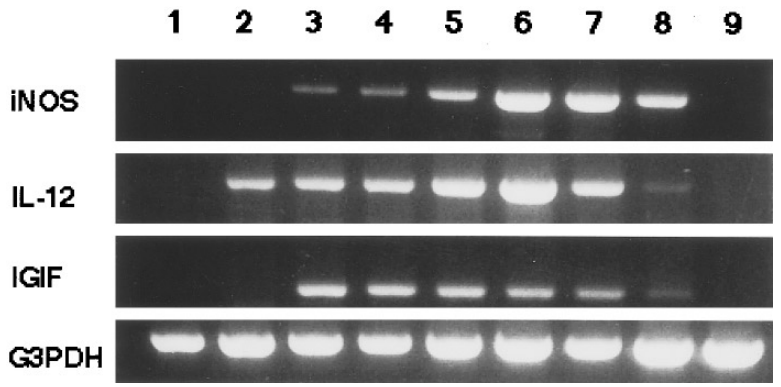

FIG. 1. Expression of iNOS, IL-12, and IGIF mRNAs in Meth A-Møs. The expression of iNOS, IL-12, IGIF, and G3PDH (as a control) mRNAs was estimated by RT-PCR. Lanes 1-9 correspond to 0 h (resident-Møs), 6 h, 1, 3, 5, 7, 11, 13 and 18 days, respectively, after Meth A transplantation.

Nuclear) for 24 h, and washed. The labeled P815 cells were added onto the insert of the Transwell under the same conditions described for ^{51}Cr release. After an 18-h incubation, the culture supernatants (M) were collected, centrifuged in a microfuge at maximal speed for 10 s, and saved. P815 cells contained in the Transwell insert were treated with ice-cold lysing buffer (25 m*M* sodium acetate, pH 6.6) for 1 h at 4 °C. DNA from these cells was separated by centrifugation in the microfuge at 14,000 × *g* for 15 min into fragmented low-molecular-weight (supernatant, S) and intact high-molecular-weight (pellet, P) fractions. Radioactivity was determined by a Hewlett Packard liquid scintillation counter. The DNA fragmentation was calculated as follows: % of specific DNA fragmentation= {[Experimental cpm (M+S)]/[Experimental cpm (M+S+P)]} − {[Spontaneous cpm (M+S)]/[Spontaneous cpm (M+S+P)]} × 100. Spontaneous release was determined in the absence of Møs and was < 20%.

DNA isolation and gel electrophoresis. Extraction of DNA was performed with a DNA isolation kit (Puregene, Minneapolis, MN). Electrophoresis was performed on a 2% agarose gel in the presence of 0.5 μg/ml ethidium bromide. A 123-base pair ladder (Gibco-BRL, Grand Island, NY) was included as a molecular weight marker.

RNA isolation, reverse transcription, and polymerase chain reaction (PCR). Total RNA was isolated from Møs by TRIzol reagent (Gibco BRL) in accordance with the supplier's instructions. The RNA was reverse-transcribed using a ''SuperScript'' kit (Gibco BRL) and amplified by PCR. The thermocycle conditions were 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, for denaturation, annealing, and extension, respectively. The primer sequences were as follows: 5′ CAAAGTCAAATCCTACCAAAGTGA-CCTG-3′ and 5′-TGCTACAGTTCCGAG-CGTCAAAGACCTG-3′ for NOS; 5′-CGT-GCTATGGCTGGTGCAAAG-3′ and 5′-GAACACATGCCCACT-TGCTG-3′ for IL-12 (p40 subunit); 5′-ACTGTACAACCGCAG-TAATACG G-3′ and 5′-AGTGAACATTAC-AGA-TTTATCCC-3′ for IGIF; and 5′-GTGAAGGTCGGTGTGAACGGATTT-3′ and 5′-TTATTATGGGGGTCTGGG-ATGGAA-3′ for G3PDH. Electrophoresis of the amplified DNAs was carried out on 2% agarose gel and stained with ethidium bromide.

RESULTS

Transient expression of iNOS, IL-12, and IGIF mRNAs in Meth A-Møs. When Meth A cells are injected *i.p.* into an allogeneic strain of mice (C57BL/6), the time-course of the numbers of leukocytes infiltrating into the peritoneal cavity shows a bell-shape, reaching the peak on day 7, and the tumor cells are rejected by day 14 (18-20). Since the infiltrating leukocytes in the peritoneal cavity are a heterogeneous population of cells which contain ≈ 60% of Møs (19), we first examined the level of activation of the infiltrating Møs during the rejection of Meth A cells using iNOS, IL-12, and IGIF expression as markers of Mø activation. Fig. 1 shows the time-course of iNOS, IL-12, and IGIF mRNA expression in Meth A-Møs during the rejection of Meth A cells. IL-12 mRNA expression was detected 6 h after the transplantation of Meth A cells, whereas the expression of iNOS and IGIF was seen after 24 h. IL-12 and iNOS mRNA expression reached their peak on day 7, whereas similar levels of IGIF mRNA expression were detected until day 11 after the transplantation (Fig. 1). Fig. 2A shows that

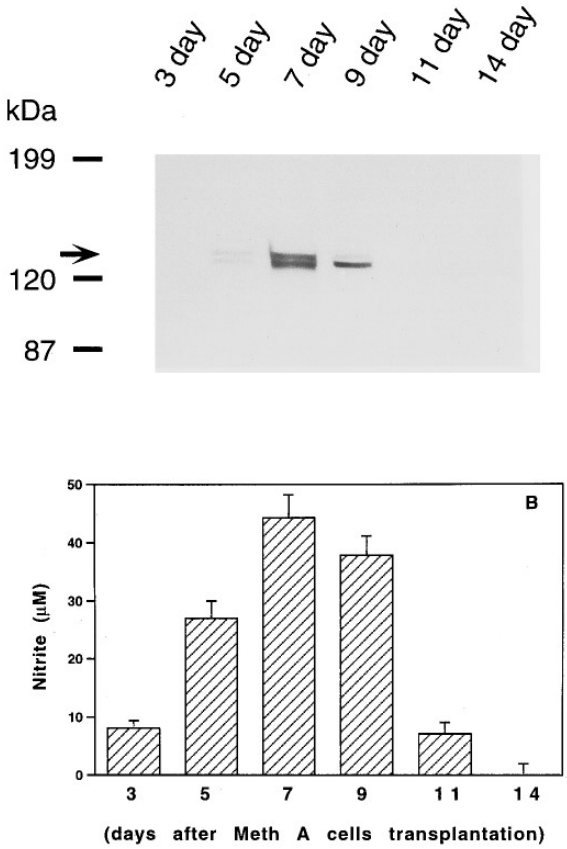

FIG. 2. Time-dependent expression of iNOS and nitrite production in Meth A-Møs. (A) SDS-PAGE of cell lysates (each 10 μg protein) of Meth A-Møs followed by Western blotting with anti-iNOS IgG antibody. The numbers on the lanes represent the days after Meth A cell transplantation. Relative molecular masses of prestained markers are shown on the left. Arrow indicates the specific iNOS doublet (≈130 kDa). Results are a representative of three experiments. (B) Nitrite production in Meth A-Møs. The values are the mean ± SD of triplicate experiments.

iNOS protein expression in Meth A-Møs was detected by immunoblot on days 5-9, reaching its peak on day 7. A similar pattern was obtained for the concentrations of NO_2^- released *in vitro* by Meth A-Møs isolated during the course of the rejection of Meth A cells (Fig. 2B).

Impairment of iNOS, IL-12, and IGIF induction in Meth A-Møs or BCG-Møs from IFN-γ −/− mice. We previously reported that the leukocytes infiltrating into the rejection site after Meth A transplantation released IFN-γ (18), one of the main cytokines that induce iNOS (3, 4) and IL-12 (2, 14) in Møs. Fig. 3 shows that the iNOS and IL-12 mRNA expression was impaired in Meth A-Møs from C57BL/6 IFN-γ −/− mice. Unexpectedly, the expression of IGIF mRNA was also completely suppressed in Meth A-Møs from IFN-γ −/− mice (Fig. 3). Similarly, these mRNAs were hardly expressed in the BCG-Mø, another well-known *in vivo*-activated Mø, from C57BL/6 IFN-γ −/− mice (Fig. 3), indicating that IFN-γ was essential for the iNOS, IL-12, and IGIF expression in the *in vivo*-activated Møs.

NO-mediated DNA fragmentation of P815 cells and ^{51}Cr release from the targets caused by Meth A-Møs. The activation of iNOS produces an L-arginine-derived NO, which has been demonstrated to be a soluble effector molecule cytotoxic against some tumor cells (5-8). Next, we tested the role of NO released *in vitro* by Meth A-Møs to assess whether or not the *in*

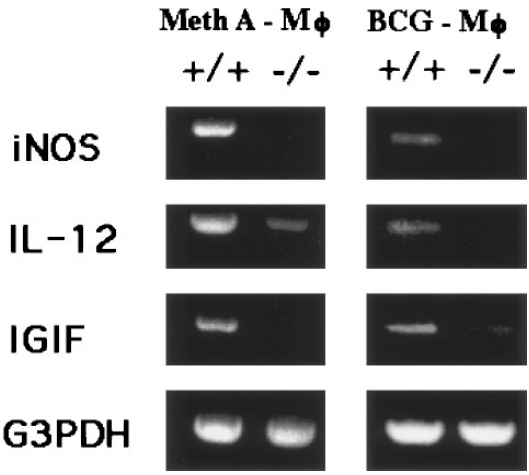

FIG. 3. Impairment of iNOS, IL-12 and IGIF induction in Meth A- or BCG-Møs from IFN-γ $-/-$ mice. The expression of iNOS, IL-12, IGIF and G3PDH (as a control) mRNAs was estimated by RT-PCR in Meth A-Møs (on day 7 after Meth A cell transplantation) or BCG-Møs (on day 5 after BCG injection) in C57BL/6 IFN-γ $+/+$ and IFN-γ $-/-$ mice. Results are representative of two experiments.

vivo activation of Meth A-Møs correlated with its cytotoxicity *in vitro.* We used P815 cells, a donor-type and NO-sensitive tumor cell line (5), as target cells. Meth A-Møs and P815 cells were cultured in different compartments of a Transwell (*see* ''Materials and Methods''), separated by a cell-impermeable membrane (insert) to prevent cell-to-cell contact. Fig. 4A shows the release of ^{51}Cr from P815 cells after an 18 h-incubation with Meth A-Møs. Under the conditions used, the fragmentation of [^{3}H]thymidine-labeled DNA of P815 cells was also detected (Fig. 4B). The extent of DNA fragmentation (27.4%) was higher than that of ^{51}Cr release from P815 cells (17.3%), implying the DNA fragmentation preceded plasma membrane damage. Both the cytotoxicity of Meth A-Møs against P815 cells and the DNA fragmentation of the targets were inhibited by the addition of N-MMA, an iNOS inhibitor, to the culture medium (Fig. 4, A and B), suggesting that these actions were mediated by NO released by Meth A-Møs. In fact, when the NO_2^- concentration in other samples from the same wells was measured, the release of NO_2^- from Meth A-Møs was also blocked by N-MMA (Fig. 4C). As expected, Meth A-Møs isolated from IFN-γ $-/-$ mice neither lysed P815 cells nor released NO (data not shown).

NO-mediated ''DNA ladder'' formation in P815 cells. To determine whether the DNA fragmentation of P815 cells detected by the use of [^{3}H]thymidine conformed to the pattern of oligonucleotides with molecular weights of 180-200 base pairs, a characteristic of apoptotic death, we cultured Meth A-Møs and P815 cells in different compartments of Transwell microtiter plates. The DNA extracted from P815 cells showed the characteristic ''ladder'' appearance that is a hallmark of apoptosis (Fig. 5, lane 3). The DNA fragmentation of P815 cells was also inhibited by the addition of N-MMA to the medium (Fig. 5, lane 4).

DISCUSSION

This study has shown that iNOS, IL-12, and IGIF mRNAs were expressed transiently in Meth A-Møs during the rejection of allografted Meth A cells in C57BL/6 mice. The iNOS protein expressed in Meth A-Møs showed a characteristic dimer (of $\approx$130 kDa) similar to that of iNOS purified from the RAW 264.7 Mø cell line (24). The peaks of iNOS and IL-12 mRNA expression in Meth A-Møs coincided in time with the maximal cytotoxic activity of Meth A-

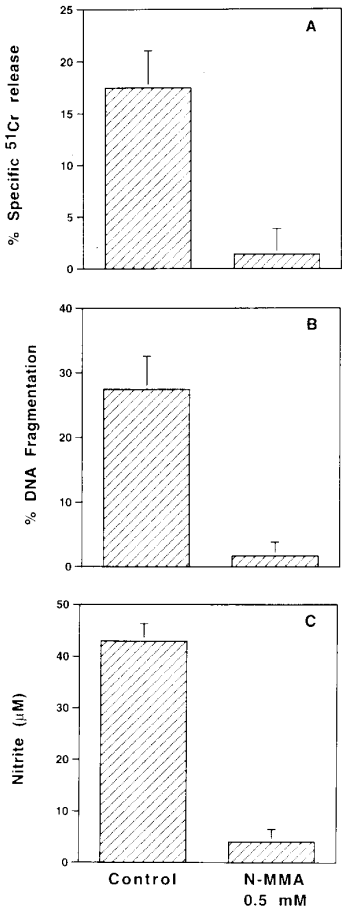

FIG. 4. Inhibitory effects of N-MMA on cytotoxicity of Meth A-Mø against P815 tumor cells (A), DNA fragmentation of the target cells (B), and nitrite production by Meth A-Møs (C). Meth A-Møs isolated as described in Fig. 2 were added to the lower chamber of a Transwell, P815 cells labelled with ^{51}Cr (A) or [^{3}H]thymidine (B) were added within the insert, and they were cultured in the presence or absence of 0.5 m*M* NMMA for 18 h. Panel C shows the nitrite concentration measured using aliquots from the same samples. Results are representative of five experiments.

Møs against Meth A cells (19) and with the maximal release of IFN-γ from the infiltrating cells (18). Therefore, Meth A-Møs induced in C57BL/6 mice 7 days after *i.p.* transplantation of allogeneic Meth A tumor cells are thought to be a type of highly activated Møs.

The expression of iNOS, IL-12, and IGIF mRNAs in Meth A- or BCG-Møs was impaired in C57BL/6 IFN-γ $-/-$ mice (Fig. 3), demonstrating the essential role of IFN-γ in the expression of iNOS, IL-12, and IGIF mRNAs in these *in vivo*-activated Møs. Similarly, the essential role of IFN-γ in the iNOS expression was earlier demonstrated by others using mice whose encoding genes for IFN-γ (25), one chain of IFN-γ receptor (26), or IFN regulatory factor (27) had been disrupted or by the injection of monoclonal antibody against IFN-γ (8). Also, since the IL-12 p40 gene promoter is known to be primed by IFN-γ in monocytic cells (15), it is reasonable that the expression of IL-12 in Meth A-Møs would be IFN-γ dependent (Fig. 3). Unexpectedly, however, the expression of IGIF mRNA in Meth A-Møs was also IFN-γ dependent (Fig. 3), although IGIF had been reported to induce IFN-γ production in T cells (13).

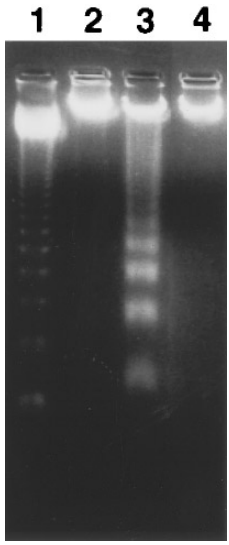

FIG. 5. NO-mediated DNA ladder of P815 cells. Meth A-Møs prepared as described in the legend to Fig. 2 were added to the lower chamber of a Transwell, and P815 cells were added onto the insert. After an 18 h-incubation, the DNA from P815 cells was extracted and subjected to electrophoresis. Lane 1, standard 123-base pair DNA ladder; Lane 2, DNA from P815 cells cultured in the absence of Meth A-Møs; Lane 3, DNA from P815 cells cultured in the presence of Meth A-Møs; and Lane 4, DNA from P815 cells cultured in the presence of Meth A-Møs and 0.5 m*M* N-MMA.

Meth A-Møs killed donor-type, NO-sensitive P815 cells *in vitro* by a mechanism that fulfill the criteria for apoptotic cell death. The extent of Meth A-Møs-induced DNA fragmentation of P815 cells was higher than that of ^{51}Cr release from the targets, implying that these two processes are sequential in time. The apoptotic type of cell death is a process that starts with fragmentation of genomic DNA into small oligomers, followed by the release of intracellular contents in the later stages (28). Both the ^{51}Cr release and the DNA fragmentation did not require cell-to-cell contact, and both were inhibited by N-MMA, an iNOS inhibitor. In addition, N-MMA inhibited the accumulation of NO_2^- in Meth A-Møs. These results demonstrate that Meth A-Møs killed P815 cells through NO as the soluble effector molecule in agreement with previous experiments that used *in vitro*-activated Møs (5, 7).

The findings that the time-course of the expression of iNOS mRNA in Meth A-Møs correlated with that of the rejection of Meth A cells by the mice (19), and that Meth A-Møs lysed P815 cells in an *in vitro* assay by an NO-dependent mechanism, could lead us to speculate that NO is involved in the rejection of Meth A cells. However, *in vitro* experiments showed that Meth A cells were insensitive to NO released from Meth A-Møs (data not shown). The role of NO in allograft rejection is somewhat controversial: Bastian *et al.* (29) have shown that the inhibition of NO production did not affect the rejection of cardiac allografts in mice. In contrast, Worral *et al.* (30), using the same cardiac allograft system, showed that treatment with aminoguanidine, an another inhibitor of iNOS, prolonged the graft survival. Since NO also acts as a scavenger of the superoxide anion released from polymorphonuclear leukocytes (31) and suppresses T cell-mediated reactions and lymphocyte proliferation (32-35), these biological functions rather than cytotoxic activity of NO may have enhanced allograft survival. Cytotoxic effects of NO released from Møs on Møs themselves have also been reported (36).

In conclusion, our results show that Meth A-Møs expressed iNOS, IL-12, and IGIF mRNAs during the rejection of allografted Meth A tumor cells in mice. At the peak of their expression,

Meth A-Møs appear to be a type of *in vivo*-activated Møs that can kill P815 cells through apoptotic cell death by a cell-to-cell contact-independent and NO-dependent mechanism. IFN-γ was essential not only for the expression of iNOS and IL-12 but also for the expression of IGIF in the *in vivo*-activated Møs (*e.g.*, Meth A-Møs and BCG-Møs), implying that IFN-γ, but not IGIF, would be the signal molecule to initiate the activation of Møs *in vivo*.

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