Differential Expression of Interferon Alpha and Beta Induced with Newcastle Disease Virus in Mouse Macrophage Cultures

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SUMMARY

Stimulation of mouse macrophages with Newcastle disease virus (NDV) leads to a rapid and high interferon (IFN) response. The magnitude of this response is influenced by the mouse genotype. We have analysed NDV-induced IFN production at the protein and mRNA levels in two different populations of macrophages derived from 'high producer' C57BL/6 and 'low producer' BALB/c mice in vitro. The data indicate that bone marrow and peritoneal macrophages from both strains grown in the presence of L cell conditioned medium (CM) as a source of macrophage colony-stimulating factor 1 (M-CSF) or purified murine M-CSF produce 10- to 50-fold more IFN on a per cell basis than cultures of resident peritoneal macrophages. These differences were also found when steady state levels of IFN mRNA were analysed. Differential analysis for the ratios of IFN-α and IFN-β showed that CM- or M-CSF-cultured macrophages produced equal amounts of both IFN species as determined by specific monoclonal antibodies and hybridization experiments using IFN-α and IFN-β DNA probes, whereas resident peritoneal macrophages induced under identical conditions produced almost exclusively IFN-β. This suggests a stimulating effect of M-CSF on IFN synthesis in NDV-induced cultures of mouse macrophages, which is in part due to additional activation of IFN-α gene expression.

INTRODUCTION

Mouse macrophages are potent producers of interferon (IFN) in response to various viral and non-viral inducers (DeMaeyer et al., 1971, 1979; Fleit & Rabinovitch, 1981a, b; Brehm et al., 1986). Two in vitro models are mainly used to analyse IFN synthesis in these cells: short-term culture of peritoneal exudate cells as a source of peritoneal macrophages, and in vitro expansion of bone marrow cells in the presence of macrophage colony-stimulating factor 1 (M-CSF), a macrophage-specific growth factor. The latter method yields cultures consisting almost exclusively of macrophages as shown by α-naphthylacetate esterase (Koski et al., 1976) and immunocytochemical staining with a monoclonal antibody to the macrophage differentiation antigen F4/80 (Hirsch et al., 1981).

Several reports have provided evidence that IFN is produced constitutively by peritoneal macrophages in vivo (Belardelli et al., 1984; Gresser et al., 1985; Proietti et al., 1986) and is able to confer an antiviral state to susceptible target cells (Lee & Warren, 1987) although the amount of IFN was below the detection limit of the antiviral bioassay. In addition, low amounts of IFN have been detected in culture supernatants from bone marrow-derived macrophages grown in M-CSF-containing media, suggesting that M-CSF is a physiological inducer of IFN in bone marrow macrophages (Moore et al., 1984).

In inbred mice, levels of circulating IFN induced by different viruses are under genetic control. For example, C57BL/6 mice are 'high producers' of IFN following induction with
Newcastle disease virus (NDV), generating 10-fold higher titres than BALB/c mice (DeMaeyer & DeMaeyer-Guignard, 1969). These differences were also observed when induction by NDV in peritoneal macrophages in vitro was studied, demonstrating that the 'high producer' allele If-1 is also expressed in macrophages in vitro (DeMaeyer et al., 1979).

This study was performed to examine in detail the enhancing effects of M-CSF on induction of IFN by NDV in macrophages from C57BL/6 and BALB/c mice. In these cells we have observed a rapid onset of IFN-α and -β mRNA and protein synthesis following induction. More importantly, we have found pronounced differences in total amounts of IFN gene expression and in the ratios of IFN-α to IFN-β between macrophages cultured in the presence of L cell conditioned medium (CM) or purified M-CSF and resident peritoneal macrophages from both C57BL/6 and BALB/c mice. Our data support the view that M-CSF is an important physiological stimulator of macrophage functions. Rapid release of high levels of IFN following viral infection clearly is one of the most prominent functions of macrophages in primary host defence.

METHODS

Culture of macrophages. Bone marrow cells were recovered from tibias and femurs of 8- to 12-week-old male C57BL/6 or BALB/c mice (obtained from Zentralinstitut für Versuchstierkunde) and seeded into bacteriological Petri dishes (Greiner & Söhne) at a density of 3 × 10⁶ cells/ml in RPMI 1640 supplemented with 10% foetal bovine serum (FBS), 5% horse serum, and 20% L cell CM. Cultures were refed with fresh medium after 4 to 5 days and then every 2 days until use. Alternatively, bone marrow cells were cultured in RPMI 1640 supplemented with 5% FCS and 1000 units (U)/ml of M-CSF purified from CM.

Resident peritoneal macrophages were obtained by culturing total peritoneal wash-out cells for 24 h in 24-well plates (Nunc) in RPMI 1640 supplemented with 5% FBS at a density of 1·5 × 10⁶ cells per culture well. Non-adherent cells were then removed by washing. The number of adherent cells varied between 2 × 10⁵ and 3 × 10⁵ per culture well and more than 95% stained for α-naphthylacetate esterase. Cultured peritoneal macrophages were obtained by incubating 5 × 10⁶ total peritoneal cells (equivalent to about 1 × 10⁵ to 1·5 × 10⁶ resident peritoneal macrophages) for 7 to 10 days in the same media as used for the culture of bone marrow cells. After this time cell numbers per culture had increased to 2 × 10⁸ to 3·5 × 10⁸ cells per culture.

L cell CM, M-CSF and M-CSF assay. CM was recovered from 7-day-old confluent monolayers of mouse L-929 cells, filtered and stored at −20 °C. Assay for M-CSF activity was performed on 35 mm Petri dishes as described (Metcalf et al., 1986). Briefly, 7·5 × 10⁴ C57BL/6 bone marrow cells in 0·5 ml Dulbecco’s modified Eagle’s medium supplemented with 20% FBS and containing 0·6% agar were mixed with an equal volume of serial twofold dilutions of CM, transferred to Petri dishes, allowed to gel at room temperature and incubated for 7 days at 37 °C in a 5% CO₂ atmosphere. Colonies of more than 50 cells were then counted microscopically at low magnification with a Leitz Diavert microscope. Colony-stimulating activity was calculated by assigning 50 U/ml to the concentration generating half maximal numbers of macrophage colonies. For CM used in these studies, half maximal numbers of colonies were counted at concentrations of 1·0 to 2·0% of CM, corresponding to 2500 to 5000 U/ml of M-CSF in undiluted CM. Murine M-CSF purified from CM was kindly provided by Dr E. R. Stanley (Albert Einstein College of Medicine, New York, U.S.A.) and served as a standard in the colony formation assay.

IFN induction of macrophages. Seven-day-old cultures of bone marrow cells were detached with a rubber policeman following incubation with ice-cold saline, washed, counted and refed into 24-well plates at a density of 2·5 × 10⁵ cells per well in RPMI 1640 supplemented with 5% FBS. They were allowed to attach for 4 to 6 h, incubated with NDV or CMA for 1 h and refed after removal of inducer with fresh, prewarmed medium.

Resident peritoneal macrophages were induced identically. Preliminary tests had shown that seeding of 1·5 × 10⁶ peritoneal cells resulted in recovery of about 2 × 10⁵ to 3 × 10⁵ adherent peritoneal macrophages. M-CSF- or CM-cultured peritoneal macrophages were induced on the 7th or 10th day of culture. Duplicate cultures were induced with NDV; a third culture was used for cell count analysis.

Culture supernatants were harvested 16 h after induction, acidified to pH 2·5, stored at 4 °C for 24 to 48 h and assayed for antiviral activity. IFN titres are expressed in international units (IU) per culture of 2·5 × 10⁶ cells.

Anti-IFN antibodies, IFN and IFN neutralization assay. Polyclonal sheep anti-mouse IFN-α/β immunoglobulin (Gresser et al., 1976) was kindly provided by Dr I. Gresser (Institut de Recherches Scientifiques sur le Cancer, Villejuif, France) and had a neutralization titre of 1:128 000 against 5 IU of mouse IFN-α/β. It was used following absorption on mouse spleen cells at a dilution of 1:100.
Monoclonal antibodies to mouse IFN-α and -β, kindly provided by Yamasa Shoyu Ltd, Japan, courtesy of Dr Y. Kawade (Kawade & Watanabe, 1987) had neutralization titres of 1:500 and 1:400000 against 10 IU of IFN-α and -β, respectively and were used in neutralization assays at final dilutions of 1:20 and 1:50 for anti-IFN-α or 1:10000 and 1:40000 for anti-IFN-β.

Neutralization assays were performed by mixing each test supernatant, diluted to a concentration of 50 IU, with two different concentrations of the monoclonal antibodies (see above) or with a mixture of both. An additional diluted sample was incubated with medium (RPMI 1640 plus 2% FBS) alone and served as an internal reference. After a 2 h incubation at room temperature, residual IFN activity in test supernatants or preparations of recombinant mouse IFN-α4 (van Heuvel et al., 1986) or purified natural mouse IFN-β (purchased from Stratech Scientific) was assayed on monolayers of L-929 cells as described (Zawatzky et al., 1982b). IFN levels were expressed in IU, based on parallel titration of a mouse IFN-α/β reference preparation (cat. no. Gu02-901-511), obtained from the NIAID repository.

**Assay of oligo-2',5'-adenylate synthetase (2-5AS).** Enzyme activities were assayed in cell extracts. Cells were harvested from 94 mm Petri dishes by scraping, and washed twice in ice-cold phosphate-buffered saline (PBS). Cell pellets were lysed in 1:5 vol. lysis buffer (0.5% v/v Nonidet P40, 90 mM-potassium chloride, 1.5 mM-magnesium acetate, 10 mM-HEPES pH 7.6). The buffer was supplemented before cell extraction with 2 mM-mercaptoethanol and 2 mM-phenylmethylsulphonyl fluoride. Post-mitochondrial supernatants were prepared by centrifugation at 12000 g for 15 min at 4 °C. Protein concentrations were determined by the Bio-Rad protein assay (Bradford, 1976). Aliquots of cell extracts were bound to poly(I)-poly(C)-agarose (Pharmacia) and incubated with buffer containing 4 mM-ATP (Stark et al., 1981). The 2-5A produced was quantified by a radioactivity binding assay as described previously (Knight et al., 1980).

**DNA probes.** Genomic DNA probes for mouse IFN α1, α2, α4 and α6 isolated by Zwarthoff et al. (1985) were described by van Heuvel et al. (1986). Inserts spanning the coding regions had sizes of approximately 700 bp and were isolated from polyacrylamide gels by electro-elution. For radioactive labelling equal amounts of each insert were mixed together. A cDNA probe for mouse IFN-β (pMβ-3) was kindly provided by Dr Y. Kawade (Higashi et al., 1983). The DNA insert spanning the coding region had a size of 680 bp and was isolated as described above.

Plasmid 27-2-86, harbouring a 10.5 kb EcoRI insert, which codes for a murine class I major histocompatibility complex (MHC) antigen, H-2k, was kindly provided by Dr B. Arnold (Arnold et al., 1984). Since the homology in nucleotide sequence to the H-2k antigen expressed by macrophages of C57BL/6 mice is 90%, there is sufficient cross-hybridization. A 2 kb cDNA coding for chicken β-actin was described by Cleveland et al. (1980). Since actin genes are highly conserved during evolution this DNA cross-hybridizes to any other vertebrate DNA.

**For in situ hybridization,** DNA probes were labelled by nick translation (Maniatis et al., 1982) with [α-32P]dATP (1000 Ci/mmol; New England Nuclear). The DNase I concentration in the reaction mixture was adjusted to generate probe fragments of 200 to 400 bases. IFN-α and -β DNA probes used in this study had similar sp. act. of 2.0 × 108 and 2.2 × 108 d.p.m./μg, respectively, for comparison of signal intensity.

For RNA blot analysis, DNA probes were labelled with [α-32P]dCTP (3000 Ci/mmol; Amersham) by random priming using synthetic oligonucleotides as described (Feinberg & Vogelstein, 1983). Again, only IFN DNA probes of similar sp. act. were used for hybridization. In general, the sp. act. obtained was 2 × 106 d.p.m./μg DNA.

**RNA extraction.** Seven- to 9-day-old bone marrow macrophage cultures were washed twice with PBS and lysed immediately with a guanidine isothiocyanate (GTC)-containing buffer (4 M-GTC, 5% w/v lauryl-l-sarcosine, 25 mM-sodium citrate pH 7, 0.7% w/v 2-mercaptoethanol) as described previously (Chirgwin et al., 1979). Total cellular RNA was recovered by centrifugation through a cesium chloride gradient. Poly(A)+ RNA was obtained by oligo(dT)-cellulose chromatography.

**RNA blot hybridization.** Denatured RNA was electrophoresed in 14% agarose gels in the presence of formaldehyde (Lerhach et al., 1977) and transferred to nylon filters (Nytran, Schleicher & Schuell). For slot blot analysis, twofold dilutions of denatured RNA were spotted onto nylon filters. The RNA was hybridized to a mixture of mouse IFN-α DNA probes, a mouse IFN-β cDNA, or a chicken β-actin cDNA labelled with [α-32P]dCTP as described above. For each hybridization only IFN probes of similar sp. act. were used. The blots were hybridized at 48 °C for 20 h in 50% deionized formamide, 10% (w/v) dextran sulphate, 1:5 × Denhardt’s solution, 5 × SSPE, 1% (w/v) SDS, 100 μg/ml salmon sperm DNA and 1 × 106 c.p.m./ml of [α-32P]dCTP-labelled DNA. The filters were washed at 68 °C for 2 h in 2 × SSC (1 × SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate, pH 7.0), 0.1% (w/v) SDS and then for 2 h in 0.2 × SSC.

**Cytocentrifuge preparations of macrophages and in situ hybridization.** Mouse bone marrow or peritoneal macrophages seeded into 35 mm bacteriological Petri dishes were infected with NDV (512 HAU/culture), resulting in infection of all the cells, as determined by immunofluorescence with a polyclonal rabbit anti-NDV antiserum, pre-absorbed onto mouse spleen and bone marrow cells (kindly provided by Dr P. van Hoogen and Dr V. Schirrmacher, German Cancer Research Center). After 2 or 6 h macrophages were detached as described above, viability was checked by trypsin blue exclusion and cell suspensions exhibiting less than 80% viability were
discarded. Cells ($2 \times 10^4$) were deposited onto aminopropylsilane-coated slides (Rentrop et al., 1986) and fixed for 10 min in 4% paraformaldehyde in PBS. Slides were then stored at $-20^\circ$C in 80% ethanol. In situ hybridization was performed essentially as described (Lawrence & Singer, 1985) with some modifications. Pretreatment with 0.5 μg/ml of proteinase K (Haase et al., 1984) often led to an increase in signal and was therefore also included in the protocol. Hybridization was conducted overnight at 42°C in 50% deionized formamide, 3 x SSC, 10 mM-dithiothreitol, 250 μg/ml carrier DNA and 0.5 μg/ml of α-35S-labelled DNA probes prepared by nick translation (Maniatis et al., 1982). For chicken β-actin and H-2k1 probes, hybridization was performed at 30°C under identical conditions. Following hybridization, the slides were washed in three changes of 1 x SSC/50% formamide at 42°C (IFN-α and -β probes) or 30°C (H-2k1 and chicken β-actin probe) for 2 h, dehydrated with increasing concentrations of ethanol and dried overnight. Autoradiography was performed as described (Zawatzky et al., 1985). For microscopic evaluation, grains on an average of 100 cells with intact morphology were counted. A cell was scored as positive when the number of grains was at least 1.5 times higher than the background level, calculated from duplicate preparations, either treated with RNase prior to hybridization (Haase et al., 1984) or washed at 42°C in 80% formamide, 0.05 x SSC (hyperstringent washing) following hybridization.

RESULTS

Requirement for L cell CM of C57BL/6 and BALB/c bone marrow cells

To determine the optimal conditions for bone marrow cells to differentiate into macrophages we incubated $6 \times 10^5$ bone marrow cells from C57BL/6 or BALB/c mice for 8 days in culture medium supplemented with various concentrations of CM as a source of M-CSF. Culturing of cells was performed in 35 mm Petri dishes and culture medium was replaced every second day. Cells were then detached and counted, and cytocentrifuge preparations were stained for macrophage-specific α-naphthyl-acetate esterase (Koski et al., 1976).

As shown in Table 1, bone marrow cells from BALB/c mice grew more slowly than those from C57BL/6 mice and did not reach the same final density per Petri dish. CM concentrations in the culture medium of 5% or less resulted in a decrease in the percentage of esterase-positive cells, whereas concentrations of 10 and 20% CM yielded pure cultures of macrophages. In view of the observation that bone marrow macrophages degrade M-CSF in vitro (Tushinski et al., 1982), we routinely cultured bone marrow cells in medium containing 20% L cell CM or 1000 U/ml of purified M-CSF in order to provide saturating amounts of M-CSF to developing macrophages.

IFN production by bone marrow and peritoneal macrophages in response to NDV

As the cell number in cultures of BALB/c macrophages was lower than in corresponding cultures of C57BL/6, cells were split on day 7 of culture, reseeded in 24-well plates at identical densities ($2.5 \times 10^5$ cells/well) and allowed to adhere for 6 h before induction by NDV.

Resident peritoneal macrophages from both mouse strains were induced in parallel for IFN production. In preliminary experiments, the percentage of plastic-adherent macrophages in total peritoneal cells had been determined in order to adjust cell densities of peritoneal- and bone marrow-derived macrophages. IFN titres in culture supernatants 16 h following NDV infection are depicted in Table 2. Ten- to 50-fold higher titres of IFN expressed as IU per culture of $2.5 \times$

<table>
<thead>
<tr>
<th>Amount of CM in macrophage culture medium (%)</th>
<th>Number of cells/dish ($\times 10^5$)</th>
<th>Esterase-positive cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>BALB/c</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>1</td>
<td>0.39*</td>
<td>ND†</td>
</tr>
<tr>
<td>2.5</td>
<td>1.5</td>
<td>0.75</td>
</tr>
<tr>
<td>5</td>
<td>1.7</td>
<td>1.0</td>
</tr>
<tr>
<td>7.5</td>
<td>3.0</td>
<td>1.4</td>
</tr>
<tr>
<td>10</td>
<td>3.1</td>
<td>1.9</td>
</tr>
<tr>
<td>20</td>
<td>3.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Mean value obtained from three culture dishes after 8 days of incubation.
† ND, Not determined.
Table 2. Dose–response analysis of IFN production* by NDV-induced macrophages

<table>
<thead>
<tr>
<th>Dose of NDV (HAU/culture)</th>
<th>Cultured bone marrow macrophages</th>
<th>Resident peritoneal macrophages</th>
<th>Cultured bone marrow macrophages</th>
<th>Resident peritoneal macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M-CSF</td>
<td>CM</td>
<td>M-CSF</td>
<td>CM</td>
</tr>
<tr>
<td>512</td>
<td>12150</td>
<td>16200</td>
<td>1350</td>
<td>4050</td>
</tr>
<tr>
<td>256</td>
<td>ND†</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>82</td>
<td>8100</td>
<td>16200</td>
<td>810</td>
<td>2700</td>
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<td>27</td>
<td>4050</td>
<td>8100</td>
<td>180</td>
<td>1350</td>
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<tr>
<td>9</td>
<td>ND</td>
<td>4050</td>
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<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>720</td>
<td>960</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>1</td>
<td>ND</td>
<td>180</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* IFN titres in culture supernatants expressed as IU per culture of 2.5 × 10⁵ cells obtained 16 h after infection. Values represent the mean of two experiments.† ND, Not determined.

Table 3. IFN production* by NDV-induced resident and M-CSF/CM cultured peritoneal macrophages†

<table>
<thead>
<tr>
<th>Resident peritoneal macrophages</th>
<th>Cultured peritoneal macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>M-CSF</td>
</tr>
<tr>
<td>900</td>
<td>8100</td>
</tr>
<tr>
<td>BALB/c</td>
<td>60</td>
</tr>
</tbody>
</table>

* IFN titres in culture supernatants expressed as IU per culture of 2.5 × 10⁵ cells obtained 16 h after infection.† Cultures of 2.5 × 10⁵ to 3.5 × 10⁵ macrophages were prepared as described in Methods and induced with NDV (256 HAU/culture).

10⁵ cells were measured for bone marrow-derived macrophages. Induction kinetics were similar for both cell types and mouse strains, with detectable levels at 3 h and peak titres at 8 to 9 h after infection (data not shown). The dose–response analysis revealed that IFN levels from bone marrow macrophage cultures were unchanged over a wide range of doses of NDV, whereas in resident peritoneal macrophages IFN titres decreased linearly with decreasing amounts of inducer.

In addition, we observed that IFN titres from bone marrow macrophage cultures of C57BL/6 and BALB/c mice only differed by a factor of 3, whereas resident peritoneal macrophages of these two strains showed at least about 10-fold differences in NDV-induced IFN levels, which is in line with published data (DeMaeyer et al., 1979). It was of interest to look at whether CM and M-CSF also enhanced the capacity of resident peritoneal macrophages to produce IFN in response to NDV. Therefore, resident peritoneal macrophages were cultured for 7 to 10 days in the presence of 1000 U/ml of M-CSF purified from CM, or 20% of crude CM. As shown in Table 3, cultured peritoneal macrophages from both mouse strains produced considerably higher titres of IFN following infection with NDV than did resident macrophages. Since we observed that M-CSF and CM also induced growth of resident peritoneal macrophages during culture, IFN titres are expressed as IU per culture of 2.5 × 10⁵ cells.

To exclude the possibility that trace amounts of bacterial lipopolysaccharide (LPS) contamination in foetal bovine serum, CM or allantoic fluid from eggs were influencing the IFN response to NDV in macrophages, additional cultures were set up in the presence of culture medium pretreated for 4 h with 2 μg/ml polymyxin B, an antibiotic known to inhibit the stimulatory activity of LPS by binding to its lipid A portion (Morrison & Jacobs, 1976). There was, however, no difference in the IFN levels in induced cultures, suggesting that there was no effect of possible contamination with LPS (data not shown).
Table 4. Characterization of macrophage-induced IFN using monoclonal antibodies to mouse IFN-α and -β

<table>
<thead>
<tr>
<th>Producer cells</th>
<th>Mouse strain</th>
<th>Inducer*</th>
<th>Anti-IFN-α</th>
<th>Anti-IFN-β</th>
<th>Anti-IFN-α + anti-IFN-β</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultured bone marrow macrophages</td>
<td>C57BL/6</td>
<td>NDV</td>
<td>50</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>C57BL/6</td>
<td>CMA</td>
<td>90</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>BALB/c</td>
<td>NDV</td>
<td>50</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Resident peritoneal macrophages</td>
<td>C57BL/6</td>
<td>NDV</td>
<td>90</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>BALB/c</td>
<td>NDV</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cultured peritoneal macrophages</td>
<td>C57BL/6</td>
<td>NDV</td>
<td>40</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>BALB/c</td>
<td>NDV</td>
<td>65</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>Recombinant mouse IFN-α</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ND†</td>
</tr>
<tr>
<td>Natural mouse IFN-β</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ND</td>
</tr>
</tbody>
</table>

* Supernatants were harvested 8 h post-induction with NDV (256 HAU/ml) or CMA (500 μg/ml).
† ND, Not determined.

Analysis of IFN species from NDV-induced macrophage cultures

Characterization of the different IFN preparations was performed using monoclonal antibodies to mouse IFN-α and -β (Kawade & Watanabe, 1987). As listed in Table 4, diluted supernatants from NDV-induced resident peritoneal macrophages of BALB/c or C57BL/6 origin contained antiviral activity which was completely neutralized by monoclonal anti-IFN-β. Surprisingly, however, cultured bone marrow and peritoneal macrophages from both mouse strains incubated for 7 to 10 days in the presence of M-CSF or CM produced comparable amounts of IFN-α and IFN-β in response to NDV. A supernatant from bone marrow macrophages treated with CMA, which induces mainly IFN-β in mouse macrophages, was completely neutralized by monoclonal anti-IFN-β.

Differential analysis of IFN-α and -β mRNA in bone marrow-derived macrophages

Northern blot and slot blot analysis using a probe mixture consisting of genomic IFN-α1, -α2, -α4, and -α6 DNA coding regions or a cDNA probe for IFN-β revealed that following infection with NDV similar amounts of IFN-α and -β mRNA were induced in bone marrow macrophages from both C57BL/6 and BALB/c mice (Fig. 1a, Fig. 2, Table 5). In addition, from a kinetic study of IFN mRNA levels it was obvious that induction of both IFN mRNA species occurred simultaneously (Fig. 1b). The corresponding culture supernatants 8 h after induction had IFN titres of 16200 and 8100 IU for C57BL/6 and BALB/c, respectively. In these blot analyses, identical amounts of RNA were applied per gel slot, as verified by rehybridization of the same blot to a chicken β-actin probe (data not shown).

Since it was reported that low amounts of IFN are generated by mouse bone marrow macrophages during culture in M-CSF (Moore et al., 1984), we analysed poly(A)+-enriched RNA preparations from non-induced bone marrow macrophages for the presence of IFN-α or -β mRNA. There was, however, no autoradiographic signal, even when 40 μg of poly(A)+ RNA was applied per lane (Fig. 1b, lane 3) and hybridization was carried out under non-stringent conditions at 35 °C followed by 3 weeks of autoradiographic exposure (data not shown).

IFN-α and -β mRNA detection in situ hybridization in individual macrophages

In addition to blotting experiments, IFN-α and -β mRNA distribution in individual cells was studied by in situ hybridization. The results are presented in Fig. 3. Non-induced cells from BALB/c and C57BL/6 mice had identical grain counts following hybridization with either probe. An enhancement of the specific autoradiographic signal compared to non-induced cells could be detected 2 h after NDV infection of C57BL/6 bone marrow macrophages.
IFN gene expression in mouse macrophages

Large numbers of grains were observed on preparations from C57BL/6 bone marrow macrophages 6 h after induction; more than 60% of the cells were identified as clearly positive according to the criteria defined in Methods. A comparison to NDV-induced BALB/c bone marrow macrophages revealed a difference in signal intensity, reflecting the difference in IFN production in vitro (Fig. 3a). For bone marrow macrophages from both mouse strains, the relative distribution of IFN-α and -β mRNA as deduced from the number of grains per cell was very similar, and hybridization with a mixture of IFN-α and -β probes resulted in a cumulative signal, suggesting that IFN-α and -β mRNA were synthesized simultaneously and in the same cells. The distribution of autoradiographic grains was quite heterogeneous, indicating considerable differences in IFN mRNA from cell to cell. In contrast, relatively homogeneous signal distribution was observed when DNA coding for chicken β-actin or for a MHC class I antigen, H-2kκ were used as a probe (Fig. 3a, 4b, c).

In some experiments, following hybridization with either IFN probe, we detected slightly higher numbers of grains on preparations of non-induced bone marrow macrophages compared...
Fig. 2. Slot blot hybridization of mRNA from bone marrow macrophages of C57BL/6 (a, b) and BALB/c (c, d) mice after infection with NDV. Total RNA from non-induced cells (lanes 1) or cells 4 (lanes 2), 6 (lanes 3), 8 (lanes 4) and 12 h (lanes 5) after induction was isolated and 5, 2.5, 1.25 and 0.6 μg of each sample was analysed. Identical amounts of RNA were loaded per slot as verified by hybridization of the same blot to chicken β-actin DNA (data not shown). The film was exposed for 3 days at -80 °C.

Table 5. Quantification of IFN mRNA from NDV-induced bone marrow macrophages*

<table>
<thead>
<tr>
<th>Total RNA† (μg/slot)</th>
<th>IFN-α</th>
<th>IFN-β</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4.5</td>
<td>4.84</td>
</tr>
<tr>
<td>2.5</td>
<td>2.34</td>
<td>2.94</td>
</tr>
<tr>
<td>1.25</td>
<td>1.09</td>
<td>1.67</td>
</tr>
<tr>
<td>BALB/c</td>
<td></td>
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<tr>
<td>5</td>
<td>4.02</td>
<td>3.02</td>
</tr>
<tr>
<td>2.5</td>
<td>2.34</td>
<td>1.45</td>
</tr>
<tr>
<td>1.25</td>
<td>1.31</td>
<td>0.72</td>
</tr>
</tbody>
</table>

* Bone marrow macrophage cultures were infected with NDV on day 7 of culture.
† Total cellular RNA was harvested 8 h post-infection, and analysed by slot blot hybridization as described.
‡ IFN-α and -β mRNA were quantified by scanning with an LKB UltraScan XL.

to duplicate preparations treated with RNase prior to hybridization (data not shown); this could indicate the presence of low levels of IFN-α and -β mRNA in these cells.

In contrast to bone marrow macrophages, NDV-induced peritoneal macrophages failed to yield a detectable signal following hybridization to the IFN-α probe mix (Fig. 3b). Furthermore, a pronounced difference in IFN-β hybridization signal intensity between NDV-induced macrophages from C57BL/6 and BALB/c was observed reflecting the differences in IFN titres measured in corresponding culture supernatants: 810 and 90 IU for C57BL/6 and BALB/c peritoneal macrophages, respectively. The long exposure time (31 days) used in this experiment and the relatively weak autoradiographic signal were in agreement with the observed differences in IFN production between bone marrow-derived and peritoneal macrophages.

Effect of anti-IFN on 2-5AS activity and on NDV-induced IFN production in cultured bone marrow macrophages

Compared to untreated bone marrow cells obtained directly from the animal, M-CSF-cultured bone marrow macrophages showed elevated levels of the IFN-induced enzyme 2-5AS (Resnitzky et al., 1986). Induction of the enzyme was, however, prevented when anti-IFN
antibodies were added to the cultures (Resnitzky et al., 1986). Since in non-induced macrophages we failed to detect IFN mRNA on Northern blots or antiviral activity in culture supernatants, we analysed cytoplasmic extracts for 2-5AS activity. As shown in Table 6, bone marrow macrophages from both C57BL/6 and BALB/c mice cultured in the presence of a 1:100 dilution of sheep anti-mouse IFN-α/β immunoglobulin had strongly reduced 2-5AS activities when compared to untreated control preparations, which is in line with the results mentioned above.

**Discussion**

Macrophages play an important role in primary host defence, and the rapid release of IFN can be considered to be part of this function (Mogensen & Virelizier, 1987). For *in vitro* studies, mature populations of macrophages can be obtained from the spleen and the peritoneal cavity of mice. Alternatively, progenitor cells from mouse bone marrow can be induced to differentiate into macrophages *in vitro* in the presence of CM as a source of M-CSF, a macrophage-specific growth factor (Bradley & Metcalf, 1976; Hume & Gordon, 1983; Stanley & Guilbert, 1981). Numerous studies have shown that M-CSF has a dual stimulatory effect *in vitro* on cells of the monocyte differentiation pathway: it induces macrophage colony formation from progenitor cells and stimulates secretory functions of mature macrophages. Since M-CSF is present in mouse sera, it is likely that it also stimulates growth, differentiation and secretory functions of...
Fig. 4. *In situ* hybridization of cytocentrifuge preparations of C57BL/6 bone marrow macrophages. DNA probes were labelled with [*-33S]*dATP by nick translation. The sp. act. of probes used in this experiment were $2.2 \times 10^8$ d.p.m./µg for mouse IFN-α DNA probe mix, $2.0 \times 10^8$ d.p.m./µg for mouse IFN-β cDNA, $9 \times 10^7$ d.p.m./µg for the H-2k DNA probe and $3 \times 10^8$ d.p.m./µg for the chicken β-actin probe. Exposure time was 11 days. (a to c) Non-induced macrophages, hybridized to (a) a mixture of IFN-α and IFN-β DNA probes, 5 ng/slide; (b) H-2k DNA probe, 5 ng/slide; (c) a chicken β-actin cDNA probe, 5 ng/slide. (d, e) IFN-producing cells 6 h after induction with NDV (512 HAU per culture of $2 \times 10^6$ cells), hybridized to IFN-α (d) or IFN-β (e) DNA probes. Bar marker represents 10 µm.

Splenic or peritoneal macrophages from bone marrow precursors *in vivo*. Accordingly, it has been shown that peritoneal macrophages also require M-CSF for long-term survival in tissue culture, as do bone marrow-derived macrophages (Tushinski *et al.*, 1982). On the other hand, bone marrow-derived macrophages cultured in the presence of M-CSF show a very high rate of consumption of M-CSF, probably due to increased receptor turnover (Tushinski *et al.*, 1982). Since high concentrations of M-CSF are also known to activate macrophages *in vitro*, it is likely that these cells are in a more active state compared to resident peritoneal macrophages.
In several studies, mouse macrophages of different sources have been used to analyse IFN production in vitro (DeMaeyer et al., 1971, 1979; Fleit & Rabinovitch, 1981a, b; Brehm et al., 1986; Domke-Opitz et al., 1987). From the study of Fleit & Rabinovitch (1981a) it is obvious that, once differentiated, mouse bone marrow or peritoneal macrophages do not require the presence of CM for optimal production of IFN in response to NDV. In this report we have found that cultured bone marrow and peritoneal macrophages were much more sensitive to IFN induction by NDV than were resident peritoneal macrophages and generated 10- to 50-fold more IFN activity on a per cell basis. These differences were also reflected in mRNA levels as shown by the in situ hybridization experiments. This indicates that there is a long-lasting stimulatory influence of M-CSF on subsequent IFN induction by NDV in mouse macrophages in vitro that cannot be reversed by its withdrawal 24 h before IFN induction. This stimulation was apparently not due to a priming effect by IFN-α/β induced during culture of bone marrow macrophages in CM, since bone marrow cells cultured from the start in the presence of potent anti-mouse IFN antibodies produced even more IFN after NDV induction. Similarly, a direct stimulatory effect of M-CSF on IFN production induced by poly I:C has been described recently for human blood monocytes (Warren & Ralph, 1986).

Interestingly, in our study we observed that the genetically determined 10-fold differences in NDV-induced IFN production between resident peritoneal macrophages from C57BL/6 and BALB/c mice (DeMaeyer et al., 1979) was diminished in bone marrow macrophages, and this is also demonstrated at the mRNA level by Northern blot or in situ hybridization experiments. A possible explanation could be that there is a genetically determined difference in the biological response to M-CSF, as we observed differences in growth characteristics of bone marrow macrophages from C57BL/6 and BALB/c mice in CM-supplemented cultures (Table 1). The differences in cell growth, however, may be due to IFN induced by M-CSF during culture, since bone marrow macrophages from BALB/c have been shown to be more sensitive to the anti-proliferative action of IFN (Dandoy et al., 1981). From our experiment, we cannot conclude whether or not BALB/c macrophages are less sensitive to the stimulatory effect of M-CSF on secretory functions in vitro. However, in this respect, it is important to remember that C57BL/6 mice are highly resistant to infection with herpes simplex virus (HSV) (Lopez, 1975) while BALB/c mice are not, and that resistance is linked to an early and high local IFN response (Zawatzky et al., 1982a). Since there is evidence that the early IFN response to NDV and HSV infection in vivo is a function of macrophages (DeMaeyer-Guignard et al., 1983), it could be possible that macrophages from BALB/c require higher levels of M-CSF for efficient production of IFN following viral infection. This requirement could only be met in vitro if macrophages are cultivated in the presence of saturating amounts of M-CSF. Since its action is restricted to cells of monocytic origin, M-CSF clearly is one of the first candidates for supporting the host defence system against viral infection.

In general, IFN-β is considered to be the major IFN species produced by macrophages (Yamamoto, 1981). Our data, however, clearly show that there is a pronounced difference in the relative amounts of IFN-α and -β produced by M-CSF-cultured macrophages and resident peritoneal macrophages. Using monoclonal antibodies to mouse IFN-α and -β we found that...
NDV-induced IFN from resident peritoneal macrophages was completely neutralized by anti-IFN-β, whereas half of the IFN activity in cultures of NDV-induced bone marrow macrophages was of the α type. These results were again substantiated at the mRNA level by Northern blot, slot blot and in situ hybridization experiments. Quantification of autoradiographic signals for IFN-α and -β mRNA by scanning and grain counting revealed similar levels for both mRNA species in NDV-induced bone marrow macrophages (Table 5, Fig. 3a).

A possible cross-hybridization between the various genomic IFN-α probes and the IFN-β cDNA can be ruled out by the fact that there is only about 30% homology at the nucleotide level between these genes. In addition, when cytospin preparations of resident peritoneal macrophages were subjected to hybridization in situ, there was only a signal for IFN-β mRNA, which also is in line with the neutralization experiments depicted in Table 4, and demonstrates that IFN-α and -β DNA probes do not cross-hybridize.

Recently, a report by Brehm & Kirchner (1986) suggested that bone marrow macrophages are almost exclusively producers of IFN-β, with IFN-α levels of less than 10%, following induction by NDV. The apparent discrepancy of the data reported here may be explained by partial inactivation of IFN-α during SDS-PAGE, since neutralization assays using polyclonal antibodies to either IFN-α or -β were not performed with culture supernatants prior to biochemical analysis. On the other hand, Bellardelli et al. (1987), utilizing the same antibodies as used in this study, reported on low levels of IFN-α induced by NDV in peritoneal macrophages. Although in our hands there was complete neutralization of antiviral activity by monoclonal anti-IFN-β, we sometimes observed a slight reduction of antiviral activity in cultures of NDV-induced peritoneal macrophages following incubation with anti-IFN-α, which could suggest a small quantity of IFN-α in these supernatants (Table 4). In addition, since we observed that NDV-induced peritoneal macrophages are able to generate considerable amounts of IFN-α when cultured in the presence of M-CSF, it may be that differences in the relative ratios of IFN-α and IFN-β produced by NDV-induced resident peritoneal macrophages in vitro reflect their state of activation induced by M-CSF in vivo.

Analysis of autoradiographic signals from in situ hybridizations with preparations of NDV-induced macrophages revealed a pronounced difference in signal intensity from cell to cell following hybridization to either IFN-α or -β probes, although at the dose of virus used for induction of IFN all cells were intensely stained for NDV as revealed by immunofluorescence (data not shown). However, when we used a DNA probe coding for a MHC class I antigen, H-2k0 or a cDNA probe for β-actin, signal distribution was much more regular. This observation is in agreement with previous studies on mouse (Zawatzky et al., 1985) and human (Enoch et al., 1986) cells and indicates that a high degree of variation in IFN mRNA content from cell to cell may be a general feature of IFN-producing cells in vitro. However, this does not necessarily imply that cells from induced cultures exhibiting only background levels of grains did not contain IFN mRNA, since in these experiments it was difficult to estimate the hybridization efficiency and accordingly the detection limit of the in situ hybridization. Thus, there is evidence for constitutive production of IFN in peritoneal macrophages and bone marrow-derived macrophages cultured in the presence of M-CSF (Moore et al., 1984; Belardelli et al., 1984, Gresser et al., 1985) and accordingly, following in situ hybridization of non-induced bone marrow macrophages, we sometimes observed a reduction in the numbers of grains per cell in preparations exposed to RNase treatment prior to hybridization, suggesting the presence of low levels of IFN mRNA (Zawatzky et al., 1986). These findings, however, could not be reproduced consistently and need further investigation.

A paradoxical finding is the apparent absence of IFN mRNA in poly(A)+ RNA preparations from CM-cultured bone marrow macrophages. Although we never detected antiviral activity in culture supernatants, there was a pronounced induction of 2-5AS activity in cytoplasmic extracts from these cells when compared to extracts from anti-IFN-treated bone marrow macrophages (Table 5). This is in agreement with results reported by Resnitzky et al. (1986), and could suggest that CM induces a factor in macrophages which cross-reacts with polyclonal anti-IFN antibodies, is capable of induction of 2-5AS activity in an autocrine manner, but does not cross-hybridize to either IFN-α or -β DNA probes. Alternatively, this unknown factor could also
be present in CM and partially purified M-CSF, emphasizing the need for pure preparations of M-CSF for future studies. However, preparation of poly(A)+ RNA from bone marrow macrophages requires large amounts of M-CSF during cell culture and is thus limited by the supply with pure material isolated from CM. In this respect, the availability of cloned human M-CSF (Kawasaki et al., 1985) which has great homology to murine L cell M-CSF (Boosman et al., 1987) and is active on mouse cells (Metcalf, 1986), could facilitate this supply.

In conclusion, the data reported here provide evidence for an enhancing effect in vitro of purified murine M-CSF on IFN synthesis in cultures of mouse macrophages following induction with NDV. This enhancing effect is in part due to additional activation of IFN-α gene expression.

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REFERENCES


IFN gene expression in mouse macrophages


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