Expression of Interferon-\(\beta\) during the Triggering Phase of Macrophage Cytocidal Activation

EVIDENCE FOR AN AUTOCRINE/PARACRINE ROLE IN THE REGULATION OF THIS STATE*

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Macrophage cytocidal activation is a pathway of development which endows macrophages with the capacity to recognize and destroy virally and neoplastically transformed cells. Observed in 1971 by Alexander and Evans (1) as a single response of mouse peritoneal macrophages to \(\mu\)g quantities of LPS† or naturally occurring and synthetic double-stranded RNAs, later work revealed this pathway to comprise two distinct stages (2–4). In the first stage, macrophages become primed upon exposure to a suitable stimulus, the most well characterized being the IFNs (\(\alpha, \beta, \gamma\)), which serve to increase the sensitivity of the cells to a second, triggering stimulus. Full cytocidal activity is expressed when primed macrophages encounter low (pg-ng) quantities of a triggering stimulus, which can take the form of LPS (2–4), double-stranded RNAs (5), heat-killed Listeria monocytogenes (6), and cytokines (7, 8).

The relationship between the single response originally observed and the two-stage response has been revealed collectively in several laboratories (5, 9, 10). The observation that most activating stimuli (e.g. double-stranded RNAs, LPS) also induce IFN expression suggested that macrophage-derived IFN may be playing a role in the regulation of cytocidal activation (9). Subsequent studies have shown that an early response of macrophages to \(\mu\)g quantities of triggering stimuli is the expression of IFN\(\alpha/\beta\) (5), and, based on the observed inhibition of cytocidal activation by anti-IFN\(\alpha/\beta\) antibodies (6, 10), local production of this cytokine appears to prime macrophages in an autocrine or paracrine fashion thereby rendering the cells sensitive to triggering agents. However, although this initial requirement for IFN expression in response to \(\mu\)g quantities of triggering stimuli can be circumvented by added IFNs, the stimuli that trigger IFN-primed macrophages to express cytocidal activity nevertheless remain prominent in their ability to induce IFN. This parallelism between triggering activity and IFN-inducing activity sug-

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‡ The abbreviations used are: LPS, lipopolysaccharide; IFN, interferon; HBSS, Hanks' balanced salt solution.
gested to us the possibility that in addition to its role in initially priming macrophages for cytocidal activity, IFN may also be expressed during the triggering phase, where it may potentially contribute to the regulation of macrophage cytocidal activity. We have explored this question in three ways. First, we attempted to dissociate triggering activity from IFN-inducing activity by exposing unprimed and IFN-primed macrophages to a wide variety of structurally related and charge-related polyanions which differ in their IFN-inducing activity, reasoning that if the hypothesis was incorrect, by priming the cells with IFN, we may circumvent the need for IFN production by the macrophages themselves. Second, we determined if macrophages express IFN during the triggering stage. Third, we explored the possible involvement of IFN in the regulation of the triggering stage by: (i) applying genetic differences in IFN production between different mouse strains to determine the capacity of macrophages to express markers of cytocidal activation, and (ii) investigating the effects of anti-IFNα/β antibodies on the expression of these markers. The markers employed were the complement component Bf and the reactive nitrogen intermediates NO2/NO3. Previous studies have shown their synthesis to be linked to the expression of cytocidal activity (10-15). Our results show that IFNβ is expressed during the triggering stage of macrophage cytocidal activation and suggest an important and previously unsuspected role in the positive regulation of this response.

EXPERIMENTAL PROCEDURES

Materials

Animals—The following strains of mice were purchased from the Jackson Laboratory, Bar Harbor, ME: C3H/HeJ, BALB/c, A/J, C57BL/6 and B10.A(2R). All mice were females and of ages ranging between 8 and 12 weeks. Upon arrival at the center, the animals were placed in a virus-free barrier room in the Animal Care Facility and were used after 2 weeks.

Reagents—Dulbecco's modified Eagle's medium was obtained from Hazleton Laboratories, Denver, PA and was supplemented immediately before use with 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Fetal bovine serum was purchased from Irvine Scientific, Santa Ana, CA. Twelve-well (22-mm-diameter) tissue culture plates and 100 × 20-mm tissue culture dishes were purchased from Costar, Cambridge, MA. Trans-S(32P)label (>1,000 Ci/mmole) and [α-32P]deoxycytidine 5'-triphosphate (>3,000 Ci/mmole) were obtained from ICN Biomedicals, Inc., Costa Mesa, CA. Goat anti-human Bf IgG was obtained from Atlantic Antibodies, Scarborough, ME. Mouse L-cell derived IFNβ (specific activity 1.3 × 107 units/mg) was purchased from Lee Biomolecular, San Diego, CA. Poly(1-C), poly(G-C), poly(A-U), poly(A-U-U), sulfanilamide, and naphthylethylenediamine hydrochloride were obtained from Sigma. Poly(dI-dC) and poly(dG-dC) were purchased from Pharmacia LKB. Hazelton Laboratories, Denver, PA and was supplemented immediately before use with 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Fetal bovine serum was purchased from Irvine Scientific, Santa Ana, CA. Twelve-well (22-mm-diameter) tissue culture plates and 100 × 20-mm tissue culture dishes were purchased from Costar, Cambridge, MA. Trans-S(32P)label (>1,000 Ci/mmole) and [α-32P]deoxycytidine 5'-triphosphate (>3,000 Ci/mmole) were obtained from ICN Biomedicals, Inc., Costa Mesa, CA. Goat anti-human Bf IgG was obtained from Atlantic Antibodies, Scarborough, ME. Mouse L-cell derived IFNβ (specific activity 1.3 × 107 units/mg) was purchased from Lee Biomolecular, San Diego, CA. Poly(1-C), poly(G-C), poly(A-U), poly(A-U-U), sulfanilamide, and naphthylethylenediamine hydrochloride were obtained from Sigma. Poly(dI-dC) and poly(dG-dC) were purchased from Pharmacia LKB Biotechnology Inc. All other reagents were of the highest possible purity.

Methods

Cells—Mouse bone marrow-derived macrophages were obtained by culturing femoral and tibial bone marrow from the various strains of mice in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 10% (v/v) heat-inactivated fetal calf serum, and 10% (v/v) L-cell conditioned medium (referred to as BM medium) using the method described by Sackstein et al. (29).

RESULTS

IFNs Prime Macrophages for Increased Bf and NO2/NO3 Expression—The capacity of IFNs to prime for the increased expression of Bf and NO2/NO3 is illustrated in Fig. 1. Macrophage monolayers were primed by incubation with IFNs (10,000 units/ml) or IFNγ (100 units/ml) for 24 h followed by immunoprecipitation of Bf with anti-human Bf antisera (10, 17). The immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis through 7.5% gels, and the radioactive bands were localized by fluorography. Each band was then excised, hydrolyzed in 18 h, and counted in a liquid scintillation spectrometer as reported previously (10, 18). Counts/min were corrected to dpm after determination of the degree of quenching within each sample and after accounting for the decay of the 35S between the experiment and the analysis. Incorporation of [35S]methionine into total protein was quantitated by precipitation of samples of the cell lysates with 10% (w/v) trichloroacetic acid in filter paper discs using the method of Robert and Peterson (19).

Quantification of NO2/NO3—The accumulation of NO2/NO3 in the culture supernatants of macrophages was quantified in 96-well plates using the Greiss reagent as described by Ding et al. (20). One hundred μl aliquots of the culture supernatants were dispensed in duplicate into Falcon 3912 Microtest III plates and mixed with 100 μl of a solution composed of 1% (w/v) sulfanilamide, 0.1% (w/v) naphthyl-ethylenediamine hydrochloride, and 2.5% (v/v) concentrated H3PO4. A standard curve consisting of 0.1-5.0 nmol of NaNO2/100-μl was prepared in BM medium. After incubation at ambient room temperature for 10 min, the absorbance of the wells were quantified at 550 nm in a Bioket Instruments enzyme-linked immunosorbent assay plate reader interfaced to an Apple IIe microcomputer. The number of cells/well was determined by lysing the cell monolayers in Zapon and quantifying the number of released nuclei with a model ZM Coulter counter. Concentrations of NO2/NO3 were interpolated from the NaNO3 standard curve, corrected for the volume of the culture supernatant, and normalized to the number of cells/well. Results are presented as nmol of NO2/NO3/106 adherent cells/unit of time (as defined under "Materials").

RNA Analyses—The expression of Bf, IFNα2, and IFNβ transcripts was determined by Northern analysis. The extraction, purification, electrophoresis, and transfer of the RNA to nitrocellulose membranes were carried out as described (10). Briefly, macrophage monolayers were extracted with 4 mM guanidine isothiocyanate (21), and the RNA was purified by centrifugation through a cushion of 5.7 M cesium chloride at 100,000 × g for 18 h. Either 15 μg of total RNA or the poly(A)+ RNA derived from 100 μg of total RNA by chromatography on oligo(dT)-cellulose (22) was electrophoresed under denaturing conditions through a 1.2% agarose-formaldehyde gel and then transferred to nitrocellulose filters (23). The blot was hybridized with 100 × 106 dpm of 32P-labeled cDNA probes (24, 25) and autoradiograms prepared by exposure to Kodak XAR-5 film at ~70 °C. The IFNβ cDNA probe was obtained from Drs. T. Maniatis and T. Enoch, Harvard Medical School, Boston (26) and was initially isolated and characterized in the laboratory of Dr. T. Taniguchi, Japanese Foundation for Cancer Research, Tokyo, Japan (27). The mouse IFNα2 cDNA probe was provided by Dr. P. Pitha-Rowe, Johns Hopkins Medical School, Baltimore, and was originally isolated and characterized by Shaw et al. (28). The mouse Bf cDNA probe has been described by Sackstein et al. (29).
Polyanions which are inactive as IFN inducers, namely poly(A-U), poly(G-C) (31-34) and four polyanions which are structurally related and active as triggering molecules in IFN-primed cells. Data similar to those were obtained when NO\textsubscript{2}/NO\textsubscript{3} was quantified in culture supernatants of macrophages exposed to poly(A·U), poly(A·U·U), and poly(G·C). Thus, the effectiveness of stimuli to trigger Bf expression could not be dissociated from their IFN-inducing activity.

**IFNβ Expression during Triggering**—The association between IFN-inducing activity and triggering activity in IFN-primed macrophages suggested the possibility that IFN expression, in addition to its clearly established role in initially priming macrophages, may be playing an important role in the triggering phase of cytoidal activation. We therefore determined the subtypes and conditions of expression of IFN by macrophages exposed to poly(I·C) and compared the temporal relationships of this response with the induction of Bf expression. Macrophage monolayers were incubated in medium alone or in medium containing 1 μg/ml poly(I·C) for 3, 6, 12, 24, or 48 h, and the levels of IFNβ, IFNγ, and Bf transcripts were determined at each time point by hybridization of equal amounts of total or poly(A+) RNA with the appropriate \textsuperscript{32}P-labeled cDNA probe. As shown in Fig. 3, under these conditions, IFNβ transcripts were detected in

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2 D. W. H. Riches, unpublished observation.

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**FIG. 1.** Priming by IFNα and IFNγ of the synthesis of the complement component Bf (panel A) and NO\textsubscript{2}/NO\textsubscript{3} (panel B) by mouse macrophage stimulated with the double-stranded polynucleotide poly(I·C). Macrophage monolayers were primed with either IFNβ (100 units/ml; panel A) or IFNγ (10 units/ml; panel B) for 4 h or were incubated in BM medium alone, washed in HBSS, and exposed to increasing concentrations of poly(I·C) for 24 h before quantifying the level of synthesis of Bf and NO\textsubscript{2}/NO\textsubscript{3}. Open circles, unprimed macrophages; closed circles, IFN-primed macrophages. TCA, trichloroacetic acid.

**FIG. 2.** Responsiveness of unprimed (panel A) and IFNβ-primed (panel B) macrophages to structurally related and charge-related polyanions. Macrophage monolayers were primed with IFNβ (100 units/ml, 6 h) or incubated in BM medium alone, washed in HBSS, and exposed to increasing levels of the indicated polyanions for 18 h before quantifying the level of synthesis of Bf. TCA, trichloroacetic acid; Mal-BSA, maleylated bovine serum albumin.
poly(A') RNA was isolated, and equal amounts were subjected to poly(1.C) (1 μg/ml) or B3 medium alone. At the indicated times, poly(A) RNA was isolated, and equal amounts were subjected to 1.2% (w/v) agarose-formaldehyde gel electrophoresis, blotted onto nitrocellulose, and probed with the appropriate 32P-labeled cDNA probe.

**FIG. 3.** Autoradiograph of a Northern blot showing the kinetics of IFNβ mRNA expression by mouse macrophages and its relationship to Bf mRNA expression after exposure to poly(I-C) (1 μg/ml) or B3 medium alone. At the indicated times, poly(A) RNA was isolated, and equal amounts were subjected to a triggering concentration of poly(I-C) (2 ng/ml) for the indicated times. Fifteen μg of total cellular RNA was subjected to Northern analysis as described under "Methods."

abundant quantities after a 3-h exposure of the cells to poly(I-C), peaked after 6 h, had declined to low levels by 24 h, and were virtually undetectable after a 48-h exposure to poly(I-C). In contrast, probing with an IFNα-specific cDNA probe failed to provide any indication that the transcript for this IFN subtype was expressed in response to challenge with poly(I-C) (data not shown). Bf transcripts were only barely detectable at 3 h and required 24 h of exposure to the stimulus before maximal expression was attained. Neither IFNα, IFNβ nor Bf transcripts were detected in unstimulated macrophages (Fig. 3). Thus exposure of unprimed macrophages to poly(I-C) led to the restricted expression of IFNβ with a time course that preceded that of Bf.

We next questioned if macrophages expressed IFNβ under triggering conditions. Macrophage monolayers were primed with 100 units/ml IFNβ for 3 h or incubated in medium alone, washed in HBSS, and exposed with increasing concentrations of poly(I-C) (0.3-1,000 ng/ml) for various time intervals up to 18 h before quantifying IFNβ mRNA by Northern analysis. As illustrated in Fig. 4A, exposure of unprimed macrophages to poly(I-C) induced a low level of IFNβ mRNA expression which was only detectable at the 3-h time point. By contrast, exposure of IFNβ-primed macrophages to poly(I-C) resulted in a dramatic increase in the rate of induction of the IFNβ mRNA, with levels being detectable after 1 h of exposure to poly(I-C). Significantly, the amount of IFNβ mRNA detected at the 3-h time point (Fig. 4B) was considerably greater than seen in unprimed cells. The response was transient in nature and had returned toward base-line levels after 6 h of triggering with poly(I-C). As will be seen in Fig. 4A and B, the kinetics of IFNβ mRNA expression also preceded that of Bf mRNA. Bf expression in unprimed cells was not detected until 6 h after the addition of poly(I-C). By contrast, the expression of Bf in IFNβ-primed macrophages was detected after only 3h, and the overall amplitude of the response was greatly increased over that seen in unprimed cells.

The ability of IFNβ to prime for its own expression by macrophages was not limited to poly(I-C) but was also seen when a triggering concentration of poly(A.U) (100 ng/ml) was employed (Fig. 5A). Furthermore, priming with IFNγ (10 units/ml) enhanced the expression of IFNβ mRNA in response to a triggering concentration of poly(I-C) (Fig. 5B). Thus IFN priming of macrophages resulted in a transient but greatly enhanced expression of IFNβ mRNA when the cells encountered a suitable triggering stimulus.

**Effect of the IFN Phenotype on the Expression of Bf**—The ability of IFNs to prime for the expression of IFNβ has been observed in other cell types, and hence it was important to determine if this response played a role in the expression of parameters associated with cytoidal activation in macrophages. To explore this issue, two experimental strategies were adopted. First, we investigated the effects of poly(I-C) on the production of Bf in mice which differ in their IFN loci and thus produce varying amounts of IFN in response to inducing stimuli. As previous studies have shown (36), B10.A(2R) mice are deficient in their ability to express IFN in response to poly(I-C) and hence comprise a low responder phenotype. However, BALB/c, C3H/HeJ, C57BL/6, and A/J mice, although differing at the If-1 locus and hence expressing different amounts of IFN in response to Newcastle disease virus (37, 38, 53), display a standard phenotype with respect to IFN induction by poly(I-C) (36). When BM macrophages established from these four strains of mouse were stimulated with increasing concentrations of poly(I-C) (0.3-1,000 ng/ml, 24 h), as expected, no significant differences were observed in the expression of Bf between macrophages derived from C3H/HeJ, BALB/c, C57BL/6, or A/J mice (Fig. 6). However, macrophages derived from B10.A(2R) mice showed a markedly diminished production of Bf in response to poly(I-C) in terms of both the sensitivity of the cells to the stimulus as well as in the overall magnitude of the response (Fig. 6). The

**FIG. 5.** Panel A, IFNβ priming of IFNβ mRNA accumulation during triggering with poly(A-U). Macrophages were primed with IFNβ (100 units/ml, 3 h) and triggered with either poly(I-C) (2 ng/ml), poly(A-U) (100 ng/ml), or BM medium alone for 4 h before harvesting the cells for RNA analysis. Panel B, IFNβ mRNA accumulation after priming with IFNγ and triggering with poly(I-C). Macrophage monolayers were primed with IFNβ (100 units/ml) or IFNγ (10 units/ml) for 3 h and triggered with poly(I-C) (2 ng/ml) for 4 h before harvesting the cells for RNA analysis. In each experiment, equal amounts of poly(A') RNA were electrophoresed through a 1.2% agarose-formaldehyde gel, blotted, and probed with the IFNβ cDNA probe.
same trends were observed for NO$_2$/NO$_3$ accumulation in the culture supernatants, indicating that these changes were not restricted to Bf (data not shown). Moreover, the diminished expression of Bf by macrophages derived from B10.A(2R) mice was also seen when the cells were primed with IFN$_\alpha$ (100 units/ml, 4 h), washed in HBSS, and triggered with increasing concentrations of poly(I-C) (Fig. 7). Indeed, approximately 10-fold greater quantities of poly(I-C) were required to induce a similar response in macrophages derived from B10.A(2R) mice compared with that seen with cells derived from C3H/HeJ mice. These data thus support the contention that the triggering-associated expression of IFN$\beta$ plays an important role in the expression of Bf.

**Effect of Anti-IFN$\alpha$/IFN$\beta$ Antibodies on the Expression of Triggering Parameters**—As a second approach to determining the potential importance of the triggering-associated expression of IFN$\beta$ on the synthesis of markers of the cytocidal state, we have determined if anti-IFN$\alpha$/IFN$\beta$ antibodies, present during the triggering stage, inhibit the expression of NO$_2$/NO$_3$. Macrophage monolayers were primed with either 100 units/ml IFN$\gamma$ or 10 units/ml IFN$\gamma$ for 4 h, washed, and then triggered with poly(I-C) (2 ng/ml) for 48 h in the presence or absence of 5,000 neutralizing units of anti-IFN$\alpha$/IFN$\beta$ antibody before quantifying the concentration of NO$_2$/NO$_3$ in the culture supernatants. As will be seen in Fig. 8A, exposure of macrophages to a triggering concentration of poly(I-C) alone resulted in the accumulation of a low level of NO$_2$/NO$_3$ in the culture supernatants which, in the absence of sufficient anti-IFN$\alpha$/IFN$\beta$ antibodies, was totally inhibited. After priming with IFN$\gamma$, the accumulation of NO$_2$/NO$_3$ in response to poly(I-C) was markedly enhanced. However, when IFN$\alpha$-primed macrophages were triggered with poly(I-C) in the presence of anti-IFN$\alpha$/IFN$\beta$ antibodies, the accumulation of NO$_2$/NO$_3$ in the culture supernatant of IFN$\gamma$-primed macrophages was dramatically inhibited (Fig. 8B). Similar results were obtained when macrophages were primed with IFN$\beta$ (data not shown). The inhibition of NO$_2$/NO$_3$ accumulation did not appear to be the result of nonspecific effects of antibody complexes since triggering in the presence of antibodies reactive against two other secretory proteins which are expressed during the triggering phase (Bf and C3) failed to inhibit the accumulation of NO$_2$/NO$_3$ (data not shown).

**Triggering Activity of the Calcium Ionophore A23187**—Recently conducted studies in our laboratory have shown that the calcium ionophores A23187 and ionomycin trigger the expression of Bf and NO$_2$/NO$_3$ in IFN$\beta$-primed macrophages but not in unprimed cells. Moreover, as will be seen from Fig. 9, in contrast to poly(I-C), triggering of IFN$\beta$ or IFN$\gamma$-primed macrophages with an optimal concentration of A23187 (0.5 $\mu$M) failed to induce the expression of IFN$\beta$ mRNA. These findings therefore provided an opportunity to determine the role of the triggering-associated expression of IFN$\beta$.

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We reasoned that since IFNβ is not produced during triggering with the calcium ionophores ionomycin and A23187, differences in the amplitude or time course of the expression of Bf or NO2/NO3 may provide an insight into the role of the IFN produced during triggering. Macrophage monolayers were therefore primed with IFNβ and were then washed and exposed to ionomycin (5.0 μM) for 24 or 48 h before quantifying the level of synthesis of Bf. As will be seen in Fig. 10, under these conditions, the synthesis of Bf is maintained for 48 h in macrophages that were triggered with poly(I-C) but had returned toward baseline in cells that were triggered with ionomycin. Identical results were obtained with the A23187. Importantly, as will be seen in Fig. 10, exposure of IFN-primed macrophages to A23187 (1 μM) in the continuous presence of purified IFNα (1,000 units/ml) approximately doubled (p = 0.003) the level of synthesis of Bf compared with macrophages triggered with A23187 in the absence of added IFN. By contrast, there was no significant effect (p = 0.512) of purified IFNβ (1,000 units/ml) on the expression of Bf in response to triggering with poly(I-C) (2 ng/ml) (Fig. 10B).

**DISCUSSION**

The question of the level of involvement of IFNs in macrophage cytotoxic activation has been wrestled with for over a decade. The possibility that IFNs may stimulate the expression of cytostatic and cytotoxic activity originally stemmed from the observations that a variety of poly-anionic agents, such as poly(I-C), and bacterial products, such as LPS, could both induce the expression of IFNa/β and activate macrophages (1, 50). Subsequently, it was shown that the addition of purified IFNs (both α/β and γ) to macrophage monolayers could directly activate macrophages to express cytotoxic activity and, moreover, that incubation of macrophages with poly(I-C) or LPS in the continuous presence of anti-IFNa/β antitogulbin led to an inhibition of the expression of cytotoxic activity (9, 40, 41). These findings led to the suggestion that IFNs, by themselves, could be inducing this important macrophage response. However, the interpretations of these early studies were drawn into question after the realization that LPS contamination of experimental materials was probably contributing to the expression of cytotoxic activity (3, 42). It has subsequently been shown that IFNs are a necessary but not sufficient stimulus for cytotoxic activation, and firm evidence has now been accrued to show that IFNs contribute to cytotoxic activation by priming macrophages and thereby increasing the sensitivity of the cells to a second triggering stimulus, such as LPS, poly(I-C), or heat-killed L. monocytogenes (2, 4, 6, 43).

However, although there is no reason to dispute the role that IFNs play in initially priming macrophages to respond to triggering stimuli, the findings reported herein emphasize the striking resemblance between triggering activity and IFN-inducing activity of most of the stimuli that are known to induce the expression of the macrophage cytotoxic state. We therefore explored the concept that IFNβ may be expressed during the triggering phase and may be contributing to the regulation of cytotoxic activity.

Exposure of macrophages to μg quantities of poly(I-C) led to an accumulation of IFNβ transcripts that peaked 6 h after stimulation and declined thereafter. IFNα2, transcripts were not found to be expressed under these conditions. These findings are consistent with previously reported studies in terms of the kinetics of induction of IFN activity in mouse peritoneal and BM macrophages during exposure to LPS (44–47), poly(I-C) (44), and Newcastle disease virus (44, 47) and in terms of the IFN subtype produced. Exposure of unprimed macrophages to a triggering concentration of poly(I-C) was found to induce a low level of expression of IFNβ mRNA. However, triggering of IFN-primed macrophages dramatically increased the level of expression of IFNβ mRNA as well as shortening the lag time required for induction. The triggering stage-associated expression of IFNβ mRNA was transient in nature, being essentially complete by 6 h. However, secreted IFNβ protein is relatively stable in culture medium and hence would be expected to be available to the macrophages after the IFNβ mRNA levels had declined. Importantly, and of relevance to the potential role of the triggering-associated production of IFNβ, IFNγ was also found to be an effective priming stimulus of this response under conditions that are optimal for the development of macrophage cytotoxic activity.

The ability of IFN to prime for its own production was originally reported by Isaacs and Burke (48) and has since been observed in many cell types (49–51) using both viruses and polyribonucleotides as inducing stimuli. In addition, Havell and Spitalny (45) have shown that crude lymphokine preparations derived from phytohemagglutinin-stimulated mouse spleen cells, which probably contain IFNγ, also prime mouse peritoneal macrophages for the increased production of an IFN activity following challenge with endotoxin. Gessani et al. (52) have recently reported similar observations using purified recombinant IFNγ. However, these observations have generally been considered in the context of the role of IFN in the induction of antiviral protection. Macrophages do differ from many of the epithelial and mesenchymal cells employed in the antiviral literature by their capacity to express many other complex functions that underlie their role in host defense and protection, including the expression of cytotoxic activity. It was important therefore to determine if the triggering stage-associated production of IFNβ contributed to the regulation cytotoxic activity or was a more general response of cells to IFNs.

First, we quantified the expression of Bf in macrophages derived from strains of mice which differed in their capacity to produce IFN in response to poly(I-C). When unprimed macrophages were exposed to increasing concentrations of poly(I-C), as expected, no differences were seen in the induction of Bf expression between macrophages derived from

![Fig. 10. Panel A, synthesis of Bf by IFN-primed macrophages triggered with ionomycin (5 μM) or poly(I-C) (1 ng/ml). At the indicated time points, the cells were biosynthetically labeled with [35S]methionine and the level of Bf synthesis determined by quantitative immunoprecipitation as described under "Methods." Panel B, reconstitution of the level of synthesis of Bf induced by A23187 during triggering in the presence of purified IFNβ. IFN-primed macrophages were triggered with A23187 (1 μM) in the presence (light shading) and absence (dark shading) of purified IFNβ (1,000 units/ml) for 36 h. TCA, trichloroacetic acid.](image-url)
C3H/HeJ, A/J, BALB/c, or C57BL/6 mice. However, the induction of Bf expression in macrophages derived from B10.A(2R) mice was markedly diminished both in terms of the sensitivity of the cells to poly(I-C) and in the amplitude of the response. Importantly, the diminished ability of macrophages derived from B10.A(2R) mice to express Bf was maintained when the cells were primed with exogenous IFN\(\beta\), washed to remove the priming stimulus, and then triggered with poly(I-C). It should be noted that H-2 linked polymorphisms in Bf functional activity have been reported (54, 55) although these genetic polymorphisms do not influence the antigenic level of Bf (55, 56). These data are thus consistent with the notion that the IFN\(\beta\) produced during the triggering stage of macrophage cytokidal activation actively contributes to the full expression of this state although the possibility that other genetic differences between B10.A(2R) mice and C3H/HeJ mice may account for these results cannot be excluded. We are currently attempting to resolve this issue since the addition of purified IFN\(\beta\) during the triggering phase was found to incompletely reconstitute the induction of Bf expression.

Second, we determined the effects of anti-IFN\(\alpha/\beta\) antibodies on the expression of the triggered state. Triggering of IFN\(\alpha/\beta\)-primed macrophages with poly(I-C) in the presence of anti-IFN\(\alpha/\beta\) antibodies was found to block the expression of NO\(\lambda\)/NO\(\lambda_3\) substantially. Furthermore, inhibition of NO\(\lambda_2\)/NO\(\lambda_3\) production by anti-IFN\(\alpha/\beta\) antibodies was equally effective when macrophages were initially primed with IFN\(\beta\) or with IFN\(\gamma\). These data thus further support the view that IFN\(\beta\) plays an active role in the expression of the triggered state. Previously reported experiments (5, 9, 10) which have probed the role of IFN\(\alpha/\beta\) in macrophage cytokidal activation have been conducted by coincubating the cells with anti-IFN\(\alpha/\beta\) antibodies and activating stimuli. Although such studies have provided important information concerning the overall necessity of IFN in cytokidal activation, such an approach has resulted in the important contribution of IFN\(\beta\) to the triggering stage being previously overlooked. Moreover, an additional implication of these findings is that the interpretation of experiments in which macrophages have been primed and triggered in vitro will be complicated by the inadvertent introduction of IFN\(\beta\).

Cytokidal activation is transiently expressed (4, 57), and we suspected that a potential role of the triggering-stage-associated IFN\(\beta\) may be to maintain macrophages in a primed state during triggering. An opportunity to investigate this question was provided by our observation that the calcium ionophores ionomycin and A23187 induce the expression of Bf and NO\(\lambda_2\)/NO\(\lambda_3\) in IFN\(\alpha/\beta\)-primed but not in unprimed macrophages. However, in contrast to poly(I-C), these ionophores do not induce the expression of IFN\(\beta\) during triggering. Thus differences in the amplitude or time course of expression of Bf or NO\(\lambda_2\)/NO\(\lambda_3\) during triggering with calcium ionophores could provide an indication of the role of the triggering stage-associated IFN\(\beta\). Triggering with poly(I-C), under conditions that were previously shown to result in the expression of IFN\(\beta\) during the triggering stage, resulted in a level of Bf synthesis that was maintained over 48 h. However, synthesis of Bf in macrophages that were triggered with ionomycin or A23187, although showing initial similarities to poly(I-C) at 24 h, had returned toward base-line levels by 48 h. Significantly, when IFN\(\alpha/\beta\)-primed macrophages were triggered with A23187 in the presence of added IFN\(\beta\), the level of synthesis of Bf was partially reconstituted compared with that seen in the absence of added IFN\(\beta\). Thus, collectively these data strongly suggest that the triggering stage-associated production of IFN\(\beta\) may play a key role in maintaining the cells in a primed state during triggering and thus may influence the duration of expression of cytokidal activity. In addition, based on observed cooperation between IFNs (39), it is possible that in the presence of activated T-cells, the triggering stage-associated IFN\(\beta\) may serve to act cooperatively with IFN\(\gamma\), leading to an amplification of cytokidal activity.

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