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Tyrosine Kinase But Not Phospholipid/Ca^{2+} Signaling Pathway Is Involved in Interferon-γ Stimulation of Ia Expression in Macrophages

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Abstract The specific signal transduction pathway(s) involved in the induction of the expression of the MHC class II molecule, Ia, on macrophages by interferon-γ (IFN-γ) is unclear. In this paper, we assessed the role of several signal transduction pathways including calcium mobilization, phospholipase C, protein kinase C and cyclic nucleotide-dependent protein kinase, and the tyrosine kinase pathways. IFN-γ was unable to mobilize intracellular calcium, unlike platelet-activating factor, which stimulated a threefold increase in cytosolic Ca^{2+} concentration in macrophages. Inhibition of the phospholipase C pathway by U73122 or ET-180CH_3 and of phosphatidic acid phosphohydrolase by propranolol did not suppress IFN-γ-induced Ia expression. In addition, inhibition of protein kinase C by calphostin C or cyclic nucleotide-dependent protein kinase by HA1004 did not suppress Ia expression. However, IFN-γ-induced Ia expression was significantly suppressed when the tyrosine kinase pathway was inhibited with herbimycin A and genestein. In addition, those two inhibitors suppressed tyrosine phosphorylation of several proteins in macrophages that may or may not be involved in the induction of Ia expression. Thus, IFN-γ used only the tyrosine kinase signaling pathway, but not the phospholipid/Ca^{2+} signaling pathways, to induce Ia expression in macrophages.

Macrophages exhibit numerous functional properties including antigen presentation to T cells [Dalton et al., 1993; Farrar and Schreiber, 1993; Huang et al., 1993]. The latter recognize antigenic peptides on the macrophage surface in association with the class II MHC molecule, the Ia molecule in mouse [Germain and Margulies, 1993]. Normally, Ia expression is low in unactivated macrophages. IFN-γ, a cytokine produced by T and natural killer (NK) cells, induces a high level expression of Ia molecules, which in turn promotes T-cell recognition [Farrar and Schreiber, 1993].

Abbreviations used: $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; CIITA, class II transactivator; DAG, diacylglycerol; EMEM, Eagle's minimum essential medium; GAF, IFN-γ activation factor; HBSS, Hank's balanced salt solution; IFN-γ, interferon-γ; LPS, lipopolysaccharide; PAF, platelet-activating factor; PI, phosphoinositide; PKA, protein kinase A; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; PLC, phospholipase C; PLD, phospholipase D; PMA, phorbol myristate acetate; TNF-α; tumor necrosis factor-α.

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The action of IFN-γ is initiated with its binding to a high affinity receptor on the target cell surface [Farrar and Schreiber, 1993]. However, the subsequent biochemical events leading to Ia expression are not completely understood. Controversies exist regarding the definitive involvement of a particular signaling pathway. Studies have shown that IFN-γ increased protein kinase C (PKC) activity in murine peritoneal macrophages after 3–4 h of stimulation [Becton et al., 1985; Hamilton et al., 1985]. IFN-γ-induced delayed PKC activation was also seen in macrophages from bacillus Calmette-Guérin (BCG)-resistant mice; however, IFN-γ did not induce PKC activation in BCG-sensitive normal mice [Brown et al., 1994]. The delayed PKC activation may be a secondary effect of IFN-γ stimulation of macrophages, because PKC activation is normally initiated within a few minutes of stimulation with an agonist [Nishizuka, 1992]. Moreover, IFN-γ was found to stimulate rapid IP3 production and PKC activation in human macrophage-like and glioblastoma cell lines [Fan et al., 1988; Klein et al., 1990; Nezu et al., 1990]. It has been shown that Ca^{2+} ionophore, PKC activa-

tors such as phorbol myristate acetate (PMA) or diacylglycerol (DAG), and intracellular injection of PKC stimulated Ia expression in murine macrophages [Smith et al., 1992; Strassmann et al., 1986]. In addition, several protein kinase inhibitors, such as calphostin C, H7, HA1004 and staurosporine, suppressed IFN-γ-stimulated Ia expression in murine macrophages [Politis and Vogel, 1990; Smith et al., 1992]. In contrast, a calcium/calmodulin-dependent protein kinase inhibitor W7, but not H7, blocked IFN-γ-induced Ia expression in murine macrophages [Celada and Maki, 1991]. Many of those inhibitors, however, are equally potent for more than one type of protein kinase [Badwey et al., 1991; Bruns et al., 1991; Elliott et al., 1990; Fallon, 1990; Herbert et al., 1990; Hidaka et al., 1984; Kobayashi et al., 1989]. Furthermore, H7, HA1004, and W7 differentially effected the IFN-γ-stimulated expression of HLA class II (human MHC class II) molecules in human macrophage-like cell lines [Fan et al., 1988; Ina et al., 1987; Klein et al., 1990; Koide et al., 1988]. Recently, an increase in intracellular pH associated with Ia expression [Prpic et al., 1989] and an increase of mitogen-activated protein kinase (MAP kinase) activity [Frendl et al., 1994] in murine macrophages were observed after IFN-γ stimulation. While those results may indicate that PKC, Ca^{2+}, or a cyclic nucleotide-dependent protein kinase could enhance Ia expression, they do not establish the specific involvement of PKC or a particular signaling pathway in IFN-γ-stimulated Ia expression.

Several previous reports have shown the involvement of tyrosine kinase in IFN-γ signal transduction in several different cell types. For example, IFN-γ induced tyrosine phosphorylation of its own receptor and a 91-kD protein, the IFN-γ activation factor (GAF), in fibroblasts [Farrar et al., 1992; Greenlund et al., 1994; Igarashi et al., 1993; Shuai et al., 1992]. Tyrosine phosphorylation was required for the activity of those proteins. Moreover, tyrosine kinase was shown to be involved in the IFN-γ-stimulated expression of HLA-DR molecules in human glioblastoma cells [Ryu et al., 1993]. In one report, IFN-γ induced the expression of *hck* and *lyn* tyrosine kinase genes in bone marrow-derived macrophages [Boulet et al., 1992]. IFN-γ–receptor interaction at the cell surface also led to activation of JAK kinases, catalyzing tyrosine phosphorylation of a 91-kD latent cytoplasmic transcription activator, STAT, in several cell lines [Darnell et al., 1994; Shual et al., 1993]. Recently, it has been shown that IFN-γ caused primary induction of class II transactivator protein in a JAK kinase-dependent way that was essential for class II MHC expression in human nonmacrophage cell types [Chang and Flavell, 1995; Chang et al., 1994; Silacci et al., 1994; Steimle et al., 1994]. In macrophages, MAP kinases at 47, 43, and 41 kD were induced by LPS [Li et al., 1994]. However, the activation of tyrosine kinase and its involvement in IFN-γ action in macrophages is unknown.

Several recent findings indicating the involvement of different signaling pathways have made the definative involvement of a particular signaling pathway in IFN-γ-stimulated Ia expression unclear. The objective of the present study was to identify one or more signaling pathways definitely involved in IFN-γ stimulation of Ia expression in murine peritoneal macrophages. Our results showed that only the tyrosine kinase signaling pathway was involved in the IFN-γ stimulation of Ia expression in macrophages, whereas others, such as phosphoinositide (PI)/Ca^{2+}, phospholipase D (PLD), and cAMP/PKA pathways, were not involved.

MATERIALS AND METHODS

Reagents

Propranolol, U73122 (1-(6-((17β-3methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5, diane), ET-18-OCH_3 (1-o-octadecyl-2-*o*-methyl-*sn*-glycerol-3 phosphorylcholine) were purchased from BIOMOL (Plymouth Meeting, PA). H7 ([1-(5-isoquinolinesultonyl)-2-methylpiperazine, 2HCl]), HA1004 ([N-(2-quandimoethyl)-5-isoquinoline sulfonamide HCl]), herbimycin A, and calphostin C were obtained from Calbiochem (San Diego, CA). Acetomethyl ester of Fura-2 (Fura-2, AM) was from Molecular Probes (Eugene, OR). Recombinant murine IFN-γ was from Gibco BRL (Grand Island, NY). Monoclonal antibody to mouse Ia antigen (anti-Ia) and biotinylated $F(ab)_2$ fragment of antirat IgG antibody (biotin-anti-IgG) were obtained from Boehringer Mannheim (Indianapolis, IN). Mouse IgG, rat IgG, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and alkaline phosphatase substrate-104 were from Sigma (St. Louis, MO). Streptavidin conjugated alkaline phosphatase was from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Anti-phosphotyrosine monoclonal antibody (clone 4G10) was obtained from UBI (Lake Placid,

NY). Horseradish peroxidase (HPO)-labeled antimouse IgG antibody (HRP-anti-IgG) and ECL Western blotting detection reagents were obtained from Amersham (Arlington Heights, IL).

Macrophages

Murine peritoneal macrophages were isolated as previously described [Hubbard et al., 1991]. In brief, 6-week-old C57BL/6N mice (Charles River, Boston, MA), housed under pathogen-free condition, were injected intraperitoneally with 2 ml of sterile thioglycollate broth. After 4 days, mice were killed and peritoneal exudate cells were removed and suspended in Eagle's minimum essential medium (EMEM) supplemented with 5% fetal calf serum (FCS), gentamycin, and L-glutamine. To each well of a 96-well microtiter plate, 2×10^5 macrophages were added. For some experiments, 2×10^7 macrophages were added to 100 mm tissue culture dishes, with or without sterile glass coverslips. For the determination of tyrosine kinase activity, 5×10^6 cells were incubated in 60-mm tissue culture dishes. After 2 hs, the nonadherent cells were removed by vigorous rinsing with Hank's balanced salt solution (HBSS). Purity of the population was greater than 95% macrophages as assessed by morphology and phagocytosis. Endotoxin level of the serum was $<0.06\%$, as determined by the supplier. All other reagents were negative for endotoxin, as determined by the *Limulus* amoebocyte lysate assay (Biowhittaker, Walkersville, MD). All glassware was heat-sterilized at 180°C for 4 h to destroy residual endotoxin.

Measurement of Ia Expression

Macrophage surface Ia was detected by an enzyme-linked immunosorbent assay (ELISA) described previously [Hubbard et al., 1991]. Briefly, adherent macrophages were stimulated with 50 U/ml IFN-γ for 24 h in serum-free EMEM at 37°C. After washing with PBS and fixing with 1% paraformaldehyde for 15 min, macrophage Fc receptors and the bare well surface were blocked by incubating in ELISA buffer (PBS, 0.02% NaN_3, and 0.5% BSA) containing 2 μg/ml mouse IgG for 60 min. Macrophages were next incubated with 1 μg/ml rat anti-Ia antibody or nonimmune rat IgG in ELISA buffer for 60 min, with biotin-anti-IgG for 60 min and with streptavidin–alkaline phosphatase conjugate for 30 min. For detection, 1 mg/ml phosphatase substrate 104 in carbonate buffer, pH 9.6, was added to the wells and incubated for 30 min in the dark. Absorbance of the reaction mixture was determined at 407 nm with an automated ELISA reader (Dynatech, Chantilly, VA).

Measurement of Cytosolic Free Ca^{2+} Concentration

Macrophages were removed from the tissue culture dishes by a gentle stream of HBSS through a 25-gauge needle. The resulting suspension of macrophages, at a density of 5×10^6 cells/ml in HEPES-buffered HBSS without phenol red, was incubated with 5 μM Fura-2, AM for 45 min at 23°C. Cells were washed, resuspended at 2×10^6 cells/ml, and measurement of Fura-2 fluorescence was initiated. After 25 sec, various agonists were added to the cells. Fluorescence was measured in a spectrofluorometer (Hitachi, F2000) with 340-nm (Fura-2-Ca complex) and 380-nm (free Fura-2) wavelengths for excitation, and 510 nm for emission. The cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$ was calculated from the ratio (R) of fluorescence from 340- and 380-nm excitation, with the equation $Kd \times (R\text{-}Rmin/Rmax\text{-}R) \times (Fmin\ \lambda 2/Fmax\ \lambda 2)$, where Kd = 285 nM for the apparent dissociation constant for Ca^{2+} and Fura-2 at physiological conditions [Groden et al., 1991]. Fmax λ2 and Fmin λ2 are the maximum and minimum fluorescence at 380 nm excitation wavelength. Maximum and minimum fluorescence, the fluorescence at the saturating and zero concentrations of external Ca^{2+} were obtained by the sequential addition of 0.02% digitonin and 10 mM EGTA. To measure Ca^{2+} level in adherent macrophages, cells on coverslips were loaded with Fura-2 in the presence of 10 μM pluronic F-127 and Fura-2 fluorescence, measured as described above.

Determination of Tyrosine Phosphorylation of Proteins

Adherent macrophages in 60-mm dishes were pre-incubated with medium alone or 0.25 μM of a tyrosine kinase inhibitor, herbimycin A. After 60 min, medium was removed, fresh prewarmed medium containing 50 U/ml IFN-γ, in the presence or absence of 0.25 mM herbimycin A, was added and the cells were incubated for various periods. After washing with chilled wash buffer (20 mM Tris–HCl, pH 8, 140 mM NaCl and 2 mM $NaOV_4$), cells were scraped into 0.25 ml chilled lysis buffer (wash buffer containing 1 mM phenylmethylsulfonyl fluoride, 20 μM leupep-

tin, 0.2 U/ml aprotinin, 10% glycerol, and 1% Triton X-100) and placed on ice for 20 min. The lysate was centrifuged at 3,200*g* for 10 min at 4°C and the supernatant stored at −80°C. The protein concentration of the supernatant was measured using a kit from BioRad (Richmond, CA). Proteins were resolved by 10% SDS–PAGE and electroblotted on to a PVD nitrocellulose membrane. The membrane was blocked with 5% skim milk in 20 mM Tris-Cl, pH 7.5, 140 mM NaCl (blocking solution) for 60 min at 23°C. After washing with Tris-buffered saline containing 0.05% Tween 20, membrane was incubated with 0.5 μg/ml antiphosphotyrosine antibody in the blocking solution for 60 min at 23°C, rewashed, and incubated with HRP-anti-IgG for 60 min at 23°C. After washing as above, blots were detected with the ECL system as described by the manufacturer.

Determination of Cell Viability

The viability of macrophages was assessed by MTT assay, a mitochondrial enzyme assay. In living cells, MTT is reduced to blue formazan by mitochondrial succinate dehydrogenase [Mosmann, 1983]. After various treatments, cells were washed and incubated with 1 mg/ml MTT in EMEM for 4 h at 37°C. The cells were washed with PBS, air dried, and the blue formazan crystals dissolved in isopropanol. Absorbance of solutions was quantitated at 550 nm in an automated ELISA reader (Dynatech). Viability was calculated as % viable = 100 × absorbance of treated samples/absorbance of untreated sample.

RESULTS

Stimulation of Ia Expression by IFN-γ

Initially, we determined the optimum concentrations required for the maximum stimulation of Ia expression by IFN-γ. In preliminary experiments, we observed that stimulation times for maximal Ia expression varied with the concentration of IFN-γ; 24 h was the time period required for the maximum induction of Ia expression with lowest amount of IFN-γ (data not shown). Stimulation of Ia expression reached a plateau with 10 U/ml IFN-γ and became maximal with 50 U/ml (Fig. 1). Based on these results, in all subsequent determinations of Ia expression, macrophages were stimulated with 50 U/ml of IFN-γ for 24 h.

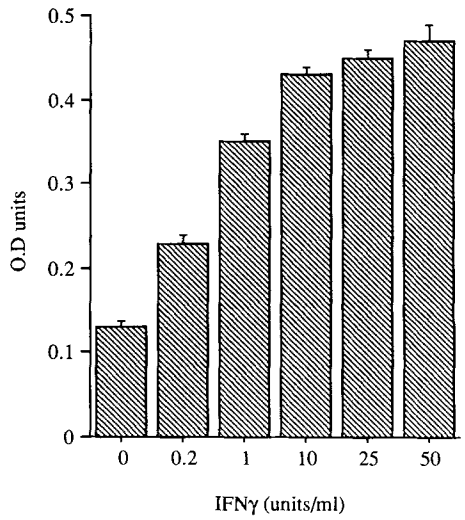
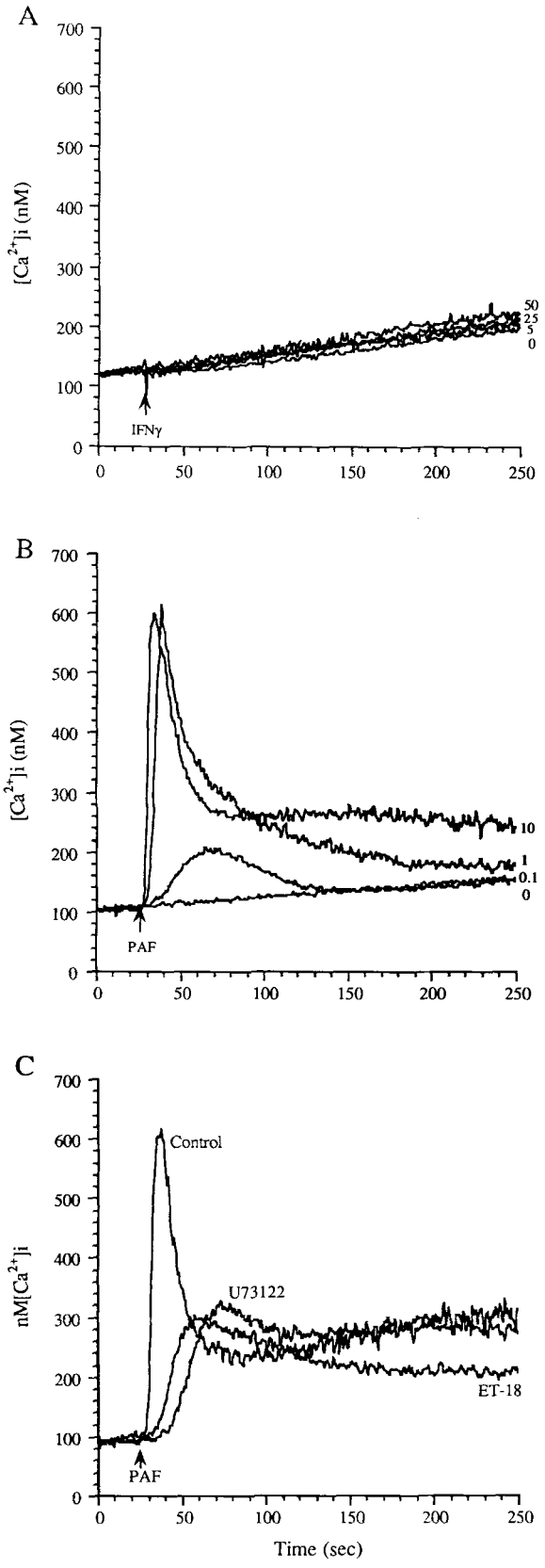


Fig. 1. Stimulation of Ia expression in macrophages by IFN-γ. Peritoneal macrophages were cultured for 24 h with various doses of IFN-γ. Cell surface Ia was measured by ELISA. Results are expressed as mean O.D. units ±SEM.

IFN-γ Did Not Stimulate an Increase of $[Ca^{2+}]_i$

IFN-γ may stimulate an increase of $[Ca^{2+}]_i$ in human macrophage-like and glioblastoma cell lines [Klein et al., 1990; Koide et al., 1988; Nezu et al., 1990]. Several studies have also indirectly suggested that IFN-γ may stimulate an increase of $[Ca^{2+}]_i$ in murine macrophages [Celada and Schreiber, 1986; Somers et al., 1986; Wright et al., 1985]. To assess the Ca^{2+} mobilizing action of IFN-γ in macrophages, we directly measured the temporal pattern of changes in $[Ca^{2+}]_i$ following IFN-γ stimulation of murine macrophages. With concentrations of 5–50 U/ml, IFN-γ did not induce any immediate changes in $[Ca^{2+}]_i$ in suspension macrophages (Fig. 2A). As a control, we stimulated a similar macrophage preparation with platelet activating factor (PAF), a Ca^{2+} mobilizing agonist in macrophages [Chao and Olson, 1993]. PAF stimulated a biphasic rise of $[Ca^{2+}]_i$ in a dose-dependent manner (Fig. 2B). Inhibition of phosphatidylinositol bisphosphate (PIP_2) hydrolysis by phospholipase C (PLC) inhibitors, U73122, or ET-18-OCH_3, greatly reduced the PAF stimulated rise of $[Ca^{2+}]_i$ (Fig. 2C). Accurate measurement of $[Ca^{2+}]_i$ in adherent macrophages was prevented due to variability in their incorporation of Fura-2, which led to altered calibration of Fmax and Fmin. For a more accurate presentation of calcium mobilization after stimulation with PAF or IFN-γ, changes in the 340/380 ratio, instead of $[Ca^{2+}]_i$, are presented. Only PAF, not IFN-γ, induced a



significant increase in 340/380 ratio in a dose-dependent manner (Fig. 3). Based on these results we conclude that IFN-γ does not stimulate an increase of $[Ca^{2+}]_i$ in macrophages under these conditions, either through the PI signaling pathway or by other mechanisms.

Inhibition of Phospholipid Signaling Pathways Did Not Suppress Ia Expression

Next, we studied the involvement of phospholipid signaling pathways in the IFN-γ stimulation of Ia expression in macrophages. To assess the PI signaling pathway, we used U73122 and ET-18-OCH_3, which inhibited PLC activity in several cell types including platelets and neutrophils [Bleasdale et al., 1990; Smith et al., 1990]. To determine the involvement of the PLD pathway we used propranolol, which blocks the PLD pathway by inhibiting phosphatidic acid phosphohydrolase [Thompson et al., 1991]. Neither U73122 nor ET-18-OCH_3 at 0.025–0.25 μM inhibited Ia expression (Fig. 4A). However, the same doses of those inhibitors were able to inhibit PLC activity, as was demonstrated by suppression of PAF-stimulated increase of $[Ca^{2+}]_i$ (Fig. 2C). Similarly, 2.5–25 μM propranolol did not suppress Ia expression (Fig. 4B). However, 2.5 μM propranolol inhibited another macrophage function which utilizes the PLD pathway such as lipopolysaccharide (LPS)-stimulated tumor necrosis factor-α (TNF-α) production by mouse macrophages (results not presented). Thus, neither the PI signaling nor the PLD signaling pathway was involved in IFN-γ stimulation of Ia expression, even though those were functional and able to respond to an appropriate stimulus.

Inhibitors of Protein Kinase Did Not Suppress IFN-γ-Induced Ia Expression in Macrophages

Several investigators have suggested the possibility that PKC may be involved in IFN-γ-stimulated Ia expression in murine macrophages [Fan et al., 1988; Klein et al., 1990; Nezu et al., 1990; Politis and Vogel, 1990; Smith et al.,

Fig. 2. Effect of IFN-γ and PAF on $[Ca^{2+}]_i$ in macrophages. Suspension macrophages were incubated with 5 μM Fura-2, AM, the cells washed, and $[Ca^{2+}]_i$ measured by a fluorescence spectrofluorometer. Agonists were added 25 sec after the initiation of Ca^{2+} measurement. Addition of **A**, various doses of IFN-γ (U/ml); **B**, various doses of PAF (nM); **C**, 0.25 μM U73122 or ET-18-OCH_3 5 min prior to the addition of 1 nM PAF.

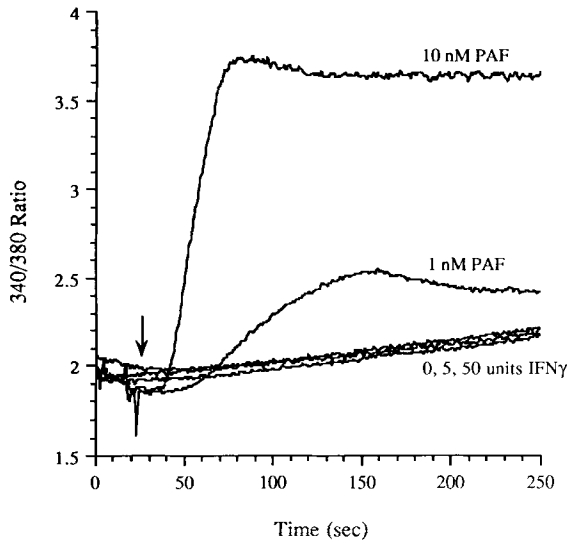


Fig. 3. Effect of PAF and IFN-γ on the cytosolic Ca^{2+} level of adherent macrophages. Macrophages on coverslips were incubated with 5 μM Fura-2, AM, in the presence of 10 μM pluronic F-127. After washing, fluorescence measurement was initiated and various agonists were added at 25 sec. Changes of $[Ca^{2+}]_i$ were expressed as the changes in fluorescence ratio from 340- and 380-nm excitation wavelengths.

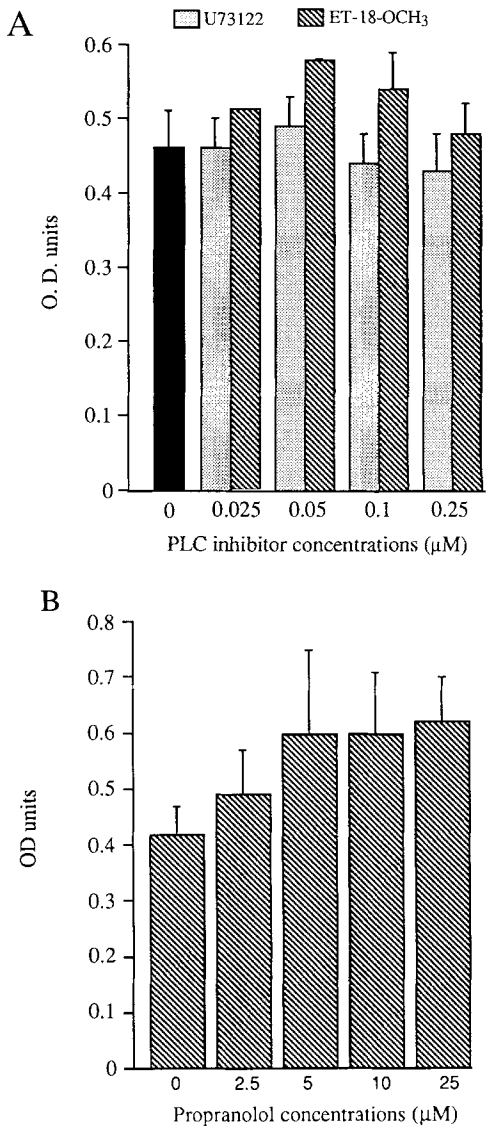


Fig. 4. Effect of U73122, ET-18-OCH_3, and propranolol on Ia expression in macrophages. Peritoneal macrophages were stimulated with 50 U/ml IFN-γ and Ia measured. Various doses of **A**, U73122 or ET-18-OCH_3, or **B**, propranolol were added to the cells 1 h before IFN-γ addition. Ia expression without IFN-γ = 0.11 ± 0.02.

1992; Strassmann et al., 1986]. By contrast, others indicated a PKC-independent pathway for Ia expression in murine macrophages [Brown et al., 1994; Celada and Maki, 1991]. In this experiment we assessed the involvement of PKC, cAMP-dependent protein kinase (PKA), and cGMP-dependent protein kinase (PKG) in IFN-γ stimulation of Ia expression. Macrophages were pretreated for 1 h with calphostin C, a specific PKC inhibitor; H7, an inhibitor of PKC, PKA, and PKG; and HA1004, a potent inhibitor of PKA and PKG but not of PKC [Bruns et al., 1991; Hidaka et al., 1984; Kobayashi et al., 1989]. The cells were then stimulated with IFN-γ. Calphostin C at 0.01–0.1 μM did not suppress Ia expression (Fig. 5A); however, 0.01 μM calphostin C suppressed another macrophage function such as LPS-stimulated TNFα production (data not shown). Likewise, H7 and HA1004 failed to inhibit Ia expression even at a maximum nontoxic concentration of 10 μM (Fig. 5B). These results demonstrated that activation of PKC, PKA, or PKG was not involved in the IFN-γ stimulation of Ia expression.

Tyrosine Kinase Inhibitors Alter IFN-γ-Stimulated Ia Expression

Previous investigators have indicated the involvement of tyrosine kinase in IFN-γ signaling in cells other than macrophages [Farrar et al., 1992; Greenlund et al., 1994; Igarashi et al., 1993; Ryu et al., 1993; Shuai et al., 1992].

We next assessed the involvement of the tyrosine kinase signaling pathway in IFN-γ stimulation of Ia expression. For this, macrophages were preincubated with the tyrosine kinase inhibitors, herbimycin A, or genistein, for 60 min before stimulation with IFN-γ. Both inhibitors suppressed IFN-γ-stimulated Ia expression in a dose-dependent manner, without affecting cell viability as assessed by the MTT assay (Fig.

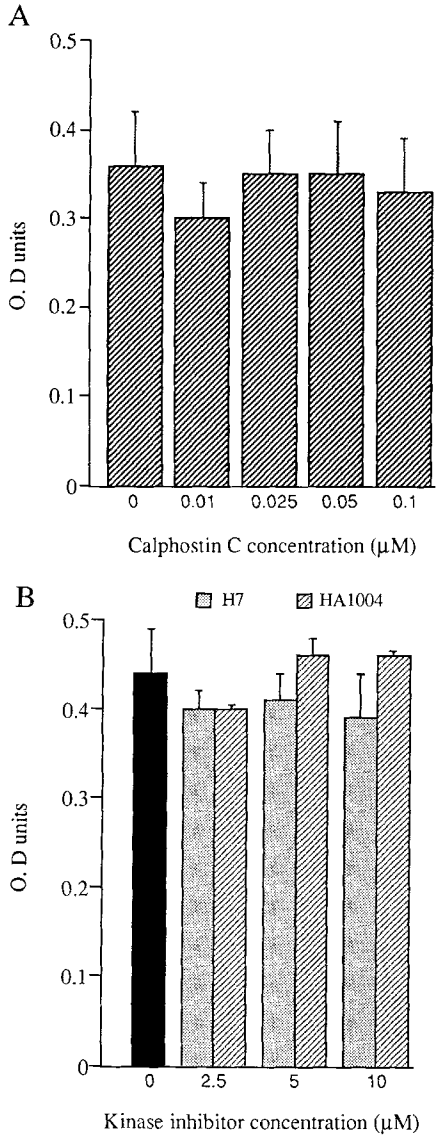


Fig. 5. Effect of calphostin C, H7, and HA1004 on Ia expression in macrophages. The inhibitors were added 1 h prior to the addition of 50 U/ml of IFN-γ and Ia expression measured. Inhibitors used were **A**, calphostin C; **B**, H7 or HA1004. Ia expression without IFN-γ stimulation = 0.096 ± 0.025.

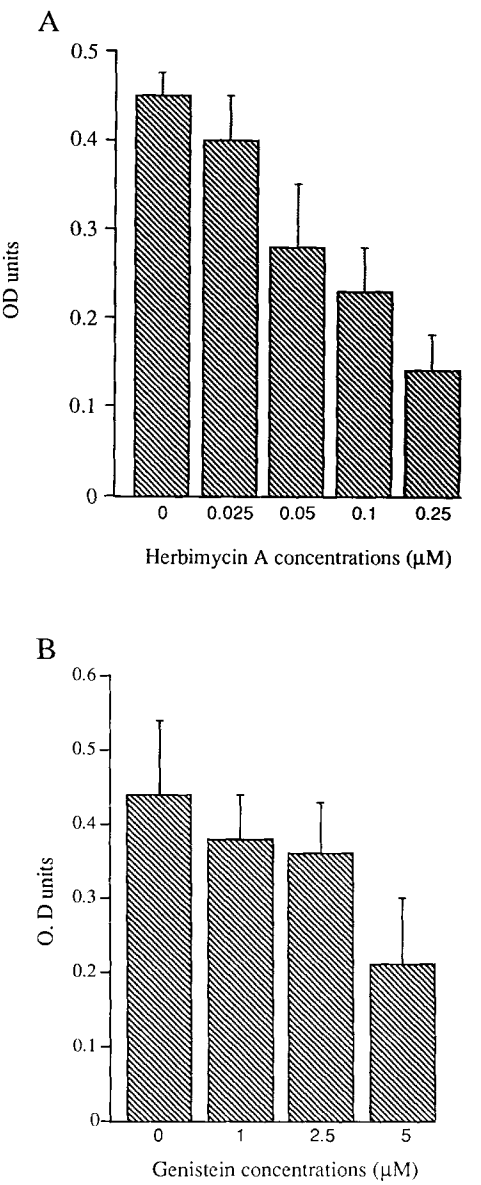


Fig. 6. Effect of tyrosine kinase inhibitors on Ia expression in macrophages. Various concentrations of herbimycin A (**A**) or genistein (**B**) were added 1 h before the addition of 50 U/ml of IFN-γ. Ia expression without IFN-γ stimulation = 0.14 ± 0.02.

6A,B). Maximum nontoxic inhibition was observed with 0.25 μM herbimycin A and 5 μM genistein. These results demonstrated IFN-γ activated tyrosine kinase as one component of inducible Ia expression in murine macrophages.

IFN-γ-Stimulated Tyrosine Phosphorylation of Proteins in Macrophages

Based on those observations, we explored whether IFN-γ was able to phosphorylate select proteins in macrophages. Stimulation of macrophages with 50 U/ml IFN-γ resulted in increased tyrosine phosphorylation of several different proteins with approximate molecular weights of 52, 43, 38, 26, 24, and 22 kD (Fig. 7). Maximum phosphorylation of p52 was observed after 10 sec of stimulation, p43 and p38 were maximally phosphorylated after 20 sec, and p22–p26 were maximally phosphorylated after 30 sec. Tyrosine phosphorylation of all the proteins significantly declined after 60 sec of stimulation. Tyrosine phosphorylation of some proteins were constitutive, because it remained the same in

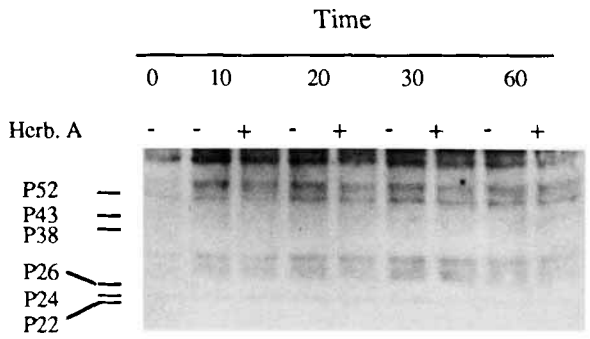


Fig. 7. IFN-γ stimulation of tyrosine phosphorylation of proteins in macrophages. Macrophages were stimulated with 50 U/ml IFN-γ for various periods (sec) and processed for the detection of tyrosine phosphorylated proteins. Lines indicate the proteins whose tyrosine phosphorylation was stimulated by IFN-γ. The corresponding numbers are the approximate molecular weight in kD.

the stimulated and unstimulated macrophages. IFN-γ-stimulated tyrosine phosphorylation of proteins was also inhibited by herbimycin. These results showed that IFN-γ stimulated a rapid increase in protein tyrosine kinase activity, resulting in the increased tyrosine phosphorylation of several cellular proteins in macrophages.

DISCUSSION

We have examined the IFN-γ signal transduction pathway leading to enhanced Ia expression in murine macrophages. We as well as others have hypothesized that IFN-γ uses the PI signaling pathway in stimulating Ia expression in macrophages [Fan et al., 1988; Klein et al., 1990; Nezu et al., 1990; Smith et al., 1992; Strassmann et al., 1986]. Several experiments were designed to test that hypothesis. First, we directly measured the changes of $[Ca^{2+}]_i$ after IFN-γ stimulation. Our results showed that IFN-γ did not induce any changes of $[Ca^{2+}]_i$. However, in the same cell preparation, PAF, a PI signaling agonist for macrophages, stimulated a biphasic rise of $[Ca^{2+}]_i$, indicating the PI signaling pathway and Ca^{2+} mobilizing system was functional in those macrophages. It was possible that IFN-γ was not able to act on suspension macrophages, due to some unknown change during suspension cell preparation. This was resolved by comparing the effect of PAF and IFN-γ on the $[Ca^{2+}]_i$-dependent changes of 340/380 ratio in adherent macrophages. Only PAF, but not IFN-γ, stimulated an increase of 340/380 ratio in the adherent macrophages. Also, the ability of IFN-γ to act on suspension macrophages was demonstrated by our preliminary results that IFN-γ inhibited PAF action on suspension macrophages (data not shown). Our findings contrast with others that have shown that IFN-γ-stimulated an increase of $[Ca^{2+}]_i$ in human glioblastoma and macrophage-like cell lines [Fan et al., 1988; Klein et al., 1990; Koide et al., 1988]. However, differences in cell types, primary cell versus cell lines and species of origin could account for these discrepancies. Collectively, our results demonstrated that IFN-γ did not activate the PI signaling or any other Ca^{2+} mobilizing pathway in primary murine macrophages. This conclusion was further substantiated by our finding that inhibition of PI signaling pathway by PLC inhibitors suppressed PAF-stimulated increase of $[Ca^{2+}]_i$, but not IFN-γ-stimulated Ia expression in macrophages. Further, we demonstrated that inhibition of PKC, PKA, PKG, or phosphatidic acid phosphohydrolase, with the appropriate inhibitors, did not suppress Ia expression. Based on these findings, we propose that PI/Ca^{2+} /PKC, cAMP/PKA, cGMP/PKG, or PLD signaling pathways were not involved in IFN-γ stimulation of Ia expression macrophages.

Alternatively, IFN-γ-stimulated rapid tyrosine phosphorylation of several proteins in macrophages, which was inhibited by herbimycin A. The identity of these proteins is not known. However, molecular weights of some of these proteins, p43, and p38, closely matches that of many proteins, such as 39–45 kD MAP kinase, known to be tyrosine phosphorylated in many cell types [Greenlund et al., 1994; Igarashi et al., 1993; Shuai et al., 1992; Weinstein et al., 1992]. In macrophages, MAP kinase was shown to be activated by LPS through tyrosine phosphorylation [Weinstein et al., 1992] and by IFN-γ through an unidentified mechanism [Frendl et al., 1994]. Consistent with these findings, inhibition of tyrosine kinase by herbimycin A or genistein suppressed the IFN-γ-stimulated Ia expression in macrophages. Inhibition of tyrosine kinase has also been shown to suppress the IFN-γ-stimulated HLA-DR expression in human glioblastoma cells [Ryu et al., 1993]. Finally, the IFN-γ-activated tyrosine kinase pathway in macrophages differed from that in other cell types in the kinetics of tyrosine kinase activation. In macrophages, maximum stimulation of tyrosine phosphorylation was observed in less than 1 min, which returned to a basal level by one min. By contrast, other cell types like fibro-

blasts required several minutes for maximum tyrosine phosphorylation, which remained at that increased level for further several min fibroblasts [Farrar et al., 1992; Greenlund et al., 1994; Igarashi et al., 1993; Ryu et al., 1993; Shuai et al., 1992]. Based on the above results, we propose that IFN-γ specifically uses the tyrosine kinase signaling pathway, in the stimulation of Ia expression in macrophages.

Several previous studies have assessed the possible signaling pathway involved in IFN-γ-induced Ia expression, using various methods. Both the PKC-dependent and -independent pathways, such as the Ca^{2+}/calmodulin pathway, may play a role. Several studies have shown an increase of PKC activity in macrophages 3–4 h after IFN-γ stimulation [Becton et al., 1985; Hamilton et al., 1985]. Normally, stimulation of PKC activation by an agonist is initiated within a few minutes of stimulation [Nishizuka, 1992]. For example, IFN-γ-induced PKC activation in human glioblastoma and macrophage-like cell lines was seen after a few minutes of stimulation [Fan et al., 1988; Klein et al., 1990; Nezu et al., 1990]. Thus, considering these time kinetics, IFN-γ-stimulated delayed PKC activation in murine macrophages must be a secondary effect of IFN-γ stimulation. It has been shown that a Ca^{2+} ionophore, a PKC activator (PMA or DAG), and intracellular injection of PKC-induced Ia expression in macrophages [Smith et al., 1992; Strassmann et al., 1986]. These findings indicate that PKC can stimulate Ia expression. However, they do not establish that PKC is involved in IFN-γ stimulation of Ia expression in macrophages. PKC may be involved in Ia expression stimulated by some PKC activating agonist, such as PAF (unpublished results).

Various protein kinase inhibitors, such as H7, HA1004, calphostin C, and staurosporine, have been reported to suppress IFN-γ stimulation of murine macrophage Ia expression [Politis and Vogel, 1990; Smith et al., 1992]. However, these inhibitors affected Ia expression differently. For example, H7, staurosporine, and calphostin C suppressed PMA- and DAG-induced Ia expression completely; IFN-γ-induced expression partially, and did not affect Ca^{2+} ionophore-induced Ia expression. HA1004 inhibited IFN-γ-induced Ia expression partially without any effect on PMA-, DAG-, or Ca^{2+} ionophore-induced expression [Smith et al., 1992]. If PKC mediated the actions of IFN-γ, only a PKC inhibitor, but not the one that inhibits many protein kinases except PKC, should inhibit IFN-γ-induced Ia expression. Similarly, if Ca^{2+} ionophore exerted its action through PKC activation, these protein kinase inhibitors should have suppress the Ca^{2+} ionophore-induced Ia expression. Some of the protein kinase inhibitors were potent for a wide varieties of protein kinases, including PKC, PKA, tyrosine kinase, and calcium/calmodulin-dependent protein kinases [Badwey et al., 1991; Bruns et al., 1991; Elliott et al., 1990; Fallon, 1990; Herbert et al., 1990; Hidaka et al., 1984; Kobayashi et al., 1989]. The role of PKC in Ia expression was further confounded, because H7 suppressed IFN-γ-stimulated Ia expression but IFN-γ did not induce PKC activation in murine macrophages [Brown et al., 1994]. Therefore, based on the above findings a specific role of PKC or other protein kinases in IFN-γ-induction of Ia expression cannot be ascertained.

We as well as others [Celada and Maki, 1991] did not observe any inhibitory effects of several protein kinase inhibitors on IFN-γ-induced Ia expression in murine macrophages. The reason for this discrepancy is not known. We have stimulated the macrophages with IFN-γ in the serum-free medium. In other studies, macrophages were stimulated with IFN-γ in serum-containing medium and Ia expression was decreased after treatment with protein kinase inhibitors [Politis and Vogel, 1990; Smith et al., 1992]. Another factor may enhance the IFN-γ-stimulated Ia expression through PKC. For example, our preliminary results showed that PAF, a PKC activating agonist, stimulated Ia expression in macrophages (data not shown). In addition, anatomical locations of the macrophages, such as peritoneal macrophages [Politis and Vogel, 1990; Smith et al., 1992] versus bone marrow-derived macrophages [Celada and Maki, 1991; Politis and Vogel, 1990; Smith et al., 1992], may also account for the difference between reports demonstrating PKC-dependent and -independent pathways of Ia expression. Moreover, the ability of H7, HA1004, and W7 to suppress IFN-γ-stimulate HLA class II expression in human macrophage-like cell lines was variable; both the PKC- as well as Ca^{2+}/calmodulin-dependent pathways has been described [Fan et al., 1988; Ina et al., 1987; Klein et al., 1990; Koide et al., 1988]. To establish the involvement of PKC in IFN-γ-stimulated Ia expression, it was necessary to demonstrate that IFN-γ induced a rapid PKC activation, the delayed PKC activation was the primary but not the second-

ary effect of IFN-γ stimulation of macrophages, and only a selective PKC inhibitor suppressed IFN-γ-stimulated Ia expression.

In the present work we have demonstrated for the first time that IFN-γ uses only the tyrosine kinase signaling pathway, but not the PI or other Ca^{2+} mobilizing pathways, to regulate Ia expression in macrophages. Although IFN-γ activation of tyrosine kinase signaling pathway and its involvement in class II MHC expression in other cells have been reported, we consider this work important, because it has resolved the controversy over the definitive involvement of a particular signaling pathway in IFN-γ-stimulated Ia expression. Because the operation of a given signaling pathway leading to a similar cellular response varies among cell types, further studies will be required to conclude whether JAK kinase/CIITA or other factors are involved in translating tyrosine kinase activation to Ia expression in macrophages. In fact, difference in the kinetics of tyrosine kinase activation indicates that the operation of IFN-γ-activated tyrosine signaling pathway in macrophages may be different from that in other cell types. Our work represents the first step toward this direction.

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