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Role of Na^+/H^+ Exchange by Interferon-γ in Enhanced Expression of JE and I-A_β Genes

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The rapid transductional sequences initiated by interferon-γ (IFN-γ) on binding to its receptor regulate functional and genomic responses in many cells but are not well defined. Induction of macrophage activation is an example of such functional and genomic changes in response to IFN-γ. Addition of IFN-γ to murine macrophages, at activating concentrations, produced rapid (within 60 seconds) alkalinization of the cytosol and a concomitant, rapid influx of $^{22}Na^+$. Amiloride inhibited the ion fluxes and the accumulation of specific messenger RNA for two genes induced by IFN-γ (the early gene JE and the β chain of the class II major histocompatibility complex gene I-A). The data indicate that IFN-γ initiates rapid exchange of Na^+ and H^+ by means of the Na^+/H^+ antiporter and that these amiloride-sensitive ion fluxes are important to some of the genomic effects of IFN-γ.

THE PLEOTROPIC ACTIONS OF IFN-γ include antiviral, antiproliferative, and immunoregulatory effects in a wide variety of responsive cells (*1*). One cellular target is the macrophage, which IFN-γ activates to present antigen to T lymphocytes and to kill facultative and obligate intracellular parasites and tumor cells (*2*). These regulatory effects are initiated when IFN-γ occupies a cell surface receptor, an event that leads to priming for protein kinase C activation, an oxidative burst, eicosanoid production, and the induction of genes coding for proteins such as class II major histocompatibility complex (MHC) molecules, also referred to as Ia molecules. Surface expression of Ia molecules is essential to functions of the activated macrophage and is stringently regulated (*3, 4*). The initial transductional events occurring after IFN-γ occupies its receptor have not been delineated (*5, 6*). We reported earlier that addition of IFN-γ to murine macrophages results in an enhanced efflux of $^{45}Ca^{2+}$ (*7*). Because this enhanced efflux was observed only after a lag of 5 to 10 min, we examined the possibility that it is preceded by more rapid ion fluxes. We now show that addition of IFN-γ to murine macrophages results in a rapid cytosolic alkalinization and an enhanced influx of $^{22}Na^+$. The ability of amiloride to inhibit these responses and to inhibit the accumulation of mRNA for the gene JE and the β chain of the class II MHC gene I-A (I-A_β), both of which are induced by IFN-γ, indicates the importance of Na^+/H^+ exchange in the IFN-γ–induced activation of murine macrophages.

IFN-γ at physiologic concentrations (10 to 100 units of recombinant IFN-γ per milliliter)—that is, at concentrations that induce macrophage activation for tumor cytotoxicity and induce surface expression of Ia—rapidly raised cytosolic *p*H by as much as 0.1 *p*H unit (Fig. 1A). An increase in intracellular *p*H (pH_i) was manifest within 1 min after IFN-γ was added to the macrophage cultures and continued for 10 to 15 min, after which pH_i began a slow decrease to basal levels (Fig. 1A). This alkalinization was inhibited by prior treatment of the macrophages with amiloride at concentrations that inhibit the Na^+/H^+ antiporter (*8*) (Fig. 1A). The increase in cytosolic *p*H was dose dependent and was observed with as little as 1 unit of IFN-γ per milliliter (*9*). In contrast, 50 units of interferon α/β per milliliter did not alter *p*H (*9*).

IFN-γ also induced the rapid influx of $^{22}Na^+$ into macrophages; this was readily observable within 2 min (176% ± 24% of the basal value; mean ± SEM, $n = 3$ experiments) after IFN-γ was added to the cultures (Fig. 1B). At these early times, the IFN-γ–induced influx of $^{22}Na^+$, when compared to the appropriate buffer control, was inhibited ~50% by prior exposure of the cultures to 0.05 m*M* amiloride and ~90% by prior exposure to 0.3 m*M* amiloride at the extracellular sodium concentrations used. Both the increase in IFN-γ–stimulated pH_i and $^{22}Na^+$ influx were also inhibited by amiloride analogs according to their relative efficacies for inhibition of the antiporter (*9*). As shown in Fig. 1, amiloride exhibited small inhibitory effects on basal $^{22}Na^+$ influx, as also described in other cells (*8*). The effects of IFN-γ on $^{22}Na^+$ influx were dose-dependent, and increases were observable with as little as 0.1 unit of IFN-γ per milliliter (*9*). The half-maximal dose for IFN-γ–stimulated, amiloride-inhibitable influx of sodium was ~7.5 units of IFN-γ per milliliter.

Addition of IFN-γ to macrophage cultures resulted in increased accumulation of mRNA for numerous genes, including the gene JE and the genes coding for Ia molecules such as I-A_β (*10*). The increased accumulation of mRNA for JE in macrophages is due to an increase in mRNA stability (*11*), whereas the increase in mRNA for Ia is transcriptionally regulated (*10*). We thus examined a potential role of Na^+/H^+ exchange in IFN-γ–mediated regulation of mRNA for JE and I-A_β. When amiloride

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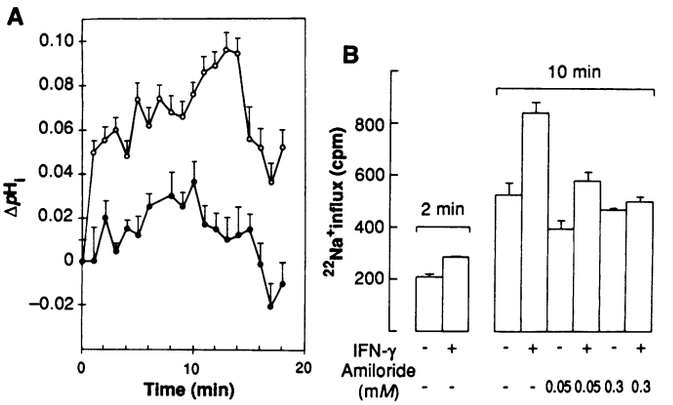

Fig. 1. Activation of Na^+/H^+ exchange by IFN-γ. (**A**) Time course of IFN-γ–induced changes in pH_i in murine macrophages. Macrophages were labeled with 2′,7′-bis-(carboxyethyl)-5(6′)-carboxyfluorescein (BCECF) (Calbiochem, San Diego) and incubated in Hanks balanced salt solution containing 10 m*M* Hepes, *p*H 7.4, at 37°C (HHBSS). Then pH_i was measured by using a digitized low-light video microscopy system (*26, 27*). (○) Data points represent the mean ± SEM of the values obtained from three individually processed cells and are shown as differences in pH_i before (time zero) and after addition of IFN-γ at 15 U/ml (1×10^6 IU/mg; Schering-Plough). (●) Preincubation of macrophages with 0.5 m*M* amiloride for 5 min before addition of IFN-γ inhibited the IFN-γ–induced cytosolic alkalinization of macrophages. This concentration of amiloride did not alter basal fluorescence monitored during the preincubation. Basal pH_i was 7.2 ± 0.01. Similar results were observed in six different experiments. (**B**) Influx of $^{22}Na^+$ into murine macrophages in the presence of buffer or IFN-γ (20 U/ml) as indicated. The data presented are for cultures of macrophages that were incubated for 5 min with 0.5 m*M* ouabain or 0.5 m*M* ouabain plus the indicated concentrations of amiloride before addition of the stimulus. The counts per minute at zero time for the experiment shown were 205 ± 3, and this value was subtracted from the values presented. Data are presented as the mean ± SD for triplicate determinations of a representative experiment. Similar results were obtained in three separate experiments (*28*).

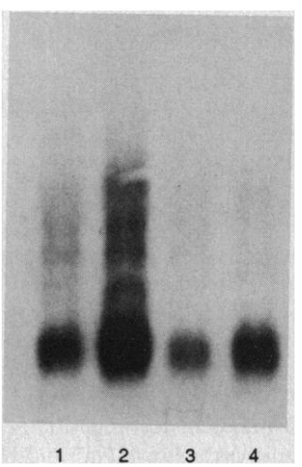

Fig. 2. Inhibition of IFN-γ–induced JE mRNA by amiloride. Thioglycolate-elicited murine macrophages (5×10^7 cells per 15 cm^2) were incubated for 3 hours with (lane 1) buffer, (lane 2) IFN-γ (50 U/ml), (lane 3) 2 m*M* amiloride, or (lane 4) IFN-γ plus amiloride before determination of JE mRNA (*10, 12*). Total cellular RNA was prepared and analyzed as described (*12, 29*). RNA was transferred from 1% agarose-formaldehyde gels to GeneScreen-Plus membranes in a capillary transfer (*29*). A JE fragment was labeled by an oligolabeling method (Pharmacia). Membranes were prehybridized and hybridized as described (*30, 31*). Blots were washed twice for 30 min each time with 0.1% SDS in 0.3*M* NaCl and 0.03*M* sodium citrate at room temperature and twice for 30 min with 0.1% SDS in 0.03*M* NaCl and 0.003*M* sodium citrate at 42°C. Blots were then dried and exposed to x-ray film at −70°C. Blots were subsequently stripped and reprobed for actin as described (*11*).

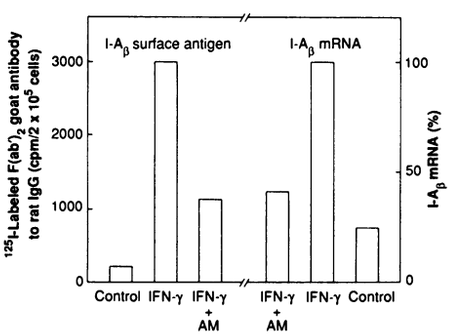

Fig. 3. Inhibition of IFN-γ–induced I-A_β by amiloride. Thioglycolate-elicited murine macrophages were treated as indicated with either no additions, IFN-γ (50 U/ml), or IFN-γ plus amiloride (AM) (0.3 m*M*) for 18 hours before determination of surface Ia at 40 hours or for 18 hours before mRNA analysis. Results for Ia mRNA are standardized to the maximum level observed in a representative experiment and are presented as a percentage of that maximum. Amiloride alone had no effect on expression of basal I-A_β mRNA or surface I-A_β antigen (*9*). I-A_β mRNA was determined as described (*13*). Accumulation of mRNA was determined from densitometric scanning of autoradiograms. Surface expression of I-A_β antigen was determined by radioimmunoassay as described (*13*). Triplicate wells were used for each point; the SEM for the triplicate determination did not exceed 10%. IgG, immunoglobulin G.

was added to the macrophage cultures before IFN-γ was added, the accumulation of mRNA for JE was inhibited at 3 hours. As determined by laser densitometry of the RNA blots, the inhibition of IFN-γ–induced mRNA for JE compared to the appropriate buffer controls was 68% ± 3% normalized to actin (mean ± range in two experiments) (Fig. 2). The presence of amiloride did not affect the ability of phorbol esters to cause mRNA accumulation for JE or the ability of endotoxin to stimulate mRNA accumulation for the gene KC (*9*). We recently presented additional evidence for the differential regulation of JE and KC in macrophages (*11, 12*).

In contrast to the rapid accumulation of mRNA for JE in response to IFN-γ, accumulation of mRNA for Ia genes is observed only after a lag of ~4 hours (*10, 13*). In the presence of 0.3 m*M* amiloride, significant inhibition of IFN-γ–induced mRNA for I-A_β was observed (61% ± 14%; $n = 3$) (Fig. 3). The inhibition of IFN-γ–induced mRNA was also evident at the level of surface expression of Ia (66% ± 10% inhibition at 0.3 m*M* amiloride; $n = 4$) (Fig. 3).

Our data suggest that (i) physiologic concentrations of IFN-γ induce rapid, amiloride-sensitive increases in cytosolic *p*H and in the influx of $^{22}Na^+$ into murine peritoneal macrophages; and that (ii) the changes in cytosolic ion concentrations appear to participate in the IFN-γ–induced increases in mRNA accumulation for JE and I-A_β. JE was originally identified as a gene in fibroblasts that was rapidly induced in response to platelet-derived growth factor (*14*). Recent data indicate considerable sequence similarity of JE to various cytokines (*15*). Transductional events involved in the platelet-derived growth factor–induced transcriptional activation of the JE gene have not been ascertained (*16*). In macrophages, increased mRNA for JE can be induced by various agents (*10*), although mRNA accumulation is not transcriptionally regulated (*10*). Class II MHC gene expression in macrophages is induced by IFN-γ (*3, 4*). The transductional mechanisms regulating these genes are not well defined (*10, 17, 18*), but inhibition of protein synthesis during the first several hours after exposure of macrophages to IFN-γ does not block accumulation of specific mRNA nor subsequent surface expression of Ia molecules (*13, 19*). Our data indicate the involvement of Na^+/H^+ exchange in IFN-γ–induced mRNA accumulation of both JE and I-A_β. The gene induction is apparently due to cytosolic alkalinization, since monensin was ineffective in inducing JE, whereas significant accumulation was observed on pharmacologic increase in cytosolic *p*H (*20*).

A model for effects of IFN-γ on macrophages can thus be proposed: occupancy of the receptor for IFN-γ leads to activation of the Na^+/H^+ antiporter and results in the efflux of H^+ from the cytosol of the cells and influx of Na^+. These changes result in increased levels of specific mRNA for at least two IFN-γ–inducible genes. The involvement of Na^+/H^+ exchange in the mitogenic response to a variety of factors has been described (*21, 22*). Our data indicate the importance of this transductional se-

quence for the cytokine IFN-γ as well. During the course of these studies, Smith *et al.* (*23*) reported that IFN-γ stimulated cytosolic alkalinization in a pre-B lymphocyte cell line, partially as a result of activation of Na^+/H^+ exchange. Because the earliest measured time was 30 min, the rapidity of activated Na^+/H^+ exchange and its importance as a genomic transductional sequence in response to IFN-γ in these cells remain to be established. In view of the pleotropic actions of IFN-γ (*5, 6*), it is likely that additional transductional sequences may mediate other actions of IFN-γ. For instance, addition of amiloride did not inhibit priming by IFN-γ for tumor cell cytolysis (*9*). Relatively high concentrations of IFN-γ can cause accumulation of labeled diacylglycerol or similar compounds in fibroblasts (*24*). In macrophages, physiologic concentrations of IFN-γ, however, induce increases in the mass of diacylglycerol only after ~4 hours, suggesting that these increases are unlikely to represent a primary transductional event (*25*). Our studies demonstrate that physiologic doses of IFN-γ induce rapid Na^+/H^+ exchange in murine macrophages and that these amiloride-sensitive fluxes are important for mediating some of the subsequent genomic responses to IFN-γ.

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28. Proteose-peptone–elicited macrophages were obtained by peritoneal lavage of pathogen-free 6-week-old inbred C57BL/6 mice (Charles River). Macrophages (1 ml containing 1×10^6 cells) were cultured for 5 hours at 37°C with 5% CO_2 on glass cover slips (22 mm by 22 mm) in RPMI 1640 containing 5% fetal calf serum, 2 m*M* glutamine, penicillin (125 U/ml), and streptomycin (6.25 μg/ml). Cultures were incubated for 30 min with BCECF-acetoxymethyl ester (2 μ*M*), and then washed three times in HHBSS. The cover slips with macrophage monolayers were then placed in a similar medium on the stage of an inverted-phase fluorescent microscope. Fluorescence of the individual cells was then measured with the use of a digitized video microscopy system described elsewhere (*26, 27*), with the following filter combination: excitation 440 or 490 nm, emission >525 nm. Values of pH_i were obtained by comparing 440/490 ratios with those obtained with BCECF-containing solutions of known *p*H. Similar pH_i values were obtained for nigericin-permeabilized (*26*) BCECF-containing cells. For $^{22}Na^+$ influx measurements, 6×10^6 peptone-elicited adherent macrophages were in modified Hanks balanced salt solution with 14 m*M* NaCl, 123 m*M* choline chloride, and 5 m*M* Na/Hepes, *p*H 7.4. Cells were warmed at 37°C for 10 min before the addition of 0.5 m*M* ouabain or ouabain plus the indicated concentrations of amiloride and then kept at 37°C for an additional 5 min. At the end of the preincubation, either buffer or IFN-γ plus 2 μCi of $^{22}Na^+$ per well was added for the indicated times after which the wells were washed five times with 0.1*M* $MgCl_2$ at 4°C during a 1-min period. Monolayers were solubilized with 1*N* NaOH and counted in a gamma counter. Efficiency of counting was ~50%.
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High Rate of HTLV-II Infection in Seropositive IV Drug Abusers in New Orleans

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Confirmed infection with HTLV-II (human T cell leukemia virus type II) has been described only in rare cases. The major limitation to serological diagnosis of HTLV-II has been the difficulty of distinguishing HTLV-II from HTLV-I (human T cell leukemia virus type I) infection, because of substantial cross-reactivity between the viruses. A sensitive modification of the polymerase chain reaction method was used to provide unambiguous molecular evidence that a significant proportion of intravenous drug abusers are infected with HTLV, and the majority of these individuals are infected with HTLV-II rather than HTLV-I. Of 23 individuals confirmed by polymerase chain reaction analysis to be infected with HTLV, 21 were identified to be infected with HTLV-II, and 2 were infected with HTLV-I. Molecular identification of an HTLV-II–infected population provides an opportunity to investigate the pathogenicity of HTLV-II in humans.

HUMAN T CELL LEUKEMIA VIRUSES type I (HTLV-I) and type II (HTLV-II) have been associated with specific forms of malignancy in humans. HTLV-I is the etiologic agent for a malignancy known as adult T cell leukemia (*1*) and has been linked to a chronic myelopathy known as HTLV-I–associated myelopathy (*2*) or tropical spastic paraparesis, which is endemic to regions of Japan, the Caribbean, and Africa (*3*). Sporadic cases of HTLV-I infection have also been reported in other areas in the world; in the United States, it has been particularly observed in the southeastern region (*4*). Recently, HTLV-seropositive individuals were identified among an intravenous (IV) drug abuser population (*5*), but infection has not been confirmed by more stringent criteria.

In contrast to HTLV-I, HTLV-II has only rarely been isolated. HTLV-II has been associated with two cases of malignancy in humans (*6*). Both patients had unusual T cell malignancies resembling hairy-cell leukemia. In one case, we showed definitively that HTLV-II was molecularly associated with a lymphoproliferative disorder involv-

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