Interferon-Resistant Cell Line Lacks Fatty Acid Cyclooxygenase Activity

Abstract. *A clone of L1210 mouse leukemia cells selected for resistance to both the antiviral and anticellular properties of mouse interferon were essentially devoid of fatty acid cyclooxygenase activity. Experiments in which broken cell preparations were mixed or the two cell types were cultivated together failed to indicate the presence of a diffusible enzyme inhibitor. Fatty acid lipoxygenase activity of similar magnitude was detectable in both cell types. A selective impairment of fatty acid cyclooxygenase in interferon-resistant cells is consistent with recently described data suggesting that this enzyme may play a crucial role in mediating the antiviral and anticellular effects of interferon.*

Many data have accumulated regarding the various ways by which interferon (IFN) inhibits viral replication (*1*). Specific inhibition of viral RNA and protein synthesis has figured prominently as the most important feature of the biochemical program induced by IFN in the treated cell (*2*). However, the impact of these data is becoming increasingly unclear since various reports have appeared recently claiming that the inhibition of the production of viral RNA and proteins by IFN is grossly inadequate to account for the observed inhibition of viral multiplication in IFN-treated cells (*3*). The induction of 2′5′-oligoisoadenylate synthetase, considered generally as a biochemical indicator of IFN action, was not correlated with the antiviral state in embryo carcinoma cells (*4*). Although several studies have implicated IFN-associated plasma membrane alterations (*5*), the exact biochemical nature of such changes is still unknown. Selective alterations in the virion envelope glycoproteins have been shown in preparations isolated from IFN-treated cells, and it has been suggested that these alterations in the membrane structure of virions might have a causative role in the subsequent infectivity of the virus particles made by IFN-treated cells (*6*).

We recently reported that a cellular enzyme present in most mammalian cells, fatty acid cyclooxygenase (prostaglandin synthase; E.C. 1.14.99.1), which is necessary for prostaglandin biosynthesis from arachidonic acid, may be required for the optimum expression of the IFN-induced antiviral state (*7*). We now report that a cell line selected for resistance to both the antiviral and anticellular effects of IFN is virtually devoid of fatty acid cyclooxygenase activity.

Cells of the IFN-sensitive S_6 clone of L1210 mouse leukemia cells and the resistant R_3 clone, selected by growth in excess IFN (*8*), were grown in suspension cultures in RPMI 1640 medium supplemented with 10 percent fetal calf serum (FCS).

Fatty acid cyclooxygenase catalyzes the oxidative cyclization of fatty acids with unsaturation in specific positions of the carbon chain, with the formation of various prostaglandins (*9*). With arachidonate and homo-γ linolenate, the well-recognized substrates of this enzyme, prostaglandin synthetase activity could be easily demonstrated in S_6 cells (Fig. 1). The IFN-resistant cells (R_3), on the other hand, had barely detectable activity. The other major enzyme system that is known to be involved in the metabolism of arachidonic acid, fatty acid lipoxygenase (E.C. 1.13.11.12), has approximately similar activity in both cell types (Fig. 1).

The inhibitors used in the experiment described in Fig. 1 have been extensively characterized with respect to the selectivity of the target enzyme (*9*). Thus, indomethacin and oxyphenylbutazone inhibit the formation of the prostaglandins PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, and hydroxyheptadecatrienoic acid (HHT), whereas the formation of hydroxyeicosatetraenoic acid (HETE), the presumed lipoxygenase product, is not affected, either with eicosatetraenoic acid (arachidonic acid) or with eicosatrienoic acid as substrate. Eicosatetraynoic acid (ETYA), which is known to inhibit both

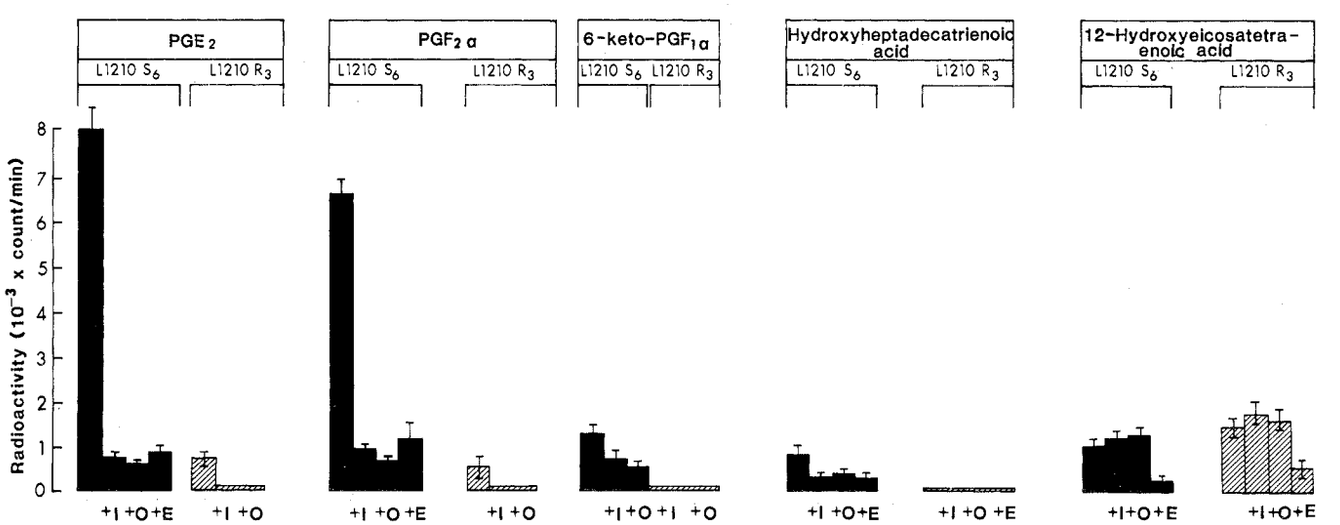

Fig. 1. Suspension cultures of L1210 cells were grown in RPMI-1640 medium, with 10 percent FCS. Cells were washed in phosphate-buffered saline (PBS) three times and resuspended in 0.1*M* tris and 0.15*M* saline, *p*H 7.5, containing 1μCi of [1-^{14}C]arachidonate at a cell density of 20×10^6 cells per milliliter. The cells were incubated at 37°C for 30 minutes. Formic acid (100 μl) was then added, and after centrifugation the cell pellet and supernatant were extracted separately with a mixture of chloroform and methanol (2:1 by volume), Folch-partitioned, and the lower chloroform layer was evaporated to dryness and chromatographed on silica gel G plates. The solvent system used was the upper layer of a mixture of ethyl acetate, isooctane, acetic acid, and water (90:50:20:100, by volume). The radioactive bands were located by autoradiography; the bands cochromatographing with authentic standards were scraped and counted as described before (*13*). For HHT and HETE, platelet HHT and HETE were used as standards. Arachidonate was used at 18 μ*M*, and inhibitors at 10^{-5}*M*. The cell pellet contained less than 2 percent of the radioactivity in metabolites given in the figure. When [1-^{14}C]palmitate was substituted for [1-^{14}C]arachidonate in the reaction mixture, radioactive compounds did not cochromatograph with the prostaglandin standards. Abbreviations: *I*, indomethacin; *O*, oxyphenylbutazone; and *E*, eicosatetraynoic acid.

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enzymes, inhibits the formation of both cyclized and noncyclized products (*10*) in S_6 cells (Fig. 1). It therefore appears clear that in R_3 cells the cyclooxygenase activity is selectively impaired with respect to arachidonate metabolism. Palmitic acid, which is not a substrate for either enzyme, produced no radioactive products, thus further substantiating the specificity of the reactions under study.

Since we found a specific absence of cyclooxygenase activity, we considered the obvious possibility that R_3 cells might be elaborating an inhibitor specific for cyclooxygenase. If this were a diffusible inhibitor, then either mixing of cultures of R_3 and S_6 cells, or mixing of the enzyme-containing subcellular fractions, would inhibit the activity in S_6 cells. However, no evidence for such an inhibitor could be obtained from such experiments (Fig. 2). The incubation mixture for determination of enzyme activity was optimized in the buffer system given in Fig. 1. To test for the formation of an inhibitor during cell growth, we added the culture medium to the incubation mixture. A nonspecific inhibitory activity toward cyclooxygenase was always present in serum-containing medium, primarily because the albumin present in serum sequesters the substrate fatty acid (*11*). Thus, the activity in S_6 cells was inhibited approximately to the same degree by culture medium from either R_3 or S_6 cells (Fig. 2). When cells were cocultivated for two or three generations and then assayed for cyclooxygenase activity in whole cells or microsomes, the activity was easily detectable (data not given). When microsomal pellets from R_3 and S_6 cells were mixed, the reduction in specific activity was that expected from simple dilution and there was no indication of the presence of an inhibitor (Fig. 2B).

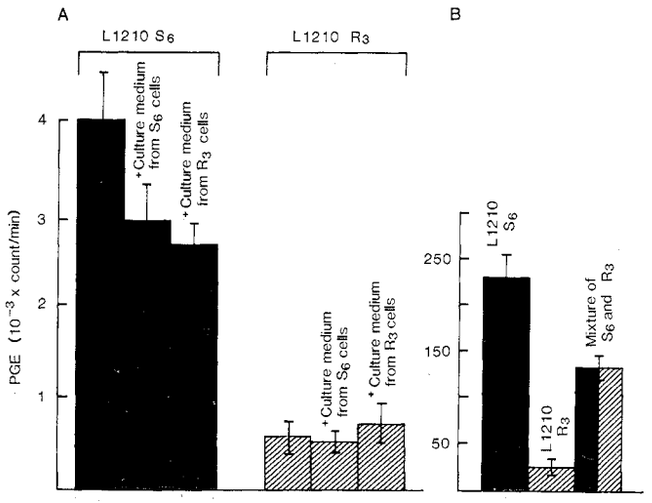

Fig. 2. (A) The absence of inhibitor in R_3 cells shown by mixing experiments with whole cells. The L1210 R_3 and S_6 cells were grown in RPMI-1640 medium with 10 percent FCS. After 24 hours of growth, 5×10^6 cells from each culture were used to inoculate a mixed R_3 and S_6 culture and were grown under identical conditions. Cells were split at a ratio of 1:4 every day and grown until 2×10^7 cells could be harvested from each culture. Cells were then washed in PBS three times, and assayed for cyclooxygenase products as given in the legend for Fig. 1. The culture medium added (at 1:5 by volume in the reaction mixture) was that from which the cells were centrifuged before being washed in PBS. The radioactivity counted is that from compounds cochromatographing with an authentic standard of PGE_2. The radioactivity pattern of two other major cyclooxygenase products, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$, resembled closely the pattern of PGE_2, giving no evidence for a diffusible inhibitor present in the supernatants from R_3 cells. The value for PGE_2 in experiments in which R_3 and S_6 cells were cocultivated was 3662 ± 489 count/min. (B) The absence of inhibitor in R_3 cells shown by mixing experiments with microsomal fractions. The cells were washed in PBS three times, homogenized by sonication in 0.25*M* sucrose (1:5, packed cells to sucrose), and centrifuged at 1000*g*. The supernatant was centrifuged at $16 \times 10^3 g$, the pellet was discarded, and the supernatant was centrifuged at $100 \times 10^3 g$ for 1 hour. The pellet was resuspended in 0.5 ml of 0.1*M* tris buffer, *p*H 7.8, and was used as the enzyme source to assay for cyclooxygenase activity. Microsomes were incubated at 37°C for 2 minutes without arachidonate. Then 18 μ*M* [1-^{14}C]arachidonic acid was added, and the reaction was terminated after 2 minutes and the mixture processed as described in the legend to Fig. 1. Data are expressed as counts per minute per milligram of microsomal protein. Whole homogenates assayed separately and in mixtures gave results similar to those of whole cells or microsomes.

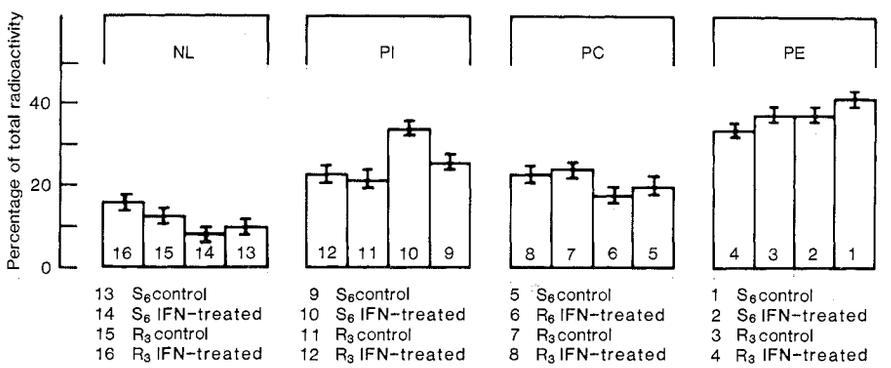

Fig. 3. Effect of IFN on the incorporation of arachidonic acid into the various lipid fractions of IFN-resistant (R_3) and IFN-sensitive (S_6) L1210 cells. The cells were labeled overnight with 1 μCi of [1-^{14}C]arachidonic acid per milliliter of medium, and were harvested and washed by centrifugation the next day. Lipids were extracted with a mixture of chloroform and methanol (2:1 by volume). The extract was Folch-partitioned and the lipids were separated by using two-dimensional chromatography on silica gel G plates (*14*). The lipids were located by autoradiography, scraped, and counted. In neutral lipids, 95 percent of the radioactivity was present in the triacylglycerol fraction. When present, mouse β IFN was used at 1000 units per milliliter of medium. Abbreviations: *NL*, neutral lipids; *PI*, phosphatidyl inositol; *PC*, phosphatidyl choline; and *PE*, phosphatidyl ethanol amine.

We have investigated the effect of IFN on the labeling pattern with [1-^{14}C]arachidonic acid of cellular lipids of R_3 and S_6 cells. The results show very little difference in the incorporation into specific lipids, except in phosphatadylinositol. A small but consistent enhancement of arachidonic acid incorporation into this phospholipid was obtained with IFN in S_6 cells, whereas no difference was detected in resistant cells (Fig. 3).

It is known that L1210 cells are sensitive to both the antiviral and anticellular effects of homologous IFN (*12*). The R_3 cells used in this study were selected and cloned in the presence of high concentrations of mouse IFN (*8*). Thus, the phenotypic transition (or selection) in this cell should reflect at least one or possibly more steps common to both anticellular and antiviral effects of IFN. We reported recently that several nonsteroidal antiinflammatory compounds which are inhibitors of fatty acid cyclooxygenase inhibited the development of the IFN-induced antiviral state (*7*). This inhibitory effect correlated well with the period of time the enzyme remained inhibited with these agents. Although none of the known prostaglandins formed by way of PGH_2 were found to substitute (when

added exogenously) for the functional presence of the enzyme, the possible need for the continued presence of an unstable prostaglandin was suggested by the data. Taken in conjunction with those studies, the present results lend further support to the hypothesis that the enzyme, fatty acid cyclooxygenase, may in some way be a necessary component for the complete expression of the IFN-mediated anticellular and antiviral states.

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Energetics of Running Cockroaches

Abstract. *Male cockroaches* Gromphadorhina portentosa *were made to run at 0.03, 0.07, and 0.12 kilometer per hour on a miniature treadmill within a small respirometer. Oxygen consumption was directly related to running velocity. The half-time necessary for oxygen consumption to reach a steady state during exercise was about 1 minute and the half-time for recovery was 4 to 6 minutes. The energetic cost of transport was comparable to that for bipedal and quadrupedal vertebrates.*

The past 10 years have seen heightened interest in comparative locomotion, with an emphasis on the energetics of vertebrates (*1–3*). Except for flying insects (*4*), however, little comparable information has been obtained concerning invertebrates. The only quantitative evaluation of the energetics of a terrestrial invertebrate under controlled conditions appears to be that of the land crab *Cardisoma guanhumi* (*5*). This large species was exercised on a treadmill at specified speeds while wearing a respiratory mask. The experiment revealed several striking parallels between land crab and vertebrate energetics, including a direct relation between oxygen consumption ($\dot{V}O_2$) and running velocity. Moreover, the crab compensated for the increased O_2 demand by increasing ventilation of its gill chamber. The minimum cost of transport (the amount of energy required to move 1 g of animal 1 km) was also similar to that for pedestrian vertebrates (*1–3*).

We studied the energetics of the large wingless tropical cockroach *Gromphadorhina portentosa* by constructing an airtight Lucite respirometer enclosing an axle-driven latex treadbelt. The belt was driven by a rheostat-controlled d-c gear motor outside the chamber and was capable of a constant speed of approximately 0.8 to 30 cm/sec. Inflow and outflow gas ports allowed for constant circulation of fresh air through the 125-ml working space of the chamber. Air was continuously drawn through the respirometer at a rate of 56 ml/min. After leaving the chamber, the air passed through a Drierite filter to absorb water before entering one sensor cell and flowmeter of an Applied Electrochemistry S-3A oxygen analyzer coupled to a Linear Instruments model 282 integrating chart recorder. For comparison, room air was drawn at the same rate through a Drierite filter and into a second sensor cell of the analyzer. This permitted us to measure differences in oxygen between room air and gas leaving the respirometer. This value, multiplied by the airflow, gave us the $\dot{V}O_2$ after being corrected to dry air under standard conditions of temperature and pressure (*6*).

Ten animals with a mean weight of 5.2 ± 0.8 g were run on the treadmill at 0.03, 0.07, and 0.12 km/hour, the latter velocity being near the maximum that can be sustained by this species. Each cockroach was exercised once per day at a single speed in a randomized order over a period of 1 week. We placed the insects in the respirometer for 60 minutes before exercising them, and during the last 15 minutes determined their rest-

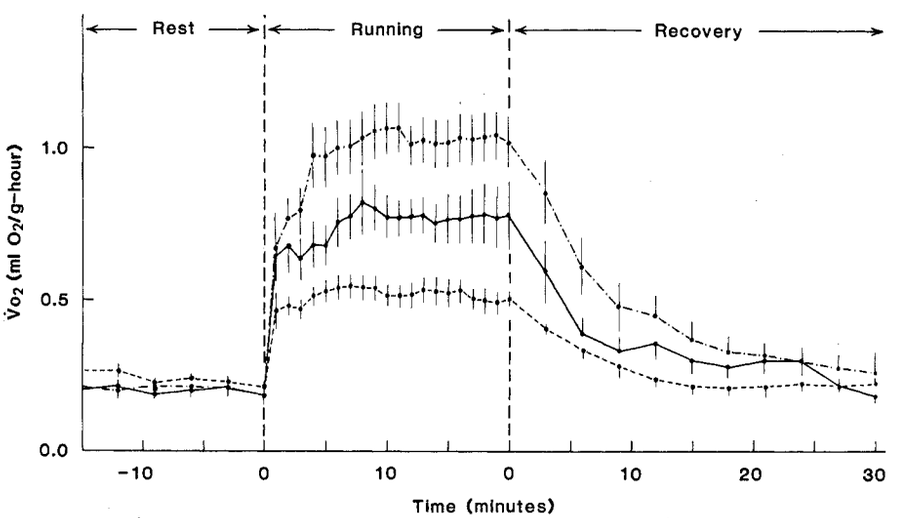

Fig. 1. Mean $\dot{V}O_2$ of cockroaches running on a treadmill for 20 minutes at 0.03 km/hour (bottom record), 0.07 km/hour (middle record), and 0.12 km/hour (top record). The vertical bars represent 95 percent confidence intervals.

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