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Inhibition of human natural killer cell activity by (14*R*,15*S*)-14,15-dihydroxy-5*Z*,8*Z*,10*E*,12*E*-icosatetraenoic acid

(15-lipoxygenase pathway/14,15-dihydroxyicosatetraenoic acid/lipoxygenase products)

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ABSTRACT **The interactions between products of the 15-lipoxygenase cascade and human natural killer (NK) cell activity have been studied. Addition of human leukocyte-derived (14*R*,15*S*)-14,15-dihydroxy-5*Z*,8*Z*,10*E*,12*E*-icosatetraenoic acid (14,15-DiHETE) to the NK cytotoxicity assay against K562 target cells resulted in inhibition of NK cell activity, whereas addition of other 15-lipoxygenase-associated metabolites [i.e., (15*S*)-15-hydroperoxy-5*Z*,8*Z*,11*Z*,13*E*-icosatetraenoic acid, (15*S*)-15-hydroxy-5*Z*,8*Z*,11*Z*,13*E*-icosatetraenoic acid, and (8*R*,15*S*)- and (8*S*,15*S*)-8,15-dihydroxy-5*Z*,9*E*,-11*E*,13*E*-icosatetraenoic acid isomers] resulted in little or no inhibition of NK function. Dose–response studies indicate that leukocyte-derived 14,15-DiHETE and 14,15-DiHETE methyl ester, at micromolar concentrations, inhibit NK function even in the presence of 2.5% fetal calf serum. Synthetic 14,15-DiHETE prepared by total organic synthesis displayed similar biological activities over identical dose ranges. These icosanoids do not inhibit NK target cell binding and they exert only a variable effect in either antibody-dependent cytotoxicity or cytotoxic T-lymphocyte assays. These results demonstrate that the 14,15-DiHETE inhibits NK cell function *in vitro*. Moreover, they suggest that activation of the 15-lipoxygenase cascade and formation of 14,15-DiHETE *in vivo* may provide a mode of immune regulation.**

Natural killer (NK) cells are a subpopulation of lymphocytes that are defined functionally as cells that can spontaneously lyse a variety of normal and malignant cells (1, 2). There has been a great deal of interest in NK cells because of their possible role in mammalian host defense against spontaneous neoplastic transformation. Despite this interest, little information is available concerning the regulatory mechanism(s) involved in NK cell activity. Results of several studies, however, suggest a role for cyclic nucleotides and oxygenation products of arachidonic acid in modulating NK cell activity (3, 4).

In general, non-esterified arachidonic acid may be oxygenated by the action of either cyclooxygenase or lipoxygenases. The lipoxygenases of mammalian tissue—i.e., the 5-, 12-, and 15-lipoxygenases—transform arachidonic acid into a number of biologically active derivatives (for review, see ref. 5). For example, the human leukocyte 5-lipoxygenase catalyzes the initial step in the formation of leukotrienes (5). These arachidonate derivatives—particularly, leukotrienes B_4, C_4, and D_4—are held to play a role in immediate hypersensitivity reactions and in inflammation (5).

In addition to initial oxygenation via the 5-lipoxygenase, human leukocytes are also capable of oxygenating arachidonate to form mono- and dihydroxy derivatives by means of the 15-lipoxygenase (6–8). Initial oxygenation at C-15 results in the formation of (15*S*)-15-hydroperoxy-5*Z*,8*Z*,11*Z*,-13*E*-icosatetraenoic acid (15-HPETE), which may be further transformed to a series of 14,15- and 8,15-dihydroxyicosatetraenoic acids (6–8). The biological role(s) of the 15-lipoxygenase cascade and its products has remained unclear. However, results of several studies suggest that the 15-lipoxygenase pathway may be of importance in several cell types (9–13).

Since some prostaglandins suppress certain immune reactivities (3) whereas leukotriene B_4 augments NK cytotoxic activity (14), it is possible that arachidonate derivatives may constitute an important group of modulators in NK cell function. Therefore, the present study was undertaken to examine the effects of arachidonate derivatives associated with the 15-lipoxygenase cascade on human NK cell activities. We report here that NK cell activity is inhibited by human leukocyte-derived (14*R*,15*S*)-14,15-dihydroxy-5*Z*,8*Z*,10*E*,-12*E*-icosatetraenoic acid (14,15-DiHETE). The stereochemistry of this compound was confirmed by total organic synthesis (15). The leukocyte-derived 14,15-DiHETE and synthetic 14,15-DiHETE appear to act directly upon NK cells in a dose-dependent fashion and their effects are reversible. These compounds inhibit NK cell activity without affecting target cell binding.

MATERIALS AND METHODS

Arachidonic acid was purchased from Nu Chek Prep and soybean lipoxygenase (E.C.1.13.11.12) type I was from Sigma. HPLC equipment was from Waters Associates.

Preparation of Cells. Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors by a Ficoll-Hypaque gradient centrifugation (16). The cells were washed and resuspended in RPMI-1640 medium (GIBCO) with 2.5% fetal calf serum and supplemented with 50 international units of penicillin per ml/50 μg of streptomycin per ml/2 mM L-glutamine. For generation of cytotoxic T lymphocytes (CTL) the PBMC from two different donors were used. PBMC from one donor were used as responder cells at a concentration of 1×10^6 per ml, and 2500 rad-irradiated (1 rad = 0.01 gray) stimulator cells (at a concentration of 1×10^6 per ml) from the other donor were suspended in RPMI-1640 medium supplemented with 5% responder plasma instead of fetal calf serum. As targets, cells from the stimulator were put in a culture of 0.5×10^6 cells per ml with 2.5 μg of Con A per ml. The Con A-induced T blasts to be used as targets did not require feeding before the cytotoxic test day 7 or 8.

Plastic adherent cells were depleted by incubating 10^6 cells in 10 ml of medium on plastic Petri dishes (diameter, 9 cm)

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Abbreviations: 15-HPETE, (15*S*)-15-hydroperoxy-5*Z*,8*Z*,11*Z*,13*E*-icosatetraenoic acid; 15-HETE, (15*S*)-15-hydroxy-5*Z*,8*Z*,11*Z*,13*E*-icosatetraenoic acid; 14,15-DiHETE, (14*R*,15*S*)-14,15-dihydroxy-5*Z*,8*Z*,10*E*,12*E*-icosatetraenoic acid; 8,15-DiHETE isomer 1, (8*R*,15*S*)-8,15-dihydroxy-5*Z*,9*E*,11*E*,13*E*-icosatetraenoic acid; 8,15-DiHETE isomer 2, (8*S*,15*S*)-8,15-dihydroxy-5*Z*,9*E*,11*E*,13*E*-icosatetraenoic acid; PBMC, peripheral blood mononuclear cells; NW, nylon wool; NK, natural killer; CTL, cytotoxic T lymphocytes; ADCC, antibody-dependent cell-mediated cytotoxicity; PGE_2, prostaglandin E_2; LGL, large granular lymphocyte(s).

for 60 min at 37°C. Nonadherent cells were collected and passed over a nylon wool (NW) column (17) to remove B lymphocytes and residual monocytes and macrophages.

NK cells were enriched by fractionation of lymphocytes on a discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden) essentially as described (18). Further enrichment of human large granular lymphocytes (LGL) was done by depletion of high-affinity sheep erythrocyte rosette-forming cells from the LGL-enriched low Percoll density fractions (18). Target cells on the NK assay were K562 (a human myelogenous leukemia cell line sensitive to NK lysis).

Cytotoxic Assays. Cytotoxicity was determined in a 4-hr ^{51}Cr release assay with 5×10^3 $Na_2{}^{51}CrO_4$ (Radiochemical Centre)-labeled target cells per microtiter well in a total volume of 120 μl as described (19). Antibody-dependent cell-mediated cytotoxicity (ADCC) was performed by incubating the target cells P815 (a NK cell resistant mouse mastocytoma cell line) with ^{51}Cr and anti-P815 rabbit antibodies at an optimal concentration for 1 hr, after which the cells were washed and used at 5×10^3 cells per microtiter well in assay. Test compounds were added in a volume of 50 μl in medium containing 0.05% EtOH, whereas controls were given 50 μl of 0.05% EtOH medium.

Single Cell Binding Assay. Effector cells (1×10^6) and target cells (1×10^6) were centrifuged 5 min at $100 \times g$ and incubated at 37°C for 10 min. The pellet was resuspended by five gentle aspirations with a Pasteur pipette and effector–target cell conjugates were determined (20).

Preparation of 15-HPETE, (15*S*)-15-Hydroxy-5*Z*,8*Z*,11*Z*,-13*E*-Icosatetraenoic Acid (15-HETE), and Human Leukocyte-Derived Dihydroxy Acids. 15-HPETE was prepared by action of the soybean lipoxygenase and 15-HETE was prepared by $SnCl_2$ reduction of 15-HPETE (21).

14,15-DiHETE and 8,15-DiHETEs were isolated from incubations containing 15-HPETE (100 μM) and human leukocytes (100 ml, 30×10^6 cells per ml) as described (6). Briefly, 15-HPETE was incubated with human leukocytes (mixed leukocyte preparations) for 30 min at 37°C. The incubations were stopped by adding 2 vol of methanol, extracted, and purified by silicic acid chromatography (cc-4, Mallinckrodt) (6). Ethyl acetate fractions were evaporated and further purified by reversed-phase HPLC with methanol/water/acetic acid, 70:30:0.01 (vol/vol), at 4 ml/min. Fractions exhibiting the UV spectrum characteristic of a conjugated triene were pooled and treated with diazomethane. Final purification was achieved on a straight phase HPLC column (Polygosil C60-10, SiO_2 10-μm particles) eluted at 2 ml/min with hexane/isopropanol/acetic acid, 92:8:0.01 (vol/vol), UV-recorded at 270 nm. The methyl esters of the dihydroxy acids were identified by gas chromatography/mass spectrometry and UV absorption (6–9). The purified material showing a UV maximum at 272 nm (in methanol), a C value of 23.8 (Se-30% on Supelcoport), and a mass spectrum similar to those previously published (6) was assigned the structure, 14,15-dihydroxy-5,8,10,12-icosatetraenoic acid methyl ester. The methyl esters were hydrolyzed to free acids, and the concentrations of dihydroxy acids were calculated by using a molar absorption coefficient of 40,000 in methanol. The complete structure of human leukocyte-derived 14,15-DiHETE and total synthesis were achieved as described (15). Synthetic 14,15-DiHETE was a gift from Upjohn.

RESULTS

Effects of 15-Lipoxygenase Products on NK Cell Activity. The effects of various products associated with the 15-lipoxygenase pathway on NK cell activity are shown in Fig. 1. Here the cytotoxic activity of NW-passed PBMC was assayed against K562 target cells. Cytotoxicity was measured at different concentrations of the various compounds by using a constant effector/target cell ratio (50:1). The free acid of leukocyte-derived 14,15-DiHETE and its methyl ester displayed marked inhibition in the cytotoxic assay over the duration of the experiment (4 hr). Inhibition was not observed with either 15-HETE or 15-HPETE [the precursor of 15-HETE and the dihydroxy acids (6)]. In addition, other dihydroxy acids (i.e., 8,15-DiHETEs) did not alter NK cell activity (Fig. 1*A*).

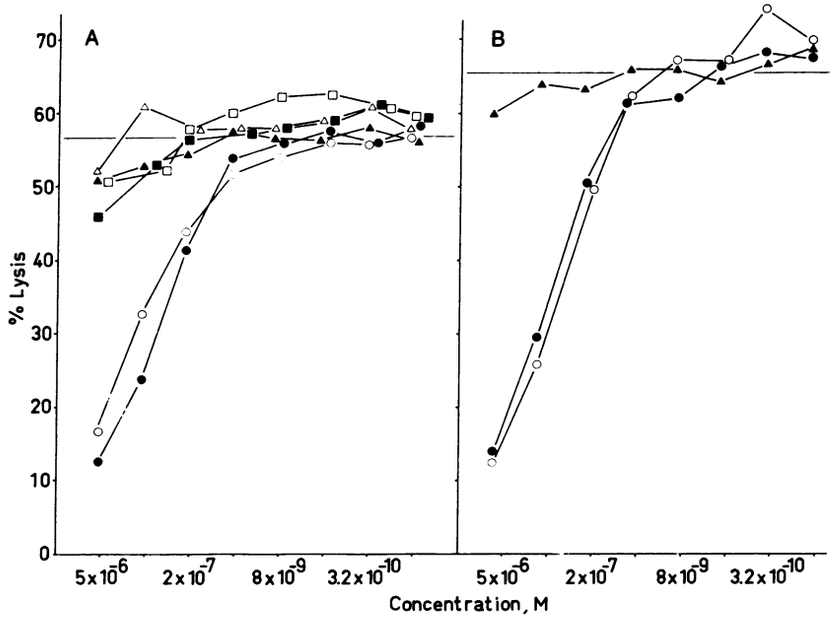

FIG. 1. Inhibition of natural killing against K562 target cells by 15-lipoxygenase products. Data are given as percent killing at an effector/target ratio of 50:1 (*A*) and 10:1 (*B*). Concentrations of the compounds are indicated on the abscissa. Control cytotoxicity levels are indicated by the horizontal line. (*A*) NW-enriched PBMC. △, 15-HPETE; ▲, 15-HETE; ○, 14,15-DiHETE; ●, 14,15-DiHETE methyl ester; □, (8*R*,15*S*)-8,15-hydroxy-5*Z*,9*E*,11*E*,13*E*-icosatetraenoic acid (8,15-DiHETE isomer 1); ■, (8*S*,15*S*)-8,15-dihydroxy-5*Z*,9*E*,11*E*,13*E*-icosatetraenoic acid (8,15-DiHETE isomer 2). (*B*) Highly purified large granular lymphocytes. ▲, 15-HETE; ○, 14,15-DiHETE organic synthesized; ●, 14,15-DiHETE methyl ester.

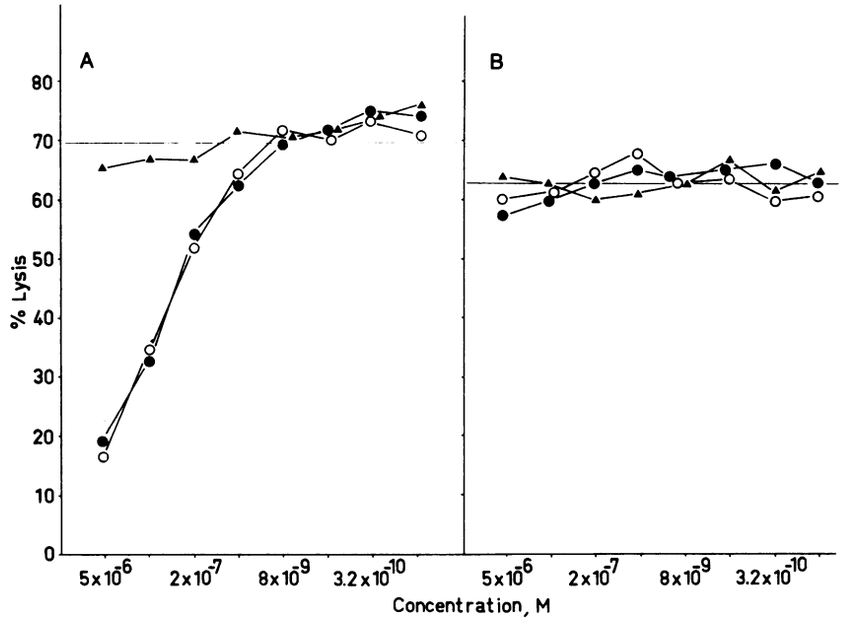

FIG. 2. Effect on NK cytotoxicity on pretreatment of the effector cells by 15-lipoxygenase products. NW-enriched PBMC were tested in a 4-hr cytotoxicity assay against K562 target cells at a 50:1 ratio. Cytotoxicity control levels are indicated by the horizontal line. Effector cells were pretreated for 2 hr (*A*) or washed twice (*B*) before assay. Concentrations of the compounds are indicated on the abscissa. ▲, 15-HETE; ○, 14,15-DiHETE; ●, 14,15-DiHETE methyl ester.

To exclude the possible presence of contaminating substances in the preparations of 14,15-DiHETE isolated from human leukocytes, which may influence the observed inhibition, the complete stereochemistry of 14,15-DiHETE was determined and synthetic material was prepared by total organic synthesis (15). When the synthetic material was compared to the biological activity of leukocyte-derived 14,15-DiHETE, it proved to be of roughly equal potency, exhibiting a similar dose–response profile. In these assays highly purified LGL cells (i.e., Percoll-enriched and sheep erythrocyte rosette-depleted LGL cells) were used as effector cells to reduce the possible effects of other cell types (Fig. 1*B*).

Pretreatment Effects on NK Cell Activity by 14,15-DiHETE. To determine whether the effects of 14,15-DiHETE were reversible, NW-passed effector cells were preincubated for 2 hr with either free acid 14,15-DiHETE, its methyl ester, or 15-HETE, and washing experiments were performed (Fig. 2). NW-passed cells preincubated with either 14,15-DiHETE, 14,15-DiHETE methyl ester, or 15-HETE for 2 hr showed a similar pattern of inhibition as in the experiments described above (compare Fig. 1 with Fig. 2*A*). When the same cells were washed twice and subsequently re-examined, cytotoxic activity against K562 returned to normal values (Fig. 2*B*). Here, again, 15-HETE displayed no activity.

Effects of Icosanoids on Conjugate Formation. To determine the level of the inhibitory effect of 14,15-DiHETE, the various compounds were tested in a standard NK target binding assay. Results presented in Table 1 indicate that these compounds do not alter the percent of conjugate formation between NW-enriched effector cells and K562 target cells.

Effects on NK Cell Activity by 14,15-DiHETE and Prostaglandin E_2 (PGE_2). To determine the inhibitory capacity of 14,15-DiHETE compared to that of PGE_2, NW-passed effector cells were incubated with the compounds at different concentrations in a ^{51}Cr release assay. Results of these studies indicate that both 14,15-DiHETE and PGE_2 inhibit cytotoxic activity over a similar dose range (Fig. 3).

Table 1. Effects of 15-lipoxygenase products on NK cell binding

Compound	% binding cells*	
	Exp. 1	Exp. 2
Control	11.8	13.1
14,15-DiHETE	14.0	13.6
14,15-DiHETE methyl ester	11.7	12.9
15-HETE	13.4	13.1

The concentration of each compound was 5×10^{-6} M. NW-enriched PBMC were mixed with K562 target cells at a 1:1 ratio and assayed for effector cells binding to target cells.
*Percent effector cells binding to target cells.

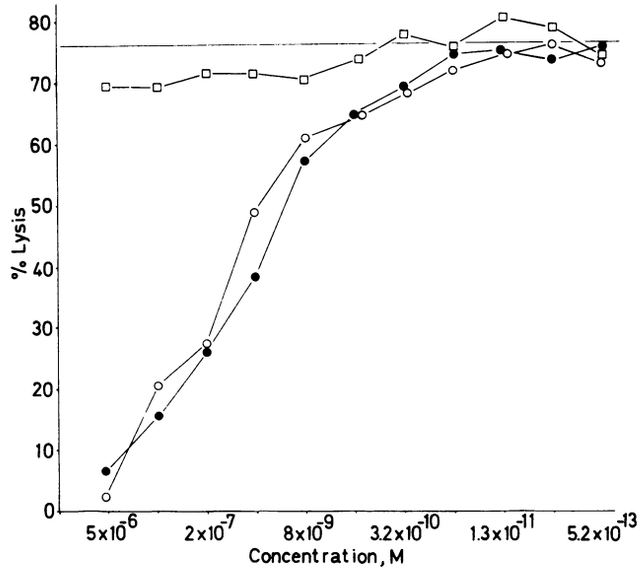

FIG. 3. Effect on NK cytotoxicity by 14,15-DiHETE (synthetic) and PGE_2. NW-enriched PBMC were tested for NK cytotoxicity against K562 at an effector/target ratio of 50:1. Control levels are indicated by the horizontal line. ○, PGE_2; ●, 14,15-DiHETE (synthetic); □, 15-HETE.

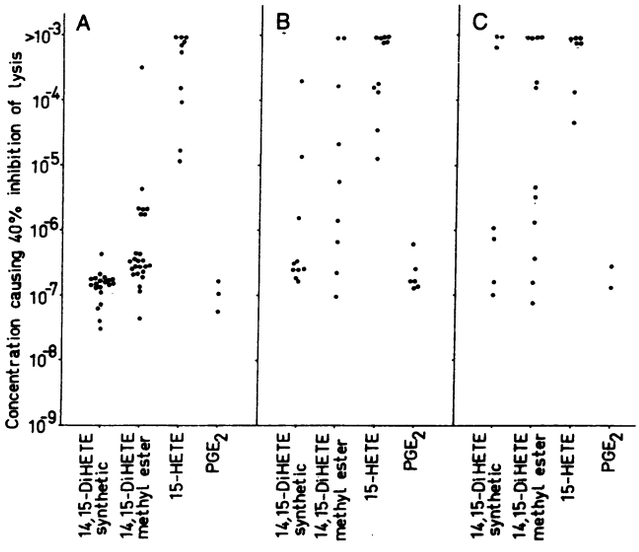

FIG. 4. Effects of 15-lipoxygenase products and PGE_2 in NK, ADCC, and CTL cytotoxicity. Data are given in concentration (M) of the compound required to give a 40% inhibition in each of the assays. Concentrations higher than 10^{-3} M are given as $>10^{-3}$ M. Each point represents a separate experiment with an individual donor. Thus, each point represents the concentration at which 40% inhibition was observed from each donor. (*A*) NK activity of NW-enriched effector cells assayed against K562 target cells at an effector/target ratio of 50:1. (*B*) ADCC activity of NW-enriched effector cells assayed against antibody-coated P815 target cells at an effector/target ratio of 40:1. (*C*) Mixed lymphocyte reaction-generated CTL assayed against Con A blasts, established from the stimulator cell population. The effector/target ratio was 50:1. The cytotoxic activity in all experiments varied between 20% and 75% in the absence of compounds.

Effects of 14,15-DiHETE, 14,15-DiHETE Methyl Ester, 15-HETE, and PGE_2 in ADCC and CTL Cytotoxicity. To determine if these compounds influence other cytotoxic effector cells and effector mechanisms, we next examined these compounds in ADCC and CTL activities as well as in NK activity. Results presented in Fig. 4 demonstrate that 14,15-DiHETE and 14,15-DiHETE methyl ester are less efficient inhibitors against CTL and ADCC effector cells than against NK cells. PGE_2 did not display this variability. In each system (i.e., NK, CTL, or ADCC) PGE_2 showed a similar pattern of inhibition.

DISCUSSION

The present results suggest that leukocyte-derived 14,15-DiHETE, its methyl ester, and synthetic 14,15-DiHETE all have a potent ability to inhibit human NK cell cytotoxicity *in vitro*. In contrast, other arachidonate metabolites associated with the 15-lipoxygenase pathway (i.e., 15-HPETE, 15-HETE, and 8,15-DiHETEs) failed to have any impact on the lytic capacity of NK cells. This inhibition could be shown to occur at the level of lysis since effector/target binding was unaffected by treatment with 14,15-DiHETE.

The inhibitory effects of 14,15-DiHETE on NK function appears to be the result of its direct effect on NK cells since similar dose–response curves were obtained regardless of whether the effector cells were highly purified LGLs or not (see Figs. 1 *A* and *B* and 2). Furthermore, the effects were reversible and optimal inhibition was obtained only when 14,15-DiHETE was present throughout the entire lytic assay. Thus, it is not possible to exclude an effect of 14,15-DiHETE on the target cells. However, when target cells other than K562 were used—i.e., Molt 4 cells—a similar inhibitory pattern was observed (data not shown). Together, these findings suggest that formation of 14,15-DiHETE by other cells would be expected to modulate NK cell activities. Our results, however, do not exclude the possibility that 14,15-DiHETE may provoke the formation of a substance that could mediate the observed inhibition. Nevertheless, at micromolar concentrations, 14,15-DiHETE can function as an efficient inhibitor of NK function.

We also analyzed the ability of 14,15-DiHETE to inhibit other lytic systems in which mononuclear cells serve as effector cells. In the present study we explored the capacity to inhibit cytolytic T cells as well as ADCC against IgG-coated nucleated target cells. In parallel studies, PGE_2 was included as a known inhibitor of all three systems (3, 22). Similarly, we found that 14,15-DiHETE as well as prostaglandin E_1 (PGE_1) and PGE_2 function as reversible inhibitors against NK cells (3). The results obtained (see comparison in Fig. 4) demonstrate that 14,15-DiHETE is most efficient as an inhibitor against NK cells, whereas the impact on both cytotoxic T cells and ADCC displayed high variability over a 100-fold dose range. PGE_2, on the other hand, did not display such variability. PGE_2 induced efficient inhibition in all three systems at roughly the same molar concentrations. In contrast to PGE_2, 14,15-DiHETE displayed a certain degree of selectivity, which, on the average, was most efficient with respect to NK inhibition.

Indirect evidence for the role of lipoxygenase products in NK cell function has been reported recently (23, 24). In these studies the authors utilized nordihydroquaiaretic acid (NDGA) and BW 755 (reported inhibitors of lipoxygenation) and observed reversible inhibition of NK cell activity (23, 24). Further support for the role of lipoxygenase products comes from the studies of Rola-Pleszczynski *et al.* (14), who found that leukotriene B_4 (LTB_4) augments NK cell cytotoxicity. More recently, studies by P. Rossi (personal communication) suggest that simultaneous addition of LTB_4 and NDGA will reverse the inhibitory effect of the lipoxygenase inhibitor. Together, these studies suggest that lipoxygenase products play a direct role in the modulation of NK cell activities (3, 14, 22, 23). Our results provide further support for this concept. Moreover, they provide evidence to suggest that 14,15-DiHETE may serve as an immune regulator. However, the mechanism(s) by which 14,15-DiHETE exerts its action on NK cell function remains to be elucidated.

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