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Inhibition of Lymphocyte Mitogenesis by an Arachidonic Acid Hydroperoxide

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Incubation of murine spleen cells with the oxidation product of soybean lipoxidase-treated arachidonic acid results in profound inhibition of induction of proliferation and maturation of these cells. The active entity was shown to be the 15-hydroperoxide of arachidonic acid (15-HPAA). Inhibition of the enzymes of the cyclo-oxygenase pathway fails to disturb this effect, indicating that 15-HPAA is not a substrate for this series of enzymes. 15-HPAA produced in this manner interfered with RNA synthesis, DNA synthesis, and blastogenesis, while failing to exert cytotoxic effects on the cells themselves. A variety of lymphocyte subpopulations, distinguished by their responsiveness to a diverse group of mitogens, were all equally inhibited by the addition of 15-HPAA to culture. Addition of this agent even as late as 24 h after initiation of culture resulted in profound inhibition of the proliferative and differentiative responses of splenic B cells to bacterial lipopolysaccharide (LPS). Exposure of cells to 15-HPAA for 10–30 min was adequate to initiate inhibition, an event that exhibited marked temperature dependence. The effects of pre-incubation with 15-HPAA could not be reversed in its absence in recovery periods of up to 6 h prior to addition of LPS. The implications of these data with reference to cellular activation mechanisms are discussed.

Key words: hydroperoxide, mitogenesis, 15-HPAA, arachidonic acid, inhibition, lymphocyte activation

The metabolites of arachidonic acid (AA) generated via the cyclo-oxygenase pathway, ie, the prostaglandins (PG), thromboxanes, and prostacyclin, have been the object of a great deal of biological and biochemical research. However, a number of biological effects have been described that are dependent upon substances produced from arachidonic acid by lipoxidation. Such effects include the stimulation of human platelet guanylate cyclase [1], modulation of human lymphocyte responses to phytohemagglutinin [2], diminution in the adherence properties of baby hamster kidney cells to substrate [3], enhancement of mediator release from activated rat mast cells [4], and enhancement of release of slow-reacting substance from ionophore-stimulated rat mast cells [5]. One such oxidation product is the 15-hydroperoxide of arachidonic acid (15-HPAA), generated by treatment of substrate with soybean lipoxidase. This agent has been used as a probe in a number of mammalian systems, where it has been shown to augment the release of anaphylactic mediators

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from guinea pig lung [6], to inhibit the formation of 6-keto $PGF_{1\alpha}$ [7], to inhibit the generation of prostacyclin [8], and to stimulate human platelet guanylate cyclase activity [1]. Because the role that these compounds play in lymphocyte activation is not yet understood, experiments were undertaken to investigate the role of the soybean lipoxidation product of arachidonic acid on lymphocyte activation.

MATERIALS AND METHODS

Mice

C3H/St male mice, 8–16 weeks of age, were obtained from the mouse breeding facility at Scripps Clinic and Research Foundation, La Jolla, CA. All mice were maintained on Wayne Lab-Blox F6 pellets (Allied Mills, Inc., Chicago, IL) and chlorinated water acidified to a pH of 3.0 with HCl [9].

Mitogens

Bacterial lipopolysaccharide (LPS) 055:B5, extracted by the Boivin technique, was purchased from Difco Laboratories, Detroit, MI. Concanavalin A (Con A) was obtained from Miles-Yeda Laboratories, Rehovot, Israel. Polyinosinic-polycytidilic acid (Poly IC) double-stranded sodium salt was purchased from P-L Biochemicals, Inc., Milwaukee, WI. 2-Mercaptoethanol (2-ME), purchased from Matheson, Coleman and Bell, Los Angeles, CA, was dissolved in phosphate-buffered saline (PBS) and sterilized by filtration. Purified protein derivative (PPD), extracted from mycobacterium tuberculosis RT33, was obtained from Statens Serum-institut, Copenhagen, Denmark. Unless otherwise specified, all mitogens were sterilized by exposure to UV light and diluted in complete medium.

Culture Reagents

Dissociated spleen cells were cultured in serum-free medium, whose constituents have been detailed elsewhere [10]. Cultures were fed daily with nutritional cocktail as described previously [11]. Arachidonic acid was purchased from the Sigma Chemical Co., St. Louis, MO; ICN Pharmaceuticals, Inc., Irvine, CA; US Biochemical Corp., Cleveland, OH; and Vega Biochemicals, Tucson, AZ. Soybean lipoxidase and indomethacin were purchased from the Sigma Chemical Co., St. Louis, MO.

Lymphocyte Cultures

Spleen cell suspensions were prepared as described previously [10]. These cells were cultured in microculture plates (#3042, Falcon Plastics, Oxnard, CA) at a cell density of 5×10^6 viable cells/ml in a volume of 0.1 ml. Microculture plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Cultures were fed daily with 8 μl of nutritional cocktail. Polyclonal activation of B cells was induced by LPS in cultures of 5×10^6 viable lymphocytes per well in a volume of 1.0 ml in tissue culture trays (#3008, Falcon Plastics). These cultures were incubated under conditions identical to those for microcultures and were fed with 60 μl of nutritional cocktail daily.

Measurement of DNA and RNA Synthesis

To evaluate DNA synthesis, cells were radiolabeled with 1.0 μCi of ^{3}H-thymidine (^{3}H-TdR) per culture (5.0 Ci/mmole, New England Nuclear, Boston, MA) during the final 24 h of culture. RNA synthesis was determined by pulsing cells with 1.0 μCi of ^{3}H-uridine

(^{3}H-UdR) per culture (5 Ci/mmole, New England Nuclear) for the final 4 hr of a 24 h culture period. Microcultures were harvested on a Brandel cell harvester, Model M24V (Biological Research and Development Laboratories, Rockville, MD) onto glass-fiber filter strips (Reeve Angel, Clifton, NJ). Filter discs were transferred to plastic scintillation vials (Kimball Products, Owens-Illinois, Toledo, OH), covered with liquid scintillation cocktail (Scintverse, Fisher Scientific Co., Fairlawn, NJ), and counted in a Beckman LS-230 liquid scintillation spectrometer.

Assay of Plaque-Forming Cells (PFC)

LPS-induced polyclonal B-cell activation was evaluated by determining the response to sheep red blood cells (SRBC). PFCs to SRBC were assayed at day 2 of culture [12], using a modification of hemolytic plaque assay of Jerne and Nordin [13]. Results are expressed as the arithmetic mean of triplicate cultures ± the standard error (SE).

Enumeration of Blast Cells

Histological preparations were generated on microscopic slides from individual lymphocyte cultures with the aid of a cytocentrifuge. Slides were stained with the methyl green-pyronin Y technique [14] to simplify the enumeration of pyroninophilic blast cells. Results are expressed as the arithmetic mean of triplicate cultures ± the standard error.

High Pressure Liquid Chromatography

Oxidation of AA by treatment with soybean lipoxidase was performed according to the protocol of Hamberg and Samuelsson [15]. Oxidation products were purified by HPLC using a Waters liquid chromatograph fitted with a 0.39 X 30 cm μ-Porasil column. The eluting solvent, n-hexane/propan-2-ol/acetic acid (994:5:1 [8]) was pumped through the column at 3 ml/min.

RESULTS

Effects of Arachidonic Acid Oxidation Product(s) on Lymphocyte Stimulation

Preliminary experiments demonstrated that neither arachidonic acid nor its lipoxidase-catalyzed oxidation product(s) were mitogenic for murine spleen cells, in spite of the reported ability of the latter product(s) to activate guanylate cyclase in human platelets [1]. Therefore, the ability of these substances to modify the mitogenic response of murine lymphocytes to LPS was evaluated. It was found that variable concentrations of arachidonic acid treated with soybean lipoxidase [15], when incubated with cultures in the presence of 100 μg/ml of LPS, led to inhibition of lymphocyte mitogenesis (Table I). Predominant conversion to 15-HPAA was verified by determining the absorbance at 234 nm [15] and by HPLC. The addition of lipoxidase alone to cultures of spleen cells incubated with LPS failed to alter the LPS response. Experiments undertaken to learn if 15-HPAA-mediated inhibition of mitogenesis was due to toxicity demonstrated that this substance caused no significant diminution of viability during the 3-day culture period (34% recovery without 15-HPAA, 37% with it).

Chromatography of 15-HPAA on HPLC produced one predominant peak; this substance reacted positively with the peroxide reagent ferrous thiocyanate. Assay of the HPLC fractions for inhibitory activity (Table II) indicated that most of the activity was attributable to 15-HPAA, eluting at 15 min. However, moderate inhibitory effects were seen with the shoulder eluting just prior to 15-HPAA, at 12.9 min.

TABLE I. Effect of 15-HPAA on LPS-Induced Mitogenesis

LPS[a]	[15-HPAA]	^{3}H-TdR uptake (cpm/culture)[b]
–	–	3,830 ± 530
+	–	26,660 ± 3,830
+	10^{-7}	23,960 ± 1,200
+	3×10^{-7}	24,540 ± 840
+	10^{-6}	23,980 ± 800
+	3×10^{-6}	24,440 ± 520
+	10^{-5}	16,400 ± 1,000
+	3×10^{-5}	3,350 ± 590
+	10^{-4}	1,650 ± 220

[a]5×10^5 viable C3H/St spleen cells were cultured with 100 μg/ml LPS in 0.1 ml of serum-free medium in the presence or absence of incremental concentrations of 15-HPAA.
[b]Cultures were pulsed with 1 μCi of ^{3}H-TdR (5 Ci/mmole) for the final 24 h of the 3-day culture period. Results are expressed as the arithmetic mean of 5 replicate cultures ± the SE.

TABLE II. Effect of HPLC Separated AA Oxidation Products on the Mitogenic Response to LPS

Peak	Retention time (min)	Inhibition of LPS response (%)[a]
–	–	0
1	3.2	0
2	5.9	1
3	7.1	0
4	12.9	36
5	15.0	87

[a]5×10^5 viable C3H/St spleen cells were cultured with 100 μg/ml LPS in 0.1 ml of serum-free medium in the presence or absence of the minimal concentration necessary for inhibition by any one component. Cultures were pulsed with 1.0 μCi ^{3}H-TdR for the final 24 h of the 3-day culture period. Results are expressed as the percent inhibition of the mean LPS response of 5 replicate cultures. ^{3}H-TdR uptake by LPS-stimulated cultures in the absence of peak fractions was 30,500 ± 750 cpm.

Inability of Indomethacin to Reverse the Effect of 15-HPAA

Prostaglandins are produced from arachidonic acid via the cyclo-oxygenase pathway, a pathway that can be blocked by the action of the "prostaglandin synthetase" inhibitor, indomethacin. Therefore, if 15-HPAA were acting as a substrate for any of the enzymes involved in synthesis of the prostaglandins, the thromboxanes, or prostacyclin, its inhibitory effect should be blocked when indomethacin is added to culture. The data presented in Table III are representative of experiments in which optimal amounts of 15-HPAA were added to cultures activated with 100 μg/ml of LPS, in the presence or absence of indomethacin. No significant difference can be seen in the presence or absence of several concentrations of this prostaglandin synthetase inhibitor, arguing that products of the cyclo-oxygenase pathway are not involved in this inhibitory phenomenon.

TABLE III. Inability of Indomethacin to Reverse the Effect of 15-HPAA

10^{-4} M 15-HPAA[a]	Indomethacin	^{3}H-TdR uptake (cpm/culture) (E-C)[b]
+	–	350 ± 90
–	–	8,540 ± 390
+	10^{-6}	470 ± 170
–	10^{-6}	9,340 ± 230
+	10^{-5}	420 ± 120
–	10^{-5}	8,620 ± 250

[a]5×10^5 viable C3H/St spleen cells were cultured with 100 μg/ml LPS in 0.1 ml of serum-free medium in the presence or absence of optimal concentrations of 15-HPAA in the presence of variable amounts of indomethacin.
[b]Cultures were pulsed with 1 μCi of ^{3}H-TdR (5 Ci/mmole) for the final 24 h of the 3-day culture period. Results are expressed as the arithmetic mean of 5 replicate cultures minus controls ± the SE.

15-HPAA Inhibits Lymphocyte Activation by a Spectrum of Mitogens

The ability of 15-HPAA to inhibit activation of a diversity of lymphocyte populations and subpopulations was investigated. It has previously been demonstrated that the B-cell subpopulations responsive to LPS and Poly IC are essentially the same, while each of those responding to PPD and 2-ME is distinct [10]. However, as shown in Table IV, activation of each of these B-cell subpopulations is completely inhibited by co-incubation with 15-HPAA. Moreover, activation of T cells by incubation of spleen cells with either the jack bean lectin Con A or with 2-ME (which stimulates both B and T cells separately) is similarly inhibited. It thus appears that 15-HPAA is capable of profoundly inhibiting the activation of a number of distinct B- and T-lymphocyte subpopulations.

Because 15-HPAA might theoretically interfere with ^{3}H-TdR uptake without otherwise altering mitogenesis, the effect of 15-HPAA on LPS-induced blastogenesis was investigated. Spleen cells from C3H/St mice were cultured with LPS under serum-free conditions in the presence or absence of 2.5×10^{-4} M 15-HPAA. After 1, 2, and 3 days of incubation, each culture was transferred to a slide by means of a cytocentrifuge, and stained as described in Materials and Methods. Enumeration of pyroninophilic blast cells under the microscope indicated that LPS-induced blastogenesis is completely inhibited by 15-HPAA regardless of the duration after which it is evaluated (data not shown).

Inhibition of RNA Synthesis by 15-HPAA

To this point, inhibition of cellular activation has been evaluated by assay of uptake of ^{3}H-TdR. Therefore, the effect of 15-HPAA on synthesis of cellular RNA was examined to determine whether earlier stages in the activation process were inhibited as well as later stages. In Table V, murine spleen cells were activated with optimal concentrations of LPS in the presence of incremental amounts of 15-HPAA. One μCi/culture of ^{3}H-UdR was added after 20 h of culture, and cells were harvested 4 h later. The data indicate that uridine uptake also is inhibitable by the action of 15-HPAA with a profile of similar shape to that for DNA synthesis.

Kinetics of 15-HPAA-Dependent Inhibition of Polyclonal B Cell Activation and Mitogenesis

The marked inhibitory effect of 15-HPAA on cellular proliferation led us to consider next whether cellular differentiation might be similarly affected. Therefore, taking

TABLE IV. Effect of 15-HPAA on Lymphocyte Activation by a Spectrum of Mitogens

Mitogen[a]	15-HPAA	^{3}H-TdR uptake (cpm/culture)[b]
–	–	1,395 ± 540
–	+	690 ± 120
LPS	–	50,650 ± 1,920
LPS	+	2,350 ± 420
PPD	–	66,150 ± 2,300
PPD	+	2,530 ± 1,190
Poly IC	–	23,790 ± 890
Poly IC	+	800 ± 180
2-ME	–	39,070 ± 2,080
2-ME	+	1,020 ± 530
Con A	–	285,180 ± 2,670
Con A	+	1,070 ± 500

[a] 5×10^5 viable C3H/St spleen cells were cultured in 0.1 ml of serum-free medium with either medium alone, 100 μg/ml LPS, 500 μg/ml Poly IC, 300 μg/ml PPD, 5×10^{-5} M 2-ME, or 1 μg/ml Con A. Each mitogen was tested in the presence and absence of 2.5×10^{-4} 15-HPAA.

[b] Cultures were pulsed with 1 μCi of ^{3}H-TdR (5 Ci/mmole) for the final 24 h of the 3-day culture period. Results are expressed as the arithmetic mean of 5 replicate cultures ± the SE.

TABLE V. Effect of 15-HPAA on Cellular RNA Synthesis

LPS[a]	15-HPAA	^{3}H-UdR uptake (cpm/culture)[b]
–	–	3,230 ± 385
+	–	10,401 ± 165
+	10^{-5}	9,049 ± 212
+	5×10^{-5}	9,906 ± 379
+	10^{-4}	8,757 ± 628
+	2.5×10^{-4}	1,061 ± 485
+	5×10^{-4}	574 ± 224

[a] 5×10^5 viable C3H/St spleen cells were cultured with 100 μg/ml LPS in 0.1 ml of serum-free medium in the presence of incremental concentrations of 15-HPAA.

[b] Cultures were pulsed with 1 μCi of ^{3}H-UdR (5 Ci/mmole) for the final 4 h of a 24-h culture period. Results are expressed as the arithmetic mean of 5 replicate cultures ± the SE.

advantage of the ability of LPS to activate lymphocyte cultures to polyclonal secretion of immunoglobulin (Ig), 15-HPAA was added at optimal inhibitory doses at various times after culture initiation. The data presented in Table VI demonstrate that this agent, added as late as 24 h after LPS, inhibits the polyclonal response as assayed against SRBC on day 2. However, at 45–48 h, when cellular differentiation to antibody secretion has occurred, 15-HPAA exerts only minimal inhibitory effects.

The ability of 15-HPAA to inhibit LPS-induced mitogenesis when added at sequential time-points after culture initiation was evaluated in experiments summarized in Table VII. The kinetic profile in this case is similar to that seen for polyclonal activation, except that the time of assay here is 72 h rather than 48 h. The data indicate that complete inhibi-

TABLE VI. Kinetics of 15-HPAA-Dependent Inhibition of Polyclonal B-Cell Activation

LPS[a]	15-HPAA added at	Anti-SRBC PFC/culture[b]
–	–	35 ± 6
+	–	427 ± 8
+	0 h	107 ± 11
+	24 h	103 ± 4
+	45 h	347 ± 12
+	Assay	348 ± 48

[a]5×10^6 viable C3H/St spleen cells were cultured in the presence or absence of 100 μg/ml LPS in 1.0 ml of 5% FCS-containing medium. At various times after culture initiation, cultures were brought to a final 15-HPAA concentration of 2.5×10^{-4} M.
[b]The direct PFC response to SRBC was assessed after 2 days of culture. Results are expressed as the arithmetic mean of triplicate cultures ± the SE.

TABLE VII. Kinetics of 15-HPAA-Dependent Inhibition of LPS-Induced Mitogenesis

LPS[a]	Time of 15-HPAA addition	^{3}H-TdR uptake (cpm/culture)[b]
–	–	3,400 ± 300
+	–	49,720 ± 1,290
+	0 h	240 ± 50
+	6 h	200 ± 90
+	12 h	770 ± 110
+	24 h	180 ± 90
+	48 h	13,350 ± 700

[a]5×10^5 viable C3H/St spleen cells were cultured with 100 μg/ml LPS in 0.1 ml of serum-free medium. At various times after culture initiation, cultures were brought to a final 15-HPAA concentration of 2.5×10^{-4} M.
[b]Cultures were pulsed with 1 μCi of ^{3}H-TdR for the final 24 h of the 3-day culture period. Results are expressed as the arithmetic mean of 5 replicate cultures ± the SE.

tion of mitogenesis occurs when 15-HPAA is added as late as 24 h after culture initiation. When added after 2 days of a 3-day culture period, 75% of the response can still be abolished. Thus, it appears that late events are important targets for the action of the inhibitory AA oxidation product.

Effect of Temperature on 15-HPAA-Mediated Inhibition

The profound nature of the inhibition of mitogenesis induced by 15-HPAA led us next to investigate whether this effect is temperature dependent. C3H/St spleen cells were incubated for variable periods of time with 15-HPAA at either 4°C or 37°C, following which they were washed in an excess of BSS 3 times, plated out in microculture wells in the presence or absence of optimal mitogenic concentrations of LPS, and incubated at 37°C for the remainder of the culture period. The results of these experiments are shown in Table VIII. It is apparent that at 37°C, a 10-min pre-incubation with 15-HPAA is sufficient to reduce the mitogenic effects of LPS to baseline levels. Pre-incubation longer than 30 min does not serve to promote additional inhibition. On the other hand, when pre-incubation is performed at 4°C, lymphocyte mitogenesis is inhibited by only approximately 50%. This inhibition also levels off after 10 min of exposure to 15-HPAA. In concert, these data suggest that the inhibitable entity is inactivated by exposure to 15-HPAA

TABLE VIII. Effect of 15-HPAA Pre-incubation on LPS-Induced Mitogenesis

LPS	Length of 15-HPAA pre-incubation[a]	^{3}H-TdR uptake (cpm/culture) (E-C)[b] 37°C	4°C
+	–	51,200 ± 2,400	51,200 ± 2,400
+	10 min	3,310 ± 1,480	23,320 ± 1,630
+	30 min	0	18,100 ± 2,670
+	1 h	0	24,730 ± 1,550
+	2 h	0	25,390 ± 3,540

[a]5×10^6 viable C3H/St spleen cells per ml were pre-incubated (in 12 × 75-mm test tubes) in the presence or absence of 2.5×10^{-4} M 15-HPAA for the time lengths shown, at either 4°C or 37°C. After pre-incubation, cells were washed 3 times in an excess of BSS. 5×10^5 viable cells were then cultured in the absence or presence of 100 μg/ml LPS in 0.1 ml of serum-free medium.
[b]Cultures were pulsed with 1 μCi of ^{3}H-TdR for the final 24 h of the 3-day culture period. Results are expressed as the arithmetic mean of 5 replicate cultures ± the SE.

very rapidly. Furthermore, this rapid inhibition includes a temperature-dependent phase resulting in a partial inhibition of cells pre-incubated at 4°C, which cannot be amended by subsequent incubation at 37°C for the duration of the culture period.

Inability of Cells to Recover From Inhibition by 15-HPAA

Finally, the possibility that cells inhibited by a brief pre-incubation with 15-HPAA might be able to regenerate 15-HPAA-sensitive function during a recovery period was tested. Therefore, cells were incubated with 15-HPAA at 37°C for 1 h, washed 3 times in an excess of serum-free medium, and cultured in the absence of inhibitor for variable periods of time before addition of LPS. As can be seen in Table IX, however, recovery periods up to 6 h were ineffectual in allowing cells to recover responsiveness to LPS. Because of the dimished stimulation obtained when addition of LPS is delayed 6 h or longer, the effects of later addition of LPS were not evaluated.

DISCUSSION

The specific introduction of a hydroperoxide group at the 15 position of arachidonic acid catalyzed by soybean lipoxidase [15] results in the creation of a compound with potent biological activities. Its abilities to enhance the release of histamine, SRS-A, and rabbit aortic-contracting substance (RCF) from guinea pig isolated perfused lungs [6], to inhibit the synthesis of 6-keto prostaglandin $F_{1\alpha}$ by aortic microsomes [7], and to inhibit conversion of prostaglandin endoperoxides to prostacyclin [8] have been well described. The current communication reports the further ability of this compound to inhibit lymphocyte mitogenesis as induced by a variety of B- and T-lymphocyte activators.

The inhibitory effect of 15-HPAA on murine lymphocytes is profound and exhibits marked dose-dependency. Inhibition does not appear to be attributable to processing by cyclo-oxygenase pathway enzymes. Indomethacin, a prostaglandin synthetase inhibitor, blocks the formation of the prostaglandins, the thromboxanes, and prostacyclin, via the cyclo-oxygenase pathway. However, this agent failed to interfere with the inhibition of lymphocyte activation mediated by 15-HPAA. Although there are differences in sensitivity of arachidonic acid metabolites to indomethacin, no effect was attributable to this agent over the entire range of concentrations that failed to alter the basic lymphocyte response itself.

TABLE IX. Inability of Cells to Recover from Inhibition by 15-HPAA

15-HPAA pre-incubation[a]	Length of recovery period[b]	LPS	^{3}H-TdR uptake (cpm/culture)[c]
–	0	–	990 ± 65
–	0	+	39,610 ± 3,130
+	0	+	2,400 ± 360
–	1 h	+	44,560 ± 1,970
+	1 h	+	3,100 ± 710
–	3 h	+	35,620 ± 5,090
+	3 h	+	3,650 ± 420
–	6 h	+	4,805 ± 530
+	6 h	+	3,900 ± 1,765

[a]5×10^6 viable C3H/St spleen cells per ml were pre-incubated (in 12 × 75-mm test tubes) in the presence or absence of 2.5×10^{-4} M 15-HPAA for 1 h at 37°C.
[b]Cells were washed 3 times in an excess of BSS, and 5×10^5 viable cells were cultured in 0.1 ml of serum-free medium in the absence of LPS. At the times shown, LPS was added to a final concentration of 100 μg/ml.
[c]Cultures were pulsed with 1 μCi of ^{3}H-TdR for the final 24 h of the 3-day culture period. Results are expressed as the arithmetic mean of quadruplicate cultures ± the SE.

The interference of 15-HPAA with cellular activities failed to result in the loss of cellular viability. Similarly, the diminution of tritiated thymidine uptake by cells was shown not to be an artifact of thymidine transport, insofar as blast transformation and RNA synthesis were similarly affected. Thus, the inhibitory effect of 15-HPAA appears to act by arresting the stimulation process without totally disrupting the function vital to cellular survival.

Activation of T lymphocytes, as well as a variety of B-cell subpopulations, were inhibited by 15-HPAA. Activation of these populations and subpopulations was elicited by the use of a number of different lymphocyte mitogens, whose cellular specificity has been previously verified [10]. These experiments, however, were carried out with whole spleen cells, and it is possible that 15-HPAA induces suppressor activity in one group of cells, ie, macrophages, resulting in an indirect mechanism of cellular inhibition.

The differentiative as well as the proliferative responses of murine spleen cells to LPS is subject to inhibition by 15-HPAA. It has been demonstrated by Andersson and Melchers [16] that maturation of resting B cells into 19S IgM-secreting plaque-forming cells (PFC) is able to occur even when DNA synthesis and proliferation are inhibited by the use of either hydroxyurea or cytosine arabinoside. Thus, differentiation can occur in the absence of proliferation, and in fact, these 2 activities have been dissociated by others [17]. Therefore, one would have to postulate that 15-HPAA exerts its effects upon a sequence of steps common to both the differentiative and proliferative pathways or that this agent interferes with a different step in each pathway. Addition of 15-HPAA to culture after LPS has effected the maturation of precursor B cells to IgM-secreting PFC failed to inhibit the development of PFC in a Jerne plaque assay. Thus, antibody secretion is not subject to the effects of 15-HPAA. Kinetic analysis of the inhibitory potential of 15-HPAA on polyclonal as well as proliferative responses to LPS indicated that this agent exerts its effects relatively late in the culture period. Others have noted that optimal activation, especially with B-lymphocyte mitogens, requires the presence of the mitogen for the first 24 h of culture [18]. Apparently, it is in this time, during which the activation signal is being received and processed by the target cell, that 15-HPAA is most effective.

Lymphocyte inhibition by 15-HPAA requires only a very brief exposure to this agent, as revealed by a kinetic analysis utilizing brief pre-incubation of spleen cells with 15-HPAA followed by extensive washing procedures. These kinetics closely resemble those described for 15-HPAA-mediated inhibition of 6-keto $PGF_{1\alpha}$ production by aortic microsomes [7]. Although the length of exposure is brief, the inhibitory event which occurs is temperature-dependent. Thus, if pre-incubation of the cells is carried out at 4°C, followed by washing and subsequent incubation at 37°C in the absence of 15-HPAA, the inhibition that follows is incomplete, averaging about 50%. This observation is taken to indicate that at least one part of the inhibitory process is dependent upon cellular metabolism while 15-HPAA is present, since later elevation of the temperature to 37°C does not correct the incomplete inhibition. However, an association of 15-HPAA with the inhibited entity seems to occur at 4°C, although one cannot determine from the data presented whether the actual process culminating in the partial inhibition observed occurs at this temperature or at the following incubation at 37°C.

Recovery periods of up to 6 h in length fail to result in the regeneration of LPS responsiveness by cells previously exposed to 15-HPAA. Further corroborating evidence for the irreversibility of this effect can be gleaned from the experiments in which cells were pre-incubated for brief periods of time in the presence of 15-HPAA followed by standard 2-day culture with LPS in the absence of this inhibitory agent. In no case was responsiveness recovered.

The reasons why inhibition of the endogenous lipoxygenase pathway with ETYA interferes with cellular activation [19], yet supplementation of cultures with exogenous products of AA lipoxidation inhibits activation, are not clear. While it is possible that the site of oxidation within the molecule may lead to the generation of products with opposing activities, that selective pathways are inhibited by a relatively large amount of exogenous hydroperoxide, or that initial exposure of these highly reactive compounds to the outside vs the inside of the cell results in very different effects, the actual cause remains to be elucidated. Further experiments, probing the mechanism by which this inhibitory action is exerted, are currently in progress.

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