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OXIDANT-MEDIATED ELECTRONIC EXCITATION OF IMIPRAMINE*

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Abstract—The interaction of imipramine with both resting and zymosan-activated human polymorphonuclear leukocytes (PMNs) resulted in the generation of chemiluminescence (CL). This CL was not accompanied, however, by an enhanced release of superoxide anion. CL was also observed following the interaction of imipramine with either a xanthine oxidase or a horseradish peroxidase catalyzed system. Collectively, these observations support the concept that the CL elicited from these interactions is reflective of the electronic excitation of the imipramine molecule. In contrast to the response seen with PMNs, addition of imipramine to resting alveolar macrophages (AMs) failed to yield CL. However, CL from imipramine was observed with resting AMs upon supplementation with exogenous horseradish peroxidase. The lack of response with control AMs and the significant inhibition of the imipramine-PMN CL by the myeloperoxidase inhibitor azide suggests that a peroxidase-derived oxidant facilitated the oxidation of imipramine, yielding a product in an electronically excited state. In addition to PMNs, CL was elicited from imipramine by rat or rabbit liver microsomes, suggesting that PMNs may be a useful model system to predict a xenobiotic effect on the CL response elicited by other cellular oxidant-generating systems. Moreover, these observations underscore the possibility that the metabolic activation of drugs by PMNs may be of pharmacologic and toxicologic importance.

It is becoming increasingly apparent that reactive forms of oxygen are involved in the interactions of drugs with cells [1–3]. For example, the NADPH cytochrome P-450-reductase catalyzed redox cycling of compounds such as adriamycin, nitrofurantoin and paraquat augments reactive oxygen-dependent microsomal lipid peroxidation [4–6]. On the other hand, reactive oxygen species can facilitate the cytochrome P-450 dependent activation of xenobiotics to reactive intermediates capable of covalently binding to protein [7, 8]. Similarly, the interaction of organic substrates with oxidant species may result in the generation of a product in an electronically excited state, which upon relaxation to ground state emits photons as indicated by chemiluminescence (CL) [9]. The *trans*-7,8-dihydroxy-7,8-dihydro metabolite of benzo[*a*]pyrene (BP-7,8-diol) is an example of such a substrate [10]. The oxidant species which mediate such CL reactions can be provided by intact cells, subcellular fractions, enzymes, or metal-containing cell-free chemical systems.

We have reported previously that the interaction of the tricyclic antidepressant imipramine with resting (i.e. metabolically unactivated) human polymorphonuclear leukocytes (PMNs) results in CL [11, 12]. Moreover, upon activation of the respiratory burst in PMNs by opsonized zymosan particles, a process which results in increased cellular generation of reactive oxygen species, a significant increase in CL (synergistic) occurs in the presence of imipramine. These observations with imipramine and PMNs are similar to those observed with the cyclic hydrazide luminol, which is an electronically excitable substrate [13, 14]. Thus, it is possible that imipramine is being oxidized to a product in an electronically excited state following the interaction with an oxidant species generated by resting and zymosan-activated PMNs. This study experimentally examines this concept, and the results demonstrate that, indeed, oxidation to a product in an electronically excited state is a property of imipramine, that the oxidant species which facilitates this reaction is primarily peroxidase derived, and that a biological system other than PMNs, namely the microsomal mixed-function oxidase system, can catalyze this process.

METHODS AND MATERIALS

Cell isolation procedures

Blood was obtained from normal, healthy volunteers by venipuncture. Similarly, blood was obtained from male New Zealand white rabbits via the ear artery. Leukocytes were isolated by a 30 min sedimentation at 37° using a 6% dextran solution (3 ml dextran/10 ml blood) [15]. Following centrifugation of the leukocyte-rich plasma layer at 100 *g* for 5 min, remaining erythrocytes were removed by lysis with

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ammonium chloride (0.85% solution for human blood) or hypotonic lysis for the rabbit RBCs (0.2% NaCl solution for 20 sec followed by 1.6% NaCl solution). Leukocytes (PMNs) were washed, counted, and then resuspended in Dulbecco's phosphate buffered saline (PBS) (pH 7.4) to the desired cell concentration.

Alveolar macrophages (AMs) were isolated from male Long–Evans hooded rats (Charles River Laboratories, Cambridge, MA) and male New Zealand white rabbits by bronchopulmonary lavage [16] using calcium- and magnesium-free Hank's balanced salt solution (pH 7.4). After sedimentation at 200 *g* for 5 min, the AMs were washed once and resuspended in PBS to a concentration of 5×10^6 AMs/ml. Microscopic examination of the AM preparations revealed that less than 1% of the cells were PMNs.

Animals and microsomal preparation

Male Sprague–Dawley rats (weighing 200–250 g) were obtained from Taconic Farms, Germantown, NY, and adult male New Zealand rabbits (2.5 to 3 kg) were obtained from Dutchland Laboratories, Denver, PA. Animals were fed standard Purina chow and allowed water *ad lib.* Rats were killed by cervical dislocation while rabbits were killed by air embolism after which the livers were removed and rinsed in chilled buffer (150 mM KCl–50 mM Tris–HCl, pH 7.4). Livers were homogenized in 2 vol. of KCl–Tris buffer using a motor-driven Teflon–glass homogenizer, the homogenates were diluted to 25% (w/v), and microsomes were isolated by differential centrifugation as previously described [5]. Microsomal protein was determined by the method of Lowry *et al.* [17] using bovine serum albumin as standard.

Determination of superoxide production by PMNs

Superoxide production by PMNs and AMs was measured by the reduction of cytochrome *c* according to Babior *et al.* [18]. One milliliter of cells (5×10^6 PMNs) was incubated in PBS for 15 min at 37°. Combinations of zymosan, imipramine, cytochrome *c* and PBS were added to the cells after this incubation period to give a total volume of 5 ml. Experimental additions yielded the following final concentrations: cytochrome *c*, 0.08 mM; zymosan, 4 mg; and imipramine, 1×10^{-4} M. The reaction was terminated by immediately placing the vials in ice and then centrifuging at 1000 *g* for 10 min at 4°. Cytochrome *c* reduction was determined by the absorbance of the supernatant fraction at 550 nm. Specificity of the reaction was indicated by the inhibition of cytochrome *c* reduction by superoxide dismutase. Vials containing cytochrome *c*, cytochrome *c* and imipramine, and cytochrome *c* and zymosan were incubated and used as reference blanks. Nanomoles of cytochrome *c* reduced was determined from the increase in absorbancy above blank using the extinction coefficient 21.0 cm^{-1} mM^{-1} [19].

Determination of malondialdehyde production by PMNs

The formation of malondialdehyde was used as an indication of the process of lipid peroxidation. PMNs 1.5×10^7 were suspended in 0.5 ml of PBS and preincubated at 37° for 15 min prior to the addition of zymosan (12 mg) and/or imipramine (3×10^{-4} M) to give a final volume of 1.5 ml. After 45 min, 1 ml of the medium was removed, added to 0.5 ml of 35% trichloroacetic acid (TCA) mixed and centrifuged at 500 *g*. Malondialdehyde in the acid solution was measured spectrophotometrically at 535 nm after reaction with 1 ml of 2-thiobarbituric acid (0.75%) for 30 min at 90° [20]. The results are expressed as the change in O.D. units minus appropriate blanks.

Monitoring of chemiluminescence responses

Cells. CL responses were measured with a Packard ambient-temperature liquid scintillation spectrometer (model 2002) operated in the out-of-coincidence mode: gain 100%, window A–∞ with discriminators set at 0–1000 and input selector 1 + 2 [15]. To begin the procedure, 1 ml of cells (PMNs and AMs) was added to previously dark-adapted polyethylene scintillation vials and incubated in PBS at 37° for 15 min, after which time the vials were transferred (every 0.5 min) to the scintillation counter and background activity was determined for each of the vials. The CL reaction was initiated by adding 1 ml of opsonized zymosan and/or 1 ml of imipramine to the vials, giving a total volume of 5 ml, and CL was monitored at 0.5-min intervals. The reaction vials were maintained at 37° between counts. In the inhibitor studies, a volume of PBS was replaced by an equal volume of the inhibitor solution. All procedures related to the measurements of CL were performed in a darkened room. The results are expressed as chemiluminescence in counts per 0.5 min above background, and the data are presented as temporal (time course) curves and peak (maximum) responses.

Enzymes. CL was also measured using a cell-free xanthine oxidase system containing 4 ml of PBS plus inhibitors when used, 1 ml of purine (final concentration of 0.9 mM), and 6 μl of xanthine oxidase (95 μg). All components of the reaction, except enzyme, were added to the vials, and background CL was determined. CL was initiated by the addition of the enzyme, and the response was followed every minute for 10 min. When the horseradish peroxidase (HRP) system was used to initiate cell-free CL, the final reaction volume was 5 ml. It contained PBS, HRP (0.05 mg/ml), imipramine (10^{-4} M) and catalase (100 μg/ml) when added and 10 μl of H_2O_2 (30% solution), giving a final concentration of 17.6 mM. The reaction was initiated by the addition of H_2O_2.

Microsomes. Microsomal CL was monitored in a model 3003 Packard liquid scintillation spectrometer operated at ambient temperature and in the out-of-coincidence mode with the following settings: gain 100%, window A–∞ with discriminators set at 0–1000 and input selector 1 + 2. The reaction mixture consisted of rat or rabbit liver microsomes (0.5 mg protein/ml), an NADPH-generating system [NADP (1.9 mM), glucose-6-phosphate (20 mM), glucose-6-phosphate dehydrogenase (1.1 I.U./ml) and magnesium chloride (4.3 mM)] and, where indicated, imipramine (1×10^{-4} M) in a total volume of 3.5 ml. Following a 10-min preincubation period, background CL was determined, the NADPH-generating

system was added to initiate the reaction, and the CL was monitored for 0.5 min after which the vials were returned to the 37° incubator. CL was then monitored at 2-min intervals. The results are expressed as counts/0.5 min $\times 10^{-3}$ corrected for background activity. After 20 min the reactions were terminated by adding 1.5 ml of a 2.0 M TCA–1.7 N HCl solution, and the supernatant fractions were analyzed for malodialdehyde as previously described [4–6].

Reagents

Dextran (100,000–200,000 mol. wt.), superoxide dismutase (bovine erythrocytes), catalase (bovine liver), cytochrome *c* (horse heart, Type VI), horseradish peroxidase, xanthine oxidase, NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and 2-thiobarbituric acid were obtained from the Sigma Chemical Co., St. Louis, MS. Sodium azide was obtained from the Nutritional Biochemicals Corp., Cleveland, OH; and sodium benzoate from the Fisher Chemical Co., Pittsburgh, PA. Imipramine HCl was a gift of the CIBA-Geigy Corp., Ardsley, NY.

Data analysis

Results were compared statistically using Student's *t*-test and considered significant if $P < 0.05$ [21].

RESULTS

Interaction of imipramine with resting or zymosan-activated PMNs

Figure 1 illustrates the response elicited by the addition of opsonized zymosan particles or imipramine to resting PMNs. As shown, CL was generated, and the maximum or peak CL to a constant concentration of zymosan or imipramine was dependent on the number of PMNs. The temporal response following zymosan addition was the same independent of cell number, with peak CL occurring at 5 min in each case; however, the temporal as well as the peak CL with imipramine varied according to cell number. The data in Table 1 demonstrate that

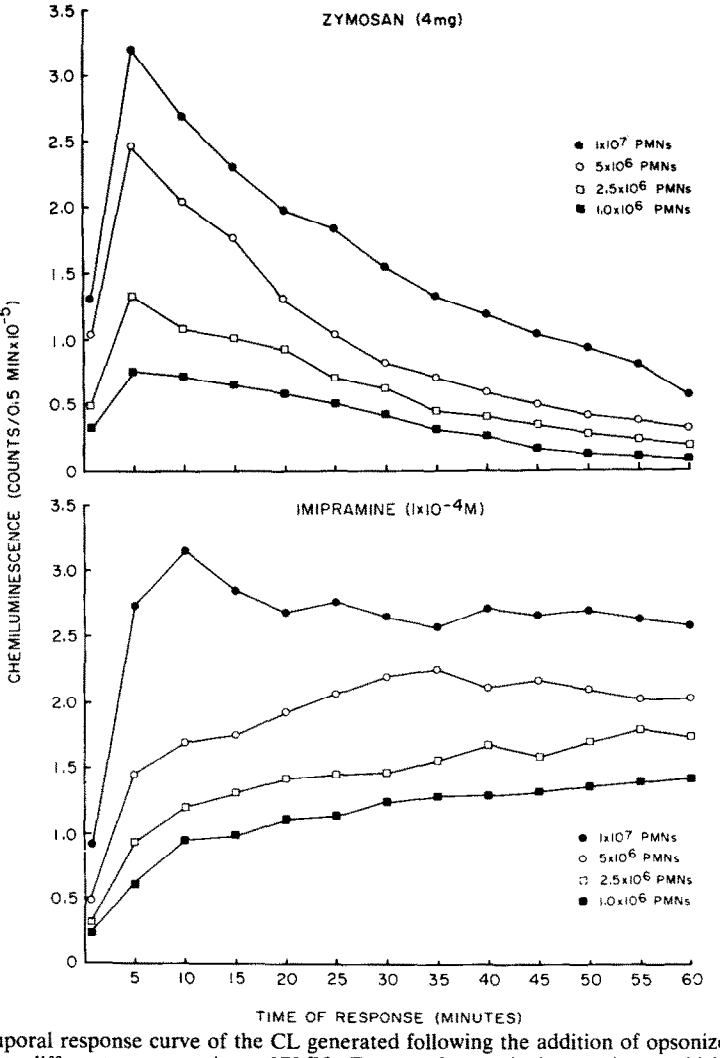

Fig. 1. Temporal response curve of the CL generated following the addition of opsonized zymosan or imipramine to different concentrations of PMNs. Data are from a single experiment which is representative of four experiments.

Table 1. CL resulting from the simultaneous or sequential addition of imipramine (1×10^{-4} M) to zymosan-stimulated PMNs (5×10^6)

Amount of zymosan (mg)	Peak zymosan CL	Time of peak response (min)	Simultaneous peak CL*	Time of peak response (min)	Sequential peak CL†	Time of peak response (min)
2	12.7 ± 0.5‡	10	176.0 ± 3.4	10	165.0 ± 1.8	10
3	17.1 ± 0.7	10	221.0 ± 2.3	10	203.0 ± 2.8	10
4	21.5 ± 0.6	5	205.0 ± 2.0	5	220.0 ± 3.6	10

* Zymosan and imipramine were added simultaneously. The characteristic peak time for imipramine CL with resting PMNs is 30–35 min.
† Imipramine was added 40 min after the zymosan.
‡ Counts/0.5 min $\times 10^{-4}$ ± S.E.M.; N = 4.

a significant increase in CL occurred when the cells were metabolically activated by various amounts of opsonized zymosan. In contrast to the slow developing response observed with imipramine and resting PMNs (Fig. 1), peak CL resulted 5–10 min after imipramine addition to zymosan-activated cells. Moreover, this enhanced CL by imipramine occurred regardless of whether it was added simultaneously with or sequentially to the zymosan (Table 1), implying that zymosan increased the cellular generation of the oxidant species which interacted with imipramine and culminated in CL.

Examination of the origin of CL from the imipramine–PMN interaction

The responses observed with imipramine with both resting and metabolically activated PMNs were similar to those of luminal [13, 14], a cyclic hydrazide that is oxidized to an excited aminopthalate anion by oxidant species. In addition to PMNs, luminol can be oxidized to its excited state by the xanthine oxidase–hypoxanthine superoxide generating system [22] or horseradish peroxidase and H_2O_2 [23]. On the other hand, CL is observed following the activation of PMNs by soluble agents, such as phorbol myristate acetate (PMA) and the calcium ionophore A23187, which elicit the same metabolic changes in PMNs as do particulate stimuli such as opsonized zymosan [24, 25]. Thus, if imipramine were acting in a fashion similar to PMA or A23187, then the imipramine–PMN interaction should engender enhanced superoxide release. Conversely, if imipramine were being oxidized, like luminol, to a product in an electronically excited state, then it should yield CL with cell-free enzyme systems but should not enhance superoxide generation from PMNs. Unlike PMA or A23187, the addition of imipramine did not enhance superoxide release from resting PMNs (Table 2); in fact, a significant decrease in both the resting and the zymosan-induced superoxide release was observed.

Another cellular process which utilizes reactive oxygen and results in CL is lipid peroxidation [6, 26, 27]. The addition of zymosan and/or imipramine did not result in a stimulation of lipid peroxidation (Table 2), which is consistent with the report of Stossel *et al.* [28] that human PMNs do not accumulate malondialdehyde. This response by PMNs to opsonized zymosan is opposite to that of rat alveolar macrophages which do exhibit measurable lipid peroxidation [20].

The data in Tables 3 and 4 demonstrate that enhanced CL was observed from imipramine with either a xanthine oxidase or a horseradish peroxidase-catalyzed system. Superoxide dismutase (SOD) significantly inhibited the CL with the xanthine oxidase system, while catalase and benzoate, a hydroxyl radical scavenger, had little effect (Table 3). Catalase, however, inhibited the CL from the horseradish peroxidase catalyzed system (Table 4).

Table 2. Release of superoxide and production of malondialdehyde from PMNs resulting from the addition of zymosan (4 mg) and/or imipramine (1×10^{-4} M)

Addition to PMNs (5×10^6)	Cytochrome *c* reduction (nmoles/10^6 PMNs/5 min)	Cytochrome *c* reduction (nmoles/10^6 PMNs/30 min)	Malondialdehyde (Δ O.D./30 min)
None	7.1 ± 1.8*	10.6 ± 0.67*	0.007 ± 0.001†
+ Imipramine	1.7 ± 0.7‡ (77%)§	6.4 ± 1.4‡ (40%)	0.009 ± 0.002
+ Zymosan	38.9 ± 2.0	71.8 ± 1.9	0.009 ± 0.001
+ Zymosan and imipramine	17.9 ± 1.4‖ (64%)	44.9 ± 3.4‖ (38%)	0.009 ± 0.002

* Mean ± S.E.M.; N = 5.
† Mean ± S.E.M.; N = 3.
‡ Significantly different ($P < 0.01$) from resting cells.
§ Numbers in parentheses represent percent inhibition.
‖ Significantly different ($P < 0.01$) from zymosan response.

Table 3. CL response resulting from the addition of imipramine (1×10^{-4} M) to a xanthine oxidase–purine superoxide generating system*

Modifications of the system	Xanthine oxidase	Xanthine oxidase + imipramine
None	36.6 ± 0.70†	215.0 ± 8.8
− Purine	1.1 ± 0.1	1.7 ± 0.1
− Xanthine oxidase	0.9 ± 0.05	1.0 ± 0.1
+ SOD (100 μg/ml)	2.3 ± 0.05	2.5 ± 0.1
+ Heat-inactivated SOD (100 μg/ml)	37.4 ± 0.7‡	216.0 ± 5.6†
+ Catalase (100 μg/ml)	39.9 ± 2.0‡	200.0 ± 8.0‡
+ SOD (100 μg/ml) and catalase (100 μg/ml)	2.9 ± 0.1§	2.9 ± 0.2§
+ Benzoate (10 mM)	34.9 ± 1.5‡	223.0 ± 1.1‡
+ Azide (1.0 mM)	39.6 ± 2.3	218.1 ± 4.6

* The xanthine oxidase system consisted of 4 ml of Dulbecco's phosphate buffered saline (pH 7.4), 1 ml purine (final concentration 0.9 mM) and 6 μl xanthine oxidase (95 μg). Modification of the system was made by substituting buffer with the addition. Superoxide dismutase was inactivated by heating at 100° for 30 min.

† Peak counts/min × 10^{-3} ± S.E.M.; N = 4.

‡ NS ($P > 0.05$).

§ $P < 0.05$ from catalase alone.

Modulation of the CL resulting from the interaction of imipramine with PMNs

The results with the xanthine oxidase and horseradish peroxidase-catalyzed systems, coupled with the absence of imipramine-induced superoxide release from PMNs, support the concept that imipramine was being oxidized to a product in an electronically excited state following the interaction with some oxidant generated by PMNs. Moreover, by metabolically activating the cells this reaction was accelerated. In an attempt to sort out the possible nature and/or origin of the oxidant species interacting with imipramine, the effects of SOD, sodium benzoate, catalase and azide, a myeloperoxidase inhibitor [29], were examined on the CL resulting from the imipramine–resting PMN interaction. For comparison, the effects of these agents on zymosan-induced CL were also assessed. As shown in Fig. 2, SOD (50 μg/ml) inhibited the imipramine CL, but not substantially; however, increasing the concentration of SOD to 100 μg/ml tended to enhance, rather than inhibit, the imipramine CL. In contrast, both of these concentrations of SOD significantly inhibited the opsonized zymosan-elicited response. SOD has been shown previously to increase, even at 5 μg/ml, several activities dependent on PMN metabolism, including myeloperoxidase-catalyzed iodination and estradiol binding [30, 31]. Unlike SOD, catalase, sodium benzoate and azide had clear inhibitory effects on imipramine CL from resting PMNs, with azide being the most effective.

Since imipramine CL was increased substantially upon zymosan activation of the PMNs, the effects of SOD and azide on the synergistic CL resulting from the simultaneous addition of zymosan and imipramine were examined (Table 5). Azide, in comparison to SOD, elicited a greater inhibition of the synergistic CL resulting from the simultaneous addition of zymosan and imipramine (89 vs 14%). These effects of SOD and azide on imipramine CL are similar to those recently observed with the CL from luminol, which has been attributed primarily to a myeloperoxidase-dependent reaction [32].

Table 4. CL resulting from the interaction of imipramine (1×10^{-4} M) with horseradish peroxidase (HRP) and H_2O_2

Reactants	Peak CL*
HRP, H_2O_2	80.0 ± 3.1
HRP, H_2O_2, catalase	3.0 ± 0.4
HRP, H_2O_2, imipramine	380.0 ± 36.0†
HRP, H_2O_2, imipramine, catalase	4.2 ± 0.7
Imipramine, H_2O_2	8.0 ± 0.4

* Peak counts/min × 10^{-3} ± S.E.M.; N = 4.

† $P < 0.01$ compared to HRP-H_2O_2. The concentration of HRP was 0.05 mg/ml in a total volume of 5 ml. The reaction was initiated by the addition of 10 μl H_2O_2 (30% solution, final concn. 17.6 mM). Where indicated, 100 μg/ml of catalase was added.

Effect of cytochalasin B incubation on imipramine CL with resting PMNs

Both the increase in CL and the shift in time at which peak synergistic CL occurred as a result of the simultaneous or sequential addition of imipramine to zymosan-activated cells suggest that some intracellular component which participated in the generation of the oxidant species that initiated the electronic excitation of imipramine was then extracellular. Incubation of PMN with cytochalasin B, an agent which enhances the release of lysosomal enzymes including myeloperoxidase, has been shown previously to increase the azide-inhibitable CL response of PMNs elicited by chemotaxic proteins [33]. Preincubation of resting PMNs with cytochalasin B (5 μg/ml) for 5 min prior to the addition of imipramine resulted in an enhancement of peak CL (from 1.9 to 3.5 counts/0.5 min × 10^{-5}) and a shift in the time that peak CL occurred (from 30 to 15 min).

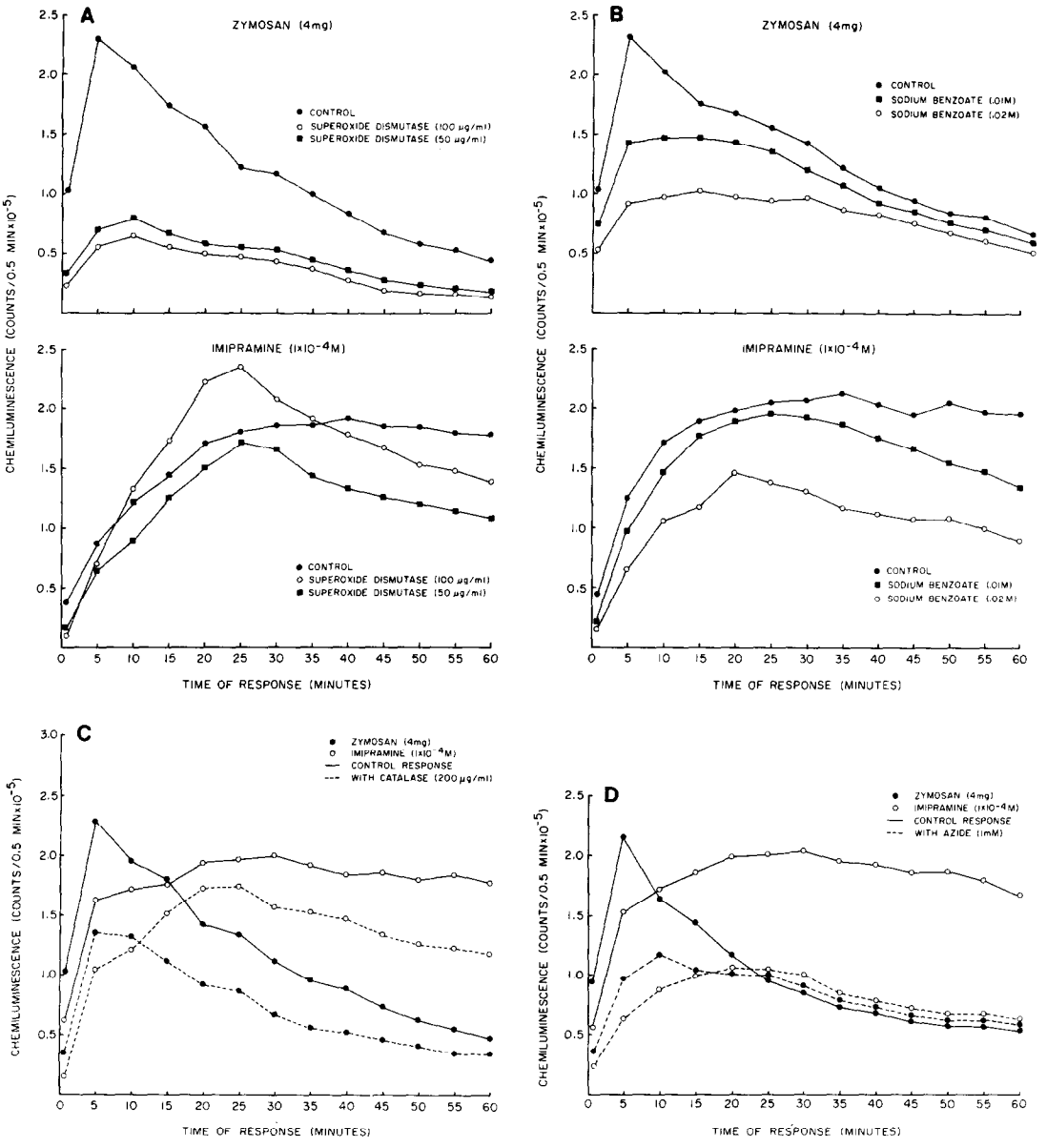

Fig. 2. Temporal response curves of the CL generated following the addition of opsonized zymosan or imipramine to 5×10^6 PMNs in the presence or absence of SOD (A), sodium benzoate (B), catalase (C) and azide (D). All agents were added simultaneously with zymosan or imipramine. Data are from a single experiment which is representative of four experiments.

Interaction of imipramine with alveolar macrophages: A cell-type deficient in peroxidase

Alveolar macrophages (AMs) release superoxide and hydrogen peroxide both at rest and following challenge by zymosan [20, 34]. Similarly, addition of zymosan elicits CL which is inhibitable by reactive oxygen scavengers, particularly SOD [20, 34]. Unlike PMNs, the CL response of AMs is neither inhibited by azide nor potentiated by cytochalasin B, an agent which increases the azide-inhibitable CL response of PMNs to chemotaxic proteins [33]. These effects of azide and cytochalasin B may be attributed to the lack of a peroxidase in AMs [35].

As illustrated in Fig. 3, the addition of imipramine to resting rat AMs failed to elicit CL, while a slight but significant increase in imipramine CL occurred following activation of the cells by zymosan. In contrast to the substantial increase in CL observed following sequential addition of imipramine to zymosan-activated PMNs (Table 1), sequential addition of imipramine to zymosan-activated AMs failed to elicit an increase in CL. Thus, the CL of human and rabbit PMNs and rabbit and rat AMs was monitored in response to imipramine, zymosan and their simultaneous addition to further examine the cellular specificity of this phenomenon (Table 6). CL from

Table 5. Effect of SOD or azide on the CL resulting from the simultaneous addition of zymosan (4 mg) and imipramine (1×10^{-4} M) to PMNs*

Addition to PMNs (5×10^6)	Peak CL response	Time of peak response (min)	Percent of control	P value
Zymosan (Z)	2.3 ± 0.1†	5	100	
Z + SOD (100 μg/ml)	0.9 ± 0.1	10	42	< 0.01
Imipramine (I)	1.9 ± 0.1	30–35	100	
I + SOD (100 μg/ml)	2.7 ± 1.5	20	131	< 0.01
Z + I	23.5 ± 3.7	5	100	
Z + I + SOD (100 μg/ml)	20.1 ± 2.6	5	86	< 0.05
Zymosan (Z)	2.1 ± 0.1	5	100	
Z + azide (1 mM)	1.2 ± 0.1	10	58	< 0.01
Imipramine (I)	2.1 ± 0.1	30	100	
I + azide (1 mM)	0.9 ± 0.1	25	45	< 0.01
Z + I	22.5 ± 1.1	5	100	
Z + I + azide (1 mM)	2.5 ± 0.1	5	11	< 0.001

* SOD and azide were added simultaneously with the zymosan and/or imipramine.
† Counts/0.5 min × 10^{-5} ± S.E.M.; N = 4.

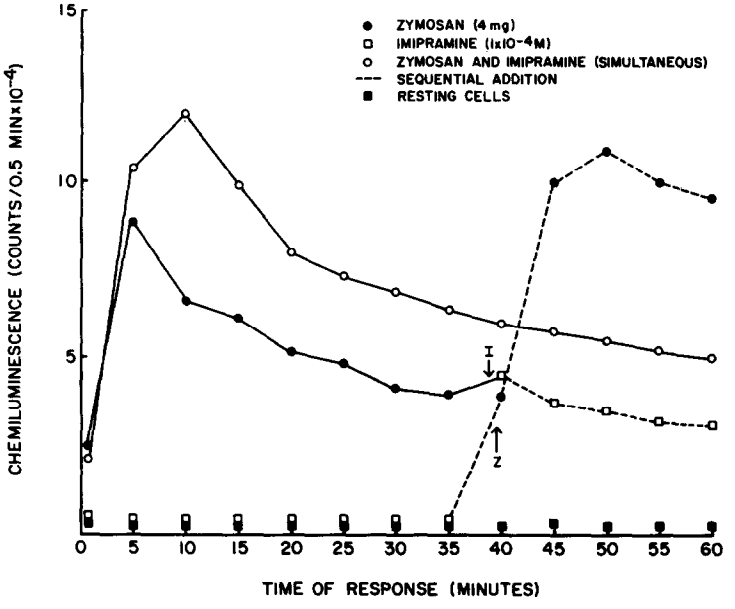

Fig. 3. Temporal response curves of the CL generated following the separate, simultaneous or sequential addition of opsonized zymosan or imipramine to 5×10^6 rat alveolar macrophages. Arrows indicate the sequential addition of imipramine (I) or zymosan (Z). Data are from a single experiment which is representative of four experiments.

Table 6. Comparison of the CL response of PMNs and AMs to zymosan (4 mg) and/or imipramine (1×10^{-4} M)

Cell type and cell number	Peak CL response to zymosan	Peak CL response to imipramine	Peak CL response to simultaneous addition of zymosan and imipramine
Human PMNs (5×10^6)	22.3 ± 0.9*	20.4 ± 0.5	222.5 ± 7.5†
Rabbit PMNs (2.5×10^6)	3.2 ± 0.3	15.6 ± 1.6	158.5 ± 12.9‡
Rabbit AMs (5×10^6)	4.5 ± 0.3	0.4 ± 0.0§	9.2 ± 1.2†
Rat AMs (5×10^6)	7.8 ± 0.4	0.0 ± 0.0§	11.2 ± 0.7†

* Counts/0.5 min × 10^{-4} ± S.E.M.; N = 4.
† Significantly different from zymosan response ($P < 0.01$).
‡ Significantly different from rabbit AM simultaneous response ($P < 0.01$).
§ Increasing the AM concentration to 1×10^7 yielded comparable results.

Table 7. Effect of horseradish peroxidase (HRP) on the CL resulting from the addition of zymosan and/or imipramine to rat alveolar macrophages (AMs)*

Initial addition to AMS (5×10^6)	Peak CL	Time of peak response (min)	Peak CL resulting from addition of the indicated sequential reactant†	
			Imipramine	Zymosan
HRP	0.7 ± 0.1‡			
Zymosan (Z)	5.0 ± 0.4	5	0.8 ± 0.1	
Z + HRP	13.2 ± 0.5§	5	190.0 ± 19.0§	
Imipramine (I)	0.3 ± 0.2			16.5 ± 3.7
I + HRP	31.8 ± 0.5‖	40		270.0 ± 22.0
Z + I	11.7 ± 0.9§	10		
Z + I + HRP	238.0 ± 30.0¶	5		

* The concentration of HRP was 0.02 mg/ml; zymosan, 4 mg; and imipramine, 1×10^{-4} M.
† Sequential reactants were added 40 min after initial additions to AMs.
‡ Counts/0.5 min $\times 10^{-4}$ ± S.E.M.; N = 3.
§ $P < 0.01$ compared to Z only.
‖ $P < 0.01$ compared to I only.
¶ $P < 0.01$ compared to the simultaneous addition of zymosan and imipramine.

imipramine was observed with resting PMNs but not resting AMs in spite of the fact that release of superoxide by resting rat AMs is similar to that of resting human PMNs [20]. While an increase in CL was observed from both cell types following the simultaneous addition of zymosan and imipramine, this response was 15- to 25-fold greater with PMNs while it was only 2-fold greater with AMs in comparison to the zymosan response.

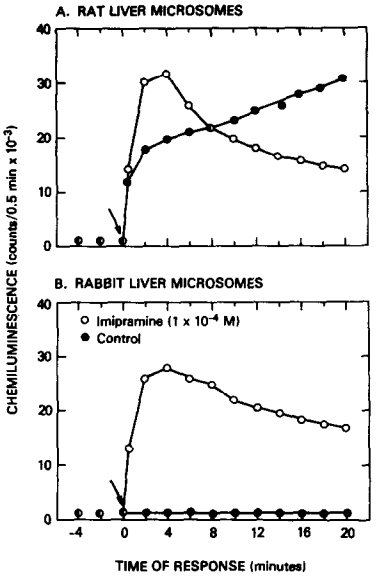

Fig. 4. Temporal response curve of the CL generated as the result of the interaction of imipramine with rat (A) or rabbit (B) liver microsomes (0.5 mg microsomal protein/ml). Arrows indicate addition of the NADPH-generating system. Analysis of the reaction mixtures for lipid peroxidation indicated the generation of 4.6 nmoles of malondialdehyde/mg protein during this 20-min incubation with control rat liver microsomes. In the presence of imipramine with either rat or rabbit liver microsomes or control rabbit liver microsomes, 1–2 nmoles of malondialdehyde per mg proetin per 20 min was measured. Each point is the mean of three separate experiments.

The quantitative difference in response between PMNs and AMs, both resting and zymosan-activated, and the significant inhibition by azide of imipramine CL from PMNs suggest that the oxidant species which interacts with imipramine may be peroxidase-derived. If so, then supplementation of AMs with a peroxidase should result in imipramine CL. Although the peroxidase present in PMNs is myeloperoxidase, Odajima and Yamazaki [36] have demonstrated that myeloperoxidase and horseradish peroxidase have similar reaction mechanisms. Thus, the effect of adding exogenous horseradish peroxidase (HRP) to rat AMs was examined (Table 7). When HRP was present, the addition of imipramine to resting AMs now resulted in significant CL. Moreover, sequential addition of imipramine to zymosan-activated HRP-supplemented AMs resulted in a 200-fold increase in CL relative to the response of control AMs with imipramine. The addition of HRP also increased the zymosan-induced CL and the simultaneous zymosan–imipramine CL. Interestingly, these responses with HRP-supplemented AMs were quantitatively and temporally similar to those observed with imipramine and resting or zymosan-activated PMNs. Addition of catalase significantly inhibited the enhancing effect of HRP supplementation with AMs (data not shown), demonstrating that resting AMs release sufficient hydrogen peroxide to serve as a cofactor for HRP.

Interaction of imipramine with rat or rabbit liver microsomes

While this study has focused on the oxidation of imipramine to an electronic excitation state by an oxidant species generated by PMNs, such a response need not be viewed as being limited to PMNs. For example, the microsomal mixed-function oxidase system generates a number of oxidant species including superoxide, hydrogen peroxide, the hydroxyl radical and possibly singlet oxygen [37]. Moreover, the interaction of imipramine with this enzyme system results in a number of metabolites of imipramine [38], one of which is of a reactive nature so as to covalently bind to protein [39]. Although not of

the magnitude observed with PMNs, Fig. 4 clearly illustrates that CL results from the interaction of imipramine with liver microsomes isolated from rat or rabbit. This CL response by imipramine was quantitatively and temporally similar with both microsomal preparations. No CL was observed in the absence of the pyridine co-factor NADPH or if heat-inactivated microsomes were used (data not shown). In the absence of imipramine, rat liver microsomes generated CL which was accompanied by the formation of malondialdehyde (MDA), indicating that this CL was associated with microsomal lipid peroxidation [6, 26, 27]; however, no MDA was measured in the presence of imipramine or with control rabbit liver microsomes, which are relatively resistant to lipid peroxidation in comparison to rat liver microsomes [40].

DISCUSSION

During investigations using CL to assess the interaction of drugs with PMNs [41, 42], we observed that addition of the tricyclic antidepressant imipramine to human PMNs resulted in CL [11]. Subsequent structure–activity studies revealed that, in addition to imipramine, desipramine, opipramol and iprindole yield CL from this interaction [12]. Unresolved, however, was the origin of this CL. Chemiluminescence can be elicited from PMNs as a result of the metabolic activation of these cells by soluble agents such as phorbol myristate acetate and the calcium ionophore A23187 [24, 25]. Alternatively, the reactive oxygen species produced by both resting and particle-activated PMNs can oxidize chemicals yielding a product in an electronically excited state, as exemplified by luminol [13, 14]. The results of the present study indicate that of these two possibilities the CL observed following the interaction of imipramine with PMNs can be attributed to the oxidation of the imipramine molecule rather than to the stimulation of PMN metabolism. This conclusion is based on two significant lines of evidence which are consistent with the behavior of an electronically excitable substrate: (1) imipramine did not enhance superoxide release from resting PMNs; and (2) it did yield significant CL with cell-free oxidant-generating enzyme systems. Further support of this conclusion is provided by the significant amplification in CL by metabolically activating the PMNs.

Resting PMNs have been shown to generate measurable quantities of superoxide anion, hydrogen peroxide and hydroxyl radical, while activation of the respiratory burst results in an increased rate in the generation of these molecular oxygen-derived oxidant species. In addition, the utilization of hydrogen peroxide, and possibly hydroperoxides [43], by myeloperoxidase results in the formation of hypochlorous acid, singlet oxygen and possibly the hydroxyl radical [43–45]. The data from the inhibitor studies and the experiments with alveolar macrophages indicate that the primary, but not necessarily the only, oxidant species which brings about the oxidation of imipramine by PMNs is a product of a peroxidase-catalyzed reaction, although the identity of this oxidant species is presently unclear. Based on the results of similar inhibitor studies combined with the lack of CL from luminol with MPO-deficient PMNs, DeChatelet *et al.* [32] have concluded recently that the oxidation of luminol is absolutely dependent upon an MPO-mediated reaction. Thus, both luminol and imipramine may be susceptible to oxidation by the same peroxidase-derived oxidant species; however, the resulting excited state intermediate does not have to be identical. In fact, the previous studies with imipramine and PMNs suggested the possibility of an epoxide intermediate [12].

While a primary goal of the present research was to define the origin of the CL from the imipramine–PMN interaction, the pharmacological significance of demonstrating that imipramine is an electronically excitable substrate may reside in the observation that this reaction can be catalyzed by liver microsomes. This observation was not surprising since the microsomal mixed-function oxidase system does generate oxidant species similar to those of PMNs, and "activated oxygen" appears to play a prominent role in the microsomal metabolism of xenobiotics [37]. Moreover, a series of papers by Seliger and coworkers [10, 46–48] have demonstrated that CL accompanies the microsomal metabolism of benzo(*a*)pyrene and that this CL is associated with the BP-7,8-diol metabolite, the precursor to the ultimate carcinogenic BP metabolite BP-7,8-diol-9,10-epoxide. Based on the specificity of a singlet oxygen-initiated CL from BP-7,8-diol, these authors have proposed that CL may be a sensitive method for measuring small quantities of BP-7,8-diol [10]. Likewise, CL may be a useful procedure for monitoring the mixed-function oxidase catalyzed metabolism of imipramine along its toxic pathway since the reactive intermediate that covalently binds to protein is believed to be an epoxide [39]. Moreover, such studies may provide further insight into the actual nature of the reactive intermediate of imipramine.

Although these studies focused on whether imipramine was an electronically excitable substrate, a conceptual extension of these studies is that the reactive oxygen-dependent metabolic activation of xenobiotics by PMNs may be of pharmacologic and toxicologic importance. For example, the interaction of benzo[*a*]pyrene-7,8-diol with PMNs could result in CL and the generation of mutagenic metabolites similar to its interaction with microsomes. Similarly, the myeloperoxidase-catalyzed activation of 9-hydroxyellipticine results in an intermediate which is cytotoxic to L1210 leukemia cells [49]. This intermediate could be in an electronically excited state and, as such, alter target molecules. The experiments described in this manuscript provide a format upon which to examine such questions.

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