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REGULATION BY SERINE ESTERASE OF HISTAMINE RELEASE FROM HUMAN LEUKOCYTES—I

DIRECT RELEASE OF HISTAMINE BY THE SERINE ESTERASE INHIBITORS DIISOPROPYL FLUOROPHOSPHATE (DFP) AND SOMAN (GD)

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Abstract—The serine esterase inhibitor diisopropyl fluorophosphate (DFP) had been reported previously to inhibit IgE-dependent histamine release. Recently, it has been demonstrated that lower concentrations of DFP enhance IgE-dependent histamine release and inhibit desensitization. This manuscript describes the abilities of several esterase inhibitors to cause release of histamine from human leukocytes (basophils), by a process that is IgE-independent. This esterase inhibitor-induced histamine release appears to be by a non-cytotoxic mechanism that requires calcium and is temperature dependent. These histamine release processes occurred over a longer period of time than IgE-dependent release. Direct release of histamine by these small molecular weight inhibitors and inhibition of desensitization both suggest that one or more serine esterases are involved in the regulation of histamine release from human basophils.

After Becker [1] demonstrated that diisopropyl fluorophosphate (DFP†) could be used to study complement activity, Austen and Brocklehurst [2] showed that DFP, when it is present at the time of challenge, completely inhibits the antigen-dependent release of histamine from chopped guinea pig lung. This effect of DFP was studied in rat mast cells and human lung fragments [3]. These findings led to the conclusion that release of mediators from these tissues requires activation of an esterase inhibitable by DFP. The work of Katz and Cohen [4] demonstrated the release of histamine when the appropriate antigen is added to human blood. Many investigators have used this model to study the mechanism of IgE-dependent histamine release [5–7]. When human leukocytes are incubated in the presence of 5×10^{-4} M or higher concentrations of DFP while being challenged, or prior to challenge, with antigen, their ability to release histamine is inhibited [5]. Histamine secretion by human basophils appears to require participation of a DFP-inhibitable esterase. However, the present results indicate that the high concentrations of DFP, which inhibited IgE-dependent histamine release in these earlier studies, caused a decrease in NAD^+, which would result in a marked change in cellular metabolism.

Further investigation of the mechanism of histamine release showed that this cellular response could be divided into two types: (a) sensitization of the cell to the appropriate signal—resulting in activation of the histamine secretory mechanism, and (b) desensitization of the cell to the same or all similar signals—resulting in termination of the secretory process [6]. Recent work, using DFP to dissect these two cellular processes, has demonstrated that desensitization is inhibited by lower concentrations of DFP (10^{-4} to 5×10^{-4} M) than used in the above investigations [7]. The inhibition of desensitization of the basophils resulted in enhancement of histamine release, suggesting that there was a DFP-inhibitable esterase that regulates the release of basophil mediators.

Until now, DFP appeared to have a dual role in antigen-dependent histamine release processes: inhibiting IgE-dependent release at concentrations of 5×10^{-3} M or higher, and enhancing IgE-dependent release at concentrations between 10^{-4} and 5×10^{-4} M. The present work indicates that DFP and other small molecular esterase inhibitors (e.g. soman) also bring about an antigen-independent histamine release. When mixed leukocytes were incubated in the presence of DFP or soman, they spontaneously released histamine.

METHODS

Materials. The following chemicals were purchased: diisopropyl fluorophosphate (DFP) (Aldrich Chemical Co., Inc., Milwaukee, WI); Tris

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† Abbreviations: DFP, diisopropyl fluorophosphate; GD, soman = pinacolyl methylphosphonofluoridate; NAD^+, nicotinamide adenine dinucleotide; LDH, lactate dehydrogenase; AgE, ragweed antigen E; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; and HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

base, potassium thiocyanate, EDTA, monobasic and dibasic sodium phosphate, sodium metabisulfite, sodium bicarbonate, dextrose, and sodium chloride (Fisher Scientific Co., Pittsburgh, PA); and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT tetrazolium), phenazine ethosulfate, alcohol dehydrogenase Cat. No. A1762, physostigmine, pyridostigmine bromide, sodium bicine, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), ethanol, bovine serum albumin, and LDH assay kit 500 (Sigma Chemical Co., St. Louis, MO). A Corning pH meter, a Yellow Springs conductivity meter, and a Beckman DU-8 spectrophotometer were used to determine pH, conductivity, and absorbance respectively. Soman (pinacolyl methylphosphonofluoridate) was obtained from the Chemical Research and Development Center, Aberdeen Proving Ground, MD, and was assayed weekly by both nuclear magnetic resonance and gas–liquid chromatography to ensure that purity was greater than 95%. Ragweed antigen E was provided by Dr. Donald MacGlashan, The Johns Hopkins School of Medicine, Baltimore, MD.

Preparation of inhibitors and analogs. Neat soman obtained from the Chemical Research and Development Center was diluted in 0.9% saline to a concentration of 10^{-2} M by the USAMRICD Analytical Chemistry Branch. The diluted soman was then divided into aliquots and frozen at −70° until use. Tyrode's buffer (4 g NaCl, 0.1 g KCl, 0.025 g NaH_2PO_4, 0.2 g $CaCl_2$, 0.1 g $MgCl_2$, 1.0 g $NaHCO_3$ and 1.0 g dextrose per liter of double-distilled H_2O [8]) plus 0.003% albumin and 10 mM HEPES, pH adjusted to 7.4, was used for all dilutions and as the incubation buffer, unless otherwise specified. The diluted soman (2 mg/ml saline) was thawed at room temperature in a fume hood and handled under USAMRICD regulations for chemical surety materiel.

Hydrolyzed soman was prepared by incubation of 1 ml of 10^{-2} M soman with 10 μl of 1 N NaOH for 30 min at 37° followed by addition of 8.8 ml of Tyrode's buffer containing 0.003% albumin and 20 mM HEPES. DFP was diluted in Tyrode's buffer containing albumin and HEPES. Hydrolyzed DFP was produced by incubating 1 ml of 10^{-1} M DFP with 200 μl of 2 N NaOH for 30 min at 37° before adding 8.8 ml of Tyrode's buffer.

Histamine release. Histamine release was investigated on dextran-EDTA isolated human leukocytes [6]. Dextran-EDTA sedimented cells were washed twice with Tyrode's buffer without $CaCl_2$ and $MgCl_2$ except where calcium dependence was studied. In the experiments designed to measure the calcium requirement, leukocytes were washed twice in Tyrode's buffer without $CaCl_2$ and $MgCl_2$ and containing 5×10^{-3} M EDTA before being suspended in Tyrode's buffer without $CaCl_2$. After washing, cells were resuspended in Tyrode's buffer and incubated with the agents of interest for 60 min at 37° unless otherwise indicated. After incubation, cells were centrifuged at 14,000 *g* for 1 min. The histamine content of 0.8 ml of the supernatant fraction was acidified with 0.2 ml of 10% perchloric acid and determined by the automated fluorometric method [9] in Dr. Lichtenstein's laboratory (The Johns Hopkins School of Medicine, Baltimore, MD). Each data point represents a single histamine determination based on the results of three separate samples. All experiments presented in this paper have been reproduced on at least three separate occasions.

Nicotinamide adenine dinucleotide (NAD^+) determinations. The cell pellet and the remaining 0.2 ml of supernatant fraction were extracted overnight with 0.8 ml of 0.5 N perchloric acid and then neutralized with 0.2 ml of 2.0 M KOH–0.66 M potassium phosphate, pH 7.8. After removal of the $KClO_4$ precipitate by centrifugation at 14,000 *g* for 2 min, the NAD^+ content was determined by an alcohol dehydrogenase cycling assay using 0.5 ml of leukocyte extract or appropriate NAD^+ standards [10].

Lactate dehydrogenase (LDH) determinations. LDH determinations were performed by use of the Sigma reagent kit [11].

Statistical analysis. Relative risk (RR) was calculated using the formula

$$RR = (n_{11}/n_1)/(n_{21}/n_2)$$

RESULTS

DFP-dependent histamine release. The leukocytes of six atopic (three with allergic asthma) and five normal volunteers were incubated with various concentrations of DFP. Leukocytes from all eleven individuals released histamine when exposed to DFP concentrations between 5×10^{-4} and 4×10^{-3} M (Fig. 1). Leukocytes from both allergic and nonallergic individuals exhibited maximum histamine release at 5×10^{-4} to 10^{-3} M DFP and inhibition of release occurred at 3×10^{-3} M DFP. Two individuals

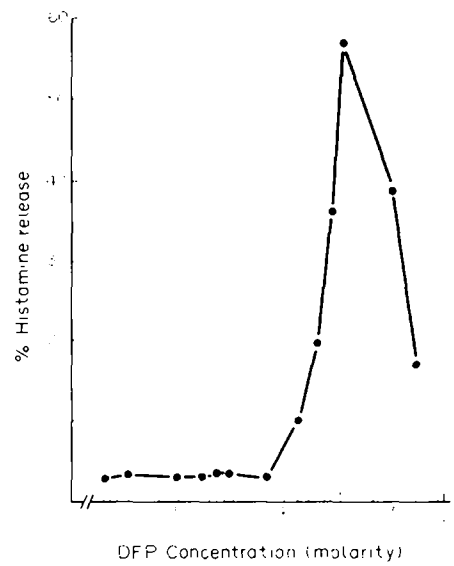

Fig. 1. DFP-induced histamine release from mixed human leukocytes. Leukocytes derived from 2 ml of blood were incubated with the indicated concentration of agent for 60 min at 37°. Each point is the average of three separate determinations and is expressed as a percentage of total histamine. Control tubes released 3% of their histamine which was not subtracted from the results obtained for the DFP-treated cells. Total histamine per sample was 36 ng/ml.

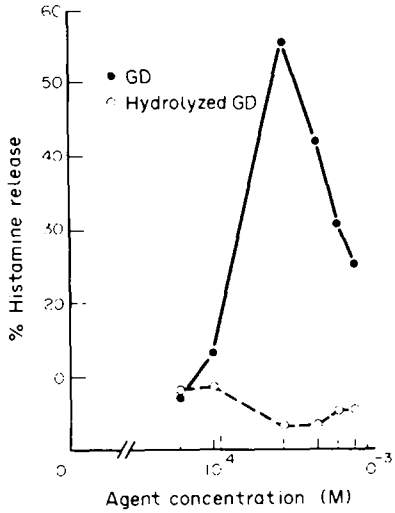

Fig. 2. Soman-induced histamine release from mixed human leukocytes. Leukocytes derived from 2 ml of blood were incubated with the indicated concentration of agent for 60 min at 37°. Each point is the average of three separate determinations. Histamine release is expressed as the percentage of total histamine with control cells releasing 6% of their total histamine (37.9 ng/ml). The control histamine release value was not subtracted from the results obtained for soman-treated cells.

with allergic asthma released 75 and 86% of their cellular histamine, compared to the other nine individuals whose histamine release ranged from 39 to 60%.

Soman-dependent histamine release. Other types of small molecular weight serine esterase inhibitors were examined to determine their abilities to release histamine from human leukocytes. An organophosphonate, soman, and two carbamates, physostigmine and pyridostigmine, were studied to measure their abilities to cause histamine release from human leukocytes. Soman (Fig. 2) caused a histamine release from human leukocytes similar to that of DFP. Soman was the most potent histamine releaser of the esterase inhibitors investigated. It caused histamine release at concentrations as low as 9×10^{-5} M and showed maximum histamine release at 3×10^{-4} M. Histamine release decreased progressively at concentrations of soman exceeding 4×10^{-4} M. Thirty-four individuals (both allergic and non-allergic) were screened to determine whether their leukocytes release histamine when exposed to soman and how this release compares to histamine release when exposed to anti-IgE (Table 1). Only one individual failed to release a significant amount of histamine. It appears that the ability of an individual's leukocytes to release histamine when incubated with soman is related to their anti-IgE stimulated histamine release. An individual who releases less than 25% of their total histamine to anti-IgE has a relative risk of 5.5 that they will release less than 25% of their leukocyte histamine to soman. An individual whose leukocytes release more than 25% of their total histamine to anti-IgE has a relative risk of 1.85 that this individual's leucocytes will release greater than 25% of their histamine to soman. Although the concentration of soman that caused maximal histamine release appeared constant for most individuals, the percent histamine release among individuals varied from 0 to 87% of the total cellular histamine content. The leukocytes from three of the individuals who were relatively good histamine releasers to soman (60–87% total histamine release) were challenged on three separate occasions and released approximately the same level of histamine (within 8%). However, soman that was hydrolyzed (as measured by a fluoride specific electrode) and possessed no esterase inhibitory activity failed to cause any histamine release (Fig. 2).

Physostigmine, a relatively weak reversible neutral esterase inhibitor, and pyridostigmine, a relatively weak reversible charged esterase inhibitor, both carbamates failed to release histamine at concentrations as high as 10^{-2} M. Figure 3 compares the abilities of

Table 1. Relationship between the histamine release induced by anti-IgE and soman

		Histamine release to anti-IgE		
		<25%	25–50%	>50%
Histamine Release To soman	<25%	6	3	3
	25–50%	1	5	4
	>50%	1	7	4

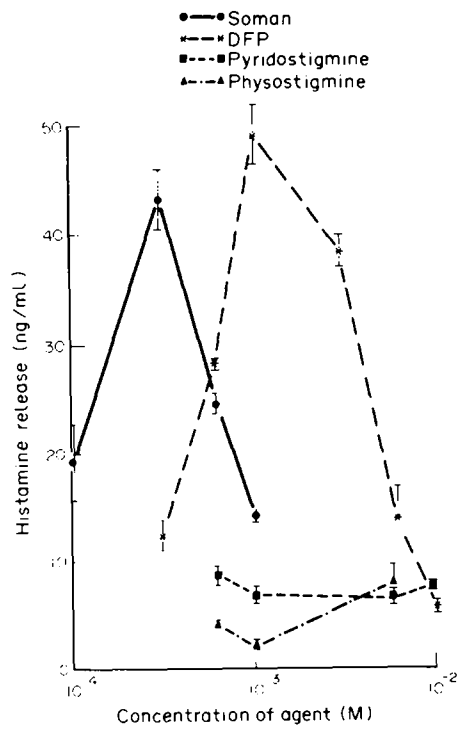

Fig. 3. Comparative abilities of DFP, soman, physostigmine and pyridostigmine to induce histamine release from mixed human leukocytes obtained from a single individual. Leukocytes derived from 2 ml of blood were incubated with the indicated concentrations of agents for 60 min at 37°. Each point is the average of three separate determinations (bars represent standard deviations). Control release of histamine was 6.70 ± 1.71 ng/ml out of a total of 86.4 ng/ml. The control histamine release value was not subtracted from the results obtained for soman-treated cells.

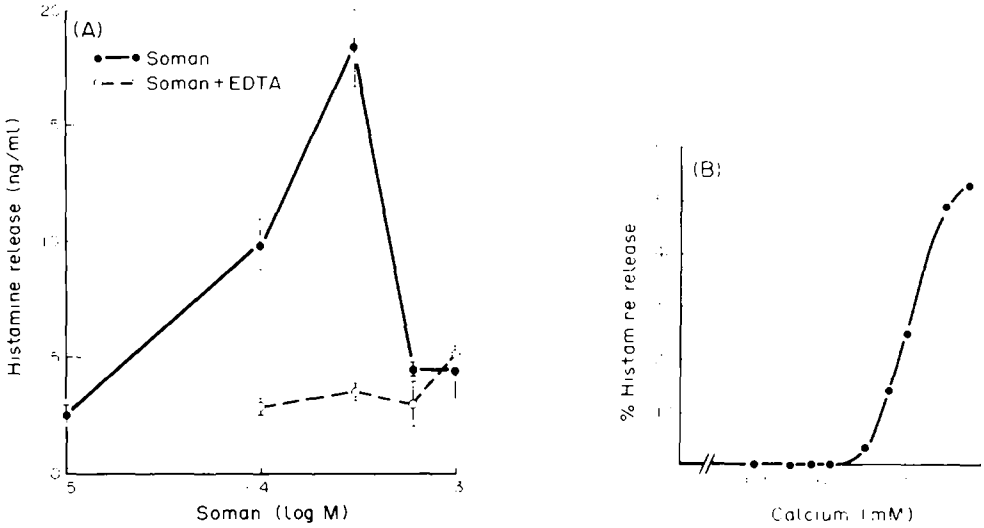

Fig. 4. (A) EDTA inhibition of histamine release induced by soman. Leukocytes derived from 2 ml of blood were incubated with the indicated concentration of agent in the presence of absence of 2×10^{-3} M EDTA for 60 min at 37°. Each point is the average of three separate determinations (bars represent standard deviations). Control histamine release was 2.5% of the total histamine (43.2 ng/ml). The control histamine release value was not subtracted from the results obtained for the soman-treated cells. (B) Calcium dependence of soman-initiated histamine release. Dependence was established by washing dextran-EDTA sedimented leukocytes twice in Tyrode's buffer containing EDTA to remove all available calcium. Cells were then added to the incubation mixture containing Tyrode's buffer without $CaCl_2$. The indicated calcium concentrations were added to the incubation mixture, and the ability of 3×10^{-4} M soman to release histamine was determined. The control histamine release value was not subtracted from the results obtained for the soman-treated cells. Total histamine per tube was 40.4 ng/ml.

these four esterase inhibitors (soman, DFP, physostigmine, and pyridostigmine) to release histamine from leukocytes derived from the same individual. Leukocytes were more susceptible to soman-induced histamine release (maximal at 3×10^{-4}) than to DFP-induced release (maximal at 5×10^{-4} to 3×10^{-3} M) but appeared to be totally insensitive to the two carbamates, pyridostigmine and physostigmine.

Because all of these compounds are potent inhibitors of acetylcholinesterase, carbachol, a non-hydrolyzable acetylcholinesterase agonist, and atropine, an antagonist of the muscarinic acetylcholine receptor, were examined for their abilities to release histamine. Neither carbachol nor atropine caused histamine release at concentrations between 10^{-7} and 10^{-2} M.

Histamine release by esterase inhibitors by a secretory mechanism. Four criteria were used to establish that the observed histamine release by esterase inhibitors was a secretory process and not the result of cell lysis: (1) lack of release of cellular constituents, (2) inhibition of histamine release by excess esterase inhibitor, (3) calcium dependence of the inhibitor-dependent release, and (4) temperature dependence of histamine release. Supernatant fractions from the histamine release mixture were assayed not only for histamine, but also for LDH and NAD^+. None of the esterase inhibitors caused release of the cytoplasmic enzyme, LDH, or of the nuclear constituent, NAD^+, into the supernatant fraction (data not shown). Soman, at concentrations higher than 2×10^{-4} (Fig. 2), and DFP, at concentrations higher than 2×10^{-3} M (Fig. 1), induced less histamine release than lower concentrations which caused peak histamine release. At concentrations higher than 10^{-2} M of both soman and DFP, the agents even inhibited spontaneous histamine release (data not shown). Histamine release by soman was inhibited by 2×10^{-3} M EDTA, indicating that bivalent cation (calcium) is required for histamine release (Fig. 4A). EDTA completely inhibited the soman-induced histamine release even at the optimal releasing concentration of soman. This inhibition of EDTA of soman's ability to release histamine extended to concentrations as high as 10^{-2} M. To corroborate that calcium was the important cation, leukocytes were incubated with soman in the presence of various concentrations of calcium (Fig. 4B). Soman did not begin to initiate histamine release until the calcium concentration reached 3×10^{-4} M. DFP-induced histamine release was also shown to be calcium dependent. The release of histamine by esterase inhibitors displayed a sharp temperature dependence. Figure 5 shows that soman, the most potent histamine releaser, failed to release histamine at 0, 22, and 46°, but was effective at 37°, releasing 40% more histamine than did untreated controls. Also noted was the inhibition by soman of the spontaneous histamine release which occurred at 46°. Thus, histamine release induced by soman, the potent esterase inhibitor, appears to be a physiological response as judged by the four above criteria.

Kinetics of histamine release by esterase inhibitors. Human leukocytes were exposed to buffer containing either ragweed antigen E or soman for 0, 1, 2, 5, 10, 15, 30, 60, 120, and 240 min to study the time course

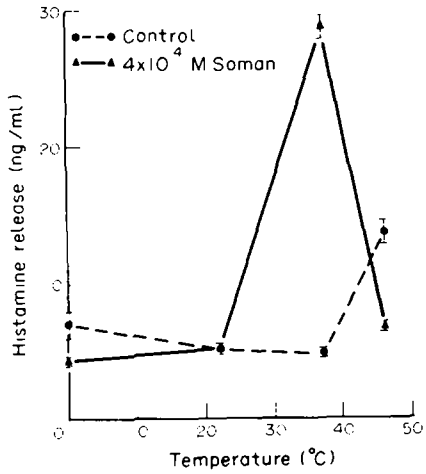

Fig. 5. Temperature dependence of soman-induced histamine release from human leukocytes. Leukocytes derived from 2 ml of blood were incubated with 4×10^{-4} M soman for 60 min at the indicated temperatures. Each point is the average of three separate determinations (bars represent standard deviations). The total histamine per sample was 83.7 ng/ml, and the values for histamine released by soman were calculated independently of controls.

of histamine release. Figure 6 illustrates some of the soman results which show a relatively slow release of histamine (compared to that of ragweed antigen E [6]). This was typical of all the esterase inhibitors studied. The release of histamine did not appear to level off until 60 min. Although histamine release by soman was slower than it was by ragweed antigen E, soman released the same amounts as, or more than, antigen E.

Concomitant inhibition of histamine release and cellular metabolism. As expected, these potent esterase inhibitors appear to have multiple effects on leukocytes. At the concentrations of esterase inhibitors where both IgE-dependent and esterase-dependent histamine release were inhibited, there were parallel, dose-dependent decreases in NAD^+ (Fig. 7). The steep decrease in NAD^+ levels indicates that, at these high concentrations of soman, there was an inhibition of metabolism which could result in the depletion of energy required for histamine secretion. The decrease in NAD^+ appears to parallel the decrease in the histamine release at concentrations higher than 2×10^{-4} M soman. DFP showed a similar relationship of decrease in histamine release with a decrease in NAD^+ levels. DFP began to cause histamine release at 5×10^{-4} M and reached its peak release at 10^{-3} M. The NAD^+ levels of leukocytes did not begin to decrease until 3×10^{-3} M, where both histamine release and NAD^+ levels dropped rapidly.

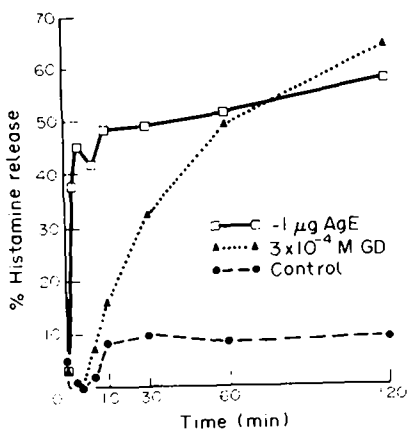

Fig. 6. Kinetics of soman-induced histamine release from human leukocytes. Leukocytes derived from 2 ml of blood were incubated with buffer containing either 3×10^{-4} M soman or 0.1 μg/ml ragweed antigen E (AgE) for the indicated times at 37°. Each point is the average of three separate determinations. Control release was the leakage of histamine with the time of incubation. The total histamine per sample was 46 ng/ml. The control histamine release value was not subtracted from the results obtained for the soman- or AgE-treated cells.

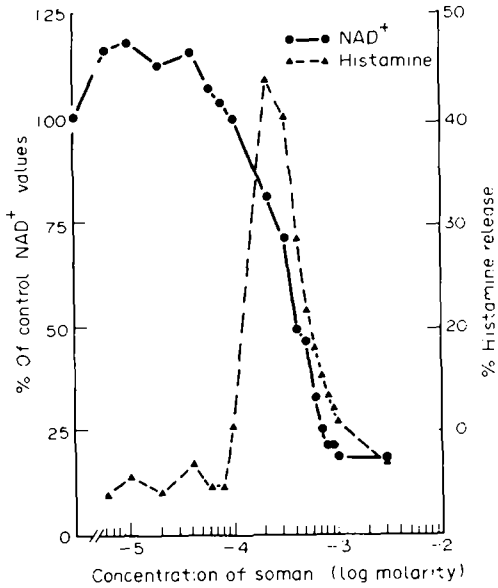

Fig. 7. Comparative effects of soman on leukocyte NAD^+ levels and histamine release. Leukocytes derived from 2 ml of blood were incubated with the indicated concentration of soman for 60 min at 37°. Each point is the average of three separate determinations for both histamine release and NAD^+. Percent histamine release was calculated independently of control histamine release which was 4.5%. NAD^+ level in control cells was 245 pmoles/ml and total histamine level was 56 mg/ml.

DISCUSSION

The small molecular weight serine esterase inhibitors have been shown to inhibit IgE-dependent histamine release (e.g. 5×10^{-3} M DFP) from many cell types [2–5]. These findings resulted in the formulation of a mechanism requiring the presence of an active serine esterase for secretion of histamine. DFP has also been shown to inhibit desensitization, resulting in enhancement of IgE-dependent histamine release ($<5 \times 10^{-4}$ M DFP) [7]. The present work demonstrates a third role of the esterase inhibitors in the release of histamine and offers an alterna-

tive explanation for inhibition of histamine release by esterase inhibitors at high concentrations as reported in the literature [3]. An alternative reason for inhibition of histamine release by high concentrations of these organophosphate serine esterase inhibitors may be that these compounds directly interfere with cellular metabolism or inhibit an enzyme required for energy production. This study also shows that these esterase inhibitors are potent histamine releasing agents. Not only was DFP, an organophosphate, a potent releaser, but soman, an organophosphonate, was also effective at liberating histamine from human leukocytes. Since soman was most effective at causing histamine release and is a more potent esterase inhibitor than DFP, it was studied as an example of this class of compounds to demonstrate that histamine release occurs by a physiological mechanism. Soman did not cause the release of either LDH or NAD^+ from leukocytes, indicating that leukocytes were not undergoing generalized cell lysis. Since basophils compose such a small percentage of the human leukocyte population, preferential lysis of basophils could not be ruled out. Therefore, other criteria had to be employed to study the release mechanism: cellular NAD^+ levels, temperature and calcium dependence, and high dose inhibition. Our data indicate that there was no decrease in cellular levels of NAD^+ until the concentration of inhibitor exceeded the concentration required for maximum histamine release (Fig. 7). However, preferential basophil lysis would not be expected to exhibit such a sharp temperature optimum as shown for inhibitor-induced histamine release (37°). Furthermore, lysis-induced release would be expected to be greater at 46°, whereas the results show that the soman-induced histamine release was, in fact, inhibited. These esterase inhibitors also exhibited a calcium dependence similar to that of other physiologic histamine-releasing agents [6]. The most important criterion considered to eliminate nonspecific cell lysis was inhibition of histamine release by an excess concentration of esterase inhibitors. The excess inhibition is not consistent with the explanation that lower releasing concentrations of esterase inhibitor would enhance lysis. However, it should be noted that high concentrations of esterase inhibitors also caused a decrease of cellular NAD^+ levels, in parallel with inhibition of histamine release (Fig. 7). All of these results are consistent with the notion that the esterase-inhibitor-induced histamine release is physiological in nature.

These esterase inhibitors appear to have multiple effects, since they enhanced, initiated, or inhibited histamine release, depending on their concentration. At concentrations where they inhibited histamine release, they also caused a depletion of leukocyte cellular NAD^+ to less than 50% of control levels. This observation indicates that these high levels of inhibitors cause profound metabolic effects [12]. It appears that these small molecular weight esterase inhibitors inhibit two or more esterases. One of the esterases appears to involve a controlling mechanism concerned with histamine release. By inhibiting this regulatory esterase, the desensitization process is inhibited. The present study also demonstrates that slightly higher concentrations of inhibitor can, in fact, induce direct histamine release. These inhibitors appear to affect another esterase which regulates histamine release. This esterase does not appear to require prior activation (unless the basophil enzyme is activated during the isolation process [13]). As a result of these studies, our conception of the mechanism of regulation of histamine release will have to be modified to include an esterase whose activity appears to control the amount of histamine which a cell will release.

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