PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 147, 681-686 (1974)

Effects of Serotonin on Platelets of Normal and *B. pertussis*-Injected Mice[1] (38416)

T. W. KLEIN, A. SZENTIVANYI, AND C. W. FISHEL

Departments of Medical Microbiology and Pharmacology, College of Medicine, University of South Florida, Tampa, Florida 33620

The addition of adenosine diphosphate (ADP) to stirred suspensions of platelets from most mammalian species induces an aggregation of these blood components (1). A similar phenomenon has been noted to occur following addition of catecholamines or 5-hydroxytryptamine (serotonin, 5-HT), although this response is much more dependent upon the species from which the platelets were obtained. For example, epinephrine (E) induces aggregation of human platelets (2) but not those derived from the rat, rabbit, guinea pig, horse, or dog (3). Aggregation responses to 5-HT have been reported for human platelets (4) as well as for a variety of other mammalian species (5). In addition, 5-HT appears to play a role in the aggregation of nucleated avian thrombocytes (6).

Mouse platelets *in vitro* also aggregate in the presence of ADP with enhancement of the response by catecholamines which, by themselves, did not induce the reaction (7). Effects of 5-HT on platelets of this species have not, to our knowledge, been examined and there are several reasons for believing such studies should be undertaken. The platelet is recognized as a storage site of the amine (1), 5-HT seems to be at least one of the mediators of allergic reactions in this species (8), the mouse is extremely susceptible to low doses of 5-HT when combined with histamine (9, 10), and this animal becomes very sensitive to 5-HT following the injection of *Bordetella pertussis* organisms (11). Therefore, the present studies were designed to determine platelet and 5-HT interaction in terms of aggregation, amine uptake and possible release during aggregation, and the influence of *B. pertussis* on the interaction.

Materials and Methods. Mice. Female DUB/ICR mice, weighing 18–20 g, were obtained from a single commercial source (Flow Research Animals, Inc., Dublin, VA). They were housed in groups of 20 animals per cage and maintained on pellets and water *ad libitum*. Five days after the injection of *B. pertussis* vaccine, the histamine LD_{50} was 0.72 mg base compared to 12.3 mg for noninjected members of this strain.

Chemicals. All chemicals and drugs were obtained from commercial sources: l-epinephrine bitartrate, l-arterenol bitartrate (NE), l-isoproterenol-D-bitartrate (ISOP), adenosine 5′-diphosphate, DL-propranolol HCl from Sigma Chemical Co., St. Louis, MO; histamine diphosphate, 5-hydroxytryptamine creatinine sulfate from Nutritional Biochemical Corp., Cleveland, OH; phenotolamine mesylate (Regitine) from Ciba, Summit, NJ; imipramine HCl (Tofranil) from Geigy Pharmaceuticals, Ardsley, NY; and morphine sulfate from Merck, Sharp and Dohme, West Point, PA. Radioactive [3-^{14}C]5-hydroxytryptamine creatinine sulfate (57 mCi/mmole) was obtained from Amersham Searle Corp., Arlington Heights, IL. Pertussis vaccine, fluid lot 4TW00, was kindly supplied by Eli Lilly and Co., Indianapolis, IN. All solutions were prepared fresh daily in concentrations which, when added in 10-μl amounts to platelet-rich plasma (0.5 ml), resulted in the desired molar concentrations.

Platelet preparation and aggregation. Normal and pertussis-injected mice were anesthetized with ether, and blood was collected from the subclavian area by means of siliconized Pasteur pipette and transferred to a polystyrene culture tube containing trisodium citrate (0.019 g/5 ml blood). Platelet-rich plasma (PRP) was

[1] These studies represent a portion of the dissertation submitted by T. W. Klein in partial fulfillment of the requirements for the Ph. D. degree from Creighton University, Omaha, NB 68131. This investigation was supported by the National Science Foundation, Grant No. GB-5707.

obtained as the supernatant fraction after centrifugation at 175*g* for 14 min at ambient temperature. Platelet-poor plasma (PPP) was derived by recentrifugation of the sedimented blood at 1100*g* for 10 min. Platelet concentrations were determined in a Coulter Thrombocounter (Coulter Electronics, Hialeah, FL) and standardized at $6 \times 10^5/mm^3$ using PPP as a diluent. Platelet aggregation was measured turbidometrically in a Chrono-Log Aggregometer (Chrono-Log Corp., Broomal, PA) with the aid of a self-balancing potentiometric recorder. An increase in light transmission is associated with platelet aggregation; a decrease from maximum indicates deaggregation.

Serotonin uptake and release. Platelet-rich plasma was obtained as described and standardized at 7.5×10^5 platelets/mm^3. All experiments were performed in 17 × 100-mm polystyrene culture tubes. To 0.5 ml PRP was added 0.5 μCi [3-^{14}C]5-HT (8.8×10^{-9} moles), incubated at 37° in a shaker bath for specified times, and then transferred to an ice bath. Where indicated, 10-μl amounts of either ADP or tris-(hydroxymethyl)aminomethane (0.02 *M*, pH 7.0) were added at 30 min. Platelets were separated from plasma by centrifugation at 1900*g* for 10 min at 4°, washed once with saline, resuspended in water, and freeze-thawed four times. Activity (^{14}C) was determined by liquid scintillation spectrometry for both platelet and plasma fractions. The sum of the total ^{14}C activity of both fractions (total recovery) of the various samples was consistently greater than 90%. The percentage of platelet associated isotope for each sample was calculated on the basis of individual total recoveries and the results given as the mean of two determinations.

Results. Effect of ADP, 5-HT, and histamine on platelet aggregation. The addition of ADP or 5-HT to stirred suspensions of platelets obtained from either *B. pertussis*-injected or normal mice and incubated at 37° induced reversible aggregation whereas histamine failed to do so (Fig. 1). One difference between preparations of the two groups of mice seemed to be the enhanced responsiveness of platelets of pertussis-injected mice to either ADP or 5-HT. For example, the aggregation pattern of "normal" platelets following 0.84 μM ADP was grossly similar to that induced by 0.42 μM ADP in preparations from pertussis-treated mice. There also seems to be a difference in the deaggregation phase following ADP and 5-HT with this phase being less rapid in the presence of amine.

Enhancement of aggregation by catecholamines. The data presented in Fig. 2 show that mouse platelets, when incubated in the presence of epinephrine, norepinephrine, or isoproterenol, did not undergo aggregation. However, subsequent addition of either ADP (Fig. 2A) or 5-HT (Fig. 2B) induced aggregation with an enhancement of the response occurring in those preparations containing E or NE. Further, the enhancement of the 5-HT response by E and NE resulted in the conversion from reversible to a nonreversible form of aggregation. The addition of E or NE to preparations (Fig. 3A) that had been aggregated by 5-HT resulted in nonreversible reaggregation of the platelets. Addition of ADP reaggregated in a reversible-type pattern; histamine and ISOP were ineffective. Platelets aggregated by ADP (Fig. 3B) were reaggregated by E, NE, and 5-HT in a reversible

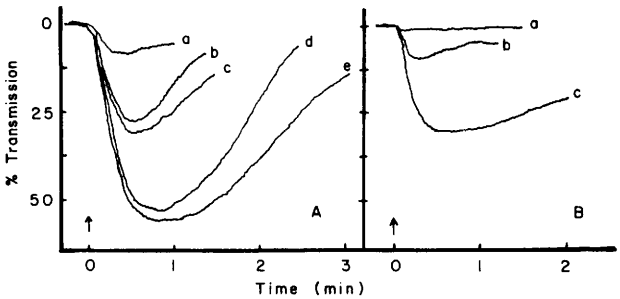

FIG. 1. In this figure, as in subsequent figures, the single tracing line before 0 time is a composite of the different tracings prior to aggregation. This figure demonstrates aggregation of normal (N) and pertussis-injected (P) mouse platelets by ADP, 5-HT, and histamine. A. ADP response: (a) 0.21 μM (N); (b) 0.42 μM (N); (c) 0.21 μM (P); (d) 0.84 μM (N); (e) 0.42 μM (P). B. Amine response: (a) histamine, 65 μM (N and P); (b) 5-HT, 49 μM (N); (c) 5-HT, 25 μM (P).

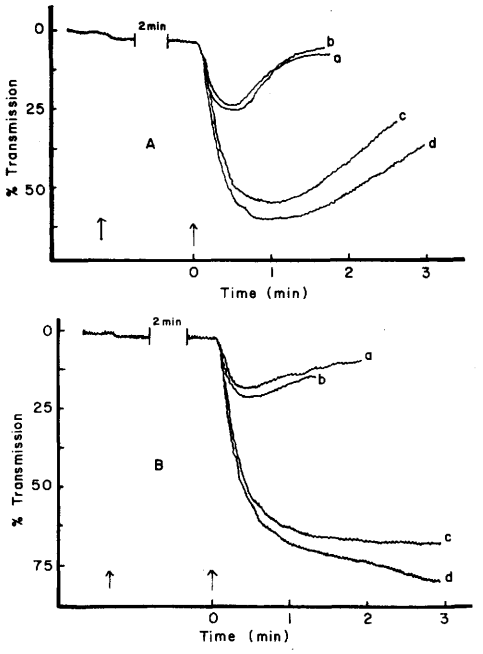

FIG. 2. Effect of catecholamines on aggregation of normal mouse platelets induced by either ADP or 5-HT. A. ADP (0.42 μM) added 3 min subsequent to (a) saline, (b) ISOP (10 μM), (c) NE (10 μM); (d) E (10 μM). B. 5-HT (49 μM) added 3 min subsequent to (a) saline, (b) ISOP (10 μM), (c) NE (10 μM), (d) E (10 μM).

fashion, but were unresponsive to histamine or ISOP. The results were the same for platelets of normal and pertussis-injected mice.

Uptake and release of 5-HT. Incubation of platelets of either normal or pertussis-injected mice in the presence of ^{14}C-serotonin resulted in an uptake of the amine as shown in Fig. 4. At 40 min, 70% of labeled 5-HT was platelet associated and, in experiments not shown, it was determined chromatographically that radioactivity represented 5-HT and not a metabolic product. There did not seem to be a difference between donors in terms of amount of platelet-associated 5-HT. Addition of ADP at 30 min and subsequent aggregation did not appear to release significant amounts of 5-HT.

Nature of the 5-HT receptor. Data presented in Fig. 5A show that the incubation of platelets from either normal or *B. pertussis*-injected mice with imipramine suppressed completely the aggregative response to subsequent addition of 5-HT, whereas morphine had no effect on 5-HT-induced aggregation. Regitine suppressed aggregation induced by 5-HT (Fig. 5B) but did not seem to affect the change in platelet shape which is shown by a reduction in oscillations in the tracing. Data were also obtained (Fig. 5C) which showed that preincubation with regitine suppressed enhancement by E or NE of ADP-induced platelet aggregation; propranolol did not appear to affect enhancement by E.

Discussion. In terms of ADP-induced aggregation and the enhancement of the response by catecholamines, present results obtained at an incubation temperature of 37° confirm those reported previously (7) which were conducted at 25°. The only difference appeared to be a failure of ISOP to enhance at the higher temperature (Fig. 2A) as it seemed to do at 25°. This would suggest that the functional characteristics of those adrenergic receptors responsive to E and NE are not as temperature sensitive as those reactive with ISOP. However, animal strain differences between the two studies place reservations on this interpretation.

The results obtained in studies of 5-HT-induced aggregation serve to point out what appear to be several unusual features of mouse platelets. As mentioned in the first section of this report, 5-HT initiates reversible aggregation in a variety of mammalian species, including rabbit, rat, and dog. In addition, the platelets of these three species fail to aggregate in response to

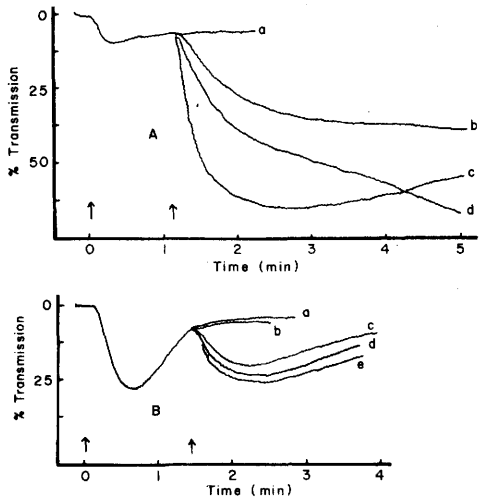

FIG. 3. Reaggregation of normal mouse platelets by various agents added subsequent to either 5-HT or ADP. A. 5-HT (49 μM) followed by (a) ISOP (10 μM) or histamine (65 μM), (b) NE (10 μM), (c) ADP (0.84 μM), (d) E (10 μM). B. ADP (0.42 μM) followed by (a) histamine (65 μM), (b) ISOP (10 μM), (c) NE (10 μM), (d) E (10 μM), (e) 5-HT (49 μM).

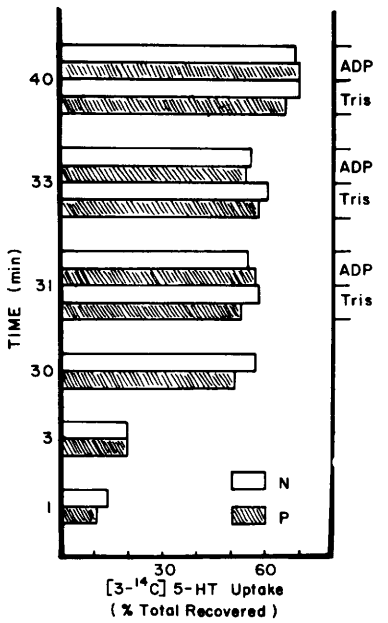

FIG. 4. Uptake of [3-^{14}C]5-HT by platelets from normal and pertussis-injected mice and the effects (measured at 31, 33, and 40 min) of either ADP (8.4 μM) or its diluent (tris buffer) added at 30 min.

epinephrine, demonstrate reversible aggregation following ADP, and display an enhanced aggregative response to ADP in the presence of epinephrine (5). Thus, in terms of these parameters, mouse platelets seem to resemble those of rabbit, rat, and dog. However, it has been reported that exposure of rabbit platelets to 5-HT for 1 min preceding E leads to a suppression of aggregation induced by the catecholamine (12).

In the present studies, exposure of mouse platelets to the amine for at least 2 min preceding addition of the catecholamine did not appear to diminish the aggregative pattern initiated by either E or NE (Fig. 3A). This difference in response may reflect a closer relationship between the 5-HT and adrenergic receptors in or on rabbit platelets than are represented on mouse components or that ratios may differ in the two species. Similar studies, to our knowledge, have not been reported with rat or dog platelets. Another unusual feature of mouse platelets is the irreversible type of aggregation induced by 5-HT in the presence of E or NE (Figs. 2B and 3A). This seems to resemble the "release reaction" involved in the biphasic response of human platelets. This reaction is currently under investigation.

It has been suggested that blood platelets contain on their membranes receptor sites specific for 5-HT and that these sites are involved with both platelet aggregation and the active uptake of the amine by the cells (13). Further work has indicated that the receptor resembles the classical D receptors for 5-HT on smooth muscle cells (blocked by dibenzyline, dihydroergotamine, but not by morphine) (14). Since platelet aggregation induced by 5-HT was found to be inhibited by phentolamine but not by morphine (Figs. 5A and 5B), present work would indicate that the receptor on mouse platelets also resembles the D-type on smooth muscle cells.

Through the use of 5-HT and several analogs, it has been shown that the structural specificity of the human platelet receptor concerned with shape change and aggregation initiated by 5-HT appears low whereas the uptake mechanism is highly specific (15). Several analogs produced a change in shape not followed by aggregation; none would produce aggregation in the absence of shape alteration. This split pattern was also noted with mouse platelets, *i.e.*, phentolamine suppressed 5-HT induced aggregation but did not affect the change in shape elicited by the amine (Fig. 5B). Although it was proposed that all active analogs elicited a response by acting on only one type of receptor, present results might be interpreted to suggest subtypes with one involving aggregation and possibly sharing configurations with alpha-type adrenergic receptors, the other manifested through induction of shape change. Suppression of both aggregation and shape change with imipramine (Fig. 5A) supports the concept that this substance exerts its effect through something other than a direct competitive action on the 5-HT receptor (15).

Although it has been shown that the metabolism of 5-HT *in vivo* was not grossly altered in the *B. pertussis*-injected mouse (16), it seemed possible that variation might exist in uptake and storage of the amine in the 5-HT sensitive animal. It is recognized that platelet uptake consists of several steps, including reaction with receptor, transport through the membrane probably by a carrier system requiring ATP, and then binding to ATP in cytoplasmic granules formed only when 5-HT is available (15). Present data would suggest that *B. pertussis* does not interfere with these steps since, under the experimental conditions employed,

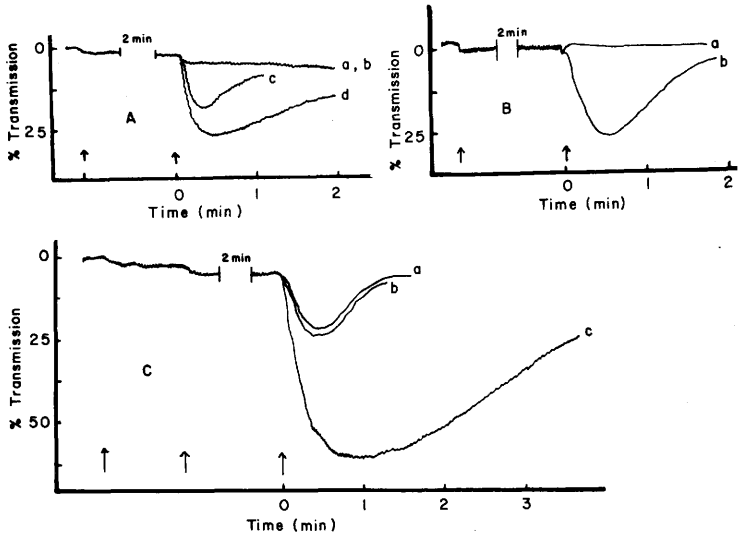

FIG. 5. The effect of various antagonists on ADP, 5-HT, and catecholamine-enhanced aggregation of normal (N) and pertussis-injected (P) platelets. A. Imipramine (130 μM) followed by (a) 5-HT (25 μM) (P), (b) 5-HT (49 μM) (N); morphine (5 μM) followed by (c) 5-HT (49 μM) (N), (d) 5-HT (25 μM) (P). B. Pertussis platelets. Regitine (100 μM): (a) 5-HT (25 μM); (b) ADP (0.21 μM). C. Normal platelets: (a) regitine (100 μM), E (10 μM), ADP (0.42 μM); (b) regitine (100 μM), NE (10 μM), ADP (0.42 μM); (c) propranolol (100 μM), E (10 μM), ADP (0.42 μM).

uptake (in the presence of excess 5-HT) was similar in the two groups of animals (Fig. 4).

Finally, 5-HT did not appear to be released from mouse platelets during aggregation induced by ADP (Fig. 4) as has been shown to occur with platelets of human subjects (17). In the latter instance, release of 5-HT and other substances is correlated with the second phase of aggregation. Failure of this release in the mouse system probably accounts for only the single phase noted with the active compounds examined.

Summary. The addition of ADP or 5-HT to stirred suspensions of mouse platelets in plasma incubated at 37° induced reversible aggregation, whereas histamine and catecholamines did not produce this response. Preparations obtained from *B. pertussis*-injected mice were more sensitive to the aggregative effect of ADP and 5-HT. Enhancement of aggregation induced by ADP or 5-HT occurred in the presence of E or NE but not ISOP. The addition of E and NE before or after 5-HT induced an irreversible type of aggregation while ISOP was ineffective. In the presence of imipramine or phentolamine, aggregation by 5-HT was suppressed; morphine did not seem to influence the response. Further, phentolamine, although inhibiting 5-HT-induced aggregation, allowed platelet shape change in response to 5-HT. Also, phentolamine inhibited the E and NE enhancement of ADP-induced aggregation but did not affect the ADP response *per se*. Finally, labeled 5-HT appeared to be taken up to the same degree by platelets from normal and *B. pertussis*-injected mice and was not released following the addition of ADP. These results are compared with those obtained in other species.

The technical assistance of Betty A. Nager and Bobbie Jean Dunathan is gratefully acknowledged.

1. Mustard, J. F., and Packham, M. A., Pharmacol. Rev. **22,** 97 (1970).

2. O'Brien, J. R., Nature (London) **200,** 763 (1963).

3. Sinakos, Z., and Caen, J. P., Thrombos. Diathes. Haemorrh. **17,** 99 (1967).

4. Mitchell, J. R. A., and Sharp, A. A., Brit. J. Haematol. **10,** 78 (1964).

5. Mills, D. C. B., Symp. Zool. Soc. London **27,** 99 (1970).

6. Belamarich, F. A., and Simoneit, L. W., Microvasc. Res. **6,** 229 (1973).

7. O'Bryan, B. S., and Fishel, C. W., Infect. Immun. **2,** 260 (1970).

8. Halpern, B. N., Neven, T., and Spector, S., Brit. J. Pharmacol. **20,** 389 (1963).

9. Iff, E. T., and Vaz, N. M., Int. Arch. Allergy **30,** 313 (1966).

10. Fishel, C. W., Keller, K. F., and O'Bryan, B. S., J. Immunol. **101,** 679 (1968).

11. Munoz, J., and Bergman, R. K., Bacteriol. Rev. **32,** 103 (1968).

12. Baumgartner, H. R., and Born, G. V. R., J. Physiol. **194,** 92(P) (1968).

13. Baumgartner, H. R., and Born, G. V. R., Nature (London) **218,** 137 (1968).

14. Michal, F., Nature (London) **221,** 1253 (1969).

15. Born, G. V. R., Juengjaroen, K., and Michal, F., Brit. J. Pharmacol. **44,** 117 (1972).

16. Fishel, C. W., Szentivanyi, A., and Talmage, D. W., J. Immunol. **89,** 8 (1962).

17. Mills, D. C. B., Robb, I. A., and Roberts, G. C. K., J. Physiol. **195,** 715 (1968).

Received May 13, 1974. P.S.E.B.M., 1974, Vol. 147.