

Platelet aggregation in atopic and normal patients

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Platelet aggregation was used as a model to evaluate the proposed beta blockade in subjects with allergic rhinitis and asthma. Studies using epinephrine, adenosine diphosphate (ADP), and streptococcal M protein as aggregating agents showed that there was no significant difference in platelet aggregation between atopics and controls. Furthermore, aggregation induced by all three agents was inhibited by propranolol and phentolamine, as well as by caffeine and dibutyryl cyclic adenosine monophosphate (d-cAMP). This inhibition was evidenced with platelets from both atopics and controls. The results suggest that with platelet aggregation the atopic population taken as a whole does not differ from the normal population. Although certain atopic subjects exhibit unique aggregation patterns, pharmacologic manipulation of this system fails to document that these unique patterns are due to beta blockade.

It has been postulated that the basic defect in the atopic state is an intrinsic blockade of the beta adrenergic receptors.¹ Investigators have made use of various systems to test this hypothesis.²⁻⁴ One of the systems used to evaluate this hypothesis involves the aggregation of platelets upon the addition of epinephrine. It has been reported that epinephrine-induced platelet aggregation is inhibited by beta adrenergic stimulation⁵ or alpha adrenergic blockade.⁶ It has also been reported that the platelets of atopic individuals demonstrate a quantitative difference in rapidity and amount of aggregation after the addition of epinephrine when compared to normal controls.⁷

This study was designed further to evaluate the platelet aggregation model in an atopic versus a control population. The platelets of 22 unmedicated atopic patients and 20 normal individuals were investigated with reference to aggregation. In order to elucidate the mechanism involved in this aggregation, adenosine diphosphate (ADP), streptococcal M protein, and epinephrine functioned as aggregation induction agents. Propranolol hydrochloride, phentolamine, caffeine, and dibutyryl cyclic adenosine monophosphate (dc-AMP) were utilized as pharmacologic antagonists of platelet aggregation.

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Supported in part by United States Public Health Service Grant AI-00320 and by Institutional Research funds from the United States Veterans Administration.

Received for publication July 3, 1972.

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MATERIALS AND METHODS

Test subjects

Subjects were chosen from volunteers. They were designated as atopic in the presence of a history of seasonal allergic rhinitis or seasonal asthma with concomitant positive immediate skin test reactions to common aeroallergens. All atopic subjects suffered from both allergic rhinitis and asthma except for 2 patients who had allergic rhinitis alone. Control subjects were selected on the basis of no clinical history of an atopic state and absence of positive skin tests. All subjects avoided any medication, including epinephrine, ephedrine, aminophylline, and aspirin products, for one week prior to testing. Coffee, soft drinks, and bananas were prohibited for 24 hours before testing. Twenty patients, 10 atopics and 10 controls, were evaluated in preliminary studies. A repeat of the studies utilized 12 atopics and 10 controls.

Preparation of platelet-rich plasma (PRP)

In preparation, all glassware was siliconized using 1 per cent Siliclad (Clay-Adams, New York, N. Y.). Two anticoagulants were used to investigate the effect of different anticoagulants on the platelet aggregation response. Thirty milliliters of blood were drawn into a plastic syringe containing 0.3 ml. of aqueous sodium heparin (1:1,000), or the blood was drawn and mixed immediately with sodium citrate (3.2 per cent, 1 ml. citrate per 9 ml. blood). The blood was transferred to a plastic tube and centrifuged at 700 g for 10 minutes at room temperature. The PRP was transferred by siliconized pipettes into colorimetric tubes. In all instances the PRP was used within 30 minutes of preparation.

Platelet aggregation agents

Epinephrine (Parke, Davis & Co. Laboratories, Detroit, Mich.) was obtained as a stock solution in a concentration of 1 mg. per milliliter ($5.4 \times 10^{-3}M$). In the aggregation studies, the final concentration of epinephrine was $2.73 \times 10^{-4}M$. ADP was obtained from Mann Laboratories, Orangeburg, N. Y., in a concentration of 2 μM per milliliter. Streptococcal M protein was an extract of a partially purified M protein prepared from type 30 group A streptococci by methods described previously.⁸ The stock solution contained 1 mg. per milliliter M protein.

Pharmacologic modifiers of platelet aggregation

Phentolamine (Ciba Pharmaceutical Co., Summit, N. J.) was obtained in a concentration of 5 mg. per milliliter ($1.78 \times 10^{-2}M$). Dose response studies were carried out with serial tenfold dilutions such that final concentrations ranged from $8.9 \times 10^{-4}M$ to $8.9 \times 10^{-8}M$. Propranolol hydrochloride (Ayerst Labs., New York, N. Y.) was obtained in a stock concentration of 1 mg. per milliliter ($3.8 \times 10^{-3}M$). Dose response studies were done using final concentrations of $1.9 \times 10^{-4}M$, $3.8 \times 10^{-4}M$, and $5.7 \times 10^{-4}M$. Caffeine (Nutritional Biological Corp., Cleveland, Ohio) was obtained in a concentration of 21.22 mg. per 100 ml. ($1.09 \times 10^{-3}M$). Dibutylryl cyclic adenosine monophosphate (dc-AMP) was obtained from Sigma Chemical Co., St. Louis, Mo., in a concentration of 41.3 μg per milliliter ($8.4 \times 10^{-5}M$). Final concentrations of caffeine and dc-AMP in aggregation tests were $5.45 \times 10^{-5}M$ and $4.2 \times 10^{-6}M$, respectively.

Platelet aggregation procedure

Platelet aggregation was performed according to the turbidimetric method of Born.⁹ Each substance to be tested was added in a volume of 0.05 ml. to 1 ml. of PRP in a siliconized colorimetric tube. Except where dose response studies were being carried out, each substance was employed in the original stock concentration to achieve the designated final concentrations. The optical density (OD) of the PRP was measured at 600 nm. in a Coleman Junior spectrophotometer adjusted to zero OD with distilled water. Throughout the readings,

the PRP was stirred magnetically at constant speed using a Teflon-coated iron bar in each tube. The OD was allowed to stabilize for 3 minutes before the aggregation study was begun. Starting time was designated as zero time, and OD readings were taken at 0.5 minute and one minute and then at one minute intervals thereafter for 8 minutes. Aggregation-inducing substances were added to PRP at zero time. When aggregation inhibitors were used, the substance to be tested was added at -3 minutes, and the aggregation-inducing agent was added at zero time. Upon completion of each test, the background OD reading obtained after centrifuging the PRP at 3,000 g for 15 minutes was subtracted from the OD readings before the data were analyzed. All studies were performed immediately after venipuncture, and all work was done at room temperature.

In analyzing the aggregation studies, the results are plotted as percentages of the initial OD reading of the PRP for each test. The student's *t* test was used to determine significant differences between means. Differences were considered significant at the 5 per cent level ($p < 0.05$).

RESULTS

Fig. 1 shows the mean results obtained upon the addition of 0.05 ml. epinephrine ($5.4 \times 10^{-3}\text{M}$) to 1 ml. PRP prepared with citrate in atopic and normal subjects. Final concentration of epinephrine in each tube was $2.73 \times 10^{-4}\text{M}$. In citrated PRP platelet aggregation occurred within 30 seconds, becoming maximal at 2 minutes before a disaggregation trend began. Disaggregation was maximal at 4 minutes, reaching a plateau level at this time. The mean curves in atopics and normals were similar, except that atopics maintained a plateau at 74 per cent of the initial optical density (OD) while the normal group plateaued at 69 per cent of the initial OD. As illustrated in Fig. 1, there was considerable variation among members of each test population. However, there was no significant difference between the mean values of atopic and control subjects.

Epinephrine induced platelet aggregation in both atopics and controls using heparinized PRP, as shown in Fig. 2. Platelets from both test groups exhibited a monophasic aggregation curve upon the addition of epinephrine (final concentration, $2.73 \times 10^{-4}\text{M}$). In all cases aggregation occurred in 30 seconds, was maximal at 5 minutes, and maintained a plateau until termination of the test. A plateau was maintained at 37 per cent of the initial OD in the atopic group, while plateau occurred at 39 per cent in the control group. There was no significant difference between the mean values of the atopic and control groups at any time interval, although there was considerable variation in each group.

Preliminary work included a dose-response study utilizing tenfold dilutions of epinephrine. In all instances, when the final concentration of epinephrine was $2.73 \times 10^{-4}\text{M}$ in citrated PRP, the response was that as shown in Fig. 1. When the final epinephrine concentrations in the tests ranged from $2.73 \times 10^{-5}\text{M}$ to $2.73 \times 10^{-8}\text{M}$, the curve as shown in Fig. 1 for citrated PRP was shifted up and to the left, indicating less platelet aggregation. In heparinized PRP, maximal aggregation, as shown in Fig. 2, was attained using the stock concentration of epinephrine (final concentration, $2.73 \times 10^{-4}\text{M}$). Serial tenfold dilutions of epinephrine again resulted in submaximal responses in platelet aggregation with a shift of the curve up and to the left. Since epinephrine-induced platelet aggregation appeared to be maximal when heparin was utilized as the anticoagulant in this system, the remainder of the studies were conducted using heparinized PRP.

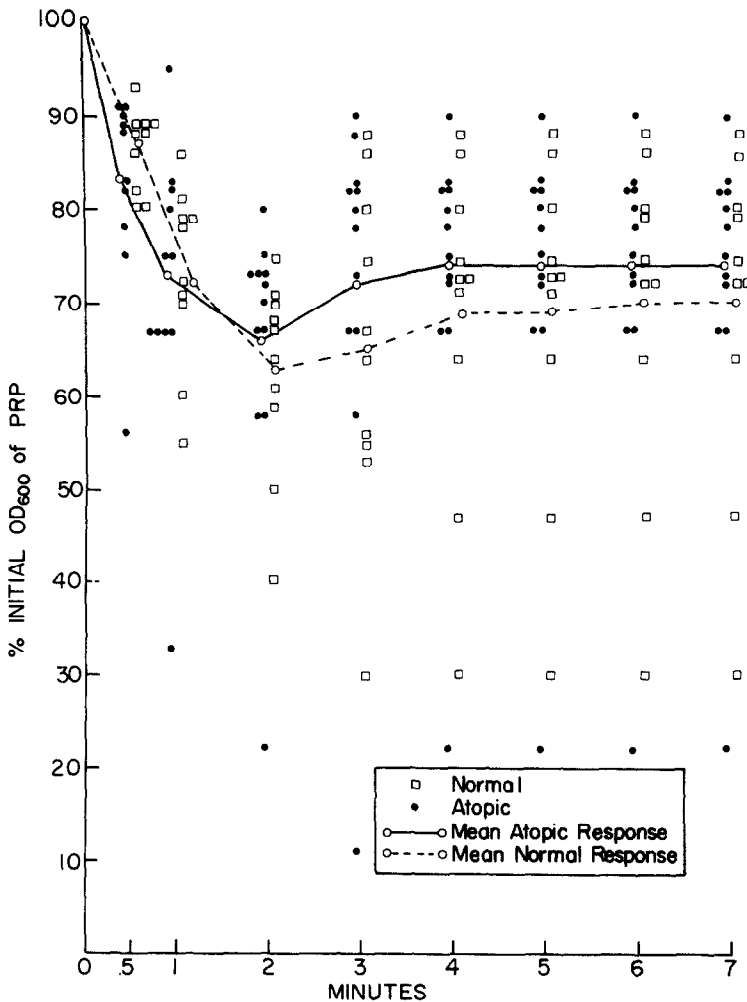


FIG. 1. Platelet aggregation responses to epinephrine using citrated PRP obtained from 10 atopics and 10 normal controls. The results are expressed as percentage of the initial optical density (per cent initial OD_{600}) at the various time intervals indicated. Epinephrine was added at zero time, and the final concentration in each test was $2.73 \times 10^{-4}M$.

Fig. 3 presents the results (mean $\pm$ 2 S.E.M.) of the addition of epinephrine to heparinized PRP of normal and atopic subjects. Final concentration of epinephrine was $2.73 \times 10^{-4}M$. These findings represent a different group of subjects from those presented in Fig. 2. However, as can be seen, the results are similar. Aggregation occurred within 30 seconds and continued for at least 3 minutes, showing no reversal trend before 6 minutes. It may be appreciated from the range of values that there was considerable variation among members of each test population. However, again the mean values showed no significant difference between normal and atopic individuals at the 5 per cent confidence level.

Fig. 4 illustrates the results obtained upon the addition of ADP to heparinized PRP of normal and atopic subjects. Platelet aggregation was demon-

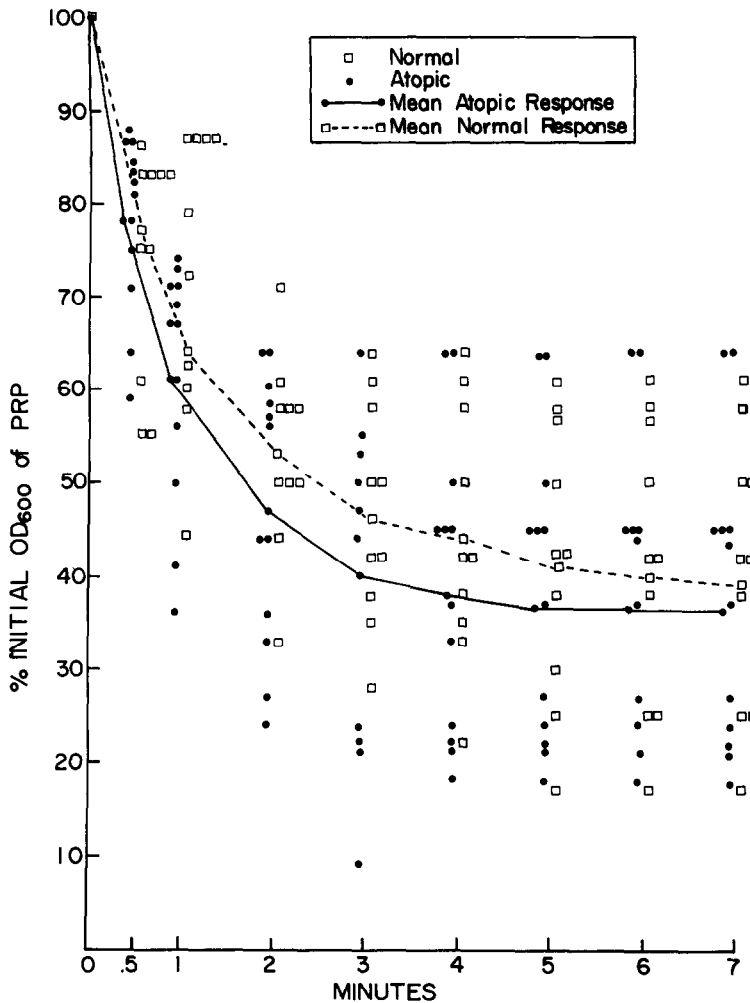


FIG. 2. Platelet aggregation responses to epinephrine using heparinized PRP obtained from 10 atopics and 10 normal controls. The results are expressed as percentage of the initial optical density (per cent initial OD₆₀₀) at the various time intervals indicated. Epinephrine was added at zero time and the final concentration in each test was $2.73 \times 10^{-4}M$.

strable within seconds, becoming maximal at about 3 minutes with minimal disaggregation over the test period of 7 minutes. No significant difference between atopic and normal subjects was noted. M protein added to PRP of atopic and normal subjects gave the results shown in Fig. 5. Aggregation commenced within 2 to 5 minutes after the addition of M protein. As with the addition of epinephrine and ADP, there was no significant difference between normal and atopic subjects.

Fig. 6 shows the results of the dose-response studies using phentolamine and propranolol to inhibit epinephrine-induced platelet aggregation. As seen in Fig. 6, A, phentolamine in a final concentration of $8.9 \times 10^{-4}M$ was 100 per cent effective in blocking platelet aggregation induced by epinephrine. Increasing

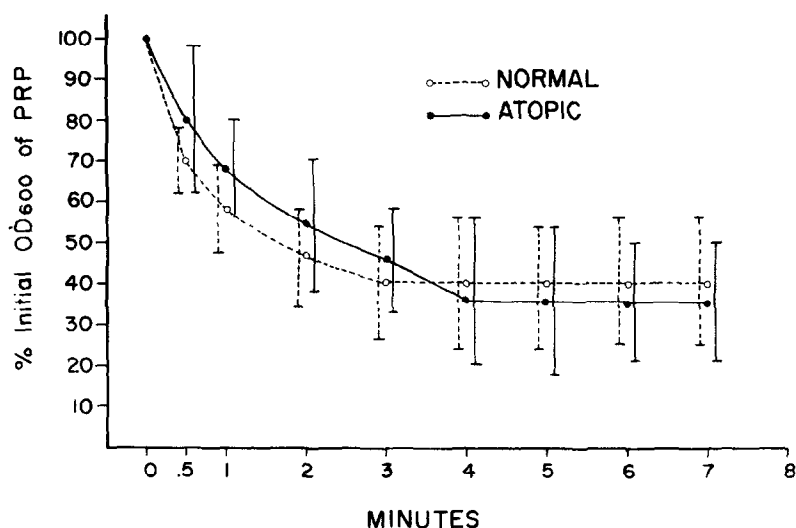


FIG. 3. Platelet aggregation responses to epinephrine using heparinized PRP obtained from 12 atopics and 10 normal controls. The results shown are means $\pm$ 2 S.E.M. and are expressed as percentage of the initial optical density (per cent initial OD₆₀₀) at the various time intervals indicated. Epinephrine was added at zero time, and final concentration was 2.73×10^{-4} M.

dilutions of phentolamine was less effective in inhibiting aggregation, and at a final concentration of 8.9×10^{-8} M it was completely ineffective. It is of some importance that the curves shown represent results using platelets from normal subjects. However, results using platelets from atopic subjects were comparable. Fig. 6, *B* shows that propranolol was not effective in inhibiting the first phase of epinephrine-induced platelet aggregation at a final concentration of 2×10^{-4} M. However, propranolol appeared to hasten platelet disaggregation during the latter part of the test period. At 4×10^{-4} M concentration, propranolol reduced the response seen during the initial phase of platelet aggregation as reflected by less change in OD during the first minute of the test period. Furthermore, the disaggregation trend occurred sooner in time and was more complete than the disaggregation that occurred at 2×10^{-4} M. Propranolol was 97 per cent effective in blocking epinephrine-induced platelet aggregation at final concentrations of 6×10^{-4} M. The two upper curves in Fig. 6, *B* also show the effect of the addition of ADP to the test system at the 5 minute interval. The addition of exogenous ADP resulted in rapid platelet aggregation as illustrated by the precipitous drop in the OD readings of the PRP. The concentration of ADP was the same as used in the tests shown in Fig. 4.

Other tests revealed that phentolamine was effective in blocking platelet aggregation induced by ADP and M protein. In these tests the final concentration of phentolamine was 8.9×10^{-4} M. In addition, as was the case with epinephrine-induced aggregation, propranolol was effective in blocking platelet aggregation induced by ADP and M protein in high doses only.

Fig. 7 demonstrates the effect of the addition of de-AMP and caffeine to platelet aggregation induced by M protein. Aggregation was completely inhibited

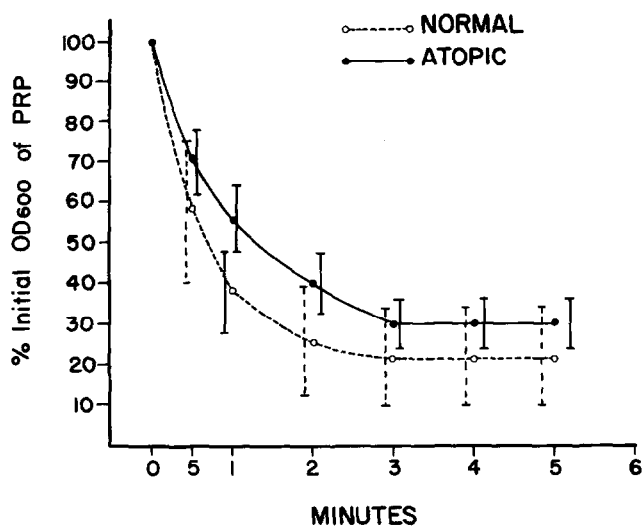


FIG. 4. Platelet aggregation response to ADP using heparinized PRP from the same subjects as in Fig. 3. The results shown are means $\pm$ 2 S.E.M. and are expressed as percentage of the initial optical density (per cent initial OD₆₀₀) at the various time intervals indicated. ADP was added at zero time, and the final concentration was 2.34×10^{-7} M.

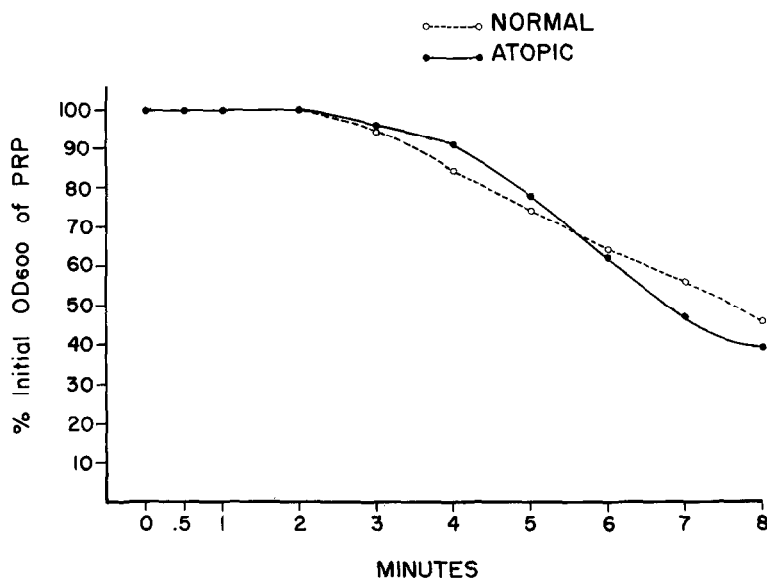


FIG. 5. Platelet aggregation response to M protein using heparinized PRP from the same subjects as in Fig. 3. The results are the mean values of 12 atopics and 10 normal controls and are expressed as percentage of the initial optical density (per cent initial OD₆₀₀). M protein (0.05 ml. of a 1 mg. per milliliter solution) was added at zero time.

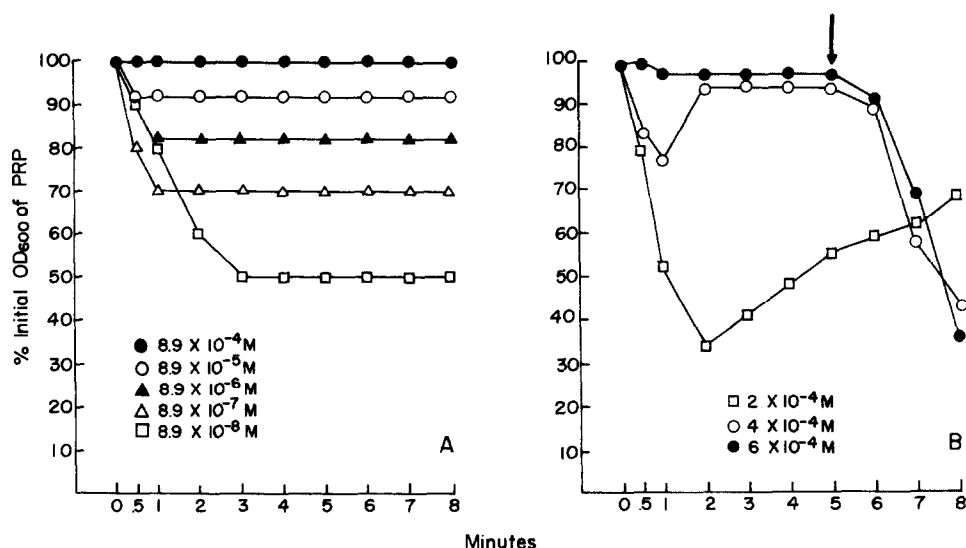


FIG. 6. Dose-response results using phentolamine (A) and propranolol (B) at various concentrations to inhibit epinephrine-induced platelet aggregation. Inhibitors were added at -3 minutes and epinephrine at zero time. Final concentrations of the inhibitors are indicated. In 2 tests using propranolol (B), ADP was added at the 5 minute interval (arrow).

by both substances. Final concentrations of caffeine and dc-AMP were $5.45 \times 10^{-5} \text{M}$ and $4.2 \times 10^{-6} \text{M}$, respectively. Similarly, dc-AMP and caffeine were equally effective in inhibiting platelet aggregation induced by both ADP and epinephrine.

DISCUSSION

The phenomenon of platelet aggregation has been studied extensively and recently reviewed.⁶ The exact mechanism involved in this reaction is unknown. However, it is thought to be mediated through the alpha adrenergic receptor via alpha adrenergic stimulation and the resultant release of ADP from platelets.^{6, 9} Since epinephrine induces aggregation and causes ADP release from platelets, it is considered an alpha agonist in this system.⁶

There is indirect evidence to indicate that beta adrenergic stimulation prevents aggregation and is therefore antagonistic to alpha stimulation. This evidence is based upon the observation that dc-AMP and substances that increase the level of platelet cyclic AMP inhibit aggregation.⁵

In view of the hypothesized role of alpha and beta receptors in the aggregation of platelets, it seemed reasonable to extend the observations of others⁷ by using this system as a tool to evaluate the postulated existence of a beta blockade in atopy. The present study was designed to do so by comparing the effect of three inducers of aggregation, epinephrine, ADP, and M protein, on platelets of atopic and normal individuals. As stated above, epinephrine is thought to act by alpha stimulation with the resultant release of ADP. ADP, via an unknown

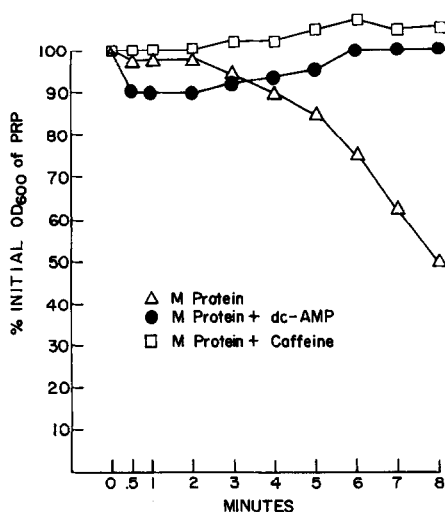


FIG. 7. Inhibition of platelet aggregation response by caffeine and dibutyryl cyclic AMP (dc-AMP). Inhibitors were added at -3 minutes and M protein at zero time. Final concentrations of caffeine and dc-AMP were $5.45 \times 10^{-5}M$ and $4.2 \times 10^{-6}M$, respectively.

mechanism, causes aggregation directly. M protein is believed to induce aggregation by ADP release via an unknown mechanism other than through the alpha receptor. In other systems this mechanism is thought to involve the activation of complement through antigen-antibody interaction.¹⁰

No significant differences between normal and atopic individuals were found in platelet aggregation due either to epinephrine, ADP, or M protein. Nor was there any detectable difference between patients with allergic rhinitis alone or those with both allergic rhinitis and asthma. These results differ from those reported in a previous study.⁷ The reason for the difference is unclear. At first the difference was attributed to a variation in technique. Citrate was used as the anticoagulant in the previous study. In the present study only heparin was originally employed. In order to obtain a more valid comparison, citrate was later utilized. There appears to be a difference in the platelet aggregation response obtained using the two different anticoagulants. Utilization of heparin resulted in a monophasic curve similar to the reports of other investigators,¹¹ and the platelet aggregation response appeared to be maximal. When citrate was used as the anticoagulant, a biphasic aggregation curve was produced. However, the aggregation response never appeared to be maximal, and the shape of the curve suggested possible inhibition in the test system. This occurred in both normal and atopic subjects. There are reports in the literature that indicate a possible inhibiting effect of ethylenediaminetetraacetic acid and large amounts of citrate on platelet aggregation.^{10, 12} There was no such indication of inhibition when heparin was used. As shown by these results, there appears to be an important difference between the effects of the two anticoagulants in platelet aggregation. However, the pertinent point in this study is that there was no significant difference in platelet aggregation between atopic and normal individuals regardless of the anticoagulant used.

To further analyze the platelet aggregation reaction in terms of its relation to the sympathetic nervous system and particularly in regard to its use as a tool to assess the beta blockade hypothesis, several pharmacologic agents were evaluated for their effect on this system. Caffeine and dibutyryl cyclic AMP were employed to elucidate the role of cyclic AMP in this system. Specific adrenergic blocking agents were used also.

Caffeine, which increases cyclic AMP levels by inhibiting phosphodiesterase activity, acted as expected to inhibit platelet aggregation. Dibutyryl cyclic AMP, a soluble form of cyclic AMP, had a similar effect. This adds support to the idea that substances that increase cyclic AMP levels inhibit epinephrine-induced platelet aggregation.^{5, 6}

As expected, phentolamine, an alpha blocking agent, inhibited epinephrine-induced platelet aggregation. Since it is felt that epinephrine-induced platelet aggregation is an alpha adrenergic phenomenon, it is assumed that phentolamine did so by alpha blockade.^{6, 14} However, not all alpha blockers exhibit this inhibitory property¹⁴; dibenamine and Dibenzyline are not inhibitors of epinephrine-induced platelet aggregation. The explanation for this is not clear.

Propranolol, a beta blocking agent, also inhibited epinephrine-induced platelet aggregation. However, the inhibitory effect of propranolol was dose-dependent, being effective in high doses only. In lower doses propranolol was not effective in blocking the first phase of platelet aggregation. This effect has been reported previously.¹³ Other studies¹⁴ have indicated that propranolol probably also functions via a mechanism other than beta blockade; i.e., it is thought to have a direct stabilizing effect on the platelet membrane, preventing release of endogenous ADP and thus resulting in inhibition of aggregation. This is important in that the addition of propranolol to normal platelets should not necessarily be interpreted as a model for beta blockade in the platelet aggregation system.

The pharmacologic schema involved in platelet aggregation becomes even more complex after the observation that propranolol and phentolamine inhibit aggregation due to all three aggregating agents. Such an effect would be expected to occur only in regard to epinephrine-induced aggregation, since epinephrine has both alpha and beta activity. It is surprising that phentolamine blocks the aggregation due to ADP and M protein since both agents are felt to act after alpha stimulation, therefore bypassing the alpha receptor completely.^{6, 10} This may indicate that phentolamine, like propranolol, may have a second direct effect as well as an alpha blocking effect. In fact, it has been reported that alpha adrenergic blocking agents inhibit aggregation induced by thrombin, the activity of which depends upon its capacity to trigger the release of endogenous ADP from platelets.^{6, 10}

The data obtained in this study indicate the platelet aggregation model is extremely complex. In the present investigation, in contrast to the work of others,⁷ no significant difference was demonstrated between the aggregation patterns of atopic and normal individuals. When the platelet aggregation model was studied by the use of inhibitors of aggregation, it was demonstrated that both alpha and beta blocking agents may inhibit the aggregation reaction. This

inhibition may be due to an effect mediated via the autonomic receptors or may be a direct effect upon the platelet membrane. However, it seems clear that cyclic AMP and agents that increase cyclic AMP levels within the platelets inhibit aggregation. On the basis of these observations one would expect that if there were a difference in aggregation patterns in atopic versus normal persons, this difference should be reflected by a more pronounced aggregation in atopics if it is assumed that the difference is due to a beta blockade in atopic subjects. This pronounced aggregation has not been demonstrated. Furthermore, there was no difference in the results obtained using standard pharmacologic blocking agents between tests with normal and atopic platelets. These observations suggest that in respect to platelet aggregation the atopic test population taken as a whole does not differ from the normal test population. Although certain atopic subjects exhibit unique aggregation patterns, pharmacologic manipulation of this system fails to document that these unique patterns are due to beta blockade.

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