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α-Adrenergic Regulation of the Secretion of an Anticomplementary Factor in Mouse Saliva[1] (39337)

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(Introduced by D. M. Woodbury)

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A potent inhibitor of complement activity has been detected in mouse saliva in the standard controls employed in a microcomplement fixation assay for nerve growth factor (1, 2). Such salivary anticomplementary activity might have physiological significance since complement has been demonstrated in both normal and inflamed gingival tissues (3) and in the crevicular fluid of patients with periodontal disease (4) or inflamed gingiva (5). In addition, anticomplementary factors have been detected in human saliva (6, 7), and it has been suggested that such factors could inhibit complement-mediated processes in diseased gingiva (6).

Secretion of the anticomplementary factor could be governed by either the sympathetic or parasympathetic nervous system. Saliva elicited in response to parasympathetic stimulation has a different protein composition from that secreted in response to sympathetic stimulation (8, 9). In order to determine which branch of the autonomic nervous system regulates secretion of the anticomplementary factor, saliva elicited by both sympathomimetic and parasympathomimetic agents was analyzed for anticomplementary activity. In addition, other pharmacological methods were employed to partially characterize the anticomplementary factor.

Materials and methods. Saliva was collected from ten- to sixteen-week-old male mice. The animals were anesthetized with pentobarbital, 60 mg/kg. Salivation was induced by the intraperitoneal (ip) administration of a secretagogue. The secretagogues and the range of doses for each were: pilocarpine, 0.1 to 0.8 mg/kg; epinephrine, 2.0 to 6.6 mg/kg; norepinephrine, 0.7 to 2.5 mg/kg; and isoproterenol, 1.0 to 5.0 mg/kg. In a few cases, about one-tenth the ip dose of secretagogue was injected under the sheath of connective tissue covering the submaxillary gland. The results were comparable to those after ip injection. In all experiments, saliva was collected in a microcapillary tube placed between the tongue and the floor of the mouth. In some cases, small aliquots of saliva were collected sequentially from a single animal given one dose of a secretagogue. In other cases, all of the saliva elicited by one dose of secretagogue was collected as one sample. Immediately after collection, the saliva was frozen at −40° until the assays were performed.

Saliva, trypsin, chymotrypsin, thrombin, Kallikrein, γ-subunit of 7S nerve growth factor (10), amylase, carbonic anhydrase, and carboxypeptidase A were tested for anticomplementary activity. This was determined following the basic procedure of Moore and Perez (11) as modified from Wasserman and Levine (12). Samples of saliva or of an enzyme preparation were diluted in sodium veronal buffer (5 m*M*, pH = 7.5) containing 0.1% gelatin and 0.005% Triton X-100. A 5-μl aliquot of each dilution was added to 50 μl of complement-containing guinea pig serum diluted 1:320 in the same buffer. The resultant mixture was incubated at 4° for 18–20 hr. After the incubation, 0.3 ml of a suspension of sheep red blood cells sensitized by treatment with hemolysin was added to each reaction mixture. The samples were incubated for 60 min at 38° to allow complement-mediated hemolysis and then centrifuged to pellet the red blood cells (12,000 *g* for 10 min). The absorbance of the supernatant at 413 nm was determined. The optical density of the blanks which have 5 μl of buffer added in

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place of the sample was usually close to 1.0. This parameter was initially set by adjusting the amount of complement in the reaction.

In order to quantify salivary anticomplementary activity, a series of dilutions of a saliva sample was assayed as described above. The dilution at which the resultant optical density was one-half that of the blanks (i.e., 50% inhibition of complement-mediated hemolysis) was determined. The reciprocal of this dilution will be defined as the number of anticomplementary units (ACU) in 5 μl of the undiluted saliva sample. Since the highest concentration of saliva examined was a 1:10 dilution, salivary anticomplementary activities of less than 10 ACU were not quantified.

In studies involving protease inhibitors, a 5-μl aliquot of inhibitor (780 ng/μl) was added to the complement-containing solution in addition to 5 μl of the diluted saliva or enzyme sample. The inhibitors studied were aprotinin, Kunitz pancreatic trypsin inhibitor, soybean trypsin inhibitor, ovomucoid trypsin inhibitor, and lima bean trypsin inhibitor.

Protein determinations were made using the method of Lowry *et al.* (13) with bovine serum albumin as a standard.

L-epinephrine bitartrate, trypsin (bovine, type III, 12,500 *N*-benzoyl-L-arginine ethyl ester units/mg), α-chymotrypsin (bovine, type II, 50 benzoyl-L-tyrosine ethyl ester units/mg), thrombin (bovine, grade I, 250 NIH units/mg), α-amylase (ovine, type I-A, 2,250 Wilbur-Anderson units/mg), carbonic anhydrase (bovine, 41 hippuryl-L-phenylalanine units/mg), trypsin inhibitor from beef pancreas (1 mg inhibits 3.1 mg trypsin), trypsin inhibitor from soybean (1 mg inhibits 1.4 mg trypsin), trypsin inhibitor from ovomucoid (1 mg inhibits 0.9 mg trypsin), trypsin inhibitor from lima bean (1 mg inhibits 4 mg trypsin), and bovine serum albumin (fraction V, 96–99%) were purchased from Sigma Chemical Co., St. Louis, Mo. Pilocarpine hydrochloride was purchased from Merck Chemical Co., Rahway, N.J., noradrenaline hydrochloride and isoproterenol hydrochloride from Regis Biochemicals, Morton Grove, Ill., and aprotinin (Trasylol, 10,000 kallikrein inhibition units/ml) from FBA Pharmaceuticals, New York, N.Y. Kallikrein (a registered trademark of Bayer AG, 1290 kallikrein units/mg) was kindly supplied by Drs. Elder and Schmidt-Kastner, Bayer A. G., Wuppertal, Germany. The γ-subunit of 7S nerve growth factor (10) was a generous gift from Dr. E. M. Shooter of Stanford University.

Results. The protein concentration, anticomplementary factor concentration, and flow rate of the saliva were measured at different times following single injections of either pilocarpine (Fig. 1) or epinephrine (Fig. 2). When epinephrine rather than pilocarpine was used, the results showed several marked contrasts: (a) the flow rates were lower, (b) the protein concentrations were approx 50-fold greater, and (c) the anticomplementary factor concentrations were more than 1000-fold greater. A change in protein concentration from one aliquot to the next appeared to be accompanied by a parallel shift in anticomplementary factor levels in

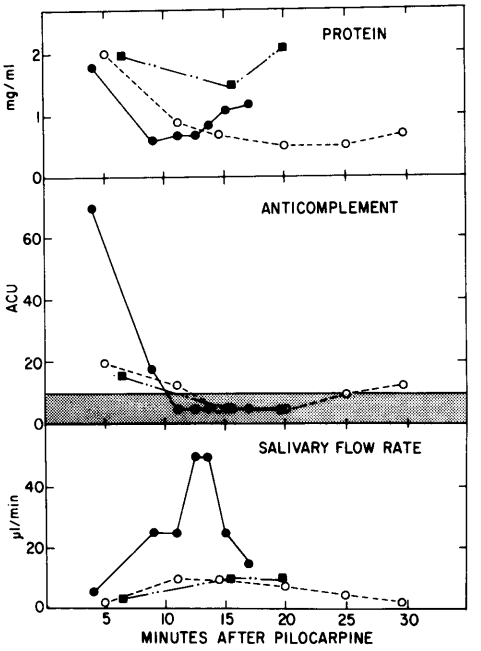

FIG. 1. Anticomplementary factor and protein concentrations of sequential samples of saliva and salivary flow rates elicited by a single injection of pilocarpine. Each set of like symbols represents data from a single animal. Values in the stippled area of the anticomplement panel represent estimates since activities of less than 10 ACU were not quantified (see Materials and Methods).

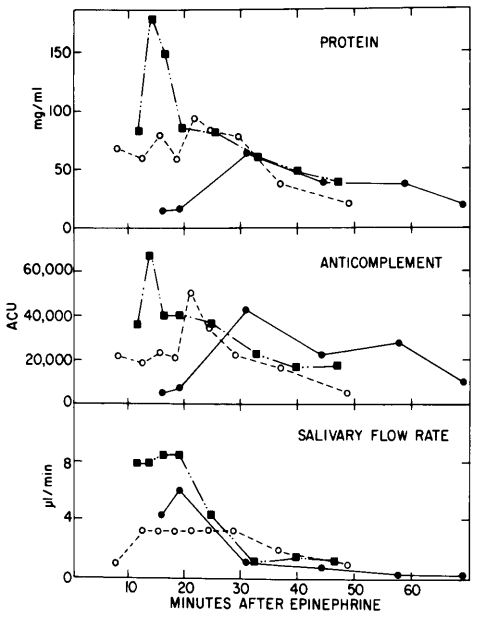

FIG. 2. Anticomplementary factor and protein concentrations of sequential samples of saliva and salivary flow rates elicited by a single injection of epinephrine. Each set of like symbols represents data from a single animal.

the epinephrine-induced saliva, but not in the pilocarpine-induced saliva.

The relationship between protein concentration and anticomplementary factor concentration with isoproterenol and norepinephrine as secretagogues was examined in order to determine whether the epinephrine-induced secretion was due to action on α-adrenergic or β-adrenergic receptors. These data along with the corresponding data from the epinephrine treatment are shown in Fig. 3. An estimate of the slopes and intercepts of these curves was obtained using linear regression analysis. These estimates of the slopes and their standard errors are shown in Table I.

Since salivary anticomplementary activity might be due to the action of salivary proteolytic enzymes on the complement proteins, several proteolytic enzymes thought to be present in saliva (14, 15) were examined for anticomplementary activity. The data in Fig. 4 demonstrate the anticomplementary activity of trypsin, chymotrypsin, thrombin, and Kallikrein, and the lack of activity of the esteroproteolytic γ-subunit of 7S nerve growth factor (16). Amylase, carbonic anhydrase, and carboxypeptidase A were also studied but lacked anticomplementary activity (data not shown).

Salivary anticomplementary activity was examined in the presence of protease inhibitors in order to determine whether the anticomplementary factor might be a proteolytic enzyme (Fig. 5). The salivary anticomplementary factor was inhibited by aprotinin and by Kunitz pancreatic trypsin inhibitor

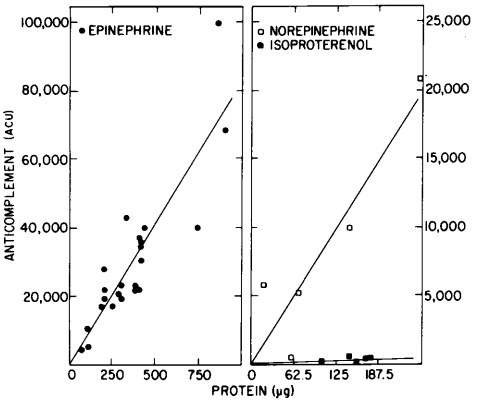

FIG. 3. Correlation between anticomplementary factor and protein in secretions elicited by adrenergic agents. The lines were determined by linear regression analysis. The correlation coefficients for epinephrine, norepinephrine, and isoproterenol were 0.88, 0.91, and 0.28, respectively.

TABLE I. SPECIFIC ACTIVITIES OF ANTICOMPLEMENTARY FACTOR IN SECRETIONS ELICITED BY VARIOUS AUTONOMIC AGENTS.[a]

Agonist	Salivary anticomplementary activity (ACU/µg protein)
Pilocarpine [cholinergic]	0.3 ± 0.1 (10)
Isoproterenol [β-adrenergic]	1.8 ± 3.6 (5)
Epinephrine [α + β-adrenergic]	85. ± 10. (24)
Norepinephrine [α-adrenergic]	78. ± 20. (5)

[a] Values are the slopes of linear regression lines ± the standard error of the slope. The regression lines for all data groups except pilocarpine are shown in Fig. 3. Number of samples is shown in parentheses. The receptors primarily stimulated by each agonist are indicated in square brackets. The following are statistically significant ($P < 0.01$): (a) pilocarpine vs both epinephrine and norepinephrine, and (b) isoproterenol vs both epinephrine and norepinephrine.

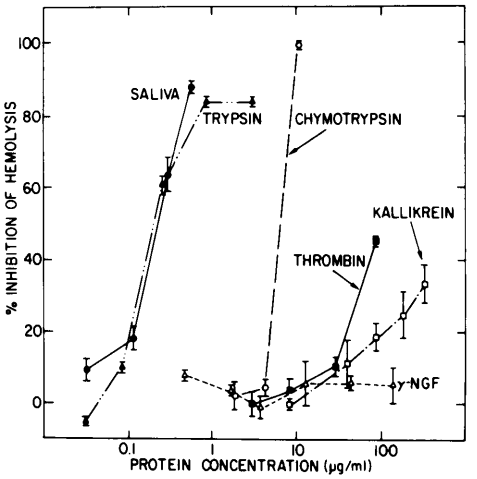

FIG. 4. Anticomplementary activity of proteolytic enzymes and saliva. Concentrations are final concentrations in the 55-μl reaction mixture. Saliva values are for total salivary protein concentrations in the 55-μl reaction mixture. Bars indicate standard errors of the means. Each point represents the mean of three to six samples.

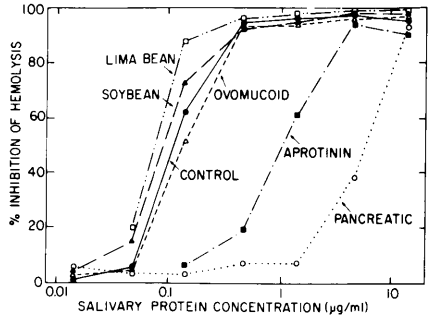

FIG. 5. Effects of protease inhibitors on salivary anticomplementary activity. The results presented were obtained using epinephrine-induced saliva from one animal. Each inhibitor has been examined with at least three different saliva samples and the same inhibition pattern was always obtained. At each concentration of salivary protein, either a 5-μl aliquot containing 3.9 μg of inhibitor or a 5-μl aliquot of buffer was added.

but not by soybean trypsin inhibitor, ovomucoid trypsin inhibitor, or lima bean trypsin inhibitor.

The ability of these protease inhibitors to block the anticomplementary activity of trypsin, chymotrypsin, and Kallikrein was examined in order to compare pharmacologically these enzymes with the salivary anticomplementary factor (Table II). The spectrum of inhibition of salivary anticomplementary activity was the same as that of Kallikrein but unlike those of trypsin or chymotrypsin.

Discussion. The present study demonstrates that mouse saliva contains one or more potent anticomplementary factors. The concentration of this factor was more than 1000 times greater in secretions elicited by epinephrine, a sympathomimetic agent, than in those induced by pilocarpine, a parasympathomimetic agent (Figs. 1 and 2). These results also show that pilocarpine elicits a rapid flow of a secretion with relatively low protein content, while epinephrine induces a slower flow of a secretion with a 50-fold higher protein content. These observations on flow rate and protein content are consistent with results obtained by other investigators (14, 17, 18).

Since both anticomplementary factor and total protein concentrations were higher in the epinephrine- than in the pilocarpine-induced secretions, specific activities were determined to compare the activity per unit protein (Table I). The mean specific activity of epinephrine-induced saliva was 283 times that of pilocarpine-induced saliva. Since high doses of pilocarpine (10 mg/kg ip) produce an indirect sympathomimetic effect in rat salivary glands (19), even the low level of anticomplimentary activity found in the pilocarpine-induced saliva might be due to this indirect adrenergic action. These data imply that secretion of the anticomplementary factor is primarily regulated by sympathetic activity.

The levels of salivary anticomplementary factor were also determined following an α- and a β-adrenergic agonist in order to characterize the adrenergic receptor involved.

TABLE II. EFFECTS OF PROTEASE INHIBITORS ON ANTICOMPLEMENTARY ACTIVITY[a].

Protease inhibitor	Anticomplementary activity			
	Trypsin	Chymotrypsin	Kallikrein	Saliva
Soybean	+	+	−	−
Lima bean	+	+	−	−
Ovomucoid	+	−	−	−
Pancreatic	+	+	+	+
Aprotinin	+	+	+	+

[a] A plus indicates a greater than 75% inhibition of anticomplementary activity, and a minus indicates a less than 15% inhibition.

The α-adrenergic agonist norepinephrine elicited saliva having a mean specific activity 43 times that of the β-adrenergic agonist isoproterenol and 260 times that of the cholinergic agonist pilocarpine (Table I). In addition, the specific activity of norepinephrine-induced saliva was not significantly different from that elicited by epinephrine, which possesses both α- and β-adrenergic activity (Table I). Thus, release of the salivary anticomplementary factor appears to be primarily regulated through the action of the sympathetic nervous system on α-adrenergic receptors within the salivary glands.

Our observation that salivary anticomplementary activity can be inhibited by both aprotinin and Kunitz pancreatic trypsin inhibitor strongly suggests that this activity might be due to a salivary protease. Such a salivary anticomplementary protease would have to be capable of selectively inactivating one or more of the complement components in the presence of a great excess of other proteins. (At 50% hemolysis, complement proteins only account for 0.06% of the total protein.)

Several highly purified proteases have been examined for anticomplementary activity in order to compare them to the salivary factor (Fig. 4). Expressed in terms of total salivary protein, the potency of the salivary anticomplementary factor was equal to that of recrystallized trypsin, the most potent enzyme examined. Thus, the salivary anticomplementary factor must be considerably more potent than any enzyme tested since it probably constitutes only a small proportion of total salivary protein.

The effects of five protease inhibitors have been determined on the anticomplementary activity of trypsin, chymotrypsin, and Kallikrein. The results show that only those inhibitors which have been reported to block the proteolytic activity of a given enzyme (20) will inhibit its anticomplementary activity (Table II). This strongly suggests that the anticomplementary activity of each of these enzymes is correlated with its proteolytic activity. To our knowledge this is the first demonstration that kallikrein possesses anticomplementary activity. This might be quite significant since kallikrein is only thought to act on a limited number of protein substrates.

The inhibition spectrum of the salivary anticomplementary factor indicates that it might be a kallikrein-like enzyme (Table II). Kallikrein is present in both animal and human saliva (21, 22) and appears to be selectively secreted through α-adrenergic stimulation (21, 23). However, the Kallikrein from ovine pancreas used in this study differed from the salivary anticomplementary factor both in potency and the slope of the dose-response curves (Fig. 4). Since others have observed marked differences between organ and salivary kallikrein (24), these observations do not demonstrate that the salivary anticomplementary factor is not salivary kallikrein. Final resolution of this question must await purification and isolation of the salivary anticomplementary factor.

Summary. Mouse saliva contains a potent inhibitor of complement activity. The secretion of this inhibitor appears to be regulated by action on α-adrenergic receptors for two reasons. First, an α-agonist (norepinephrine) elicited saliva with a 260-fold higher specific activity of the inhibitor than that obtained with a cholinergic agent (pilocarpine). Second, the α-agonist elicited saliva having a 43-fold greater specific activity than that obtained following administration of a β-adrenergic agonist (isoproterenol). This anticomplementary factor probably proteolytically degrades one or more of the complement components since it is inhibited by several protease inhibitors. The salivary anticomplementary factor is more potent than trypsin, chymotrypsin, thrombin, or Kallikrein. The anticomplementary factor has a pattern of inhibition like that of Kallikrein but unlike those of trypsin or chymotrypsin.

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