was no apparent correlation between numbers of cells injected and speed of onset of hyperglycemia. Of the 32 control rats that received an injection of medium alone, two were killed within 2 weeks for evaluation of pancreatic morphology, which appeared normal. Of the remaining 30 rats, ten eventually became diabetic, with a mean age of onset of 86 ± 5 days. The other 20 animals were followed for at least 118 days and remained normoglycemic.

Most of the animals were killed within 1 week of onset of hyperglycemia in order to assess the presence of insulitis (*20*). This was present in each of the 12 animals examined. The insulitis appeared similar to that in spontaneously diabetic animals.

Of the two diabetes-prone recipients that failed to develop hyperglycemia when injected with cells from donors with acute diabetes one appeared unhealthy and died 31 days after transfer. Autopsy was not performed because of autolysis. The second animal was killed at 120 days of age, and the pancreatic morphology appeared normal.

We also investigated a number of other donor-recipient combinations. We were unable to transfer diabetes to partially inbred BB/W rats in which there was a low incidence of the disease (*21*). Transfer to immunologically deficient athymic nude mice was also unsuccessful. Furthermore, to date we have been unable to transfer diabetes using diabetes-prone donors prior to the onset of hyperglycemia.

Further experiments must be conducted to determine the precise role of Con A in activating cells so that they transfer insulitis and diabetes, and to identify the specific cell type or types responsible for transfer. The question of whether transferred cells become localized in the pancreatic islets and whether there is recruitment of host cells in the resultant insulitis and β cell necrosis must also be examined. The model described in this report may furnish a means for delineating the β cell antigen against which presumptive pancreatic autoreactive T cells are directed in the spontaneously diabetic BB rat.

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16. Experimental allergic encephalomyelitis is an inflammation of the central nervous system produced in susceptible strains of mice or rats by injection of myelin basic protein. Passive transfer of the clinical disease is possible with the use of $10^8$ freshly isolated lymph node cells but not with freshly isolated spleen cells. As few as $2 \times 10^7$ spleen cells that have been cultured with Con A for 3 days are capable of transferring the disease.
17. Spleen and pancreatic plus cervical node cells were prepared by teasing tissue apart. The cells were cultured in RPMI-1640 medium containing Con A (5 μg/ml), 10 percent heat-inactivated fetal calf serum, 5 m*M* L-glutamine, $5 \times 10^{-5}$ *M* β-mercaptoethanol, and sodium penicillin (400 U/ml). Spleen and node cells from individual animals were cultured separately for 3 days in flasks containing 150 ml and 100 ml of medium, respectively. Cells were collected, washed, resuspended in 1 to 2 ml of serum-free RPMI-1640 medium, and injected into the tail vein. Recipients received either spleen cells ($50 \times 10^6$ to $100 \times 10^6$), node cells ($5 \times 10^6$ to $20 \times 10^6$), or a combination of the two ($55 \times 10^6$ to $120 \times 10^6$).
18. BB/W designates Bio Breeding/Worcester rat. Donors with acute diabetes and diabetes-prone recipients were produced by random matings between diabetic males and nondiabetic females. Male and female rats with acute diabetes, 56 to 115 days old, were used within 4 days of the onset of hyperglycemia. The term "diabetes-prone" refers to rats that may eventually become hyperglycemic.
19. Although our first experiments suggested that a combination of spleen and node cells produced more rapid onset of disease than spleen cells alone, this was not borne out in subsequent experiments. Lymph node cells alone from donors with acute diabetes failed to produce hyperglycemia in the young diabetes-prone recipients ($N = 7$). However, the number of node cells harvested from each of the animals with acute diabetes was only 10 to 20 percent of the number of spleen cells.
20. Tissue was fixed in Bouin's solution and embedded in paraffin, and sections were stained with hematoxylin and eosin.
21. This low-incidence line, designated VB, was produced by ten generations of inbreeding of nondiabetic BB/W rats. There have been no diabetic VB animals for the past three generations.
22. We thank K. Bernard for technical assistance. This work was supported in part by NIH grants AM-30846 and AM-25306. S.K. is the recipient of a Juvenile Diabetes Foundation postdoctoral fellowship.

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## Monocyte Chemotaxis: Stimulation by Specific Exosite Region in Thrombin

Abstract. *Human α-thrombin is a potent chemoattractant for human monocytes, with optimum activity occurring at about 10 nanomoles per liter. A variety of thrombins that were chemically modified to alter procoagulant or esterolytic functions showed a similar optimum activity, but complexes of prothrombin or α-thrombin with either antithrombin III or hirudin did not. These findings indicate that the regions in thrombin responsible for monocyte chemotaxis are proximate to those involved in certain protein recognition interactions of α-thrombin (for example, hirudin binding) but are distinct from the catalytic site and from certain exosites required for clotting.*

Thrombin (E.C. 3.4.21.5) is generated from its circulating zymogen, prothrombin, during blood coagulation and, once activated, plays multiple roles in hemostasis (*1*). In addition to clotting fibrinogen and enzymatically activating other parts of the plasma-clotting system (for example, factors V, VIII, and XIII), thrombin stimulates the aggregation of release of platelets (*2*), various endothelial cell functions (*3*), smooth muscle contraction (*4*), and mitogenesis (*5*). We reported that thrombin, at concentrations consistent with those generated in vivo (*6*), also elicits a chemotactic response from human peripheral blood monocytes and that this activity does not require a catalytically functional enzyme (*7*).

A variety of thrombins with modifications of the catalytic site and exosite were tested in an effort to identify the domain or domains in thrombin that are responsible for stimulating monocyte chemotaxis. Chemotaxis was assessed in Boyden-type chambers by the double filter method (*7, 8*). In brief, mononuclear cells were isolated from peripheral blood (*9*) drawn from ten healthy donors and transferred in equal portions into the upper compartment of the chemotaxis chambers. Native or modified thrombins were added to the upper compartment, lower compartment, or both compartments. After 2 hours of incubation at 37°C, the pairs of filters were removed, stained, mounted on slides, and scored microscopically for the number of mono-

cytes that had migrated into the area between the two filters. These results were then corrected for the random, unstimulated movement of cells and were compared with the response recorded in the presence of a known monocyte chemoattractant, the formylated peptide fMet-Leu-Phe (*10, 11*). Results were expressed as the mean of three trials, and each experiment (Table 1) was repeated at least twice with cells from different donors.

The results show that alteration of the catalytic site or certain procoagulant exosites had little effect on chemotactic activity (Table 1). Indeed, inactivation of the thrombin-active serine with either the $MeSO_2$ group (7, Table 1) or the larger, more sterically hindering $iPr_2P$ group (6, Table 1) elicited an even greater chemotactic response, as judged by the optimal dose, than did native α-thrombin. A similar enhancement was noted in thrombin reacted with the exosite affinity label, D-Phe-Pro-Arg chloromethylketone (9, Table 1).

The possibility that the differences in chemotactic stimulation between α-thrombin and the modified forms was due to interactions with different receptor subsets was examined by competition studies (Fig. 1). Equimolar concentrations of α-thrombin (3, Table 1), $iPr_2P$–α-thrombin (6, Table 1), or the chemotactic peptide fMet-Leu-Phe (1, Table 1) were placed in the upper compartment of the Boyden chamber with α-thrombin in the corresponding lower compartment. The data show that although net cell movement did not change in the case of the fMet-Leu-Phe and α-thrombin combination, chemotaxis was inhibited when the upper compartment contained either $iPr_2P$-thrombin or α-thrombin. These observations indicate that $iPr_2P$- and α-thrombins compete for the same binding site or receptor on the monocyte cell membrane, whereas chemotaxis induced by thrombin and fMet-Leu-Phe are mediated by different receptors. Enhanced chemotaxis can there-

Table 1. Chemotactic response of human monocytes to active-site modified thrombins. Human prothrombin complex and α-thrombin were prepared as previously described (*14, 15*). Active-site modified forms were derivatives of α-thrombin. A molecular weight of ~ 36,500 is assumed for all thrombin forms. Isolation of peripheral blood monocytes (*9*) and cell migration assays in Boyden filter chambers were carried out according to established procedures (*8*). Abbreviation: hPF, high power field.

| Chemotaxin | Description | Characterization* | Mobility (cells/hPF) | Optimal dose (*M*) |
|---|---|---|---|---|
| | | *No modification* | | |
| 1. fMet-Leu-Phe (*10*) | Control chemotaxin | | 110 ± 8 | $10^{-8}$ |
| 2. Prothrombin complex (*14, 15*) | Barium citrate eluate > 90 percent prothrombin† | < 0.7 U/mg | 10 ± 1.5‡ | $> 10^{-6}$ |
| 3. α-thrombin (*14, 15*) | Enzyme form with high procoagulant and esterolytic activities | 94.4 percent α-, 2.8 percent β-, and 2.9 percent γ-thrombins; 91.7 percent active enzyme; 3,975 U/mg | 120 ± 10 | $10^{-8}$ |
| | | *Fibrin exosite* | | |
| 4. $NO_2$–α-thrombin (*13, 16*) | ~ 4 nitrotyrosines per mole; procoagulant activity reduced relative to esterolytic activity | 57.3 percent active enzyme; 34.3 U/mg | 120 ± 12 | $10^{-7}$ |
| 5. γ-thrombin (*15, 17*) | β chain proteolytically modified; absent procoagulant activity with retention of esterolytic activity | 0.0 percent α-, 15.7 percent β-, and 84.3 percent γ-thrombin 80 percent active enzyme; 1.74 U/mg | 142 ± 8 | $10^{-7}$ |
| | | *Catalytic site* | | |
| 6. $iPr_2P$–α-thrombin (*16*) | Catalytically inactivated with $iPr_2P$-conjugated active-site serine | < 0.1 percent active enzyme; < 0.5 U/mg | 170 ± 9 | $10^{-10}$ |
| 7. $MeSO_2$–α-thrombin (*16*) | Catalytically inactivated with $MeSO_2$-conjugated active-site serine | < 0.1 percent active enzyme; < 0.5 U/mg | 90 ± 8 | $10^{-10}$ |
| 8. Tos-Lys-$CH_2$–α-thrombin (*18*) | Catalytically inactivated α-thrombin with Tos-Lys-$CH_2$–conjugated active-site histidine | 8.0 percent active enzyme; 1.04 U/mg | 80 ± 9 | $10^{-7}$ |
| | | *Fibrinopeptide exosite* | | |
| 9. D-Phe-Pro-Arg-$CH_2$–α-thrombin (*19*) | Catalytically inactivated fibrinopeptide exosite affinity label | 0.0 percent active enzyme; < 0.1 U/mg | 130 ± 12 | $10^{-10}$ |
| 10. *m*CP(PBA)–α-thrombin (*17*) | Catalytically inactivated fibrinopeptide exosite affinity label | < 0.0 percent active enzyme; < 0.1 U/mg | > 200 | $10^{-8}$ |
| | | *Inhibitor complex* | | |
| 11. Hirudin–α-thrombin complex (*13*) | Small (~ 7,200 daltons) peptide inhibitor forming high-affinity ($K_i$ = ~ 63 p*M*) noncovalent complex, not requiring active site | Complex formed in situ with excess inhibitor two times that of enzyme | 9 ± 3‡ | $> 10^{-6}$ |
| 12. AT III–α-thrombin complex (*20*) | Large (~ 58,000 daltons) protein inhibitor masking thrombin active site | Complex formed in situ in the presence of heparin (0.5 U/ml) with molar excess inhibitor ten times that of enzyme | 0‡ | $10^{-6}$ |

*Percentages of α-, β-, and γ-thrombins are determined from the distribution of [$^{14}$C]$iPr_2P$-F–labeled components in preparations following electrophoresis are 0.1 percent SDS-containing 10 percent cross-linked polyacrylamide gels. The percentage of active enzyme is established by active-site titration with NPGB and are expressed as the total fraction of esterolytically active enzyme within this reagent. Specific clotting activities are given in U.S. NIH clotting units per milligram of protein (*14, 15*). †Prothrombin complex proteins are passed through Amberlite CG-50 resin to remove trace amounts of thrombin or thrombin-like materials (*14, 15*). ‡Indicates response at the highest concentration chemotaxin tested.

fore be explained if it is assumed that large, sterically hindering groups at or near the catalytic site either (i) induce conformational changes in thrombin, rendering the chemotactic site more accessible to the monocyte receptor, or (ii) alter receptor occupancy by modifying ligand affinity.

Other than prothrombin, the only thrombin derivatives devoid of chemotactic activity are the complexes that thrombin forms with the inhibitors AT III and hirudin, which places the thrombin chemoattractant site in proximity to its catalytic and exosite regions. Unlike AT III, hirudin will form complexes with thrombin forms that are affinity-labeled at the fibrinopeptide exosite regions (9 and 10, Table 1) as well as with various catalytically inactivated α-thrombin forms (6 through 8, Table 1) (*12*). However, both γ-thrombin (5, Table 1) and $NO_2$–α-thrombin (4, Table 1), which show reduced fibrinogen clotting activity independent of synthetic substrate esterolytic activity, have significantly reduced affinity for hirudin (*12, 13*). This suggests that hirudin noncovalently binds to an exosite in thrombin removed from its catalytic site but vicinal to its fibrin recognition exosites—that is, to the regions in thrombin that are altered in γ- and $NO_2$–α-thrombin (Fig. 2). Our finding of a loss of chemotactic activity in complexes of thrombin and hirudin (Table 1) therefore suggests that the thrombin chemoattractant site and the hirudin binding site are closely associated.

Finally, it is apparent from our studies that thrombin is a potent chemotaxin and may therefore be an important physiological stimulator of inflammatory responses at sites of tissue injury. By use of modified thrombins, we were able to show that this potentially important function of thrombin is mediated through a specific region on the thrombin molecule that is independent of the sites required for esterolytic activity and fibrinogen recognition. Our data further suggests that AT III, a potent circulating thrombin inhibitor, may also modulate these thrombin-induced chemotactic responses in vivo.

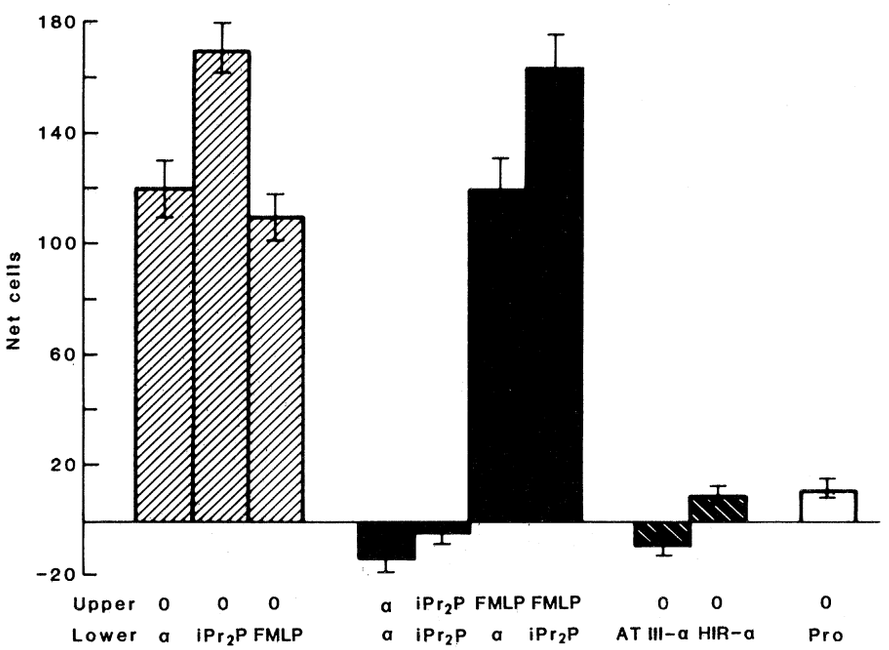


Fig. 1. Comparison of relative chemotactic activity of α-thrombin (α) with that of $iPr_2P$-α-thrombin (*$iPr_2P$*), fMet-Leu-Phe (*FMLP*), and prothrombin complex (*Pro*). The activity of α-thrombin complexes with the inhibitors AT III (*AT III*-α) and hirudin (*HIR*-α) is also shown. Control experiments showed that neither inhibitor affected monocyte movement stimulated by the formylated peptide (data not shown). The doses tested produced optimal cell migration (see Table 1). Data represent the mean of three trials; error bars represent standard error of the mean. Results were essentially identical in three separate experiments.

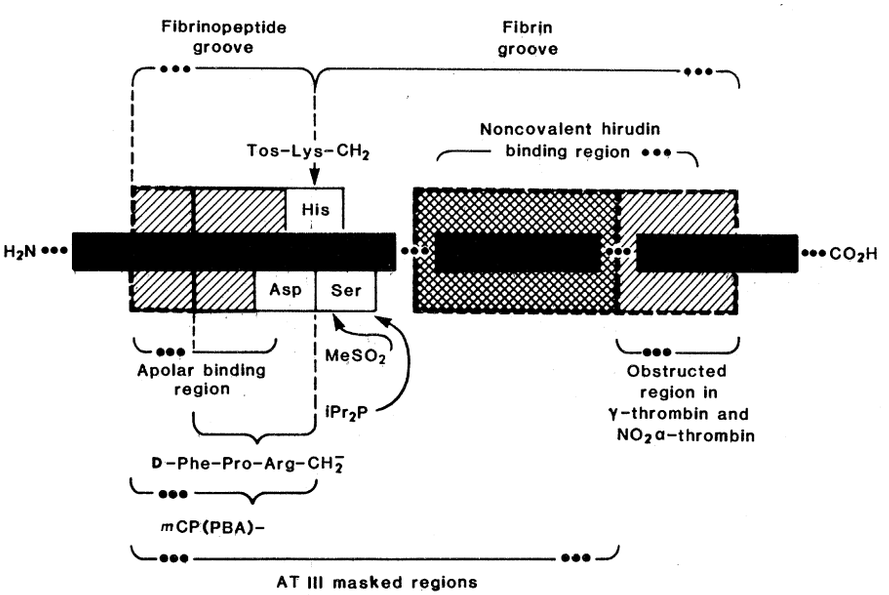


Fig. 2. A depiction of the α-thrombin active site showing specific amino acid residues or regions modified in the various enzyme forms. The catalytic site consists of the functional triad [histidine (*His*), aspartic acid (*Asp*), and serine (*Ser*)]with the fibrinopeptide groove extending to to the left and the fibrin groove to the right. The relative locations of various active-site regions are shown.


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## A Direct and Active Influence of Gravity on the Behavior of a Marine Invertebrate Larva

Abstract. *Larvae of the bryozoans* Bugula neritina *and* Bugula stolonifera *exhibit an apparent negative geotaxis under conditions of darkness and constant temperature. This behavior cannot be accounted for by buoyancy since the larvae are negatively bouyant, nor is it a consequence of gradients in the partial pressures of dissolved gases since the response occurs under conditions where the gradient is reversed or when experiments are conducted in chambers with interfaces of only glass and water. Pressure bomb experiments indicate that the behavior is not a barokinesis. Centrifuge experiments, however, showed that larvae of* Bugula stolonifera *orient directly and actively to gravity, while those of* Bugula neritina *have some other measure of geographic up. Since bryozoan larvae lack statocysts, the sensory apparatus mediating the gravity response in* Bugula stolonifera *is still unknown.*

The vertical distributions of planktonic organisms and the settlement patterns of larvae of benthic organisms are determined by various biological and physical environmental cues. Responses to the perception of geographic up and down, generally referred to as geotaxes (*1*), are commonly reported behaviors among planktonic marine invertebrate larvae. The larvae of some marine invertebrates possess either statocysts or statocytes that are thought to function in the detection of gravity (*2*). An apparent geotaxis has been observed both in larvae that are known to have gravity receptors and those that do not, but there is no evidence that the movement is accomplished through an active, direct response to gravity (*3*). Bryozoan larvae lack statocysts (*4*), yet some exhibit an apparent negative geotaxis prior to settlement (*5*). Explanations that may account for this behavior include (i) orientation to the geomagnetic field; (ii) response to a partial pressure gradient of dissolved gases; (iii) positive buoyancy, a passive direct effect of gravity; (iv) high barokinesis, an active indirect effect of gravity; and (v) orientation to a gravitational force, an active direct response to gravity. Two or more of these alternatives acting in concert may account for an observed geotactic behavior. We report that the negative geotaxis of larvae of the bryozoan *Bugula stolonifera* is an active, direct, true gravity response. Although the mechanism of gravity reception is not known, neither positive buoyancy nor high barokinesis can account for the response.

Under laboratory conditions, an apparent negative geotaxis occurs in larvae of the cellularioid cheilostome bryozoans *Bugula neritina* and *B. stolonifera*. We analyzed responses of larvae of both species to four environmental cues that might explain this behavior (*6*).

We did not test for the influence of magnetic fields. It seems unlikely that species such as *B. neritina* and *B. stolonifera*, with wide latitudinal distributions, including in the case of *B. neritina* a distribution that spans the magnetic equator, could effectively use geomagnetic cues to control vertical distribution unless different populations orient at radically different angles to the field.

Orientation along a gradient in partial pressures of gases dissolved in the seawater was discounted on the basis of qualitative observations. First, free-swimming larvae of *B. neritina* (*7*) and *B. stolonifera* distributed themselves near the top of a vessel kept in darkness, even when it was sealed in such a way that gases could only enter from the bottom. Second, larvae of both species settled near the top of a vertical column of water that was kept in darkness, at constant temperature, and sealed to present an equivalent glass-water interface at both ends of the chamber.

The observed distribution of *Bugula* larvae might result from the larvae being positively buoyant. Buoyancy is known to be a contributing factor in determining the vertical orientation and distribution of certain aquatic organisms (*8*). However, *Bugula* larvae were found to be negatively buoyant when swimming was arrested by lowering the temperature or by adding 1 percent Formalin or 1 percent sodium azide to the seawater.

To test for a barokinetic response of *Bugula* larvae, we used a pressure bomb in conjunction with a photocell counting device. The experimental device consisted of a rectangular Plexiglas chamber (inside dimensions, 5 by 3 by 3 cm) connected at the inflow to a filtered compressed air supply and at the outflow to a reservoir of distilled water at the base of a 1-m graduated column. The four vertical faces of the chamber were blackened except for a pinhole aperture 1 cm from the floor at the center of each of the two ends through which passed a beam from a fiber-optic light source. Opposite the light was a silicon phototransistor (Radio Shack 276-130) whose current output through a resistor was measured by a single-channel penwriter. Each time a larva crossed the light beam, the penwriter recorded a potential drop