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Thrombin Generation in a Reconstituted System: A Comment

In the article of Butenas et al. (1) like in a number of similar publications of these authors on the same subject (2–6), the authors observe a relatively good agreement between the behavior of a model system of the thrombin generating mechanism, reconstructed from a set of purified proteins, and the data obtained from clotting whole blood.

This is an extremely interesting result because it strongly suggests that the model system realistically represents the essence of the physiological mechanism. If this is true, then the data from the model system should automatically also have to be in accordance with observations on thrombin generation in platelet poor and in platelet rich plasma (PPP/PRP).

Comparison with data obtained by others in PPP and PRP therefore should be an essential element in the discussion. In the last decade much new information on thrombin generation in these systems has been obtained by various groups [e.g. (7–12), see (13) for more examples]. However, in the articles of Butenas and coauthors, all references to recent work on thrombin generation in PPP and PRP are carefully avoided.

If we do attempt such a comparison, several discrepancies appear:

The most obvious is that thrombin generation in the reconstituted system with added procoagulant phospholipids (PS/PC) is about three times as high as that in normal plasma. Calculation of prothrombin conversion and thrombin decay from the curves of fig. 1 from (1) using the figures for the concentrations previously published (4) and the algorithm of (14), shows that thrombin is inactivated at about half of the rate observed in fresh normal citrated plasma [i.e. in the absence of heparin, see (15)], despite the presence of purified antithrombin at a concentration that is about 1.4 times higher than the physiological one (15, 16). Physiological concentrations of purified proteins obviously do not guarantee physiological activities. Calculation of prothrombin conversion also reveals that the prothrombin present must be (almost) entirely converted into thrombin. In whole blood, according to the same authors (17) 25% of prothrombin remains unconverted; in our experience in PPP and PRP about 10% residual prothrombin remains. Under near to physiological conditions prothrombinase activity therefore stops before its substrate, i.e. prothrombin, is exhausted. In the reconstituted system it does not, despite the presence of TFPI, protein C and thrombomodulin. In normal PPP, even without added TM, the APC system effectively limits the amount of prothrombin converted, as can be judged from the fact that more thrombin is generated in APC resistant plasma than in normal plasma (18). Apparently a phenomenon of recognised pathophysiological importance is not reflected in the reconstituted system. This might, for example, be caused by biological activity of the proteins added and/or the absence of phosphatidyl etha-

nolamine (PE) that is known to be required for protein C activation by TM (19). Platelets do contain PE, which may (partly) explain why thrombin generation in the reconstituted system with platelets is so much lower than with added PCPS (Butenas *op. cit.* figs 1 and 2).

When comparing observations made in fresh PRP with those in the reconstituted system with isolated platelets it strikes that thrombin formation occurs almost immediately. In fresh PRP (7), and in whole blood (8), it is characteristically preceded by a lag time of several minutes, also observed in minimally altered whole blood by the authors et al. (17). During this lag, thrombin mediated reinforcement loops switch on (7, 20) and in this respect PRP is fundamentally different from PPP to which procoagulant phospholipids and TF are added. The mechanism involves platelet receptors GPIIb/IIIa (21) and GPIb in combination with von Willebrand Factor (vWF) and fibrin (22). Its physiological importance is illustrated by the fact that in fresh PRP, thrombin generation is seriously impaired in von Willebrand disease (independent of the concomitant lack of factor VIII) as well as in congenital a- and hypofibrinemia (13, 22). Due comparison to pre-existent literature would have learned that the lag-time tends to disappear when isolated platelet are added back to plasma [cf. figures 5 and 3 in (21)] and gradually disappears by the addition of traces of thrombin or of TF concentrations equal to or higher than that in human brain thromboplastin diluted 1:240 (7), a dilution that roughly corresponds to the 15 pM used by Butenas et al. Probably slight platelet damage during isolation, or TF concentrations above a certain very low level, generates sufficient thrombin to short-circuit one or more of the physiological mechanisms that trigger platelet procoagulant activity. This explains why the activity of the reconstituted system has a superficial resemblance to normal function even in the absence of fibrinogen and von Willebrand factor.

It is known for over half of a century – and reconfirmed in the present article – that the platelet number does not influence the clotting time of blood unless below ~10% of normal e.g. (23). Extensive experiments on the relation between platelet number and thrombin generation in fresh or reconstituted PRP exist in the literature (21, 24). From these results one can deduce that only a small amount of the platelet procoagulant material is required for the onset of thrombin generation. In our experience, even in citrated or ACD PRP, the minimal platelet damage that suffices to facilitate thrombin generation already is observable within two hours after venapuncture. A fortiori, in thrombin generation experiments in which isolated platelets are used, one has to reckon with such damage and a control that determines how much procoagulant phospholipids are present in the unactivated platelet preparation is obligatory. In summary: Maintenance of the claim that the results in the model system of Butenas et al. represent more than a superficial similarity to a physiologically relevant situation would require careful comparison to the body of knowledge on thrombin generation in PPP, PRP and blood as built up in recent years by other groups, as well as control experiments that show 1: Normal activity of the thrombin inhibiting system; 2: Normal activity of the TM-protein C system; 3: Normal activity of the TFPI system; 4: An estimate of procoagulant phospholipids present in the platelet preparation used and 5: Dependency of the system on vWF and fibrinogen.

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Thrombin Generation in a Reconstituted System: A Reply

Dear Sir,

The aim of our laboratory is to provide a biologically relevant, quantitative, mechanistic description of the blood coagulation process. Toward this end, we have developed two biological systems, which involve non-anticoagulated, non-chilled whole human blood: a) Phlebotomy blood contact pathway suppressed by corn trypsin inhibitor

is initiated to clot *in vitro* by a fixed concentration of a chemically defined tissue factor (TF) preparation (1-6); b) Blood exuding from a microvascular wound is evaluated for composition by sequential sampling (7, 8).

We attempt to emulate these biological models using mixtures of highly purified reaction components and examine the individual and combined mechanistic contributions of substrates, intermediates and products in the reaction (6, 9-17). Finally, we simulate these reactions using computer-based models employing defined chemical parameters for the individual reactions of the blood coagulation process and its regulation (18, 19). Each person has an individual phenotype which may or may not represent mean population values either quantitatively or qualitatively. Therefore we use convergence of results from biological,

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