

Studies, with a luminogenic peptide substrate, on blood coagulation Factor X/Xa produced by mouse peritoneal macrophages

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The formation and secretion of coagulation Factor X/Xa by mouse peritoneal macrophages was studied with a luminogenic peptide substrate (S-2613; t-butyloxycarbonylisoleucylglutamyl- γ -piperidylglycylarginylisoluminol). Amidolysis was quantified by measuring the light emitted during oxidation of isoluminol, released by Factor Xa. A lower detection limit of about 0.5 ng of Factor Xa was established; the assay was linear with enzyme concentration up to at least 100 ng/ml. Factor X was determined after treatment with the Factor X-activating component of Russell's-viper (*Vipera russelli*) venom. Macrophages, cultured in the absence of serum, released Factor X/Xa into the culture medium. The concentration of coagulation enzyme in the medium increased in an essentially linear fashion over a period of at least 3 days, at a rate corresponding to 6–8 ng produced/24 h per 10^6 cells. The ratio of Factor Xa/X + Xa varied from about 60 to 100%, showing that activation of Factor X to Xa is not prerequisite to release of the enzyme from the cells. Factor Xa activity was suppressed in the presence of warfarin [3-(α -acetylbenzyl)-4-hydroxycoumarin; 12.5 μ g/ml of medium], but could be restored by adding vitamin K (0.1 μ g/ml) along with the warfarin. Cultures to which Sepharose beads containing covalently bound anti-(Factor X) antibodies had been added showed decreased amounts of free Factor X/Xa in the culture medium. The missing activity could be demonstrated by incubating the recovered conjugate with the substrate peptide S-2613. Factor Xa produced by the macrophages was efficiently inactivated by heparin in the presence of antithrombin, heparin with high affinity for antithrombin being more effective than the corresponding low-affinity species.

The coagulation of blood involves the sequential activation of a series of serine proteinases, culminating in the conversion of fibrinogen, a soluble plasma protein, into insoluble fibrin. The proteinases are synthesized in the liver and are generally contained within the circulating blood. However, the so-called extrinsic pathway of coagulation is initiated by an extravascular factor, thromboplastin or tissue factor [for a review see Jackson & Nemerson (1980)]. The procoagulant properties of macrophages have been ascribed to the ability of these cells to produce thromboplastin and to present this initiator

molecule at the cell surface (Schwartz *et al.*, 1981; B. Østerud and E. Bjørklid, unpublished work), after exposure to certain stimuli [for references see Geczy & Hopper (1981)]. The expression of thromboplastin activity depends on an interaction between the macrophages and T-lymphocytes (Østerud *et al.*, 1981) that apparently involves transfer of a stimulatory signal from the activated lymphocytes to the macrophages (Levy & Edgington, 1980; Geczy & Hopper, 1981). The thromboplastin thus formed and/or exposed as a consequence of an inflammatory reaction may initiate the local deposition of fibrin, provided that the appropriate coagulation proteinases and fibrinogen are available. Increased vascular permeability that accompanies inflamma-

Abbreviations used: S-2613, t-butyloxycarbonylisoleucylglutamyl- γ -piperidylglycylarginylisoluminol; RVV-X, Factor X-activating component of Russell's-viper venom.

tory reactions allows leakage of such components into the interstitial spaces.

Novel aspects on the relation of macrophages to the coagulation system emerged with the finding that mouse peritoneal macrophages produce not only thromboplastin but also the vitamin K-dependent coagulation Factors VII, IX and X and prothrombin, as well as Factor V (Østerud *et al.*, 1980, 1981). Taken together, these proteins constitute all the components involved in the extrinsic coagulation pathway. Secretion of coagulation enzymes by macrophages *in vitro* was initially detected by spectrophotometric determination of *p*-nitroaniline released from chromogenic peptide substrates added to culture media (Seljelid *et al.*, 1980), and subsequently confirmed by using specific one-stage clotting assays (Østerud *et al.*, 1980). However, neither of these assays could be adopted to a more detailed characterization of the coagulation factors; the former system lacked sensitivity, the latter accuracy. In the present investigation the secretion and some properties of Factor X/Xa from mouse peritoneal macrophages were studied by using a highly sensitive assay based on a luminogenic peptide substrate.

Experimental

Materials

The luminogenic peptide substrate S-2613 was kindly donated by Dr. Leif Aurell, AB Kabi Peptide Research, Mölndal, Sweden. Stock solutions (0.1 mM) were stored at -70°C .

Coagulation Factor X was isolated from human plasma (Østerud & Rapaport, 1977); Factor Xa was obtained by activating Factor X with Russell's-viper (*Vipera russelli*) venom (Lindquist *et al.*, 1978). The Factor X-activating component of Russell's-viper venom (RVV-X), purified as described by Kiesel *et al.* (1976), and bovine antithrombin, were kindly given by Dr. I. Björk of one of our institutions (Uppsala). The heparin preparations used were as described by Bengtsson *et al.* (1980). β 1,3-Glucan from *Saccharomyces cerevisiae* (baker's yeast) was isolated as described by Manners *et al.* (1973). Goat anti-(human-Factor X) antibodies (active also against Factor Xa) were prepared (Seligsohn *et al.*, 1979) and covalently bound to Sepharose 2B (March *et al.*, 1974).

Haemin chloride (Fluka AG, Buchs, Switzerland), human serum albumin and transferrin (Sigma Co., St. Louis, MO, U.S.A.), warfarin [3-(α -acetoxybenzyl)-4-hydroxycoumarin (Nycos; Nyegaard and Co., AS, Oslo, Norway)] and vitamin K (Merck, Sharp and Dohme, West Point, PA, U.S.A) were obtained from the commercial sources indicated.

Methods

Macrophages from hybrid C_3D_2 ($\text{C3H}/\text{Tif}\varphi \times \text{DPB}/2\sigma$) mice were collected by peritoneal washing with phosphate-buffered saline (Cohn & Benson, 1965). Cells were seeded in either 16 mm wells in Costar plastic plates (Costar, Broadway, Cambridge, MA, U.S.A.; 0.7×10^6 cells/well in 0.5 ml of medium) or in Falcon plastic dishes (Falcon, Oxnard, CA, U.S.A.; 3×10^6 cells/well in 1 ml of medium). The cells were cultured in Medium 199 with Earle's salts (Gibco Bio-cult, Paisley, Scotland, U.K.) supplemented with human albumin (0.4 mg/ml), human transferrin (10 $\mu\text{g}/\text{ml}$), vitamin K (0.1 $\mu\text{g}/\text{ml}$, unless otherwise stated); and 100 i.u. of penicillin and streptomycin/ml. Human albumin and transferrin had been treated as described by Iscove & Melchers (1978). Incubations were conducted in a humidified atmosphere of CO_2/air (1:19) at 37°C . Non-adherent cells were washed away after 2 h in culture. The media were routinely collected after 24 h of incubation, centrifuged at 500 g for 10 min and stored at -70°C until analysed. In some experiments, anti-(Factor X)-Sepharose conjugate was added to the culture as described in the Results section.

For assays of Factor Xa, 100 μl of culture medium was mixed with 50 μl of 0.05 M-Tris/HCl, pH 8.0, containing 0.15 M-NaCl and 30 mM- CaCl_2 , in plastic Ellerman tubes. After the addition of 50 μl of 0.1 mM-S-2613, the reaction mixtures were incubated in a water bath at 37°C for 15 min. Inactive Factor X was determined in parallel incubations containing 50 ng of RVV-X; these samples were preincubated for 5 min before the addition of S-2613 in order to convert any Factor X present into Xa. After the 15 min reaction period, amidolysis was interrupted by adding 20 μl of 20% acetic acid. The liberated isoluminol was determined by chemiluminescence measurements, using a Lumac Biocounter M2010 luminescence photometer. From each incubation mixture samples of 50 μl were transferred to cuvettes containing 0.7 ml of 0.05 M-NaOH and 25 μl of 25 μM -haemin. Immediately after mixing, the cuvettes were placed in the counting chamber of the photometer and the luminescent reaction (oxidation of isoluminol; see Roswell & White, 1978) was initiated by injecting 100 μl of 80 μM - H_2O_2 . The light emitted was monitored over a 30 s period, during which the reaction was essentially completed. Assays were performed in triplicate with an error (s.d./mean) of 5%.

In order to determine the effects of heparin and antithrombin on Factor Xa activity these components were added to the incubation mixtures for a 3 min preincubation period before the addition of S-2613. Polybrene (*NNN'N'*-tetramethylhexane-1,6-diamine polymer with 1,3-dibromopropane; 2 μg)

was added along with the substrate to bind free heparin.

Results

Assay of Factor X/Xa

Luminogenic peptides with isoluminol as chemiluminescent leaving groups have been previously employed in studies on serine proteinases such as trypsin, chymotrypsin and thrombin (Branchini *et al.*, 1980). In the cited study, background light emission of unchanged substrate was considered a serious limitation to the potentially greater sensitivity of luminescence over absorbance- or fluorescence-based methods, and was therefore eliminated by use of immobilized substrates that could be removed by centrifugation after incubation with the enzymes. In the present work the background emission, although considerable (see the legend to Fig. 1), did not interfere to any significant extent with the determination of Factor X/Xa, in the concentrations relevant to the test system studied. Under the conditions of the assay the release of isoluminol by Factor Xa from the substrate, S-2613, remained linear with time over a period of 60 min (result not shown). After a 15 min incubation period, the amount of free isoluminol was proportional to enzyme concentration, up to at least 100 ng/ml (Fig. 1). Although the presence of RVV-X had no effect on the reaction with Factor Xa (Fig. 1a), it was a prerequisite to activity with Factor X (Fig. 1b). When mixtures of Factor X and Factor Xa are assayed, the former component is thus determined by the difference in luminescence obtained with and

without RVV-X respectively in the incubations. The lower limit of detection for Factor Xa corresponded to about 5 ng/ml (0.5 ng/0.1 ml of sample). The luminogenic substrate thus affords an approx. 100-fold increase in sensitivity compared with chromogenic substrates.

Secretion of Factor X/Xa by macrophages

Medium collected from a 24 h culture of mouse peritoneal macrophages catalysed the cleavage of the luminogenic peptide substrate, S-2613 (Fig. 2). Similar activity was observed regardless of whether vitamin K had been added to the cultures or not. However, amidolysis was greatly decreased in cultures lacking exogenous vitamin K and to which warfarin had been added (cf. Østerud *et al.*, 1980). This inhibitory effect of warfarin was largely eliminated by concomitant administration of vitamin K. These results indicate the formation and secretion of a vitamin K-dependent proteinase, presumably Factor Xa, by the macrophages. Although the supply of endogenous vitamin K would seem to be sufficient to sustain production of this proteinase for at least 24 h, exogenous vitamin was routinely added to the cultures as described in the Experimental section.

The specificity of the assay was further ascertained by adsorbing the factor secreted from the cells

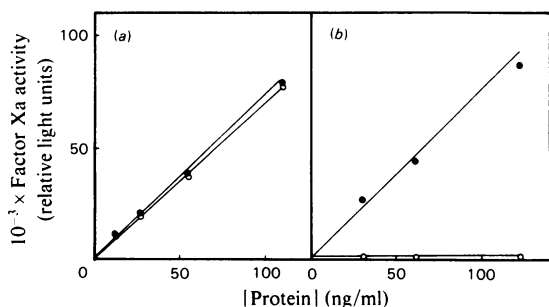


Fig. 1. Assays of Factor Xa (a) and of Factor X (b) with the luminogenic substrate S-2613

The assays were performed, as described in the Experimental section, in the presence (●) or absence (○) of RVV-X. The values obtained for blank incubations lacking Factor X/Xa (5640 ± 280 relative light units) have been subtracted. The specific activities of the enzyme preparations used as standards were 80 units/mg of protein for Factor X and 90 units/mg for Factor Xa.

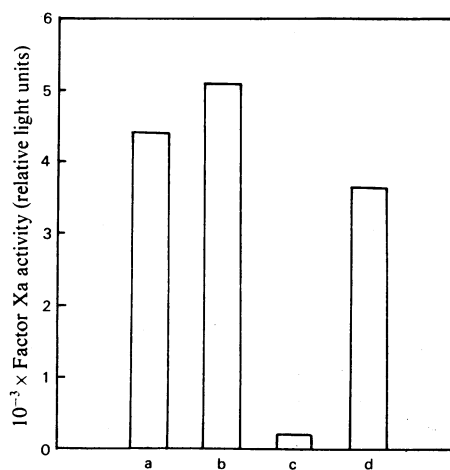


Fig. 2. Effects of warfarin and vitamin K on Factor Xa in macrophage cultures

Macrophage cultures were established in Costar plastic plates as described in the Experimental section. The culture media contained (per ml): (a) $0.1 \mu\text{g}$ of vitamin K (standard incubation conditions); (b) no vitamin K; (c) $12.5 \mu\text{g}$ of warfarin, no vitamin K; and (d) $12.5 \mu\text{g}$ of warfarin and $0.1 \mu\text{g}$ of vitamin K. After 24 h, 0.1 ml of medium was assayed for Factor Xa (without RVV-X).

to Sepharose beads containing covalently bound anti-(Factor X) antibodies (Fig. 3). Microscopic inspection of macrophage cultures containing such beads showed no apparent derangement of cellular morphology, nor any attachment of cells to the beads. The amounts of free Factor X/Xa remaining in the cultures varied inversely with the amounts of immobilized immunoglobulin present, to the extent of being completely sequestered from the medium. Similar results were obtained by incubating harvested culture medium with the immobilized antibodies at 37°C for 60 min, with continuous shaking, followed by centrifugation. Model experiments with human standard coagulation enzymes revealed that not only Factor Xa, but also Factor X, could be determined, after adsorption to the beads, by incubation with the luminogenic substrate S-2613; apparently, the effect of RVV-X on Factor X was not seriously impeded by the bound immunoglobulin molecules. In contrast, clotting assays for either of Factor X or Factor Xa were completely negative in the presence of the antibodies, free (Seligsohn *et al.*, 1979) or conjugated (B. Østerud, unpublished work). In macrophage cultures contain-

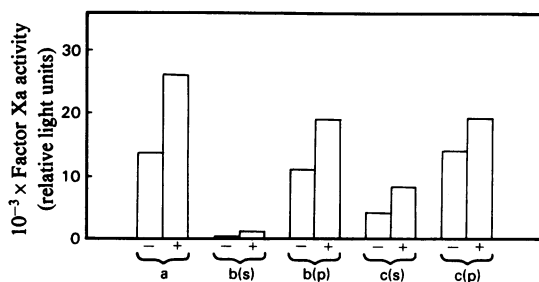


Fig. 3. Factor X/Xa in macrophage cultures containing immobilized anti-(Factor X) antibodies

Macrophage cultures were established in 35 mm Falcon plastic Petri dishes as described in the Experimental section. To cultures b and c were added 0.1 ml of suspensions containing anti-(Factor X)-Sepharose conjugate and medium, in the proportions (v/v) 1:1 and 1:3 respectively. Culture a served as control. After 24 h the cultures were agitated to suspend Sepharose beads and the media were harvested and centrifuged. Samples of 0.1 ml of the resulting supernatants [a, b(s) and c(s)] were assayed for Factor Xa activity, in the absence (-) or presence (+) of RVV-X (50 ng). The precipitates [b(p) and c(p)] were washed and suspended in 0.5 ml of medium; 0.1 ml portions were quickly withdrawn and analysed for Factor Xa activity, in the absence (-) or presence (+) of RVV-X (250 ng). During the 15 min incubation with S-2613 the samples were shaken in a water bath, and were then centrifuged to remove the Sepharose beads. All results are expressed corresponding to 0.1 ml of culture medium.

ing anti-(Factor X)-Sepharose beads, the total X/Xa activities associated with the antibodies were in fair agreement with those expected from a comparison with a control culture lacking antibodies against Factor X (Fig. 3). Furthermore, in experiments where the control culture showed only partial activation of Factor X into Factor Xa, a significant proportion of the material bound to the immobilized antibodies could be activated by treatment with RVV-X.

Cultured mouse peritoneal macrophages continuously release Factor X/Xa into the medium over a period of at least 3 days (Fig. 4). The amounts produced and secreted over a 24 h culture period would correspond to approx. 6–8 ng/10⁶ cells. In cultures maintained under standard conditions, the ratio of activated Factor Xa to native Factor X in the culture media varied from one experiment to another, in a seemingly non-systematic manner. With some cell preparations, such as that used in the experiment illustrated in Fig. 4(a), the native factor consistently accounted for a major proportion, up to 30–40%, of the total Factor X/Xa secreted into the medium. Activation of Factor X is thus not a prerequisite to its release from the macrophage. However, with other cell preparations, isolated under virtually identical conditions, treatment with RVV-X failed to increase the Xa activity of spent medium (not shown in the Figure). Nevertheless, a correlation of the X/Xa ratio to the functional state of the cells was clearly suggested by experiments in which cultures established from the same preparations of

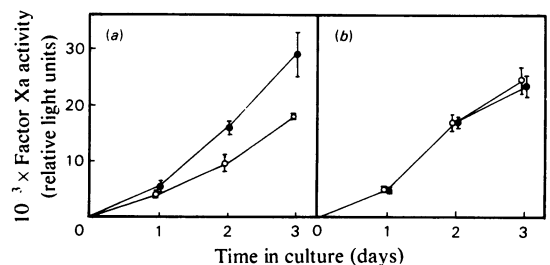


Fig. 4. Release of Factor X/Xa from mouse peritoneal macrophages *in vitro*

Macrophage cultures were established in Costar plastic plates (0.7×10^6 cells seeded/16 mm circular well containing 0.5 ml of medium) as described in the Experimental section. The cultures, all derived from the same pool of mouse peritoneal cells, were maintained either in the absence (a) or in the presence (b) of β 1,3-glucan from baker's yeast (40 μ g/culture). After the periods indicated, 0.1 ml samples of media from duplicate cultures were assayed for Factor Xa activity, with (●) or without (○) the addition of RVV-X.

peritoneal cells were maintained either in the absence (Fig. 4a) or in the presence (Fig. 4b) of β 1,3-glucan from baker's yeast. This polymer, which is known to 'activate' macrophages (Seljelid *et al.*, 1981), consistently induced complete activation of Factor X. It should be emphasized in this context that the variability with regard to the X/Xa ratio (observed in the absence of added glucan) generally applied to comparisons between different cell preparations, but not to individual cultures established from a single preparation.

Effect of heparin and antithrombin on Factor Xa produced by macrophages

The Factor Xa produced by macrophages was further studied with regard to the effects of antithrombin and heparin (Fig. 5). Under the conditions of the assay, antithrombin alone (1 μ g) decreased the Xa activity of spent culture medium by about 10%. Heparin with high affinity for antithrombin greatly potentiated the effect of the inhibitor, such that the addition of 0.1 μ g resulted in almost complete loss of activity. Significant inhibitory effect was observed with less than 1 ng of polysaccharide. Heparin with low affinity for antithrombin also potentiated the inhibition of Factor Xa, but was about one order of magnitude less potent than the high-affinity species. These findings conform to the general concept of

antithrombin-mediated inhibition of coagulation enzymes (Nordenman *et al.*, 1980). Furthermore, at higher heparin concentrations (1 μ g/incubation mixture), significant inhibition of Factor Xa was noted, even in the absence of added antithrombin, high-affinity heparin again being more potent than low-affinity heparin (Fig. 5). This observation, made in three separate experiments, is compatible with the occurrence of an endogenous antithrombin-like component secreted by the macrophages into the medium (see the so-called 'protease-nexin' described by Low *et al.*, 1981).

Discussion

Under the experimental conditions employed, the ability of mouse peritoneal macrophages to produce and secrete Factor X/Xa *in vitro* was a constant finding. Whether this property is typical of macrophages in general, including cells from other tissues of the mouse, or from other species, is at present unclear. It should be emphasized that although the 'procoagulant activity' detected in mononuclear phagocytes from various sources, including monocytes from blood, has generally been ascribed to the presence of tissue thromboplastin, i.e. an initiator of the extrinsic pathway of coagulation (Rickles & Rick, 1977; Prydz & Allison, 1978; Levy & Edgington, 1980; Geczy & Hopper, 1981; Levy *et al.*, 1981; Schwartz & Edgington, 1981), the methods used in those studies were not designed to detect the actual coagulation enzymes. Preliminary experiments in our laboratory indicate that human blood monocytes do not produce Factor X/Xa *in vitro*, at least not in short-term culture. This observation suggests that the ability to produce coagulation enzymes may be a feature of the differentiated macrophage.

The introduction of the luminogenic peptide substrate S-2613 has permitted quantitative assays of Factors X and Xa with a sensitivity and accuracy that permit analysis on a cellular level. The application of such assays to cultured mouse peritoneal macrophages demonstrated that, under the conditions employed, the factor released from the cells occurred largely in activated form. However, it was evident that Factor X may be secreted from the cells without concomitant activation to Factor Xa. These observations raise a number of questions regarding the mechanism of activation. Does activation take place before secretion into the medium, in the course of secretion, or extracellularly? Further, if activation is a cell-associated process, can once-secreted Factor X be recaptured by the cells and then activated? Release from the cells of exclusively non-activated Factor X, followed by activation, would presumably be reflected by a progressive decrease in the X/Xa ratio with increasing time in

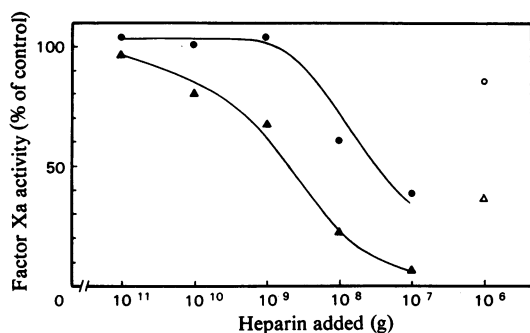
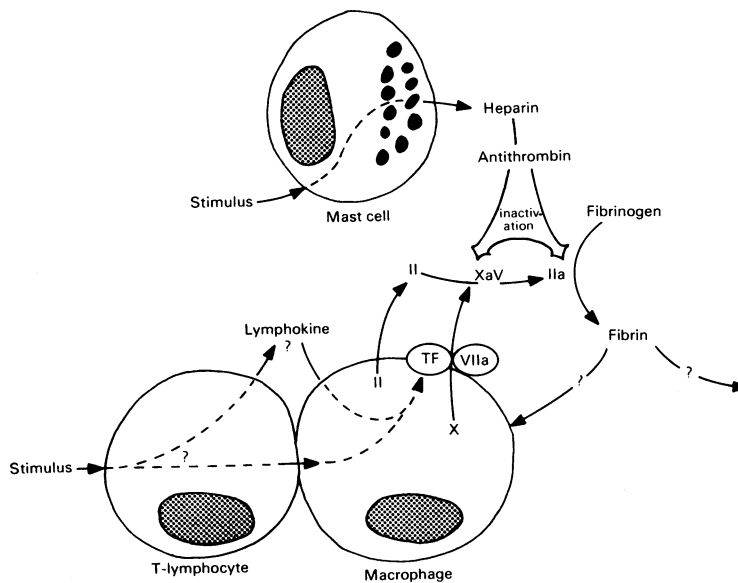


Fig. 5. *Effects of heparin and antithrombin on Factor Xa secreted by macrophages*

Samples of 0.1 ml medium from 24h macrophage cultures were incubated for 3 min at 37°C with 50 μ l of Tris/NaCl/CaCl₂ buffer (see the Experimental section), containing 1 μ g of antithrombin and heparin with high (▲) or with low (●) affinity for antithrombin, in the amounts indicated. The corresponding open symbols denote samples incubated in the absence of added antithrombin. The samples were then assayed for residual Factor Xa-activity as described in the Experimental section. The results are expressed as a percentage of the activity recorded after incubation with 1 μ g of antithrombin without added heparin; the antithrombin alone brought about a 9% decrease in activity.



Scheme 1. Scheme for the regulation of fibrin formation through interaction between lymphocytes, macrophages and mast cells

In response to certain stimuli (for instance, a bacterial lipopolysaccharide or an antigen-antibody complex), T-lymphocytes generate a signal that induces synthesis (or presentation at the cell surface) of tissue thromboplastin (TF) in macrophages. The signal is transmitted either via a soluble lymphokine or by direct cell-cell contact. Subsequent to thromboplastin formation, Factor X may become activated, leading ultimately to the conversion of fibrinogen into fibrin. All the coagulation factors involved (except fibrinogen) have been shown to be produced by macrophages. According to recent reports the fibrin may be deposited at the surface of the macrophage. Given the appropriate stimulus (for example, an anaphylatoxin or cross-linking of immunoglobulin E-receptors), mast cells may release heparin that will, together with antithrombin, interrupt the process by inhibiting Factor Xa and thrombin (IIa). For additional information, see the text.

culture. The results shown in Fig. 4(a) are not in accord with such a concept, but rather suggest that Factors X and Xa are both secreted as such without any further conversion of Factor X into Xa. However, more refined quantitative data are required to settle this matter.

Numerous observations have appeared in the literature linking the coagulation system to various types of inflammatory reactions involving the immune system (for references, see Levy & Edgington, 1980; Hopper *et al.*, 1981; Geczy & Hopper, 1981). Extravascular fibrin deposition is often a prominent feature of such reactions. Fairly recently, interaction between stimulated lymphocytes and macrophages was found to result in the deposition of fibrin on the macrophage cell surface and in changed functional properties of these cells (Hopper *et al.*, 1981; Geczy & Hopper, 1981). It seems reasonable to assume that the procoagulant properties displayed in this manner by the macrophages reflect not only the production of thromboplastin, but also the formation of the actual coagulation enzymes by the

same cells. The overall process, which may thus be regarded as an expression of an inflammatory reaction, is liable to regulation at various levels. Two potential regulatory mechanisms, with support in experimental data, are illustrated in Scheme 1. The formation of thromboplastin, presumably essential for the activation of Factor X, appears to be under control of lymphocytes; mechanisms based either on direct contact between lymphocytes and macrophages (Levy & Edgington, 1980), or on lymphokine action (Geczy & Hopper, 1981), have been postulated. An opposing effect is exerted by heparin released from mast cells; in the present study, heparin, together with antithrombin, was found to effectively inhibit Factor Xa produced by the macrophages.

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