Interference with Fibrin Stabilization by Polymyxin B Sulfate. (32109)

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One of the more recent and significant contributions to the understanding of the blood coagulation process is the evidence that fibrin undergoes a cross-linking or "stabilization" under the influence of a transpeptidating enzyme. The earlier work of Laki and Lorand(1) showed that clots formed by the action of thrombin upon purified fibrinogen in the presence of calcium ion were readily soluble in 5 M urea and in 1% monochloroacetic acid. Clots formed by recalcification of oxalated plasma were not soluble in these solvents. From this, it was inferred that the soluble gel was composed of fibrin which was not covalently cross-linked whereas the insoluble gel was a truly polymeric structure. More recently, Lorand(2) has reviewed the subject of the fibrin cross-linking process. It now appears widely held that thrombin and calcium ion are required for activation of a plasma protein found in the alpha-2 globulin fraction which has been referred to as fibrin stabilizing factor, FSF, Factor XIII, fibrinase and Laki-Lorand factor. Active FSF is considered to be a calcium ion requiring, transpeptidating enzyme which is responsible for the production of cross-linked or "stabilized fibrin".

The physiological importance of FSF has been pointed out in several clinical reports describing congenital absences of FSF activity which resulted in severe hemorrhagic diatheses. Nussbaum and Morse(3) have described diminished FSF activity in a number of patients with various diseases.

Lorand and Jacobsen(4) demonstrated that the cross-linking of fibrin can be inhibited by certain amines and by some compounds with carbonylamide functions. Morse and Nussbaum(5) showed mercurial diuretics to interfere with FSF activity in man. This study was initiated to determine the effects of the administration of certain nitrogenous antibiotics on plasma FSF activity.

Materials and methods. Fibrinogen solution containing 6 mg/ml protein in 0.3 M potassium chloride at pH 7.4 was prepared from bovine fibrinogen, Cohn fraction 1 (Nutritional Biochemicals Corp., 60% clottable protein) according to the method of Laki(6). This procedure yielded fibrinogen which was at least 80% clottable. Fibrinogen solutions were stored at −90°C until used. Solutions of bovine thrombin containing 25 units/ml (Parke-Davis, Topical) were freshly prepared in 1% calcium chloride solution. Cysteine solution (pH 7.4) was prepared immediately before use from cysteine hydrochloride (Fisher, reagent grade) by neutralization with sodium hydroxide. Platelet poor plasma was prepared by centrifugation after mixing 9 volumes of blood with 1 volume of 0.1 M sodium oxalate solution. Mongrel dogs of either sex were anesthetized with pentobarbital sodium (30 mg/kg, I.V.) and the femoral vein cannulated with polyethylene tubing for withdrawal of blood samples and the administration of drug. Polymyxin B sulfate ("Aerosporin", Burroughs Wellcome Co., 500,000 units equivalent to 50 mg) and other antibiotics were dissolved in isotonic sodium chloride solution and administered intravenously over a period of 5 minutes.

Plasma fibrin stabilizing factor (FSF, Factor XIII) activity was measured in dogs by the method of Lorand and Dickenman(7) as modified by Nussbaum and Morse(3). Two-tenths, 0.4 or 0.6 ml of oxalated plasma was placed in conical centrifuge tubes and sufficient 0.85% sodium chloride solution added to obtain a total volume of 0.7 ml. For the *in vitro* experiments in which the effects of the addition of cysteine to polymyxin B sulfate treated dog plasma was investigated, 0.4 ml of plasma was used and a total volume of 0.8 ml was obtained. One ml of fibrinogen solution was then added with gentle mixing. This was followed with addition of 1 ml of thrombin-calcium solution to produce clotting. The tubes were then incubated at room tem-

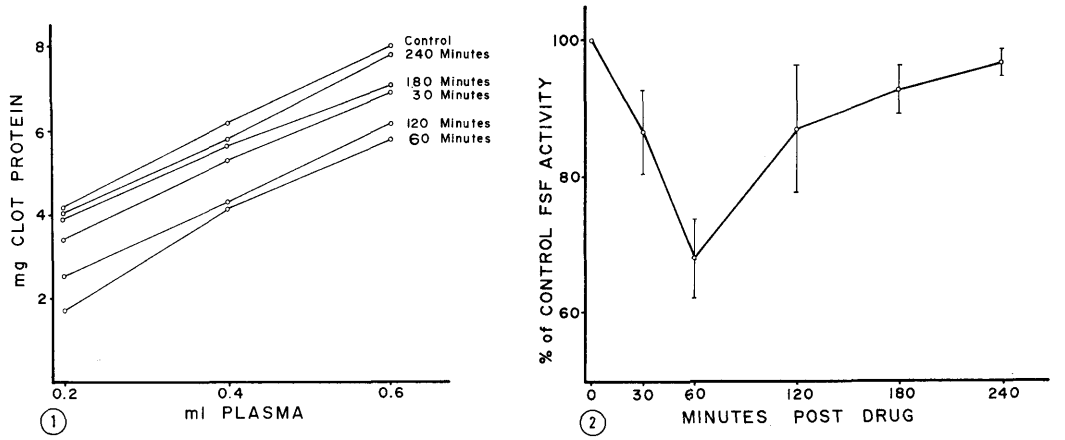

FIG. 1. Effect of 2.5 mg/kg of polymyxin B sulfate administered to an anesthetized dog on plasma FSF activity. Ordinate indicates mg of monochloroacetic acid insoluble clot protein (*i. e.*, FSF activity). Abscissa indicates ml of oxalated plasma used in the assay for FSF activity. Blood samples were drawn at the indicated times following administration of polymyxin B sulfate.

FIG. 2. Summary of time-effect data from 5 experiments in which anesthetized dogs received 2.5 mg/kg of polymyxin B sulfate intravenously and plasma FSF activity was measured. 0.4 ml of oxalated plasma was used in measuring FSF activity. Ordinate indicates the per cent of control activity where each animal served as his own control. Abscissa indicates the times at which blood samples were drawn following administration of polymyxin B sulfate. Vertical bars indicate standard deviation.

perature for 30 minutes before addition of an equal volume of 2% monochloroacetic acid solution with vigorous mixing on a vortex mixer. In some experiments the effect of prolonged incubation (4.5 hours), before addition of monochloroacetic acid, on plasma FSF activity following polymyxin B sulfate administration to dogs was determined. In this case, only 0.4 ml of plasma was used. The clot and monochloroacetic acid solution was incubated for 18 hours at 37°C. At the end of this time the acid solution was drawn off and the insoluble protein washed twice with 0.85% sodium chloride solution and once with distilled water with the aid of centrifugation. The monochloroacetic acid insoluble protein was determined using the Folin phenol reagent by the method of Lowry *et al*(8) after hydrolysis in 1.5 N sodium hydroxide solution. The optical density values obtained were converted into mg of monochloroacetic acid insoluble clot protein from standard curves constructed using various concentrations of crystallized human albumin (Dade Reagents, Inc.).

Results. Fig. 1 illustrates the effect of the intravenous administration of 2.5 mg/kg of polymyxin B sulfate to an anesthetized dog on plasma FSF activity. When 0.2, 0.4 and 0.6 ml are used from each sample of plasma it appears a linear relationship to FSF activity can be demonstrated.

Fig. 2 summarizes time-effect curves from 5 experiments in which anesthetized dogs received 2.5 mg/kg of polymyxin B sulfate intravenously and plasma FSF activity was measured using 0.4 ml of plasma. Results from the use of 0.2 and 0.6 ml of plasma in these experiments gave nearly identical curves. The dose of 2.5 mg/kg was chosen for all experiments with polymyxin B sulfate since this is the dose recommended for clinical use in patients with relatively good renal function. In all experiments, anti-FSF activity appeared in the 30-minute post drug sample. Near normal activity was found in the 240-minute sample. Peak activity was routinely found in the 60-minute sample and averaged 67.9% of normal activity with a standard deviation of ± 5.9%. Included in each experiment, fibrinogen was clotted with thrombin in the presence of calcium ion but in the absence of plasma. These clots always dissolved completely in the monochloroacetic acid solution.

The results of 3 experiments in which the

TABLE I. Effects of Addition of Cysteine and Polymyxin B Sulfate on FSF Activity of 0.4 ml of Oxalated Dog Plasma *in vitro*. Duplicate determinations were done in each of the 3 experiments. The concentrations of cysteine and of polymyxin B sulfate in the clotting mixture were 0.02 M and 5.0 mg/ml, respectively.

	% of Control FSF Activity			
	I	II	III	$\bar{X} \pm$ S.D.
Addition of Cysteine	+80.7	+107.9	+97.7	+95.4 ± 13.7
Addition of Polymyxin B sulfate	—50.6	— 52.4	—51.6	—51.4 ± 0.9
Addition of Polymyxin B sulfate and Cysteine	+77.1	+109.5	+99.2	+95.3 ± 16.6

effect of cysteine was determined on the FSF activity inhibiting properties of polymyxin B sulfate *in vitro* are summarized in Table I. In these experiments, heated fibrinogen was used. The process of heating reduced the cysteine activated FSF activity in the fibrinogen by an average of over 80% but did not abolish it completely. Fibrinogen not treated with cysteine dissolved completely in monochloroacetic acid after clotting with thrombin in the presence of calcium. The final concentration of cysteine in the clotting mixture was 0.02 M and of polymyxin B sulfate, 5.0 mg/ml. Frozen, oxalated dog plasma in the amount of 0.4 ml served as the source of FSF. It was found that the addition of cysteine increased the FSF activity of normal dog plasma by 95.4 ± 13.7% (S.D.). The addition of polymyxin B sulfate decreased the FSF activity of normal dog plasma by 51.4 ± 0.9% (S.D.). Cysteine added to the dog plasma before polymyxin B sulfate preserved completely the FSF activity of the plasma. The amount of monochloroacetic acid insoluble clot protein found in the latter case was essentially the same as that found when cysteine alone was added. In each experiment, clots were formed in the presence and absence of polymyxin B sulfate and not subjected to treatment with monochloroacetic acid. The former procedure was found to yield no less clot protein than the latter.

In 3 experiments the effect of prolonged incubation of the clotting mixture on the plasma FSF activity of anesthetized, polymyxin B sulfate treated dogs was studied. After drawing of a control blood sample, 2.5 mg/kg of polymyxin B sulfate was administered intravenously and subsequent blood samples were drawn at the same intervals of time previously described. FSF activity was determined in 2 sets of duplicate determinations, each with 0.4 ml of plasma. In one set, the clotting mixture was allowed to incubate at room temperature for the usual 30 minutes whereas the second set was allowed to incubate for 4.5 hours before the additon of solvent. Prolonged incubation gave rise to only a small increase in monochloroacetic acid insoluble clot protein with each sample of plasma. In pre-drug controls, this increase averaged about 15%. The effect of polymyxin B sulfate was evident in each experiment. However, prolonged incubation did not give rise to any more increase in acid insoluble clot protein than seen with pre-drug control plasma samples.

Discussion. The foregoing illustrates that the antibiotic polymyxin B sulfate inhibits FSF activity following intravenous administration to dogs at a dose similar to that used clinically. The effect appeared within 30 minutes, was maximal at about one hour and near normal activity was usually found 4 hours post drug. Anti-FSF activity for polymyxin B sulfate was also demonstrated *in vitro*. This activity was shown not to affect total fibrin yield. Furthermore, it appears that cysteine is capable of completely antagonizing the anti-FSF activity of the antibiotic.

Nussbaum and Morse(3) were able to show in patients with diminished FSF activity that prolonged incubation of the clotting mixture before addition of solvent corrected the diminished activity in the same individuals whose FSF activity could be corrected by addition of cysteine. In the case of polymyxin B sulfate inhibition, prolonged incubation had relatively little effect. The data suggest that the antibiotic interferes with fibrin stabilization by a reversible inactivation of FSF.

Polymyxin B sulfate therefore, appears not to be acting by the same mechanism described by Lorand and Jacobsen(4) for certain amine and carbonylamide inhibitors namely, the incorporation of the inhibitor into the fibrin molecule as a result of the action of FSF. In view of the effect of polymyxin B sulfate described here, it may be worthwhile to caution clinicians administering this drug parenterally to be alert for abnormalities in blood coagulation in their patients.

Several other antibiotics were studied for *in vivo*, anti-FSF activity in dogs. The antibiotics, the dose administered intravenously and the number of animals treated are as follows: neomycin sulfate ("Micifradin", The Upjohn Co.), 15 mg/kg—2 dogs, 50 mg/kg—1 dog, vancomycin hydrochloride ("Vancocin", Eli Lilly and Co.), 12 mg/kg—3 dogs, kanamycin sulfate ("Kantrex", Bristol Labs.), 7.5 mg/kg—3 dogs and colistimethate sodium ("Coly-Mycin", Warner Chilcott Labs.), 5 mg/kg—3 dogs. No evidence of anti-FSF activity could be demonstrated with any of these antibiotics.

Summary. Of several antibiotics investigated, polymyxin B sulfate showed anti-FSF activity following intravenous administration to dogs. Peak activity was found at approximately one hour and the effect persisted through the third hour post drug. *In vitro* experiments, in which cysteine was found to antagonize the anti-FSF activity of polymyxin B sulfate, suggest that the antibiotic interferes with fibrin stabilization by a reversible inactivation of FSF.

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Received February 17, 1967. P.S.E.B.M., 1967, v125.

Morphological and Quantitative Aspects of Mycoplasma-Human Cell Relationships.*† (32110)

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The method for direct microscopical demonstration of mycoplasma (Pleuropneumonia-like organisms, PPLO) in cultured cells(1), has been used extensively in diagnostic work to test for contamination of tissue and cell cultures; it has proven rapid and reliable. The technique, involving hypotonic treatment of the cells, air drying, and staining with orcein, has permitted detailed observations of the characteristic morphology of the mycoplasma-mammalian cell association. Such observations can be applied quantitatively to an evaluation of the amounts, or to actual counts, of mycoplasma attached to the mammalian cells. The present report is based upon an analysis of the correlation between mammalian cell-associated mycoplasma and mycoplasma present in the fluid phase of cell cultures under various experimental conditions.

Materials and methods. Cells. FL human amnion cells cultured serially in LY Medium (2), with 20% human serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were transferred with trypsin, 0.05%, and for experiments seeded and infected in 1 ml medium in "Demuth cups" (Demuth Glass Works, Division, Brockway Glass Co.) with

* This investigation was supported in part by NCI grants CA-05883 and CA-08748 and Research Career Award K6-CA-21607.

† The authors acknowledge the valuable technical assistance of Ralph Ibe.