PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 146, 904–907 (1974)

Effect of Leukocytic Endogenous Mediator on Plasma Fibrinogen and Haptoglobin (38216)

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During the acute phase of inflammation the liver shows increased rates of synthesis of a number of carbohydrate-containing proteins (1, 2). Although years ago sterile pus (3) and products of tissue disintegration (4) were suggested as mediators, the humoral mechanisms involved in inflammation are still unknown (1, 5). Recently it was shown that injections of leukocytic endogenous mediator (LEM) increased the serum levels of α-macrofetoprotein (6), ceruloplasmin (7), and glycoproteins (8). It was suggested that LEM, a low molecular weight protein, from phagocytic cells may be the mediator for the synthesis of many of these acute phase proteins (6, 7).

The present investigation was undertaken to determine whether LEM would increase the serum levels of fibrinogen and haptoglobin and to investigate the possibility that LEM acted directly upon the liver to alter the synthesis of these proteins.

Materials and Methods. Animals. Female Holtzman rats weighing 180–200 g were fed Rockland rat diet and water *ad libitum*. They were routinely maintained at 72°F with 12 hr of light and 12 hr of darkness. New Zealand white rabbits weighing 3–4 kg were purchased locally.

Preparation of leukocytic endogenous mediator. LEM was prepared from rabbit peritoneal granulocytes (9), and the crude material was partially purified by the method of Rafter *et al.* (10). Doses employed are expressed in terms of the number of granulocytes in the original preparation. Partially purified LEM from 1×10^7 granulocytes contained approximately 12 μg of protein. The endotoxin source was *Escherichia coli* 055:B5, lot 524181, purchased from Difco Laboratories, Detroit, Michigan.

Determination of fibrinogen and haptoglobin. In the *in vitro* synthesis experiments fibrinogen was determined by the colorimetric method of Ratnoff and Menzie (11). During *in vivo* experiments plasma fibrinogen was measured by a heat turbidity method (12). The resulting turbidity was plotted vs the concentration of fibrinogen determined by the colorimetric method. Serum haptoglobin was determined by the method of Roy *et al.* (13). Cyanmethemoglobin was prepared from washed defibrinated rat red blood cells. The hemoglobin versatol reagent was prepared by adding freshly drawn rat plasma to cyanmethemoglobin.

Fibrinogen biosynthesis in vitro. The rate of glycine incorporation into fibrinogen was based on slight modifications of the method of Pilgeram and Pickart (14). Slices of rat liver weighing 100–300 mg were incubated 4 to 6 hr in 2 ml of freshly drawn, heparinized plasma from rats containing 1.25 μCi of glycine-2-^{14}C, specific activity 30 mCi/m*M* (CalAtomic, Los Angeles, California). After incubation, the slices were removed, the loose debris was separated by centrifugation, and the fibrinogen precipitated with 25% $(NH_4)_2SO_4$. Precipitated fibrinogen was dissolved in citrate–saline buffer at pH 6.0. Fibrin was precipitated by adding 8 units of thrombin (Parke-Davis) and the resulting clot collected on a small glass stirring rod. The clot was thoroughly washed with and dialyzed overnight against distilled water, then placed in 1 ml of 10% NaOH

and heated at 80° for 10 min. Fibrinogen was determined on one 0.5 ml aliquot and the other aliquot was adjusted to approximately pH 7 and diluted with a xylene, dioxane, cellosolve cocktail (15). Radioactivity was determined by liquid-scintillation counting with quenching corrected by the channels-ratio method (16). The specific activity was calculated (14) and the results expressed as a percent of the normal samples which were run at the same time.

Results. The effects of injecting varying doses of LEM on the plasma levels of fibrinogen and haptoglobin in the rat are shown in Table I. The concentration of both proteins increased at the smallest dose tested, and further increases were observed as the amount of injected LEM was increased.

The time course of increased fibrinogen after LEM or endotoxin injections is presented in Fig. 1. Doses of endotoxin and LEM were selected so they gave approximately equal elevations in plasma fibrinogen 24 hr after injection. When endotoxin was injected ip into rats, a slight increase in serum fibrinogen was observed 6 hr after injection. There was a significant increase in fibrinogen 4 hr after LEM and the maximum seemed to occur before 24 hr.

The influence of LEM or endotoxin injected *in vivo* upon fibrinogen synthesis *in vitro* is shown in Table II. At varying times after injecting either LEM or endotoxin, the liver was removed and slices incubated for 4 hr in the presence of radioactive glycine and normal rat plasma. The fibrin was then isolated and the specific activity determined. One hour after injecting LEM and 2 hr after endotoxin, the synthesis of fibrinogen

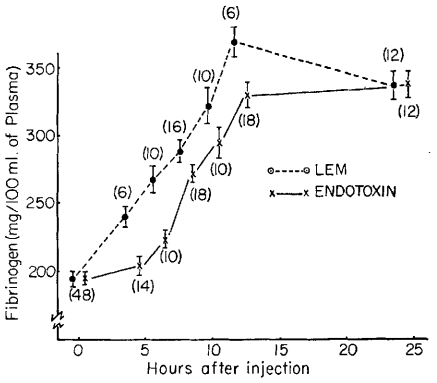

FIG. 1. Time course of changes in plasma fibrinogen after injecting either 1 μg of endotoxin or leukocytic endogenous mediator (LEM) from 5×10^7 granulocytes into normal rats. The number of rats used for each time period is shown in parentheses. The brackets indicate the standard error.

TABLE I. Fibrinogen and Haptoglobin Concentration in the Plasma of Rats 24 Hr after an ip Injection of LEM.

Dose of LEM[a]	No. rats	Fibrinogen (mg/100 ml)	Haptoglobin (mg/100 ml)
None	46	196 ± 6[b]	16 ± 3[b]
1×10^6	12	235 ± 9[c]	26 ± 4[c]
1×10^7	7	232 ± 7[c]	49 ± 2[c]
5×10^7	14	339 ± 12[c]	60 ± 4[c]
1×10^8	14	410 ± 14[c]	72 ± 4[c]
2×10^8	3	430 ± 5[c]	84 ± 6[c]
5×10^8	3	457 ± 19[c]	81 ± 2[c]

[a] Number of rabbit peritoneal granulocytes from which the leukocytic endogenous mediator (LEM) was derived. The partially purified LEM from 1×10^7 granulocytes had a protein content of approximately 12 μg.

[b] Mean ± SE.

[c] Significantly different from normal, $P < 0.01$.

TABLE II. Incorporation of Radioactive Glycine into Fibrinogen Using Rat Liver Slices Obtained at Varying Periods after Injecting LEM or Endotoxin.[a]

Time after ip injection (hr)	No. rats	% of Normal (cpm/mg fibrin) LEM[b]	Endotoxin[b]
0	26	100 ± 14[c]	
0.5	6	121 ± 18	101 ± 9
1	6	171 ± 17[d]	137 ± 16
2	8	190 ± 24[d]	194 ± 23[d]
4	6	217 ± 26[d]	195 ± 21[d]
8	10	279 ± 43[d]	280 ± 48[d]

[a] Liver slices incubated 4 hr with normal rat plasma and 1.25 μCi of glycine-2-^{14}C.

[b] Liver slices taken at varying times after injecting either LEM from 5×10^7 granulocytes or 1 μg endotoxin.

[c] Mean ± SE.

[d] Significantly different from normal $P < 0.01$.

TABLE III. Incorporation of Glycine-2-^{14}C into Fibrinogen Using Normal Liver Slices.[a]

No. trials	Source of rat plasma	Additions to incubation flask	% of Normal (cpm/mg fibrin)
32	Normal rat	None	100 ± 4[b]
14	Normal rat	1 μg endotoxin	97 ± 5
14	Normal rat	1×10^8 LEM	94 ± 5
7	2 hr after 10 μg endotoxin	None	172 ± 21[c]
12	2 hr after 50 μg endotoxin	None	216 ± 18[c]
9	2 hr after 1×10^8 LEM	None	133 ± 6[c]
7	2 hr after 5×10^8 LEM	None	174 ± 18[c]

[a] Normal liver slices were incubated at 37° in either normal rat plasma or plasma obtained 2 hr after an ip injection of LEM or endotoxin.

[b] Mean ± SE.

[c] Significantly different from normal $P < 0.01$.

was significantly different from the normal controls. There was again a suggestion of an earlier increase after LEM injection.

When LEM or endotoxin was added to normal liver slices *in vitro* and incubated for 6 hr in normal plasma, the rate of glycine incorporation into fibrinogen was not increased above normal (Table III). This was also true in a few experiments, not shown in the table, in which the whole liver was perfused with solutions containing either LEM or endotoxin. The *in vitro* synthesis of fibrinogen by normal liver slices was increased using plasma obtained from rats injected 2 hr earlier with either LEM or endotoxin (Table III).

Discussion. A number of investigators have suggested that the stimulation of glycoprotein synthesis during cancer, inflammation, or infection was due to the release of humoral factors from the injured tissue (17–20). The wide variety of stresses which will promote a selective increase in plasma proteins (1) suggests that a common endogenous mediator may be involved. A logical choice for a common mediator seemed to be LEM, since it has been shown to produce many of the "acute phase" reactions (9, 21). The present investigation demonstrates a progressive increase in both plasma fibrinogen and haptoglobin in rats receiving increasing doses of LEM. The increase in fibrinogen occurred sooner after injections of LEM when compared to endotoxin, suggesting but providing no definitive evidence that LEM was an intermediate for increased fibrinogen synthesis during stress. Previous investigations (12) with slightly higher doses of endotoxin also showed a delay before plasma fibrinogen increased in the rat.

Neither endotoxin nor LEM stimulated fibrinogen synthesis when incubated directly with the liver *in vitro,* but both of them when injected into the rat caused the rapid release of a humoral material into the plasma that stimulated *in vitro* fibrinogen synthesis. A number of possible mechanisms for humoral mediation have been suggested, e.g., labilization of lysosomes (1), phagocytosis by the reticuloendothelial cells (22), movement of amino acids and zinc into liver (23), increased plasma fatty acids (14), or release of a supernatant protein from polymorphonuclear leukocytes (24). The liver slice technique seems to be a rapid and easy method for checking these suggested humoral factors. This *in vitro* technique appears to be free from interference by endotoxin or LEM.

Summary. A progressive increase in plasma fibrinogen and haptoglobin occurred in rats receiving increasing doses of leukocytic endogenous mediator. The increases in fibrinogen occurred sooner after injections of leukocytic endogenous mediator than with endotoxin injections. Neither endotoxin nor leukocytic endogenous mediator caused increased radioactive glycine incorporation into fibrinogen when incubated directly with the liver *in vitro.* Both of them when injected into the rat caused a rapid

release of a humoral material into the plasma which stimulated *in vitro* glycine incorporation into fibrinogen.

1. Koj, A., *in* "Energy Metabolism in Trauma" (R. Porter and J. Knight, eds.), p. 79. J. and A. Churchill, London (1970).

2. Weimer, H. E., Humelbough, C., and Benjamin, D. C., Lab. Invest. **12**, 948 (1963).

3. Homburger, F., J. Clin. Invest. **24**, 43 (1945).

4. Chanutin, A., and Ludewig, S., J. Biol. Chem. **167**, 313 (1947).

5. Weimer, H. E., Roberts, D. M., and Comb, J. C., J. Nutr. **102**, 873 (1972).

6. Eddington, C. L., Upchurch, H. F., and Kampschmidt, R. F., Proc. Soc. Exp. Biol. Med. **139**, 565 (1972).

7. Pekarek, R. S., Powanda, M. C., and Wannemacher, R. W., Jr., Proc. Soc. Exp. Biol. Med. **141**, 1029 (1972).

8. Cockerell, G. L., Proc. Soc. Exp. Biol. Med. **142**, 1072 (1973).

9. Kampschmidt, R. F., Upchurch, H. F., Eddington, C. L., and Pulliam, L. A., Amer. J. Physiol. **224**, 530 (1973).

10. Rafter, G. W., Cheuk, S. F., Krause, D. W., and Wood, W. B., Jr., J. Exp. Med. **123**, 433 (1966).

11. Ratnoff, O. D., and Menzie, C., J. Lab. Clin. Med. **37**, 316 (1951).

12. Wycoff, H. D., Proc. Soc. Exp. Biol. Med. **133**, 940 (1970).

13. Roy, R. B., Shaw, R. W., and Connell, G. E., J. Lab. Clin. Med. **74**, 698 (1969).

14. Pilgeram, L. O., and Pickart, L. R., J. Atheroscler. Res. **8**, 155 (1968).

15. Bruno, G. A., and Christian, J. E., Anal. Chem. **33**, 1216 (1961).

16. Bush, E. T., Anal. Chem. **35**, 1024 (1963).

17. Weimer, H. E., and Benjamin, D. C., Amer. J. Physiol. **209**, 736 (1965).

18. Gordon, A. H., and Darcy, D. A., Brit. J. Exp. Pathol. **48**, 81 (1967).

19. Sarcione, E. J., Cancer Res. **27**, 2025 (1967).

20. Kushner, I., and Somerville-Volanakis, J., Proc. Soc. Exp. Biol. Med. **142**, 112 (1973).

21. Wannemacher, R. W., Jr., DuPont, H. L., Pekarek, R. S., Powanda, M. C., Schwartz, A., Hornick, R. B., and Beisel, W. R., J. Infect. Dis. **126**, 77 (1972).

22. Ohara, H., Nishio, H., Watanabe, K., and Wada, T., J. Reticuloendothel. Soc. **11**, 301 (1972).

23. Powanda, M. C., Cockerall, G. L., and Pekarek, R. S., Amer. J. Physiol. **225**, 399 (1973).

24. Darcy, D. A., Brit. J. Exp. Pathol. **49**, 525 (1968).

Received Apr. 1, 1974. P.S.E.B.M., 1974, Vol. 146.