

THE ACTIVE PRINCIPLE IN THE LEUKOCYTOSIS-PROMOTING FACTOR OF EXUDATES

By VALY MENKIN, M.D.

THE earlier studies of the writer have demonstrated that the leukocytosis accompanying many inflammatory processes is referable to the liberation at the site of inflammation of a factor closely associated with the pseudoglobulin fraction of exudates.^{1, 2, 3} The factor per se offers a reasonable explanation for the mechanism of leukocytosis with inflammation. This factor not only induces a discharge of immature leukocytes into the circulating blood,¹ but it also is capable of producing a hyperplasia of granulocytes and of megakaryocytes in the bone marrow.⁴ The factor (abbreviated as the LPF) is active on human beings, thus suggesting possible clinical application.⁵ It has been found to be active on guinea pigs. This animal may well serve as a convenient assay animal.⁶ In as yet unpublished studies, it has been found that the LPF reinforces the leukocytosis caused by an already existing inflammation. This may be significant in the usage of the material in clinical cases with inflammatory processes. It is quite conceivable that the factor may be utilized as an adjunct to the antibiotics.

The leukocytosis-promoting factor has always been recovered in the form of a pseudoglobulin. Recent studies in association with Dr. G. Cooper and Mr. M. L. Dillon at Duke University suggest that the LPF seems to be distributed primarily between the α_1 and α_2 globulins of exudates. In the present communication evidence is furnished which suggests that the active group in the pseudoglobulin molecule of exudates is a relative simple polypeptide.

EXPERIMENTAL

The leukocytosis-promoting factor (LPF) utilized in the following observations has been obtained from pleural exudates in dogs previously injected with 1.5 cc. of turpentine as described in the past.⁷ The scheme of extraction can be briefly restated by referring to the diagram on p. 940.

The material when freshly obtained appears as a fluffy, white powder which is extremely soluble in an aqueous medium. It is active in dogs, inducing a discharge of immature white cells into the circulation.

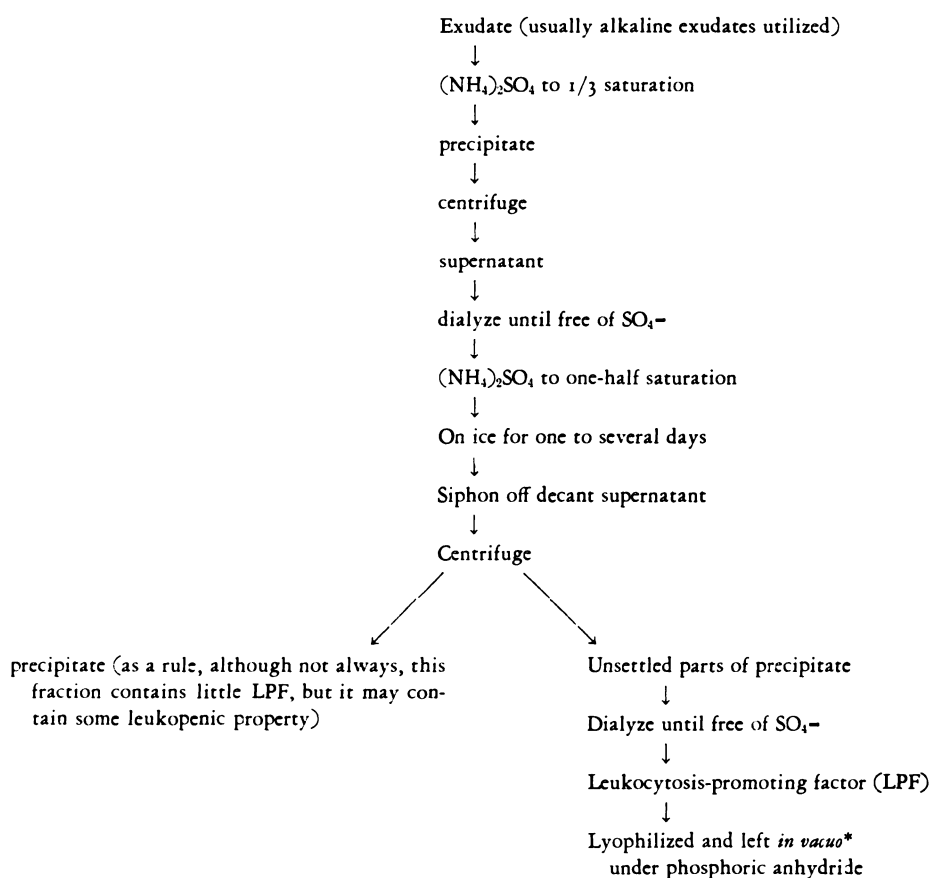
After several months, a curious change occurs in the material. It seems to lose its solubility, becoming quite insoluble in physiologic saline, and at the same time the material loses its biologic activity. It now has either no or little activity in causing a rise in the number of circulating leukocytes. It seems as if a spontaneous

From the Agnes Barr Chase Foundation for Cancer Research, Temple University School of Medicine, Philadelphia. Presented before the Philadelphia Pathological Society on March 13, 1947.

The aged samples of leukocytosis-promoting factor used in this study were for the most part prepared at Duke University School of Medicine. Aided also in part by a grant from the Bristol-Meyers Company of New York.

This is No. 37 of a series entitled "Studies on Inflammation".

denaturation has occurred. If the material is then centrifuged, the now insoluble part of the precipitate may even induce a leukopenic effect in contrast to the original leukocytosis-promoting property which it possessed. This, however, is not consistently true. At times it has either no activity, or at most a weak activity. On the other hand, if the supernatant phase of the insoluble aged LPF is injected



into the blood stream of a normal dog, considerable activity is obtained. The supernatant fraction induces a rise in the number of circulating leukocytes. It appears as if aging of the LPF causes a spontaneous denaturation into a relatively inactive and insoluble part. It splits off the active principle in the form of soluble component. The data of several such experiments appear in table I. It is clear that when 10 to 20 milligrams of aged LPF (3-6 months old) is treated with about 10 cc. of saline, stirred, and centrifuged, the supernatant part yields considerable activity when injected into dogs. There is an increase of about 64 per cent in the number of circulating leukocytes (table 1). The evidence indicates that the active

* Determination on three samples of LPF have yielded a recovery on the average of 12.8 milligrams of LPF per cc. of exudate.

principle is liberated *in toto* as a soluble component from the now insoluble and aged sample of leukocytosis-promoting factor. The course of one such experiment is shown in figure 1.

TABLE 1.—Effect of a Soluble Fraction Derived from Aged LPF (3-6 months old) on the Leukocyte Level

Dog No.	Amount of original LPF from which soluble fraction derived mg.	Basal no. of white blood cells cu. mm.	Maximum no. of white blood cells within 3-6 hours following administration of material cu. mm.
3-T	10	18,850	30,250
3-T	14.5	19,000	46,125
8-D	14	11,000	27,175
5-T	13.5	16,975	24,050
6-T	14	19,000	22,250
8-T	11.5	16,950	27,300
8-T	17	18,700	20,100
9-T	10	16,000	25,700
10-T	20	9,800	16,850
11-T	20	14,700	24,450
Average.....		16,097	26,425*

* Percentage increase in leukocyte level = 64.2%.

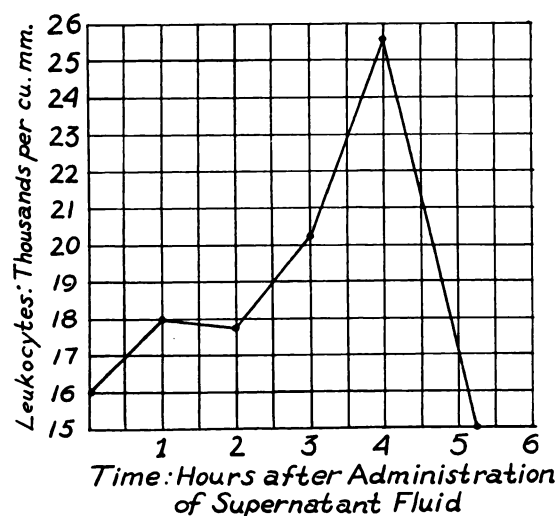


FIG. 1. EFFECT OF SUPERNATANT FRACTION FROM AN OLD SAMPLE OF LPF ON THE LEUKOCYTE LEVEL

Dog 9-T: Supernatant from 10 milligrams of 5 months old LPF employed.

In view of this observation, it became of interest to determine whether a proteolytic enzyme would inactivate the leukocytosis-promoting factor when freshly recovered as a soluble pseudoglobulin from exudates. The LPF was extracted from exudates of dogs, as described above. Various quantities of the factor in the fluid

state were treated with crystalline trypsin in amounts varying from a mere pinch of the enzyme to 2 milligrams. The length of incubation with the LPF was also variable, lasting from about one hour to over twelve hours. The treated LPF failed to be inactivated by tryptic digestion. The observations are assembled in table 2. It is quite clear that the addition of trypsin has failed to inactivate the factor. Following such digestion the injection of the treated material still caused a rise of 111.5 per cent in the number of circulating leukocytes (table 2). The course of an experiment is graphically shown in figure 2. Trypsin is known to hydrolyze complex proteins, and also the products of peptic digestion to the peptide stage.⁸

TABLE 2.—Effect of Tryptic Digestion on the Activity of the Leukocytosis-Promoting Factor

Dog No.	Material injected	Basal no. of white blood cells	Maximum no. of white blood cells within 2-6 hours following injection of treated LPF with enzyme
		<i>cu.mm.</i>	<i>cu.mm.</i>
11-T	13 cc. LPF + pinch crystalline trypsin	12,100	24,600
12-T	18 cc. LPF incubated overnight with trypsin	7,225	11,300
16-T	20 cc. LPF incubated with trypsin 2 hours	12,275	25,900
9-T	18 cc. LPF incubated with trypsin 2 hours	10,200	30,900
17-T	17 cc. LPF incubated 2 hours with 2 mgm. trypsin	6,300	8,950
20-T	10 cc. LPF incubated 1 hour and 5 minutes with 1 mgm. crystalline trypsin	10,900	24,100
9-T	10 cc. LPF incubated 1 hour and 25 minutes with approximately 1 mgm. crystalline trypsin	10,750	21,750
Average		9,964	21,071*

* Percentage increase in leukocyte level = 111.5%.

On the basis of the foregoing evidence, it is conceivable that the leukocytosis-promoting factor is not a protein at all, and therefore it remains intact when subjected to the influence of a proteolytic enzyme.

Yet when the newly obtained and active leukocytosis-promoting factor is heated for 30-35 minutes at 100 C., the whole molecule appears to be denatured and the LPF is likewise inactivated. The results of these experiments are summarized in table 3. When the LPF is exposed to such temperature, the material is completely inactivated. The LPF now yields a rise of 6 per cent in the number of circulating leukocytes in contrast to its original activity of 104 per cent. (table 3). The course of such an experiment is illustrated in figure 3.

These observations simply indicate that the LPF is thermolabile, but neverthe-

less that it may be merely associated with the pseudoglobulin molecule without being an actual part of it.

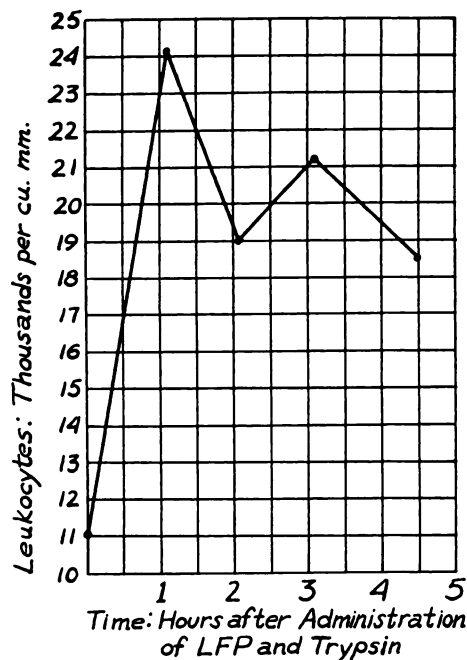


FIG. 2. THE FAILURE OF TRYPSIN TO INACTIVATE THE LPF

Dog 20-T: 10 cc. of LPF + 1 milligram of trypsin incubated for 1 hour and 5 minutes before administering to the animal.

TABLE 3.—Effect of Heat (at 100 C. for 30-35 minutes) on the Activity of the Leukocytosis-Promoting Factor

Experiment No.	Amount of LPF used	Basal white cell count in experiment with unheated LPF	Maximum white cell count 3-6 hours following administration of unheated LPF	Basal white count in experiment with heated LPF	Maximum white cell count 4-6 hours following administration of heated LPF
	mg.	cu.mm.	cu.mm.	cu.mm.	cu.mm.
1	20	8,500	17,400	9,375	9,550
2	19	9,425	19,500	7,350	8,350
3	17	10,575	16,550	8,825	8,400
4	25	11,800	27,950	9,425	10,600
5	23	11,000	23,250	13,275	14,150
Average		10,260	20,930	9,650	10,210

Percentage increase in leukocyte level with unheated LPF = 104%.

Percentage increase in leukocyte level with heated LPF = 6%.

Such an interpretation is, however, not consistent with subsequent facts. When now the active supernatant or soluble fraction obtained from aged LPF is evaporated to dryness on a steam bath, its activity remains essentially intact. Evapo-

ration of the active supernatant material even over a steam bath fails to inactivate the principle. These experiments are collected together in table 4. When such brittle, dried material obtained by evaporation over a steam bath is heated for 30-40 minutes at 100 C., the active principle fails to be inactivated (table 4). The active supernatant material when evaporated to dryness forms brittle flakes which

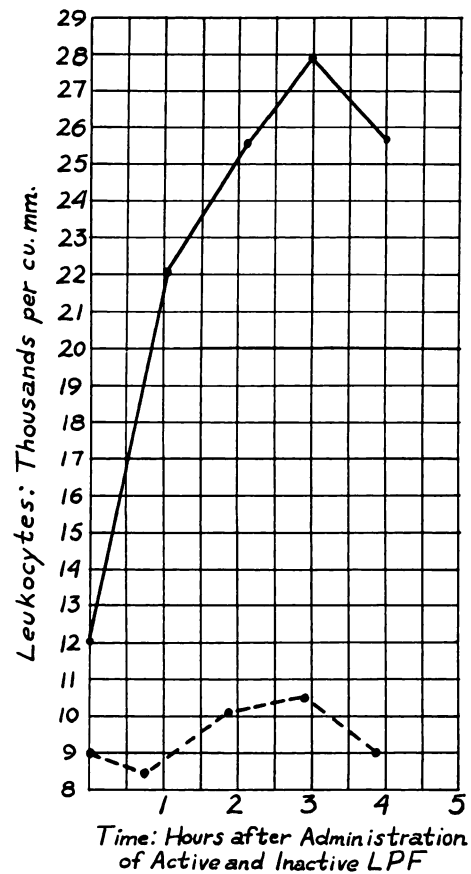


FIG. 3. THE INACTIVATION OF THE LPF BY HEAT

Solid line indicates Dog 33-T: 25 milligrams of LPF administered. Broken line indicates Dog 30-T: 25 milligrams of LPF inactivated by heating at 100°C. for 35 minutes.

are insoluble in an aqueous medium. Heating again to 100 C. such insoluble material fails to decrease its potency. Its injection in dogs induces a rise of 114.7 per cent in the level of circulating leukocytes. This observation definitely indicates that the LPF can be recovered from aged exudates as a highly thermostable substance. Experiments of this sort appear in figure 4. Such observations would preclude the interpretation that the leukocytosis-promoting factor is a thermolabile substance adsorbed to the pseudoglobulin molecule.*

* The insoluble brittle flakes obtained when evaporating the active supernatant to dryness on a steam bath are biuret and ninhydrin positive.

TABLE 4.—Effect of the Soluble Fraction Derived from Aged LPF When Evaporated to Dryness on Steam Bath and also When That Dried Fraction is Boiled for 30-40 Minutes

Dog No.	Amount of dried supernatant material derived from aged LPF injected into heart	Basal white cell count	Maximum white cell count following administration of either evaporated material derived from aged LPF or following boiling of such evaporated dried material
	mg.	cu.mm.	cu.mm.
9-T		7,625	17,500
11-T		11,050	24,200
16-T	10	9,350	20,650
29-T	20	12,950	18,400
Average.....		10,244	20,188*
9-T†	20	13,500	20,350
11-T†	23	11,575	22,100
39-T†	28	14,750	43,050
Average.....		13,275	28,500‡

* Percentage increase in leukocyte level = 97.1%.

† The evaporated material to dryness has in addition been subjected to boiling for 30-40 minutes.

‡ Percentage increase in leukocyte level = 114.7%.

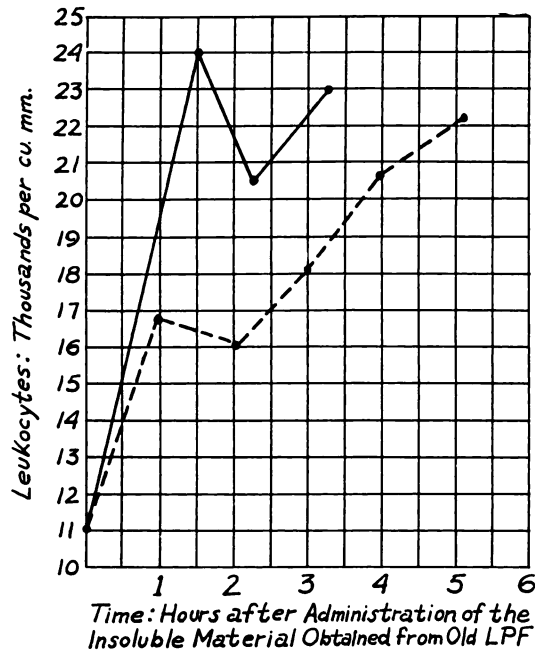


FIG. 4. THE ACTIVITY OF THE EVAPORATED SUPERNATANT FRACTION OF OLD LPF

Solid line indicates Dog 11-T: Suspension of supernatant fraction from old sample of LPF; evaporated to dryness and suspended as insoluble material. Broken line indicates Dog 11-T: the insoluble material was obtained by evaporating to dryness the supernatant fraction of an old sample of LPF. This was then heated for 30 minutes at 100°C. The procedure essentially failed to inactivate the material.

Polypeptides are known to be highly thermostable.⁹ For this reason the amino acid nitrogen before and after hydrolysis was determined on several samples of the active supernatant phase from an aged sample of LPF. These observations are as yet preliminary, but the measurements from such samples indicate in each case a rise in the amino acid nitrogen following acid hydrolysis. The actual figures obtained on such samples before and after hydrolysis are listed in table 5. These

TABLE 5.—*The Amino Nitrogen Content in the Active Principle of the LPF before and after Hydrolysis*

	Before hydrolysis	After hydrolysis
	mg./cc.	mg./cc.
Colorimetric method (modified O. Folin method ¹⁰)	0.09	0.2
	0.15	0.37
	0.16	0.51
	0.22	1.00
Average.....	0.155	0.520
Copper method (method of A. A. Albanese and V. Irby ¹¹)	0.009	0.066
	0.009	0.053
	0.009	0.095
	0.026	0.106
Average.....	0.013	0.080

observations suggest very strongly that the active principle is a relatively simple polypeptide.

DISCUSSION

The foregoing observations are consistent with the interpretation that the active principle in exudates, which reasonably explains the mechanism of leukocytosis with inflammation, is a relatively simple polypeptide.*

Besides the theoretic significance of this fact there is a possibility that the above observations may have practical application. The canine leukocytosis-promoting factor has been shown to be effective in human beings.⁵ The active factor has, however, been shown not to be too stable with time. A spontaneous denaturation occurs whereby the material loses its initial solubility. The present observations indicate that in spite of these changes the LPF is split off as a soluble and thermostable component. Its biologic activity remains undamaged. In this way it is conceivable that the leukocytosis-promoting factor of exudates can be preserved for long intervals in spite of age. These facts definitely suggest further practical use of this factor.

CONCLUSIONS

The leukocytosis-promoting factor of exudates appears to be a relatively simple polypeptide attached to the pseudoglobulin of exudates when extracted from the latter.

* The active principle is primarily nondiffusible from the active supernatant fraction of an aged sample of LPF. This would tend to indicate that the factor is somewhat larger than an amino acid.

This has been found by a study of samples of leukocytosis-promoting factor which have become insoluble by aging. Nevertheless, the active material has been found to be liberated as a water soluble component which is highly thermostable.

REFERENCES

- ¹ MENKIN, VALY: *Am. J. Path.* 16: 13, 1940.
- ² —: *Arch. Path.* 30: 363, 1940.
- ³ —, AND KADISH, M. A.: *Am. J. M. Sc.* 205: 363, 1943.
- ⁴ —: *Am. J. Path.* 19: 1021, 1943.
- ⁵ —: *Arch. Path.* 41: 376, 1946.
- ⁶ —, MATTISON, M. D., AND E. ULLED, E.: *Proc. Soc. Exper. Biol. & Med.* 61: 318, 1946.
- ^{6a} —: *Arch. Path.* 43: 566, 1947.
- ⁷ —: *Am. J. Path.* 10: 193, 1934.
- ^{7a} DILLON, M. L., COOPER, G. R., AND MENKIN, VALY: *Proc. Soc. Exper. Biol. & Med.* 65: 187, 1947.
- ⁸ HARROW, BENJAMIN, AND SHERWIN, C. P.: *A Textbook of Biochemistry*. Philadelphia, W. B. Saunders Company, 1935, p. 331.
- ⁹ GREENSTEIN, J. P.: *J. Biol. Chem.* 118: 321, 1937.
- ¹⁰ FOLIN, O.: *J. Biol. Chem.* 51: 377, 1922.
- ¹¹ ALBANESE, A. A., AND IRBY, V.: *J. Biol. Chem.* 153: 583, 1944.