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Monocytin, a Protecting Substance Produced by Murine Monocytes. (30090)

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Bactericidal substances were isolated from white blood cells by several authors. The results of investigations performed from the very beginning of the bacteriological era(1) and those of later investigations were reviewed(2). A substance named "phagocytin" was extracted from rabbit granulocytes with a salt solution buffered at pH 5.6(3) and an arginine-rich fraction (histone B) was isolated from calf thymus(4). Phagocytin and histone B were identified as separate substances(5). A bactericidal substance had been accumulated in Eagle's medium incubated with murine granulocytes and living Gram-negative bacteria(6). Three lysozomal fractions rich in arginine and acting on *Escherichia coli in vitro* were isolated from guinea pig granulocytes(7).

The results presented here demonstrate that several bactericidal substances were freed by monocytes in the course of the phagocytic process. One of them was able to prevent infection, when administered at least 4 hours before intraperitoneal inoculation of a lethal dose of *Salmonella typhi* (strain Ty_2).

Materials and methods. Eighteen-hour cultures of *E. coli, S. paratyphi A, S. typhi* (strain Ty_2), *Shigella dysenteriae* and *Staphylococcus aureus* on nutrient agar were employed. Murine peritoneal exudate monocytes were used for production of bactericidal substances. Mice were pretreated by intraabdominal injections of 3 ml of 1% sodium caseinate 3 days before the harvest of the monocytes. The cells were collected in phosphate buffer saline (pH 7.4) and washed with the same buffer 3 times. Monocytes were incubated in Eagle's medium (pH 7.4) in the presence or absence of *S. typhi* (strain Ty_2). The phagocytosis experiments *in vitro* were carried out as previously described(6). Supernatant fluids were separated by centrifugation and discarded. The monocytes were washed with buffer and destroyed by 3-fold freezing and thawing. Debris was discarded by suitable centrifugation. The resulting cell extracts were dialyzed against 0.05 M buffer phosphate (pH 7.0), or against distilled water. Bactericidal substances were separated from infected dialyzed extracts by preparative electrophoresis. The bactericidal activity of their graded dilutions was examined by incubation with 10^5 bacteria in a total volume of 1.0 ml 0.05 M buffer phosphate (pH 7.0). The number of surviving bacteria was counted after 2 hours of incubation at 37°C. Mouse protective activities were examined by intraperitoneal or intramuscular administrations of various samples prior to or simultaneous with an intraperitoneal challenge of 2×10^8 microorganisms of *S. typhi* (strain Ty_2).

Preparative electrophoresis was carried out according to Osborn(8) with some modifications. The eluates were dialysed against 0.05 M buffer phosphate (pH 7.0) and concentrated by pervaporation. Analytical electrophoresis was carried out on 30/5 cm cellulose acetate strips (Oxoid-Sigma). The electrophoresis was carried out in TRIS-EDTA-

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TABLE I. Bactericidal Activity of Products Isolated from Murine Monocytes Incubated in Eagle's Medium for 2 Hours at 37°C. Inoculum for bactericidal test 10^5/ml. Bacterial count performed after 2 hours contact.

Origin of bactericidal substance	Concentration of bactericidal substance (μg/ml)	% of surviving bacteria after 2 hr at 37°C				
		S. typhi	*S. paratyphi A*	*E. coli*	*Sh. dysenteriae*	*Staph. aureus*
Supernatant of	10^3	1	2	2	1	11
infected	10^2	12	11	16	9	37
monocytes	10^1	87	90	98	80	94
Supernatant of	10^3	2	2	2	2	11
non-infected	10^2	19	21	25	18	38
monocytes	10^1	100	96	100	90	93
Extract of infected	10^3	31	24	30	31	33
monocytes	10^2	89	83	97	100	89
Extract of non-in-	10^3	55	47	50	43	100
fected monocytes	10^2	100	100	100	100	100

Borate (TEB) (pH 8.9) ionic strength 0.08 (9), during 9 hours at 0.5 mA/cm width and 150 V. In addition, films of agar prepared similarly to that used for preparative electrophoresis were employed. For determination of isoelectric points paper eletrophoresis was performed on 30/4 cm Whatman No. 1 paper strips in glycine buffer pH 9.6 or 10.5, ionic strength 0.07.

Several samples were treated with trypsin or pepsin twice crystallized (Nutritional Biochemicals Corp., Cleveland). The enzymes were diluted 1:100 in buffer solutions and the enzymatic treatment was continued for 2 hours at 37°C. The samples to be trypsinized were heated for 1 hour at 100°C. Trypsin treatment was performed as previously described(5). The enzymatic processes were stopped by neutralization and the enzymes destroyed by heating for 30 minutes at 100°C. The samples were dialyzed against 0.05 M buffer (pH 7.0).

To exclude the possibility of an increase in resistance due to lipopolysaccharides (LPS), the various samples were examined for traces of LPS on the basis of their ability to produce the Shwartzman phenomenon(10) or cutaneous injuries(11) and their ability to produce anti-LPS antibodies in mice. Leucocytosis and leucopenia production were examined by a method already described(12). Lysozyme activity was determined by a standard method(13) and extraction of histone B was carried out as previously described(5).

Results. The experiments summarized in Table I show that during 2 hours incubation of monocytes in Eagle's medium and after removing the cells by centrifugation, bactericidal substances appear in the supernatant fluids which in a concentration of 10^3 μg/ml are able to kill about 99% of each Gram-negative organism tested and about 90% of 10^2 μg/ml. The bactericidal action on *Staph. aureus* was weaker, the respective percentages of bacteria killed equalling 89% and 63%. Weaker bactericidal effects were obtained with cell extracts. It seems that the substances liberated from non-infected cells were almost as active as those liberated from infected ones. Though the supernatant fluids of infected and non-infected cells were equally active when equal quantities were tested, the yield of the active dried substance was at least 6 times higher in the supernatant fluid derived from infected cells as compared with that obtained from non-infected ones.

Fig. 1 and 2 show that 2 fractions appear in the supernatants of infected murine monocytes running to the cathode, not existing in normal mouse serum. Table II and Fig. 1 show that by electrophoresis 4 bactericidal substances were separated—2 running to the anode and 2 others to the cathode. All these substances were tested *in vivo,* but only one which migrated faster than the other three has proven to exert a strong protective activity. This *in vivo* effect was exerted only if fraction 8 was given at least 4 hours before the bacterial inoculum. When the bacteria and fraction 8 were injected together intraperitoneally at different sites of the body

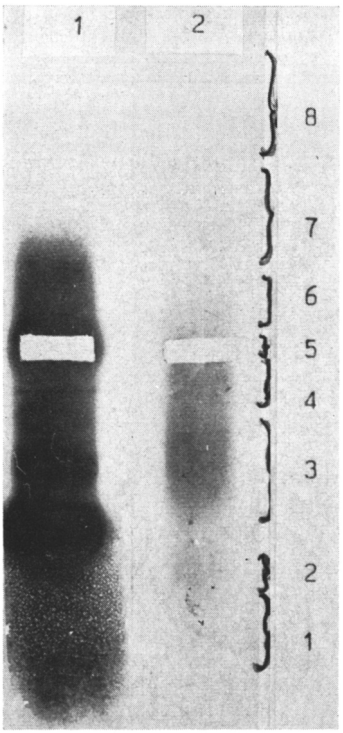

FIG. 1. Electropherograms of whole mouse serum and whole supernatants of infected murine monocytes in agar films: 1. whole serum, 2. whole supernatant.

(fraction 8 intramuscularly and the bacteria intraperitoneally) no protecting effect was observed. Furthermore, when the bacteria were injected before fraction 8 no protecting effect was seen.

To exclude any protecting effect exerted by some residual LPS of *S. typhi*, the experiments presented in Table III were carried out. These experiments showed that the protection by LPS did not start after an interval of 4 hours between its abdominal injection and inoculation of the challenge dose, while at the same time the protecting effect of fraction 8 was well marked. The protecting effect of residual LPS was also excluded by the fact that extracts of non-infected monocytes were protective, though *in vitro* they were never brought in contact with Gram-negative microorganisms. Further tests were carried out to exclude the presence of LPS. In the Shwartzman test fraction 8 did not act either as preparing or as injury producing factor. After an intravenous injection of 10.0 μg/kg into rabbits fraction 8 produced within 45 minutes a strong leucocytosis lasting for at least 48 hours, while injection of the same quantity of LPS produced a leucopenia lasting at least 4 hours followed by leucocytosis. Intracutaneous injection of 1.0 μg LPS produced local inflammation in rabbits, while the same quantity of fraction 8 did not produce any macroscopic lesions. Sera of mice immunized with fraction 8 did not produce anti-Ty_2 antibodies.

The protecting effect *in vivo* was not paralleled by the bactericidal effect *in vitro*. As shown in Table II, fractions 1, 2 and 7 of infected cells were *in vitro* highly bacteri-

TABLE II. Bactericidal Activity of Electrophoretic Fractions Obtained from Supernatant of Infected Monocytes. Test organisms *S. typhi* (Ty_2), bacterial inoculum 10^8/ml. Time of preincubation and bactericidal test as in Table I.

Fraction No.*	% of surviving bacteria after 2 hr contact at concentrations of (μg/ml) 100	10	1
1	1	42	75
2	2	70	100
3	61	87	89
4	67	73	77
5	16	22	44
6	42	70	100
7	5	58	90
8	1	24	80

* Nos. of fraction according to Fig. 1.

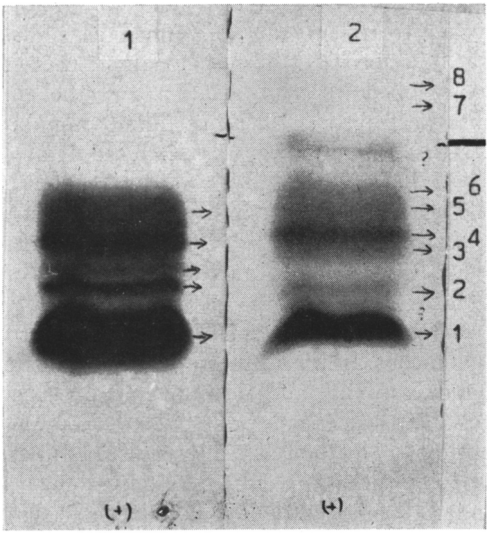

FIG. 2. Electropherograms of whole mouse serum and whole supernatants of infected murine monocytes in cellulose acetate strips: 1. whole serum, 2. whole supernatant.

TABLE III. Protective Action of Purified Substances Against an Intraperitoneal Inoculation of Strain Ty_2 (2×10^8 Microorganisms). Time interval between protective injection and challenge dose 4 hours.

Substance	Percentage of survivors after injection of protective substance (μg)							
	.01	.1	.2	.5	1.0	10.0	100.0	1000.0
Supernatant of infected monocytes	–	0	0	0	33	100	100	100
Fraction 8 of supernatant of infected monocytes	–	0	33	66	100	100	100	100
Supernatant of non-infected monocytes	–	0	0	0	0	0	0	33
Extract of infected monocytes	–	0	0	0	33	66	100	100
Extract of non-infected monocytes	–	0	0	0	0	33	100	100
Lipo-polysaccharide of *S. typhi* 0901	0	0	–	–	0	0	–	–

cidal, though *in vivo* they did not exert any protective effect. We also observed the same difference with the supernatant of non-infected monocytes, which *in vitro* were bactericidal but *in vivo* did not provide protection except in high concentrations. It seems therefore that this protecting effect *in vivo* is not identical with the bactericidal power *in vitro*. A positive protecting effect exerted by the infected or non-infected monocyte extracts or by their supernatants, could be observed during a period of 7-10 days following their administration.

The supernatants of infected or non-infected monocytes were stable to heating at 100°C for one hour, not losing their bactericidal activity or their protecting power, while by trypsin treatment they lost only their bactericidal activity *in vitro* but not their protective effect *in vivo*. The same results were obtained with the purified fractions 7 and 8 which by trypsinization have lost their bactericidal potency, while fraction 8 retained its protective effect. Pepsin treatment could not be estimated as both activities were destroyed by incubation at pH 2.0 for 2 hours.

Fractions 7 and 8 have proven to be basic proteins by determination of their amino acid compositions. Their isoelectric point turned out to be about 9.6 for fraction 7 and about 10.5 for fraction 8. These 2 fractions differed from histone B by their lability to acid treatment and from phagocytin by their lability to trypsin and their higher resistance to heat(5). They did not show any lysozyme activity. In fact, the crude supernatants of infected or normal monocytes contained very small quantities of histone B, while in fraction 8 its presence was no longer evident.

Summary. Several bactericidal substances were isolated from the supernatant of murine monocytes incubated for 2 hours at 37°C in Eagle's medium *in vitro* with addition of *S. typhi* (Ty_2). Smaller quantities of the same substances were liberated from monocytes incubated without addition of bacteria. An *in vivo* protecting substance has also been isolated. This substance protected mice if injected at least 4 hours before the bacterial inoculum, and the protection lasted for about 10 days, but when administered simultaneously with bacteria or after onset of infection no protection could be achieved. The substance turned out to be a basic protein, labile to acid treatment and trypsin and stable to heating at 100°C for one hour.

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Isolation and Properties of Human Leukocyte Lysosomes *in vitro*.* (30091)

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Cohn and Hirsch(1) have isolated the cytoplasmic granules of rabbit polymorphonuclear (PMN) leukocytes, demonstrating that in composition and behavior they resembled lysosomes of rat liver described by De Duve (2). These granules, like lysosomes, were rich in acid hydrolases (cathepsins, DNAase, acid phosphatase and beta glucuronidase), but also contained the basic anti-bacterial substances lysozyme and phagocytin. Enzymes associated with the granules could be released from their limiting membranes by mechanical trauma, hypotonic or acid media or by surface active agents. Subsequent studies on optically homogeneous suspensions of such granules have shown that other agents which labilize lysosomal membranes (lysolecithin, streptolysins, ultraviolet irradiation, vitamin A(3) and etiocholanolone(4) also release enzymes from PMN granules *in vitro*.

Heretofore it has not been possible to study isolated human PMN granules in similar fashion, mainly because methods employed for isolation of the relatively larger granules of rabbits damaged the smaller leukocyte lysosomes of human cells. The experiments to be described below outline a general method for isolation of human PMN granules in good yield from 25 ml of peripheral venous blood and describe some of their properties *in vitro*.

Materials and methods. Separation procedure. Twenty-five ml of blood, drawn in a heparinized ('liquaemin' Organon, 5,000 U/ml) syringe, were allowed to sediment 1-1½ hours at 37°C in an 18 × 125 mm screw cap tube. Approximately 6 ml of plasma, containing 40-60 million cells, 60-75% of which were PMN's, were removed with a Pasteur pipette inserted near the red cell-plasma interface and centrifuged at 110 *g* for 5 minutes in a 10 ml conical centrifuge tube. The cellular pellet was resuspended by slowly swirling cells off the pellet with fluid ejected from a rubber-bulbed Pasteur pipette and washed, first in 5 ml Earle's Balanced Salt Solution, then in 3 ml 0.34 M sucrose and finally very slowly resuspended, so as to avoid clumps, in 5 ml of 0.34 M sucrose with 0.5 ml heparin. The cell suspension (in an ice water bath) was then pipetted with rapid, sudden ejection of the suspension through a Pasteur pipette; a considerable increase in the viscosity of the solution was observed. Pipetting was discontinued just before the viscosity began to drop (approximately 15-20 times). A measured aliquot (original volume) of the suspension was centrifuged in 5 ml lusteroid tubes in a Servall Automatic Superspeed Centrifuge at 2,000 *g* for 10 minutes and the supernatant carefully poured off into another lusteroid tube. The remaining 'nuclear' pellet was washed in 2 ml of 0.34 M sucrose and the supernatant was added to the first. The combined supernatants were centrifuged at 27,000 *g* for 20 minutes, resulting in a white pellet with a viscous supernatant in which there was a thin, fluffy, strand of material. The supernatant and strand were carefully poured off

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