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Leucotoxic Substance Produced by *Bacillus Anthracis*

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SUMMARY

A new quantitative method is presented for the *in vitro* assay of aggressin of *Bacillus anthracis*. Aggressin was shown to contain a leucotoxic factor, which inhibits the chemotaxis of leucocytes of sensitive animals to *B. anthracis*. The leucotoxic factor could be completely neutralized by therapeutic anthrax antisera.

The leucocytes of resistant animals were completely insensitive to the leucotoxic factor. The difference in susceptibility to the factor of the leucocytes of sensitive and resistant animals could well explain the difference in the histo-pathological findings with regard to the infiltration of leucocytes.

The leucotoxic factor was produced *in vitro* and also by attenuated strain Sterne cells.

INTRODUCTION

Bacillus anthracis infection is one of the most interesting problems in the analysis of host-parasite relationships. To study host-parasite relationships, two aspects have been investigated; anthracidal substances and aggressin. With regard to anthracidal substances, Gruber and Futaki (1907) first reported on plakin extracted from blood platelets. The nature and mode of action of plakin has been extensively studied in this laboratory (Amano *et al.*, 1952, 1953a, 1953b, 1953c, 1953d, 1956, 1957, 1958; Kato *et al.*, 1954). Watson and his associates made extensive studies (Bloom, Watson, Cromartie and Freed, 1947; Skarnes and Watson, 1956) on the anthracidal substances from leucocytes and various tissues. Bail made observations on aggressin in 1904, but its nature remained obscure until just recently.

Attempts were made in this laboratory to elucidate the nature of aggressin in relation to the peculiar histo-pathological finding that anthrax lesions in sensitive animals were characterized by paucity of polymorphonuclear leucocytes (Cromartie, Bloom and Watson, 1947), and that in resistant or immune hosts there was abundant infiltration of leucocytes as in other acute bacterial infections. We regarded aggressin as a leucotoxic substance, which inhibits the chemotaxis of leucocytes toward infected foci. In 1954, the authors demonstrated inhibition of chemotaxis of leucocytes in an *in vitro* medium containing sterilized aggressin. Further, inhibition of leucocytic chemotaxis was specifically neutralized by a therapeutic antiserum for anthrax (Amano *et al.*, 1954). The experimental techni-

ques employed at that time were too complex to differentiate live and dead leucocytes. Shortly after our results were reported, we knew that Smith and Keppie (1954) found lethal and oedema-producing toxin in plasma of animals dying from anthrax and the plasma also contained antiphagocytic activity.

Recently, we developed a modification of Harris' method (1953), which permits rapid multiple assay of samples for inhibition of chemotaxis of leucocytes. The results of the study are described in this paper. The oedema fluid of anthrax lesions definitely contains a substance which is toxic to leucocytes of sensitive animals but not to those of resistant animals other than rats. The histo-pathological differences between sensitive and resistant animals could be well explained by different sensitivity of the leucocytes. The leucotoxicity of the oedema fluid could be specifically neutralized by therapeutic antisera for anthrax.

MATERIALS AND METHODS

Strains of microorganisms:

B. anthracis strain Vollum: The virulent strain was kindly given by Dr. J. Tomcsik.

B. anthracis strain N.P.: A non-proteolytic virulent derivative of strain *Vollum,* was kindly furnished by Dr. F. C. Belton.

B. anthracis strain Sterne: An attenuated, non-capsulated and still toxigenic strain was kindly given by Dr. H. Smith.

B. anthracis strain H. M.: An avirulent, encapsulated and nontoxigenic strain was also kindly supplied by Dr. H. Smith.

Therapeutic antisera for anthrax: Two batches of antisera, a highly immunized horse antiserum and a highly immunized bovine antiserum, containing no preservative, were kindly supplied by the Japanese Government Experimental Station for Animal Hygiene through the courtesy of Dr. H. Kawashima. The antisera were heated at 56°C. for 30 minutes and stored in a deep freezer.

Preparation of aggressin from infected guinea pigs: An 18 hour slant culture of *B. anthracis* strain *Vollum* was suspended in sterile saline at a concentration of one loopful per ml. Two ml. of this suspension were injected intraperitoneally into a guinea pig and the inflammatory oedema fluid was collected from the pleural and peritoneal cavities just before death. The fluid was centrifuged at 4,000 r.p.m. for 30 minutes, and the supernatant fluid was sterilized by passage through a Millipore filter membrane. Three to 5 ml. of filtrate were usually obtained.

RESULTS

Method of observation of leucocytic chemotaxis:

A modification of the method of Harris (1953) was used. This method, enabled the quantitative estimation of aggressin and also of the potency of the therapeutic antisera for anthrax. The details of the procedure are as follows:

Two or three drops of blood of test animals were ejected by syringes onto a clean coverslip. The coverslip was then placed in a moist chamber and incubated at 37°C. for 30 minutes. After incubation, the coverslip was transferred to a Petri dish containing balanced salts solution (Hanks et al., 1949). As the coverslip was immersed in the solution, the blood clot floated off and was sequently removed. The surface of the coverslip was irrigated until all red cells had been washed off.

A heavy suspension of heat killed nonpathogenic staphylococci in distilled water containing 0.02 per cent agar was streaked on a straight line crossing the long axis of a clean

slide glass, dried at room temperature and fixed over a flame. A drop of a medium consisting of an equal amount of aggressin and of inactivated normal guinea pig serum was placed on the slide and covered with a coverslip, to which the leucocytes had adhered. A control with 0.5 per cent gelatin dissolved in saline in place of the aggressin was always included. The resulting chamber was sealed with melted paraffine and incubated horizontally at 37°C. for 1 hour. After incubation, the slide was examined under a phase contrast microscope at a magnification of 200 at room temperature.

In the control, polymorphonuclear leucocytes were found to have migrated to two or three lines along the linear edge of the mass of staphylococci and in the neighborhood of the mass of the bacteria many elongated leucocytes were found to have been moving toward the mass of the bacteria as shown in Fig. 1. Such a pattern of leucocytes was designated as (+). When aggressin was added to the medium, neither migration of leucocytes to the edge of the bacterial mass nor the elongated forms near the bacterial mass could be found; only rounded and somewhat shrunken leucocytes were found randomly distributed as shown in Fig. 3. Such a pattern was recorded as (−).

Quantitative assay of the leucotoxic activity in the aggressin:

The new method enabled the quantitative assay of the leucotoxic action of the aggressin.

Serial two fold dilution of the aggressin were made in 0.5 per cent gelatin dissolved in saline. All the slides were kept at room temperature after incubation at 37°C. for 1 hour.

The end point for inhibition of chemotaxis was the final dilution of the aggressin, at which there was no migration of leucocytes to the edge of the bacterial smear but nearly half the leucocytes had moved slightly showing pseudopods as shown in Fig. 2. Such a pattern was designated as (±). On further dilution of the aggressin, complete chemotaxis was observed. At the previous dilution of the aggressin there was complete inhibition of chemotaxis as shown in Table 1. The titer of the aggressin used in this experiment was 1 : 32, final dilution.

Table 1. Quantitative assay for the leucotoxic activity in the aggressin

	1	2	3	4	5	6	7	8
Aggressin (0.1 ml. resp.)	1 : 1	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 2 (60C° 30 min.)	—
Inactivated normal g. p. serum	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
0.5% Gelatine-saline	—	—	—	—	—	—	—	0.1
Chemotaxis of leucocytes	—	—	—	—	±	+	+	+

The heat stability of the leucotoxic factor was also tested at a final dilution of 1 : 4. As can be seen from Table 1, it was denatured by heating at 60°C. for 30 minutes.

Neutralization of the leucotoxic factor by therapeutic antisera:

To study the leucotoxic factor further specific neutralization was examined

with therapeutic antisera for anthrax.

Aggressin active to a final dilution of 1:32 was diluted to 1:2. 0.1 ml. of the diluted aggressin was added to each 0.1 ml. of serial dilution of horse hyper-immunized antiserum, as shown in Table 2. The tubes were stoppered and incubated at 37°C. for 30 minutes. To each of these tubes were added 0.2 ml. of inactivated normal guinea pig serum. Single drops of these mixtures were placed on slides, and covered with coverslips as described above. Following procedures were also the same as described above. The results are shown in Table 2.

Table 2. The neutralization of the leucotoxic factor by therapeutic antisesa

	1	2	3	4	5	6	7	8	9	10	11	12	13
Aggressin (1 : 2)	0.1	—	0.1	—	0.1	—	0.1	0.1	0.1	0.1	0.1	0.1	—
0.5% Gelatine-saline	—	0.1	—	0.1	—	0.1	—	—	—	—	—	0.1	0.2
Antiserum (0.1 ml.)	1:1	1:1	1:10	1:10	1:100	1:100	1:200	1:400	1:800	1:1600	1:3200	—	—
	incubated at 37°C for 30 min.												
Inactivated normal g. p. serum	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Chemotaxis of leucocytes	±	+	+	+	+	+	+	+	+	+	±	—	+

The leucotoxic action of the aggressin was completely neutralized up to a serum dilution of 1 : 1,600. Neutralization in the tube containing undiluted antiserum appeared incomplete. However, when a mixture of the aggressin and the undiluted antiserum was further kept overnight at 4°C., or when the antiserum were absorbed with guinea pig leucocytes before the neutralization experiment, the neutralization by undiluted antiserum was as complete as that with diluted antiserum. Though a protocol is not given, normal horse sera or bovine sera could not neutralize the leucotoxic action of the aggressin. From these results it can be stated that a specifically neutralizable leucotoxic factor was present in the inflammatory oedema fluid produced by *B. anthracis* infection.

Effect of aggressin produced in vitro on leucocytic chemotaxis:

Harris-Smith, Smith and Keppie (1958) recently showed that an oedema producing and lethal toxin of *B. anthracis* conld be detected when the cultural conditions were adequately controlled. Attempts were made to reproduce their results and the authors confirmed the presence of aggressin in our assay method.

One half ml. of meat infusion broth was inoculated with 1×10^7 spores and incubated at 37°C. for 30 minutes. After almost all the spores had germinated, 4.5 ml. of inactivated normal guinea pig serum were added to the medium and incubated on a shaker at 37°C. for 4 hours. The centrifuged supernatant was sterilized by a Millipore filter membrane. The leucotoxic activity of the filtrate was assayed by the method described above. The results are presented in Table 3.

The leucotoxic factor was produced by attenuated strain Sterne cells at the same titer as produced by highly virulent strain N. P. cells, however it was not

Table 3. Effect of aggsessin produced *in vitro* on leucocytic chemotaxis

Strain used	N. P.		Sterne		H. M.
Dilution of aggressin (final concentration)	1 : 8	1 : 16	1 : 8	1 : 16	1 : 2
Chemotaxis of Leucocytes	—	±	—	±	+

produced by avirulent strain H. M. cells.

Though a table is not presented in this report, the leucotoxic factor produced *in vitro* could also be neutralized by the therapeutic antisera for anthrax. Harris-Smith *et al.* also reported the same titer of the oedema producing and lethal toxin in the culture filtrate of the strain Sterne cells and in strain N. P. cells and that their toxin was neutralized by therapeutic antisera. From these two facts the authors assume that the leucotoxic factor we found was the same as the toxin found by Harris-Smith *et al.*

Demonstration of the leucotoxic factor at an early stage of infection:

Since no infiltration of leucocytes was found even at early stage of infection of *B. anthracis* and the leucotoxic factor was produced *in vitro* even after 2 hours of the incubation, the leucotoxic factor seemed to be produced at an early stage of the infected foci. To demonstrate the early production of the leucotoxic factor, the following experiment was performed.

An overnight slant culture of *B. anthracis* strain *Vollum* was harvested and suspended in saline to a concentration of one loopful per ml. Five ml. of the suspension were inoculated into the peritoneal cavity of a male guinea pig weighing 500 g. After 6 hours the guinea pig was sacrificed and the peritoneal and pleural exudates were collected. The pooled exudate was sterilized by a Millipore filter membrane. The leucotoxic factor was assayed and the results are shown in Table 4.

Table 4. Demonstration of the leucotoxic factor in early stage of infection

	from guinea pig 6 hrs after infection			from guinea pig just before death		
Final dilution of aggressin	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64
Chemotaxis of leucocytes	—	±	+	—	±	+

The leucotoxic activity was detected at a final dilution of 1 : 4. Though it was weaker than that obtained from guinea pig just before death, the leucotoxic factor was clearly demonstrated in early stage of infection. Therefore the paucity of leucocytes in the infected foci of *B. anthracis* is due to the leucotoxic factor produced.

Susceptibility of leucocytes of various animals:

The susceptibility of human, cow, horse, sheep, rabbit, mouse, dog, hog and rat leucocytes were tested with the leucotoxic factor produced in guinea pig.

Human, bovine, horse, sheep, dog and hog leucocytes like those of guinea pig readily adhered to the clean surface of a coverslip. The leucocytes of other species did not adhere from blood clots deposited on coverslips. Rabbit leucocytes were collected as a buffy coat from heparinized whole blood by mild centrifugation and the buffy coat was suspended in

Hanks' solution. A coverslip was mounted with the suspension and kept in a moist chamber at 37°C. for 30 minutes. The coverslip to which the rabbit leucocytes now adhered was irrigated until all red cells had been washed free by the Hanks' solution. It was subsequently used for study. Mouse or rat leucocytes suspension was removed by syringe from the peritoneal cavity 16 hours after administration of 10 per cent glucose (1.0 ml. for a mouse and 10 ml. for a rat) and a coverslip covered with the suspension. The following procedure was the same as for rabbit leucocytes. The results are presented in Table 5.

Table 5. Susceptibility to aggressin of leucocytes of several species of animals

Leucocytes	human	bovine	horse	sheep	rabbit	guinea-pig	mouse	dog	hog	rat
Maximal final dilution of positive inhibition	1:32	1:32	1:32	1:32	1:32	1:32	1:16	N.I.*	N.I.*	1:4

*N. I.: No inhibition even at 1:2 dilution.

The leucocytes of animals susceptible to *B. anthracis* (human, horse, sheep, rabbit and mouse as well as guinea pig) were very sensitive to the leucotoxic factor. The leucocytes of resistant animals (hog and dog) were completely insensitive. Unexpectedly rat leucocytes were somewhat sensitive. However their susceptibility can be explained from the description of Metschnikoff (1902). "In den meisten Fällen kommt es bei den infizierten Tieren zu einer schweren Erkrankung, die Milzbrandbazillen vermehrten sich an der Injektionsstelle und führen zur Bildung eines grossen Oedems, welches an flüssigen, durchscheinenden Serum sehr reich und an Leukozyten sehr arm ist. Erst später kommen die Leukozyten in grösserer Menge hinzu; das Exsudat wird dick und trübe, die zahlreichen weissen Blutkörperchen verzehren und zerstören die Milzbrandbazillen."

Thus, the susceptibility of leucocytes of these animals is closely related to the sensitivity of the animals to the infection of *B. anthracis*.

DISCUSSION

Results have been given here on a leucotoxic factor of the inflammatory oedema fluid caused by *B. anthracis*. The leucotoxic factor is an antigenic substance and can be neutralized by an antibody contained in therapeutic antisera for anthrax.

Leucocytes of animals sensitive to *B. anthracis* infection were very susceptible to the leucotoxic factor. However those of animals insensitive to the bacterial infection were completely resistant to the leucotoxic factor. The leucocytes of rat relatively resistant to the infection, were sensitive when tested against more concentrated leucotoxic factor. The result observed on rat leucocytes coincides with the fact that the infected foci are initially free from leucocytes and only later leucocytes infiltrate. The difference in susceptibility to the leucotoxic factor of leucocytes of sensitive and resistant animals can well explain the different pathologic pictures of infected foci with regard to the abundance or paucity of leucocytes.

Using guinea pig leucocytes, the neutralization of the leucotoxic factor by undiluted therapeutic antisera was not complete in a short time but was complete

only after further overnight incubation at 4°C. or by antisera previously absorbed with guinea pig leucocytes. The reason for this is not yet clear. Probably some additional factor other than antibodies or a relatively high concentration of antigen-antibody complexes disturbs the chemotactic migration of guinea pig leucocytes to the focus.

It is striking that *in vitro* attenuated strain Sterne cells produce the leucotoxic factor at the same titer as the virulent strain N. P. The same phenomenon was observed by Harris-Smith *et al.* (1958) and they explained this curious phenomenon by postulating that the capability of the bacteria to produce toxin and capsules *in vivo* is the essential factor for the full virulence of *B. anthracis*. In this laboratory other groups are studying the mechanism of decapsulation of organisms in resistant and immune hosts and also anthracidal factors in various animals. Their results will be helpful for the study of the virulence of *B. anthracis*.

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REFERENCES

Amano, T., Kato, K. and Shimizu, R. (1952). Studies on the rôle of plakin. *Med. J. Osaka Univ.* **3**, 293–311.

Amano, T., Kato, K. and Shima, M. (1953a). Studies on the rôle of plakin. II. *Med. J. Osaka Univ.* **3**, 695–710.

Amano, T., Shima, M. and Kato, K. (1953b). Studies on the rôle of plakin. III. The damage of semipermeability of bacteria caused by plakin. *Med. J. Osaka Univ.* **4**, 277–284.

Amano, T., Kato, K., Morishima, T. and Okada, K. (1953c). Studies on the rôle of plakin. IV. The effect of plakin on the respiratory enzyme system of *Bacillus megatherium*. *Med. J. Osaka Univ.* **4**, 285–289.

Amano, T., Kato, K. and Okada, K. (1953d). Studies on the rôle of plakin. V. On the correlationship of phosphate metabolism to the maintenance of the organization of bacterial cells. *Med. J. Osaka Univ.* **4**, 291–297.

Amano, T., Kato, K., Shima, M., Morishima, T., Okada, K. and Torii, M. (1954). Studies on the rôle of plakin VI. presented at the 7th meeting of Kansai branch of the Society of Japanese Bacterologists held in October, 1954.

Amano, T., Kato, K., Okada, K., Tamatani, Y. and Higashi, Y. (1956). Studies on the rôle of plakin. VII. Effects on the protoplast of *Bacillus megatherium*. *Med. J. Osaka Univ.* **7**, 217–231.

Amano, T., Tamatani, Y., Kato, K., Higashi, Y. and Toyoda, K. (1957). Studies on the rôle of plakin. VIII. The mechanism of activation by serum. *Med. J. Osaka Univ.* **7**, 829–832.

Amano, T., Kato, K., Okada, K., Higashi, Y., Tamatani, Y. and Toyoda, K. (1958). Studies on the rôle of plakin. IX. Effect on the respiratory activity and on the macromolecular structure of *Bacillus megaterium*. *Biken's J.* **1**, 5–12.

Bail, O. (1904). Untersuchungen über natürliche und künstliche Milzbrandimmunität. XI. Erster Bericht über Milzbrandschutzimpfungen an Schaffen. *Zentr. Bacteriol., I. O.* **37**, 270–280.

Bloom, W. L., Watson, D. W., Cromartie, W. J. and Freed, M. (1947). Studies on infection with *Bacillus anthracis*, IV. Preparation and characterization of an

anthracidal substance from various animal tissues. *J. Infect. Dis.* **80,** 41–52.

Cromartie, W. J., Bloom, W. L. and Watson, D. W. (1947). Studies on infection with *Bacillus anthracis*. I. Histopathological study of skin lesions produced by *B. anthracis* in susceptible and resistant animal species. *J. Infect. Dis.* **80,** 1–13.

Gruber, M. and Futaki, (1907). Ueber die Resistenz gegen Milzbrand und über die Herkunft der milzbrandfeindlichen Stoffe. *Münch. med. Wochenschr.* **54,** 249–265.

Hanks, J. H. and Wallace, R. E. (1949). Relation of oxygen and temperature in the preservation of tissues by refrigeration. *Proc. Soc. Exp. Biol. Med.* **71,** 196–200.

Harris, H. (1953) Chemotaxis of granulocytes. *J. Path. Bact.,* **66,** 135–146.

Harris-Smith, P. W., Smith, H. and Keppie, J. (1958). Production *in vitro* of the toxin of *Bacillus anthracis* previously recognized in vivo. *J. gen. Microbiol.* **19,** 91–103.

Kato, K., Shima, M., Okada, K. and Morishima, T. (1954). Studies on the rôle of plakin. VI. Calcium, magnesium or manganese as a co-factor of plakin. *Med. J. Osaka Univ.* **5,** 291–300.

Metschnikoff, E. (1902). *Immunität bei Infektionskrankheiten* (einzig autorisierte Uebersetzung von J. Meyer), Jena, Gustav Fischer, p. 124.

Sakarnes, R. C. and Watson, D. W. (1956). Characterization of leukin: an antibacterial factor from leucocytes active against Gram-positive pathogens. *J. Exp. Med.* **104,** 829–845.

Smith, H. and Keppie, J. (1954). Observation on experimental anthrax: Demonstration of a specific lethal factor produced in vivo by *Bacillus anthracis*. *Nature* **173,** 869–870.

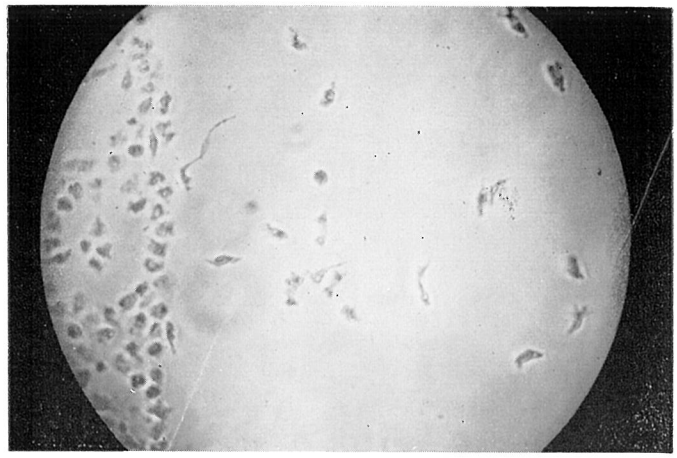

Fig. 1. The phase contrast micrograph of leucocytic chemotaxis, designated as (+)

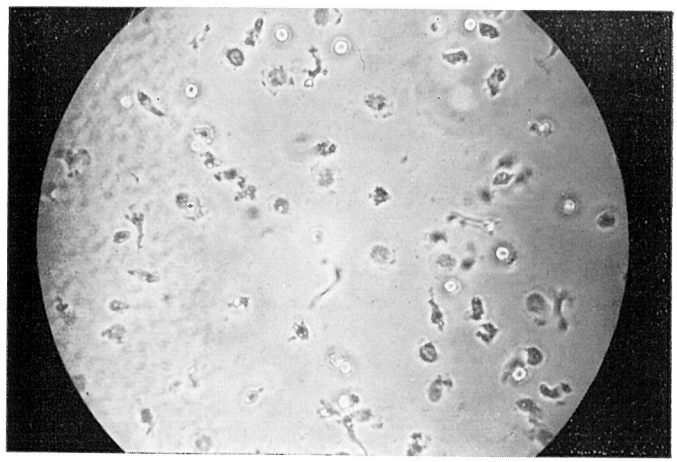

Fig. 2. The phase contrast micrograph of leucocytic chemotaxis, designated as (±)

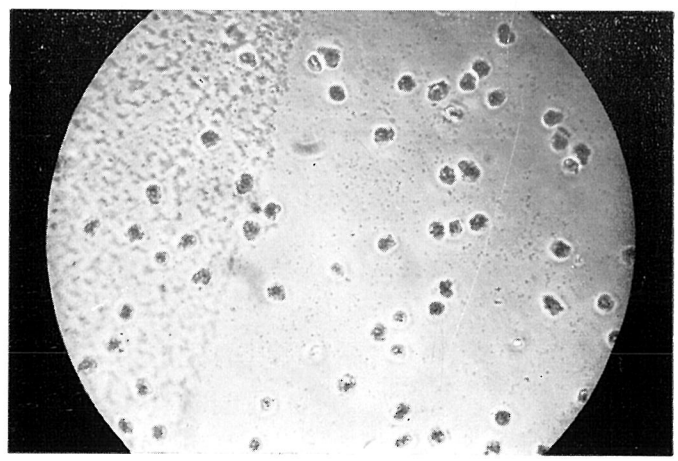

Fig. 3. The phase contrast micrograph of leucccytic chemotaxis, designated as (−)