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# PHAGOCYTOSIS OF BACTERIA BY POLYMORPHS IN SUSPENSION OR ADHERENT TO A SURFACE

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The results of investigation of phagocytic activity of polymorphonuclear leukocytes (polymorphs) essentially complement other laboratory and clinical data and are now widely used to assess the condition of a patient and the prognosis of his disease [1, 4, 7]. There is no doubt that the closer the conditions of performance of the *in vitro* test to conditions actually created in the inflammatory focus, the more fully and precisely does the information thus obtained reflect the pathogenesis of the infectious process. Many different versions of the method of studying phagocytosis *in vitro* have been described. These versions differ in many respects, and this is reflected in the end result of analysis. Only those distinguishing features of the method which can affect passive or active movements of polymorphs will be discussed. Soviet workers usually allow the mixture of leukocytes and microorganisms to stand in a tube placed in an incubator [2, 3, 5, 6]. It is stated in one Western publication that if the test is carried out in this way the number of ingested bacteria is very small [15], and in order to increase the uptake of bacteria the tubes in the incubator are placed in an apparatus which spins them at a speed of 8-20 rpm [10, 12, 13]. In both these versions of the test the conditions created *in vitro* differ sharply from the situation in an inflammatory focus. In particular, so important a factor of the phagocytic reaction as chemotaxis cannot play its proper role, because polymorphs, which are capable of active movement only along a surface, and not of "swimming" either remain suspended in the fluid or are impelled by external sources in different directions.

In this investigation the level of ingestion of bacteria in the two versions of the technique described above was compared with their ingestion in a third version, which the present writers proposed, which provides for the possibility of chemotaxic movements of polymorphs.

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## EXPERIMENTAL METHOD

Polymorphs from normal healthy blood donors were studied. Blood in a volume of 5 ml was poured into a tube containing 0.5 ml of a 6% solution of trilon B and 0.5 ml of 10% gelatin solution. Erythrocytes were sedimented for 60 min at room temperature. The suspension of leukocytes in plasma formed in the top part of the tube was sedimented by centrifugation at 1000 rpm for 10 min. The supernatant was recentrifuged for 10 min at 3000 rpm to separate plasma from platelets thrown down into the residue. The plasma was used subsequently for opsonization of bacteria and for addition in a proportion of 10% to medium 199 in which the leukocytes were resuspended. The concentration of the latter on resuspension was adjusted to $5 \cdot 10^6$ cells/ml and they were mixed with an equal volume of a suspension of *Staphylococcus aureus* $2 \cdot 10^8$ cells/ml. To prepare this suspension a 24-h agar culture of bacteria was washed off and made up to the necessary concentration in medium 199. Next, 10% of plasma of the leukocyte donor was added and the tube was incubated for 10 min for opsonization. The bacterial suspension was then sedimented at 3000 rpm for 15 min and resuspended in the original volume of medium 199. The mixture of leukocytes and bacteria was incubated for 30 min in test tubes, divided into four groups: 1) tubes standing still in a stand; 2) tubes in the same stand, but into which a partition made of reinforced photographic film was lowered lengthwise; 3) tubes which differ from those of group 2 in that the partition was made of film folded double, with the backing on the inside and the photographic emulsion on the outside; 4) tubes fixed in an apparatus spinning them at a speed of 15 rpm. At the end of incubation the leukocytes in all the tubes were sedimented at 1500 rpm for 15 min and, after removal of the supernatant, they were fixed with 4% glutaraldehyde in cacodylate buffer and 1% $OsO_4$. The material was embedded in epoxide resin as described in [14]. The partitions from the tubes of group 2 were fixed in methyl alcohol and stained on both sides with toluidine blue and azure. In three of the six cases in this group the partitions were removed from the test tubes before sedimentation of the mixture of leukocytes and bacteria, and in the other three cases after sedimentation. Semithin sections were cut from each block embedded in epoxide resin, stained with a mixture of toluidine blue and azure, and used to determine the phagocytic index — the percentage of polymorphs containing bacteria, and the phagocytic number — the mean number of bacteria per phagocyte (only polymorphs containing bacteria were counted in this case). Electron-microscopic sections were cut from some blocks of each series and examined in the JEM-100B microscope. In the 1st, 3rd, and 4th groups eight blood samples were tested in each case. The results were subjected to statistical analysis by Wilcoxon's test.

## EXPERIMENTAL RESULTS

Quantitative data reflecting the intensity of uptake of staphylococci by polymorphs of the different groups are given in Table 1. When the mixture of leukocytes and bacteria was incubated in a stationary tube (group 1) injection of bacteria was insignificant. In groups 3 and 4, both parameters studied (phagocytic index and phagocytic number) were much higher. The data for group 2 were not used for statistical calculations because they were divided into two numerically small subgroups, but these results do explain the reason for the difference between group 1 and groups 3 and 4. The partitions in the tubes of group 2 were cut out of reinforced photographic film and, consequently, one side of the partition consisted of backing, the other of the layer of photographic emulsion. During treatment with fixer, crystals of silver bromide were removed from the layer of emulsion and it thus became a simple surface covered with a layer of gelatin. Examination of stained partitions, removed before centrifugation, under the microscope showed that the surface of the backing was essentially empty, and single cells were found very rarely on it. The gelatin surface, on the other hand, was covered with a dense layer of polymorphs, many of which contained staphylococci. Investigation of partitions removed after centrifugation showed that both surfaces were practically free from polymorphs; consequently, the centrifugal force created by spinning at 1500 rpm was stronger than the force of adhesion of the polymorphs to the gelatin surface and they were sedimented to the bottom of the tube. Addition of polymorphs saturated with staphylococci from the partition to the residue led to a marked increase in the index of phagocytic activity in these tubes compared with those from which partitions with active polymorphs adherent to them were removed after centrifugation.

The surface covered with protein (gelatin) used in these experiments was evidently adequate for the polymorphs and simulated to some degree the surface of an inflammatory focus. At least after introduction of a partition containing such surfaces on both sides into the test tube, maximal values of phagocytic activity were obtained (group 3).

TABLE 1. Ingestion of *Staph. aureus* by Polymorphs Depending on Conditions of Incubation of Leukocyte-Bacterial Mixture

| Parameter | Group of tubes | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | | 3 | 4 |
| | | sedimentation of leukocytes in tubes with partition | sedimentation of leukocytes in tubes without partition | | |
| Mean phagocytic index, % | 1,0 | 25,7 | 4,5 | 50,1 | 30,1 |
| Mean phagocytic number | 1,4 | 3,2 | 1,8 | 3,5 | 2,4 |
| Significance of difference from group 1 in phagocytic index, *P* | | | | <0 01 | <0 01 |
| Significance of difference from group 1 for phagocytic number, *P* | | | | <0 01 | <0 01 |
| Significance of differences between groups 3 and 4 for phagocytic index, P | | | | <0,05 | |
| Significance of differences between groups 3 and 4 for phagocytic number, P | | | | <0,01 | |

In group 1, polymorphs not adherent to the surface and unable to perform chemotaxic movements ingested bacteria only rarely, evidently because of infrequent direct contact with them. This is shown by the sharp increase in parameters of phagocytic activity if bacteria and polymorphs were continually colliding with one another during spinning of the tubes (group 4). The phagocytic index was very high in this group, only a little below that in group 3 (the difference is not statistically significant). Meanwhile the phagocytic number in group 4 was significantly lower than in group 3 (Fig. 1). This shows that even in cases when contact between bacteria and polymorphs was brought about artificially, the ingestive capacity of the polymorphs was still not exhibited to the full. Investigation of phagocytosis in rotating tubes thus does not reflect the exact behavior of polymorphs in an inflammatory focus, for active movement of these cells, which, in the light of data on the development of septic complications in burned patients [9, 11] and worsening of the state in other diseases [8] attended by disturbances of chemotaxis, plays an essential role, is not taken into account.

In conclusion, the disparity between these results in group 1 and data obtained by many other Soviet workers for the intensity of ingestion of bacteria in stationary tubes must be explained. The reason for this disparity is evidently that those data were obtained by investigation of much smaller volumes of blood (sufficient only for preparing films), and with a correspondingly high ratio between the surface area of glass to which the leukocyte-bacterial mixture was applied and the volume of the mixture. Polymorphs can adhere to glass and can migrate along it. Consequently, as a result of analyses of this kind chemotaxis makes its own contribution, although the authors cited do not discuss it. In our own version of the test, when a sufficiently large number of leukocytes were studied, sufficient for obtaining preparations for electron-microscopic examination, the ratio of the surface area (the walls of the test tube) to the volume of leukocyte-microbial mixture was very small, and this has a substantial influence on the results.

The results of the present experiments show that phagocytic activity of polymorphs is realized to the full in an inflammatory focus, where factors preventing adhesion of the cells

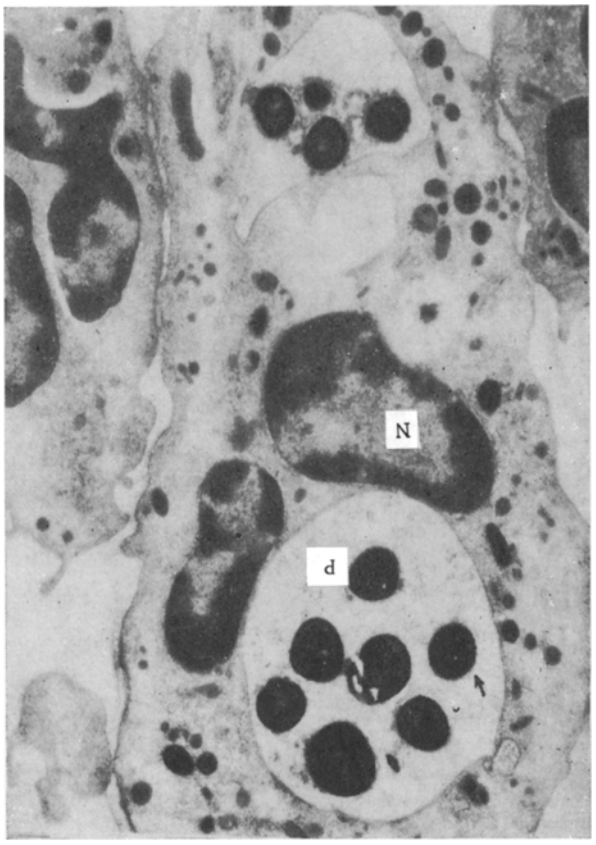


Fig. 1. Polymorphs from a tube of group 3, with the maximal phagocytic number, contains 11 bacteria in two phagosomes (arrow). N) Leukocyte nucleus, P) phagosome. 12,000×.

with one another and with bacteria are absent, and where polymorphs can adhere and migrate by means of chemotaxis. In blood, on the other hand, their phagocytic activity must evidently be either absent or, at least, very sharply reduced.

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