A Phagocytosis Inhibition Test in Infection Hypersensitivity.* (19526)

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(Introduced by W. J. Nungester.)
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A distinctive feature of infection hypersensitivity is cell response *in vitro*, as demonstrated by Holst(1), Rich and Lewis(2), Aronson(3), Moen and Swift(4), and others. In reporting their studies of sensitization in infection, most investigators have used tissue culture technics and have emphasized the importance of using cells from sensitized rather than normal animals. The addition of specific antigen to sensitized cells results in cessation of cell migration, along with necrotic changes. Favour and his co-workers(5) have suggested that the chief factor in these cell changes may be a component of serum from sensitized animals or man. These investigators have shown that serum from a hypersensitive individual plus the specific sensitizing antigen will cause lysis of either normal or "sensitized" leukocytes *in vitro*. Moreover, Favour(6) has reported that the serum factor responsible for cell changes is located in the globulin fraction, and that it is readily destroyed by heating at 56°C for 15 minutes.

The leukocytolysis reaction, while useful in studying infection allergy, requires an hour to complete and necessitates making two white cell counts. The work reported here is the result of efforts to devise a more rapid and more sensitive *in vitro* test for infection hypersensitivity. The possibility of inhibiting the phagocytic action of polymorphonuclear leukocytes with a serum factor and specific antigen was suggested by the fact that lysis of white blood cells undoubtedly is preceded by fundamental changes in structure of the cell membrane. Such changes might be expected to result in loss of specific cell functions before lysis would occur.

Materials and methods. Polymorphonuclear leukocytes were obtained by stimulating the production of an exudate in the peritoneal cavity of normal guinea pigs as described by Nungester and Ames(7). The cells were collected from the exudate, washed and diluted to a count of 25000/mm^3. Their phagocytic action was tested in a system containing serum from animals sensitized to a beta-hemolytic streptococcus of Group B or to a Type I pneumococcus, and a filtrate of a 24 hour broth culture of the respective organism. The material to be phagocytized was a heat killed suspension of *Bacillus anthracis*. In making the tests 0.3 ml of sensitized serum was mixed with 0.1 ml of filtrate and 0.1 ml of the leukocyte suspension. The tubes were incubated with continuous agitation for 15 minutes at 37°C after which 0.1 ml of anthrax suspension was added with thorough mixing. A drop of this material was placed within a ring of petrolatum on a glass slide and overlayed with a coverslip. After an additional 15 minutes of incubation at 37°C, the slide was examined with the aid of darkfield illumination or a phase contrast microscope. Percentage phagocytosis was calculated according to the formula:

$$\frac{\text{No. of cells containing bacteria}}{\text{Total No. of cells counted}} \times 100$$

Normal serum, uninoculated broth, or 0.85% NaCl (in the combinations indicated in Tables I, II, III), were used as controls.

Results. Streptococcal filtrate alone had a slight toxic action but the combination of sensitized serum and specific filtrate caused a definite decrease in phagocytic action (Table I). Results of experiments using a Type I pneumococcus were essentially the same as those with the streptococcus (Table II). The specificity of the reaction is indicated by the failure to demonstrate inhibition of phagocytosis with sensitized serum and heterologous filtrate. Heating the sensitized serum at 56°C for 30 minutes destroyed its ability to prevent phagocytosis.

To demonstrate the association of phagocytosis inhibition with infection hypersensitivity, an attempt was made to block phago-

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TABLE I. *In vitro* Inhibition of Phagocytosis in Hypersensitivity to Group B, β Hemolytic Streptococcus (Summary of 8 Exp.).

	Streptococcal filtrate, %	Uninoculated broth, %	.85% NaCl, %
Normal serum	76*	80	80
Sensitized serum	66	80	
" " (heated at 56°C)	79		

* % phagocytosis.

TABLE II. *In vitro* Inhibition of Phagocytosis in Hypersensitivity to Type I Pneumococcus (Summary of 3 Exp.).

Serum	Pneumococcal filtrate, %	Streptococcal filtrate, %	Uninoculated broth, %	.85% NaCl, %
Normal	86*		84	85
Sensitized	75	85		88

* % phagocytosis.

TABLE III. *In vitro* Inhibition of Phagocytosis in Hypersensitivity to Egg Albumin (Summary of 3 Exp.).

	Egg albumin 1:100, %	Egg albumin 1:1000, %	.85% NaCl, %
Normal serum	68*	73	85
Sensitized serum	75	84	83

* % phagocytosis.

cytic action with serum from guinea pigs sensitized to egg albumin. The results indicate that egg albumin, by itself, blocks phagocytosis (Table III). A similar observation has been reported by Ouweleen(8) in the phagocytosis of carbon particles but he could not show this blocking action of egg albumin when starch granules were used. In the present study, serum from sensitized animals neutralized the inhibitory action so that normal engulfment of the bacteria could take place.

Discussion. From the data presented it is seen that a factor present in the serum of guinea pigs sensitized to certain species of bacteria is capable of inhibiting the phagocytic action of normal guinea pig leukocytes in the presence of a filtrate of the specific organism. The materials used in this test are the same as those used in the leukocytolysis reaction, except that a suspension of bacilli was needed to demonstrate phagocytosis. This suggests that lysis results as an extension of the cellular changes which interfere with phagocytosis. The fact that the total white cell counts made at the beginning and at the end of some of our experiments showed a loss of 10-20% of the cells is further evidence for this view. Jung(9) and Hanks(10) in studies of quantitative phagocytosis, suggested that an appreciable drop in numbers of leukocytes would lead to an increased percentage phagocytosis, since the ratio of white cells to phagocytizable particles is altered. For this reason the 10% decrease (mean) which was actually observed in our experiments becomes even more significant.

All of the values presented in the tables are mean values for a series of experiments. To determine the possible significance of the 10% difference observed between sensitized serum plus filtrate and normal serum plus filtrate the standard error of the difference between the means (S. E.). was determined for the systems testing sensitization to streptococcus (Table I) and to pneumococcus (Table II). In the former instance the S. E. was 2.05 and in the latter was 1.89, indicating that the differences observed are statistically significant.

The specificity of phagocytosis inhibition is indicated by the results of tests using a heterologous filtrate (Table II). Failure to block phagocytic action with serum from guinea pigs sensitized to egg albumin in the presence of the specific antigen is in accord with findings using the leukocytolysis and tissue culture methods to study *in vitro* cell damage.

Summary. A phagocytosis inhibition test is described for infection hypersensitivity. It is similar to the leukocytolysis reaction and to studies of cell damage in tissue culture in that it is not readily demonstrated in hypersensitivity to egg albumin. The test is simple to perform and can be carried out very rapidly. These features, along with its specificity, make it a useful procedure for the *in vitro* evaluation of infection allergy.

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Modification of Gomori Method for Alkaline and Acid Phosphatase Avoiding Artefact Staining of Nucleus.* (19527)

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The histochemical phosphatase methods originally outlined by Gomori(1,2) have been critically studied in this laboratory. Consistently reproducible results with Gomori's acid phosphatase method have been obtained during the past two years by adhering to certain details ascertained to be necessary in the procedure(3). By incubating the sections in the paraffin ribbons directly in substrate, the known harmful effect of exposure of the contained enzyme to various concentrations of alcohol incident to deparaffinization and rehydration was eliminated. Not only do the resultant preparations show greater and more constant preservation of phosphatase activity but also its localization is much sharper and appears in finer detail. In addition, the apparent phosphatase activity of the nucleus as seen in conventional preparations is largely if not entirely absent, as Ruyter and Neumann(4) have shown with the alkaline phosphatase technic. The discrepancy between the results obtained from cellular fragmentation experiments and from histochemical determinations is therefore resolved. As noted by several investigators (5,6), nuclei isolated by cellular fragmentation technics show chemically only a small fraction of the total phosphatase activity demonstrable. Histochemically this is confirmed by the Menten(7) or other similar methods which use the alcoholic end of the phosphate ester to produce a color reaction by formation of a diazonium salt. This is clearly seen in illustrations in the articles by Danielli (8) and Manheimer and Seligman(9) although no statement to this effect appears in their respective papers. Novikoff(10) however, has recently emphasized this point. The results obtained with the conventional Gomori method for alkaline or acid phosphatase(1,2) using deparaffinized tissue sections are in direct contrast to these data in that all nuclei in tissues showing phosphatase activity have also shown marked "phosphatase activity". This discrepancy is eliminated by the use of non-deparaffinized tissue sections in which it is found that the nuclei show very little activity. Since all 3 methods using entirely different approaches now agree it would seem that this localization is a true finding.

The validity of this unconventional method depends upon the assumption that the substrate reaches every part of the cell in which phosphatase activity may exist. This has been put to test by observing the penetration, or loss of substances of different molecular size into or from tissue sections within paraffin ribbons.

1) *Penetration of water.* As noted by Harrison and Bunting(11), ferrocyanide in paraffin sections of kidney is immediately removed by contact of the ribbon with water. Similarly, glycogen leaches out of paraffin sections mounted on a glass slide and immersed in distilled water for 2 hours, parallel-

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