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In vitro Phagocytosis by Amoebocytes from the Haemolymph of *Helix aspersa* (Müller)

I. EVIDENCE FOR OPSONIC FACTOR(S) IN SERUM

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Summary. *In vitro* phagocytosis of foreign particles by amoebocytes from the haemolymph of *Helix aspersa* (Müller) was studied.

Results showed the presence of an opsonin in *Helix* serum. This opsonic effect of serum is due to the adsorption of serum component(s) on to the foreign particles. No phagocytosis occurs in the absence of these factors.

There was some degree of specificity of the opsonin for two different foreign particles: formalized yeast cells (*Saccharomyces cerevisiae*) and formalized sheep red blood cells.

INTRODUCTION

Understanding of the discriminatory process by which phagocytic cells identify foreign material is a very crucial point in the formulation of a theory of immunity. Boyden (1962) suggested that the mechanism whereby the phagocytic cells distinguish between indigenous and foreign material may be mediated by soluble macromolecules in the body fluids. There is a dearth of information concerning the mechanism of recognition by phagocytic cells from invertebrate species. Yet responses, which find their full expression in vertebrates, may be an evolutionary extension of a recognition mechanism already established in lower phyla. For this reason it seems valuable to study intensively invertebrate systems, which may be less complex, but still offer similar general principles.

The experiments described were designed to investigate the question whether there exist in the serum of invertebrates substances performing a function similar to that of opsonins in the vertebrates. The system employed in this study was the *in vitro* phagocytosis of foreign particles by amoebocytes from the haemolymph of the snail, *Helix aspersa* (Müller). There are several approaches to this problem. The one chosen in this study was to presume that some serum factor(s) adsorb on to the foreign particle and can be removed from the serum by absorption with that particle. The phagocytic activity of amoebocytes regarding a foreign particle could then be contrasted in the presence of normal serum and of absorbed serum.

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EXPERIMENTAL METHODS

Foreign particles

Formalized yeast cells and sheep red blood cells (SRBC) were used as the foreign particles. A packed volume of baker's yeast (*Saccharomyces cerevisiae*) was fixed for 24 hours in 3 volumes of 10 per cent formaldehyde in 0·85 per cent saline at 4°. Sheep red blood cells were fixed in formaldehyde according to the method of Butler (1963). Following fixation, particles were washed and dialysed against running tapwater for 72 hours.

Collection of haemocytes and serum

A clean suspension of large numbers of haemocytes in haemolymph was obtained by dissecting open the mantle cavity of the snail to expose the roof of the perivisceral haemocoele. The latter was punctured and the haemolymph collected in a beaker kept at 0° to prevent adherence of cells to the glass. Fresh haemolymph was used in all experiments. In addition to amoebocytes this suspension contained low numbers of smaller round non-phagocytic cells. In counts of pooled haemolymph these consistently made up 12–14 per cent of the total cells.

To obtain serum no dissection was carried out. Haemolymph was withdrawn by syringe directly from the haemocoele situated immediately below the shell; it was freed of cells by centrifugation. This serum will be referred to subsequently as normal serum. This technique enabled one to repeatedly bleed individual snails.

Absorption of serum

Absorbed serum was prepared by incubating 1 volume of foreign particles (packed) with 4 volumes of serum for 15 minutes. The suspension was centrifuged and the supernatant removed. The procedure was then repeated with fresh foreign particles for a total of four absorptions.

'Opsonization' of foreign particles

Particles were 'opsonized' by incubating them in normal serum for 1 hour at room temperature, then washing three times to remove all traces of excess serum.

Preparation of Hédon Fleig salt solution

Hédon Fleig salt solution, prepared according to Gatenby (1937), was used in all cases for washing of cells and diluting serum. Antibiotics (penicillin 100 units/ml of medium and streptomycin 100 μg/ml of medium) were added. With this precaution and sterilization of all glassware, cultures remained clean over the term of the experiment.

Technique for quantitative determination of phagocytosis

Phagocytosis experiments were carried out in small flat-bottomed glass tubes of base diameter 1 cm. A suspension of haemocytes was placed in each tube and allowed to settle for 1 hour at 25°, during which time the amoebocytes adhered to the glass bottom of the tube. The amoebocytes were then washed three times with Hédon Fleig salt solution to remove all traces of serum and non-phagocytic haemocytes. Foreign particles suspended in various media were then added to the tubes; the ratio of foreign particles to amoebocytes was approximately 10 : 1. Sufficient tubes for each treatment were prepared to allow individual tubes to be examined for phagocytosis at various time intervals. The extent of

phagocytosis at various times was determined in each tube after resuspending unphagocytosed foreign particles on a 'Vortex-Genie' (Scientific Industries Inc.). A comparison was then made of haemacytometer (Resistance: improved Neuerbauer ruling) counts of these resuspended particles with a count made in a control tube containing particles but without amoebocytes. Four replicates were made at each time interval and of each control tube.

Technique for observing phagocytosis

Wells similar to those described by Boyden (1964) were used for observing phagocytosis. They were made by waxing glass coverslips to one side of Perspex slides of 0·25 cm thickness with two holes of diameter 1 cm cut in them. Amoebocytes could attach to the glass coverslip forming the base of the well. The well was then filled with a suspension of foreign particles in various media. Following incubation, the well was sealed with a coverslip and the slide inverted and the amoebocytes viewed under phase contrast.

RESULTS

Experiment 1

The first experiment was set up to investigate the role of serum factors in phagocytosis of formalized yeast cells by snail amoebocytes. Yeast cells suspended in one of various media, as outlined below, were added to glass tubes containing 20,000 amoebocytes. The latter had previously been allowed to settle for 1 hour at 25° in 0·2 ml of 50 per cent normal serum in Hédon Fleig salt solution. The amoebocytes were then washed three times in Hédon Fleig salt solution prior to addition of yeast particles.

Treatment 1

Yeast cells were allowed to incubate in normal serum 50 per cent in Hédon Fleig salt solution for 1 hour. A 0·4-ml volume of this suspension containing 200,000 yeast cells was added to each tube (yeast in normal serum).

Treatment 2

Yeast cells were allowed to incubate in absorbed serum 50 per cent in Hédon Fleig salt solution for 1 hour. A 0·4-ml volume of this suspension containing 200,000 yeast cells was added to each tube (yeast in absorbed serum).

Treatment 3

Yeast cells were allowed to incubate in normal serum 50 per cent in Hédon Fleig salt solution for 1 hour as in Treatment 1 above. They were then thoroughly washed and resuspended in absorbed serum 50 per cent in Hédon Fleig salt solution. A 0·4-ml volume of this suspension containing 200,000 yeast cells was added to each tube (opsonized yeast in absorbed serum).

Fig. 1 shows the results of this experiment, which was repeated three times using different batches of pooled serum and of phagocytic cells. Figures for recovered yeast cells are expressed in terms of mean number of yeast cells phagocytosed per amoebocyte for various intervals up to 6 hours.

There was no significant phagocytosis of yeast cells in a medium of absorbed serum. On

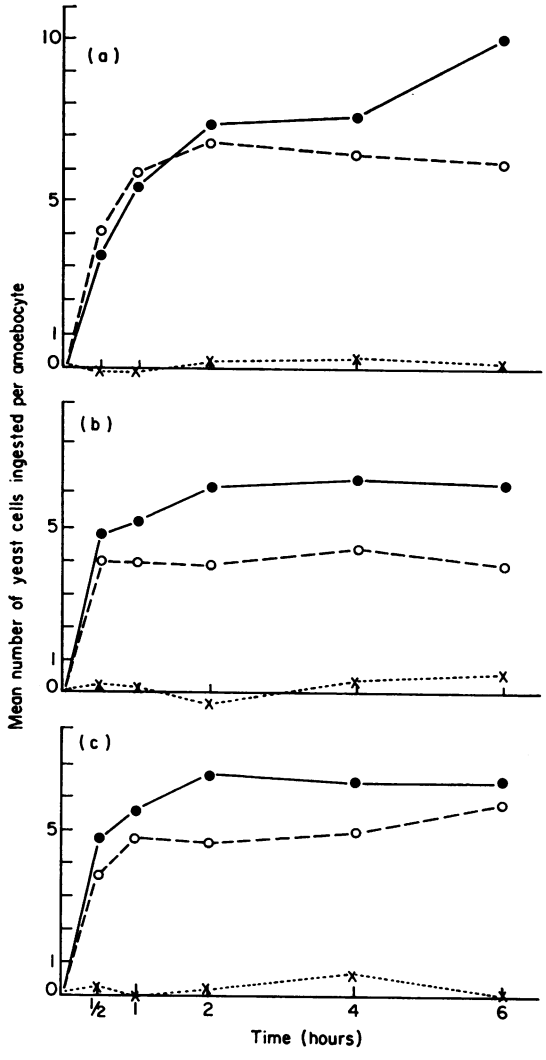

FIG. 1. The rate and extent of phagocytosis of yeast by snail amoebocytes, ●. Yeast in normal serum; ×, yeast in absorbed serum; ○, opsonized yeast in absorbed serum.

the other hand, yeast cells suspended in normal serum and opsonized yeast cells in absorbed serum were phagocytosed. Opsonized yeast cells in absorbed serum were not phagocytosed to quite the same extent as yeast cells in normal serum. The differences were statistically significant at 6 hours in the first experiment (Fig. 1a), at 2, 4 and 6 hours in the second experiment (Fig. 1b) and at 2 hours in the third experiment (Fig. 1c).

Examination of the wells set up to observe phagocytosis showed that this activity occurred when yeast cells were suspended in normal serum and when opsonized yeast were suspended in absorbed serum. Amoebocytes suspended in a medium of absorbed serum appeared healthy, but no phagocytosis was observed. Fig. 2 shows yeast particles phagocytosed by amoebocytes in the presence of normal serum.

The results of this experiment suggest that there is a substance present in snail serum which adsorbs on to the surface of the yeast cells, thereby rendering them susceptible to phagocytosis. The results of preliminary experiments to determine the optimum conditions for complete absorption of this substance from snail serum suggested that the adsorption on to the yeast cells was to some extent reversible. This finding could possibly account for the lower phagocytic activity of amoebocytes presented with opsonized yeast cells in absorbed serum as compared with yeast cells suspended in normal serum. If there were a reverse action in which opsonins were slowly being detached from the yeast cells, loss of opsonins might occur during the washing procedures.

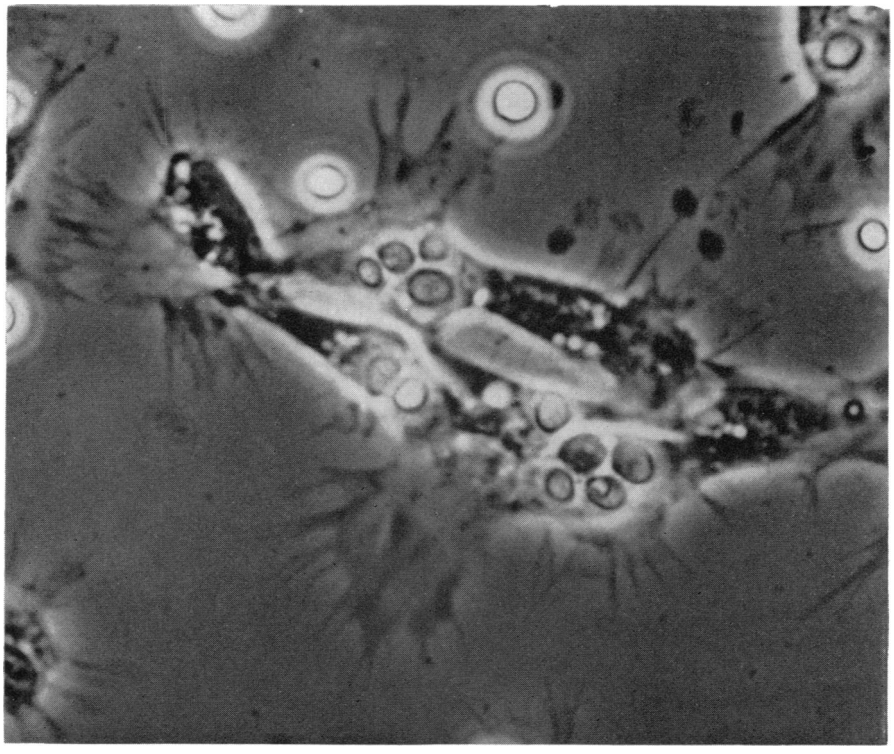

FIG. 2. Yeast particles phagocytosed by amoebocytes in the presence of normal serum. × 1040.

EXPERIMENT 2

This experiment was designed to determine the specificity of this opsonic factor in snail serum. Using a similar system to the above, a comparison was made of the phagocytosis by snail amoebocytes of two different foreign particles. The extent of phagocytosis of formalized yeast cells and formalized SRBC was compared when they were suspended in snail serum absorbed with either the homologous or the heterologous foreign particle. The experiment was repeated using different batches of pooled serum and of phagocytic cells.

The results of this experiment are given in Fig. 3.

No significant phagocytosis of yeast or of SRBC occurred in a medium containing serum absorbed with the homologous foreign particle. Phagocytosis of each foreign particle

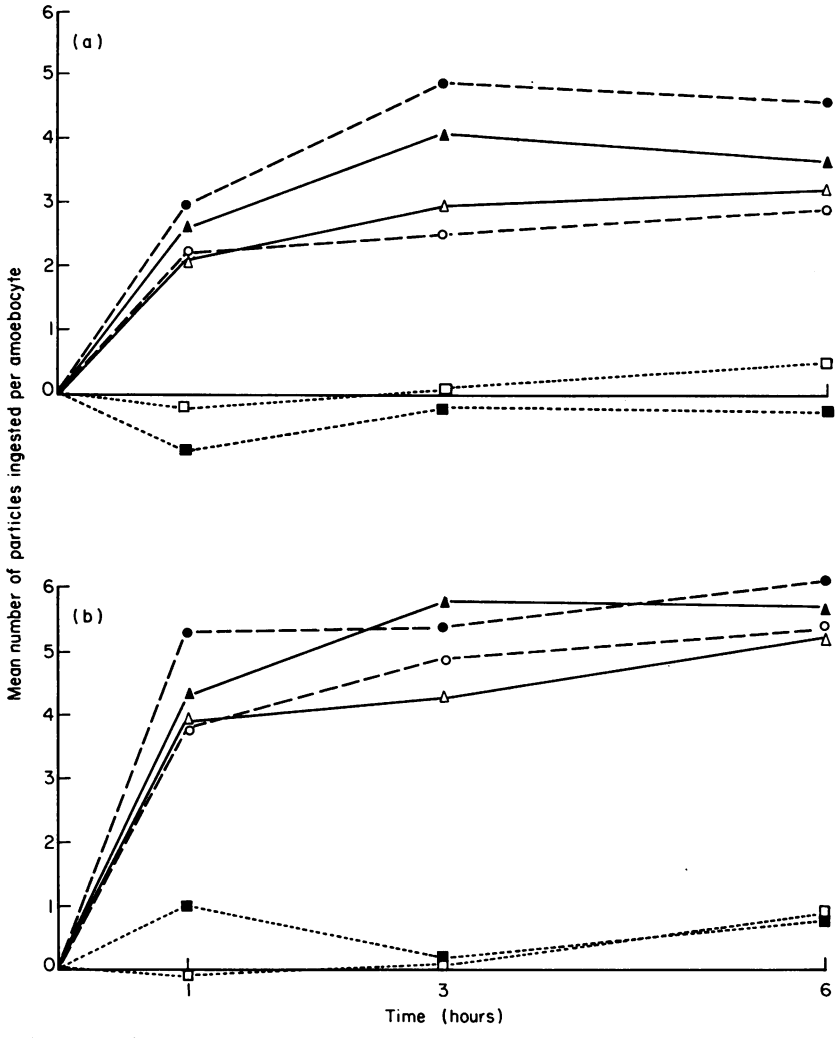

FIG. 3. The rate and extent of phagocytosis of yeast and sheep red blood cells in serum absorbed with either the homologous or the heterologous particle. ▲, Yeast in normal serum; △ SRBC in normal serum, ●, yeast in serum absorbed with SRBC; ○, SRBC in serum absorbed with yeast; ■, yeast in serum absorbed with yeast; □, SRBC in serum absorbed with SRBC.

occurred in normal serum and in serum absorbed with the heterologous particle. There was no significant difference between the extent of phagocytosis of yeast cells in normal serum and in serum absorbed with sheep red blood cells. The same was true of SRBC in normal serum and in serum absorbed with yeast. Within the range of foreign particles used, this experiment shows that there is some degree of specificity of the opsonic factor(s).

DISCUSSION

The evidence presented in this paper strongly suggests an essential role of serum factor(s) in the phagocytosis of foreign particles by amoebocytes from the haemolymph of the snail *H. aspersa*.

The method employed to quantify phagocytosis by estimating the number of particles not ingested had the advantage of rapid counting and accurate distinction between phagocytosed particles and particles merely adherent to the surface of the amoebocytes. The technique had the disadvantage that visual verification of phagocytosis could not be made. However, the visual method, using wells, did not allow rapid and accurate counting of ingested particles. A combination of these two techniques—quantitative and qualitative—provided the most satisfactory assay.

Numerous reports have been made claiming the occurrence, in the fluids of many invertebrate species, of substances having some of the serological activities of antibodies in vertebrates (Good and Papermaster, 1964). However, few reports have been concerned with elucidating the role of these serum factors in phagocytosis. Studies have been carried out on the oyster, *Crassostrea virginica*, claiming that serum factors influence the *rate*, rather than the extent of phagocytosis (Tripp, 1966). On the other hand, our results indicate that, in the snail, serum factors are necessary for any phagocytosis at all to take place.

Our findings are consistent with a view that there is a factor(s) in snail serum which is adsorbed on to the surface of foreign particles, rendering them susceptible to phagocytosis. Perhaps an analogy may be drawn between this substance and an opsonin in the serum of a vertebrate. Further support for this interpretation is provided by the finding that this interaction was specific for the two foreign particles tested. The specific nature of the opsonin rules out the possibility that this phenomenon is due to a non-specific activity of normal serum, such as, an alteration of charge on the surface of the foreign particle or an enzymic removal of an 'antiphagocytic' material.

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