2 μCi ^{3}H-thymidine (Radiochemicals Centre, Amersham) was added to each culture. 3 h later the cell deposit was washed consecutively in PBS, 5% trichloracetic acid (3 times), absolute methanol then dissolved in 0.2 ml 0.1 *M* NaOH. The ^{3}H content was determined by scintillation counting after mixing NE 260 scintillation fluid (Nuclear Enterprises Ltd.).

Results. Figure 1 shows that the level of PFC production 96 h after SRBC injection was dependent on the time of carrageenan administration in relation to antigen. Antibody production in mice receiving a single injection at −24 or 0 h were significantly lower than control values ($p < 0.005$ and $p < 0.0025$ respectively, according to Student *t*-test), the effect being most marked when carrageenan was given at −24 h. Carrageenan treatment at any other time did not significantly affect the response.

The effect of carrageenan on cultures of in vivo sensitized spleen cells is shown in Figure 2. Notably, the presence of carrageenan alone did not significantly affect the amount of thymidine incorporated. Although pretreatment of antigen-stimulated cells with carrageenan significantly depressed the response ($p < 0.025$), the addition of this agent 6 h after antigen challenge did not affect uptake of radiolabel. The level of stimulation obtained to the polyclonal mitogen PHA was not significantly altered by pretreatment of the spleen cells with carrageenan.

Discussion. From this study it is clear that the efficacy of carrageenan as an immunosuppressant both in vivo and in vitro depends on the temporal relationship between treatment and antigen administration. The fact that carrageenan caused marked suppression of the PFC response only when given 24 h before or together with SRBC suggests that it acts on the inductive phase of the immune response and not on antibody-producing bone-marrow derived (B) cells. Macrophages are known to be involved in the induction of immune reactivity to a variety of antigens[7] and our observations are therefore consistent with the known in vitro selective cytotoxic effect of carrageenan for these cells. This argument gains further support from our finding that treatment of sensitized spleen cell cultures with carrageenan before, but not 6 h after antigen challenge impaired the blastogenic response.

It is unlikely that carrageenan is toxic to thymus-derived (T) lymphocytes, since it failed to suppress lymphocyte transformation by PHA, a known T cell mitogen. This finding is in agreement with the observation of previous authors using cultured human and guinea-pig lymphocytes[2,8]. Further, we have shown elsewhere[9] that in the rat, carrageenan does not affect T cell function as measured by the graft versus host reaction. In conclusion, we believe that these observations are consistent with the hypothesis that in vivo, carrageenan is toxic to macrophages but non-toxic to lymphocytes.

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Production of Heteroagglutinins in Haemocytes of *Leucophaea maderae* L.

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Summary. It was our purpose to study the immunological activity of circulating fluid cells in the blattoid insect *Leucophaea maderae* L. These cells are generically called haemocytes; after a preliminary morphological study under optical microscope, they were treated with anti-heteroagglutinins serum marked with fluorescein isothiocyanate. Thus, it was possible to show the presence of heteroagglutinins into the cytoplasm, as regards one group of haemocytes, and on the cell membrane for the second group.

For some years the attention of many researchers has been drawn more and more to the immunological phenomena to be found in invertebrates.

The substances present in the fluids of many invertebrate classes and in nearly all species, are the object of studies which more and more frequently appear in the literature. These substances are commonly known as heteroagglutinins, due to their capacity for reacting more or less specifically with heterologous cells or with bacteria.

Such substances have been isolated from worms[2,3], molluscs[4,5] arthropods[6–10], echinoderms[11,12], and protochordates[13,14], and many authors consider them to be similar to or comparable with vertebrate immunoglobulins. Some authors also suggest that the immunoglobulinic chains of vertebrates may originate from these simpler proteins. In this connection, the heteroagglutinins of some crustacean and mollusc species have been studied particularly from a structural, immunochemical and biophysical point of view[15].

On the other hand, nothing is known about organs, tissues or cells that may be involved with, or responsible for their synthesis, except for MARCK's studies[8] concerning

[1] Acknowledgment. The author wishes to express his sincere thanks to Prof. R. MILANI, Director of the Zoological Department of the University of Pavia, who kindly supplied the insects to carry out the present research.
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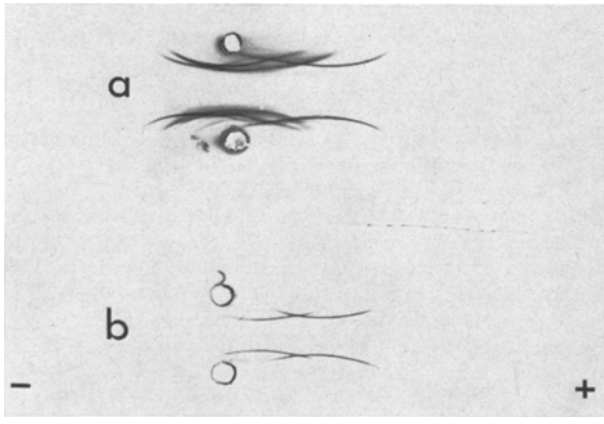

Fig. 1. Immunoelectrophoresis of *Leucophaea* haemolymph against serum anti-haemolymph (a). Immunoelectrophoresis of *Leucophaea* haemolymph against serum anti-heteroagglutinins (b); only 2 bands of precipitation representing the 2 purified heteroagglutinins are seen.

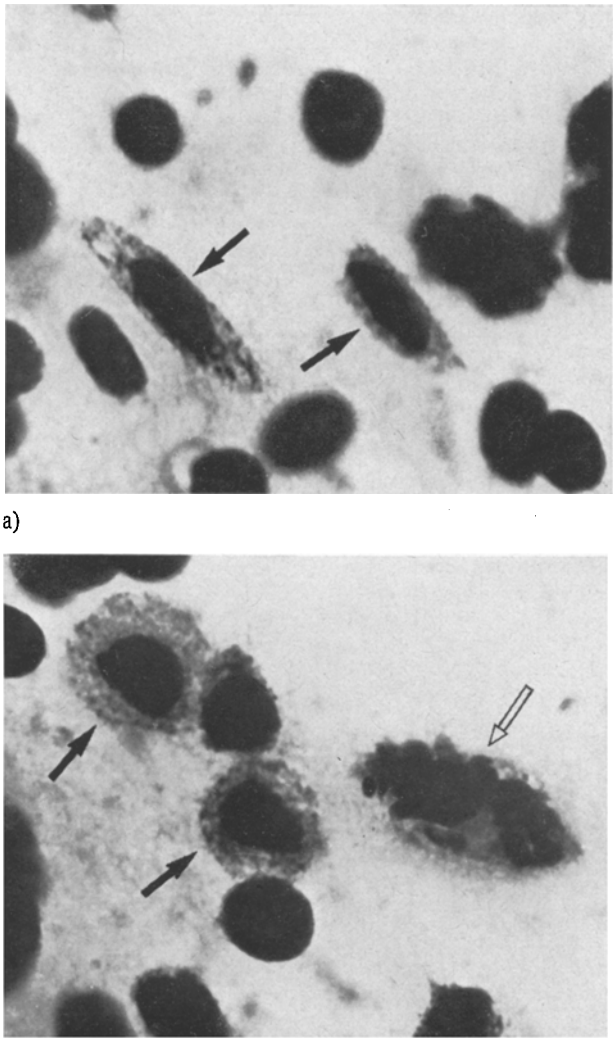

Fig. 2. Strips of haemocytes stained according to May Grünwald-Giemsa. a) Two differently sized elliptical α-cells (see arrows) showing various vacuoles. It is possible to see β-cells nuclea. b) Sheroidal α-cells with cytoplasmatic granulations (black arrows) and big ellipsoidal α-cells with cytoplasm full of dense inclusions (white arrow).

appearance of heteroagglutinins in *Galleria mellonella* after a thermal shock; in this research MARCK hypothesizes that haemocytes, i.e. the cells present in the invertebrates haemolymph, may be somehow correlated with the heteroagglutinins synthesis, since after their appearance haemocytes increase in number.

On the other hand, the activity of haemocytes, or at least of some of them, is well known in relation to the identification of extraneous corpuscles and fragments of heterologous tissues, this reaction taking place both in vitro and in the tissues experimentally grafted in the animal. In fact, in anellids[16], molluscs[17] and arthropods[18], it was observed that the presence of heterologous bodies leads to a rheotaxis of these cells, which first isolate the foreign body by incapsulating and then destroying it.

The purpose of the present work is to carry out a more thorough investigation on the role that the haemocytes present in the blattoid insect *Leucophaea maderae* L. play in this animal immunological response, and particularly to ascertain whether any of them may be response, and particularly to ascertain whether any of them may be responsible for the natural heteroagglutinins synthesis.

Materials and methods. Extraction of haemolymph. Adult specimens of *Leucophaea* (supplied by the Zoological Department of Pavia University) were immobilized, and haemolymph was removed from the haemocoel with a micropipette. The haemolymph of about 80 animals was immediately collected and centrifuged in order to remove all haemocytes. Natural haemagglutinins were extracted by the haemolymph so obtained by making them react with a suspension of rabbit red blood cells.

The resulting agglutinate was centrifuged and then washed several times with saline solution to remove every haemolymphatic component that had not specifically reacted with erythrocytes. The heteroagglutinins-red cell complex was emulsified with Freund's complete adjuvant (1:1) and injected to rabbits. This treatment was repeated 4 times, and soon after a good production of heteroagglutinin specific antibodies was obtained. To test the specificity of such antiserum, immunoelectrophoresis against haemolymph or hydrosoluble extract of *Leucophaea*, various organs homogenate were prepared. The results have always confirmed a positive reaction only against the 2 heteroagglutinins (Figure 1) that are present in haemolymph, and which we isolated and studied from an immunochemical point of view in a precedent work[19].

Furthermore, if antiserum is first treated with the heteroagglutinins-red cell complex, the 2 immunospecific bands do not appear on immunoelectrophoresis.

Conjugation of antiserum with fluorochromes. The anti heteroagglutinin antiserum so obtained was marked, according to NAIRN[20], by conjugating it with fluorescein isothiocyanate purified on a DEAE Sephadex A25 column, and then concentrated by ultradialysis.

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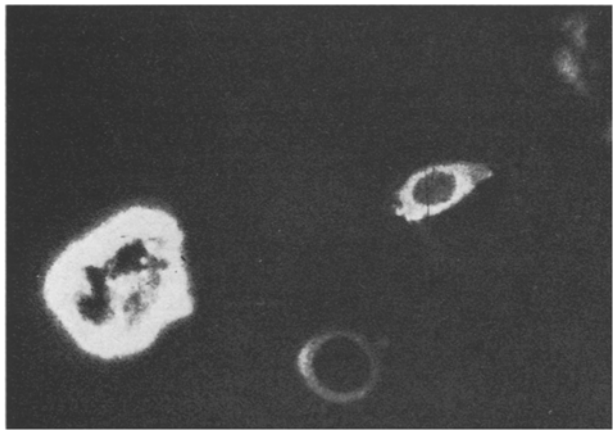

Fig. 3. Haemocytes treated with marked anti-heteroagglutinin serum. The green fluorescence is present at the level of the cytoplasmatic membrane in some cells; in others the fluorescence is diffused throughout the cytoplasm, with the exception of the vacuoles and nuclea.

Extraction of haemocytes and their treatment with marked antiheteroagglutinins. The haemocytes present in haemolymph were taken from the haemocoel with a micropipette, as described above. A drop of this haemolymph containing haemocytes was immediately laid on a slide and dried. Some strips were stained according to May Grünwald Giemsa or according to Wright, and were observed under optical microscope; others were fixed with methanol for 5 min, washed 3 times with PBS pH 7.2 and treated with marked antiheteroagglutinin antiserum for 50 min. The slides were then washed 3 times with PBS and prepared with buffered glycerin in order to be observed under UV microscope. As a control, some slides were treated with unmarked antiserum for 20 min, washed and then treated for 50 min with marked antiserum; furthermore, the marked serum that had been treated beforehand with the heteroagglutinins-red cell complex and then was made to reach with haemocytes strips, always give a negative response.

Results and discussion. The haemocytes extracted from haemolymph and observed under optical microscope were found to be very numerous. As regards their morphology, observing them superficially they can be distinguished into 2 classes of cells. These classes may be compared with the 2 groups of haemocytes that were studied and described by JOHNSTON et al.[21] in *Carcinus moenas*. In our case also, the cells of the first group, which JOHNSTON called α- cells, have the highest numerical percentage and are represented by a fair variety of forms. In fact, some are smaller (about 6 μm) and have a generally spherical or elliptical shape and little cytoplasm, others are larger (about 10 μm) and have a more abundant cytoplasm in which acidophil granulations can be seen, finally some are still bigger (about 12 μm) and have less regular cellular outline than the foregoing ones; their cytoplasm abounds in vacuoles which are optically empty or full of inclusions (Figure 2). The characteristic common to these cells are a spherical or ovoid form, the basophily of cytoplasm and the presence of a strongly basophil nucleus which is spherical and eccentric.

The haemocytes, belonging to the second group which observed, can also be compared with the β-cells described by JOHNSTON. Less numerous in percentage, they have larger dimension than the ones of the first class; their nucleus is roundish and less basophil, and their cytoplasm is much clearer. Very seldom, these cells were observed uninjured. Often the cell membrane appears broken, so that only nuclei can be seen. As JOHNSTON and TAIT and GUNN[22] suggest, these cells, may be responsible for the haemolymph coagulation.

In the slides treated with marked anti-heteroagglutinin serum, we found that about 95% of the first group of haemocytes (α-cells) were fluorescent. This fluorescence is very clear and outstanding and, furthermore, is highly specific. In fact, the haemocytes treated before with unmarked antiserum and then with marked antiserum, never showed fluorescence. Examination of Figure 3 reveals that fluorescence is localized on the cell membrane, as regards the smallest group of haemocytes, while in the largest cell fluorescence is clear and diffused all over the cytoplasm, except vacuoles and inclusion that look dark and non-fluorescent spots. The nucleus never shows fluorescence. From these preliminary data, we can infer that α-haemocytes are responsible for the natural heteroagglutinins synthesis. That, in these cells, the synthesis of heteroagglutinins takes place (at least in the biggest haemocytes) is proved by the fact that fluorescence is uniformly diffused all over cytoplasm, always excepting the vacuoles. This hypothesis will obviously have to be supported by further observations and tests, which are being carried out in our laboratory.

The fact that heteroagglutinins are differently localized in small and large haemocytes, suggests that these two groups of cells do not belong to the same class of α-cells (young and adult forms), as observation under optical microscope gives reason to suppose, but rather that they constitute 2 quite different groups: the former, in which heteroagglutinins are localized on cell membrane, may be responsible for cellular immunological reactions; the latter, in which there is a considerable concentration of heteroagglutinins in cytoplasm, may be responsible for their synthesis and subsequent excretion in haemolimph. One one hand, we propose to extent our knowledge about immunochemical and biophysical properties of heteroagglutinins, while on the other hand we intend to investigate the behaviour of *Leucophaea* haemocytes in vitro and in vivo, as well as their role in biosynthesis mechanism of such substances.

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