SECONDARY IMMUNOLOGICAL REACTIVITY IN INVERTEBRATE ANIMALS

O. E. Vyazov and L. S. Volkova

Laboratory of the Immunology of Embryogenesis (Head—Senior Scientist O. E. Vyazov), Institute of Experimental Biology (Dir.—Prof. I. N. Maiskii) of the AMN SSSR, Moscow
(Presented by Active Member AMN SSSR, N. G. Khlopin)
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The possibility of reproduction of immunological reactions in lower animals is not without grounds, for we know that various forms of immunity, like infectious diseases, are encountered at all levels of the zoological scale. A. A. Bogomolets [1] successfully immunized a mussel (Mytilus galloprovincialis) by injection of cultures of Escherichia coli. Ishimori [2, 3] and other authors have reported successful experimental immunization of insect larvae with Galleria melonella, a species of bacteria pathogenic towards them. Several papers indicate the possibility of the production of immunity in members of the phylum Arthropoda.

It is of practical importance to obtain a firm answer to the question of the secondary immunological reactivity of invertebrate animals, for the lower animals may be a very convenient object for the study of certain general problems of immunobiology.

METHOD AND RESULTS

The research into secondary immunological reactivity in invertebrates, which we carried out from a base at the White Sea Biological Station of Moscow State University, was of the character of a preliminary investigation. In the first place a number of technical problems had to be solved: the choice of experimental animals, the method of preparation of the antigen, the mode of immunization and dose of antigen injected, and the choice of reactions to be used for determining the immunological response in the recipients. Initially for the immunization experiments we used four phyla of marine animals, occupying close but different levels on the biological scale: the Chordata, Mollusca, Echinodermata and Annulata. From the phylum of the Chordata, 4 species of ascidia were used in the experiments—Styela rustica, Molgula retortiformis, Hyalocenthia pyriformis and Boltenia echinata; from the phylum of the Mollusca one species was studied—Mya arenaria; from the Echinodermata—Asterias rubens; from the Annulata—2 species, Nereis pelagica and Arenicula marina. Antigens for immunization of the animals were prepared from the tissues of ascidia and molluscs. For control and cross-testing purposes antigens were used from the tissues of all the phyla and species of animals listed above.

Antigen was prepared in the form of a tissue extract in sea water and, in a parallel series, in sea water with physiological saline (0.6% NaCl). The antigen was administered (in a dose of 0.3-0.5 ml per animal) by two methods: by injection with a syringe (0.5 ml) or in sterile glass capillary tubes (0.3 ml). Since the worms died 24-48 hours after receiving the foreign protein and sea water, they were not used in the subsequent experiments, and the "antigen" prepared from the tissues of the Annulata was used only in the performance of control reactions.

The experimental animals were kept in natural or close to natural conditions: molluscs in soil; littoral animals, ascidia—in a tank, kept in the sea; the starfishes in an aquarium with frequently changed sea water. In

Results of the Ring-Precipitation Test Between the Celomic Fluid of Molluscs Immunized with Tissue Proteins of Ascidia ("Antibodies") and Tissue Extract of Ascidia ("Antigens")

Series of experiments	Celomic fluid of molluscs immunized by single or repeated injections of antigen (0.3 ml of tissue extract each injection)	Result of investigation of "antibodies" in celomic fluid of molluscs, taken at different times after immunization of animals	
		after 24 hours	after 7 days
First	Immunization of animals with one injection of antigen	–	+
Second	Daily injection of antigen on 3 consecutive days	–	+
Third	Daily injection of antigen on 5 consecutive days	–	+
Fourth	Molluscs kept continuously in "ascidium" medium for 4 days (oral immunization).	–	–
Fifth	Continuous oral immunization for 4 days and one injection of antigen from syringe	–	+

Note. The sign – denotes a negative reaction, + a positive reaction

order to determine the presence or absence of an immunological response in the recipients, the ring-precipitation test and the precipitation test in agar capillary tubes were used. The animals to be immunized were sacrificed and investigated at different periods: from 2 to 30 days after immunization. The celomic fluid of the animals was tested with tissue proteins (antigens) of various species of invertebrate animals – members of the four above-mentioned phyla.

In the preparation of the antigen all the soft tissues of the animal were used (usually pooled tissues from 5-10 animals of the same species were taken together; tissue "antigens" of the Chordata were prepared from a mixture of the organs of the 4 species of ascidia). The tissues taken were washed once with sterile sea water, minced with scissors and then thoroughly ground in a porcelain mortar. The minced tissue was mixed with an equal volume of sea water, the mixture centrifuged for 40-50 min at 2000 rpm and the supernatant fluid was investigated. The celomic fluid of the immunized animals, to be tested for the presence of precipitins, was extracted as follows: from the body of the ascidia – with a Pasteur pipette (after preliminary removal of sea water from the animal's stomach), and from the molluscs and starfish by thorough mincing and expression of fluid from the tissues of the animal. The fluid obtained in this manner contained, besides tissue proteins and juices, cells of different organs and sea water. The fluid was centrifuged and its supernatant portion was investigated – whole, or diluted 1:2 and 1:4. A disadvantage of this method of obtaining fluid is that the dose of "antibodies" used in the tests cannot be determined or measured. It is obvious that this method can only be used when the question to be solved is merely whether a secondary immunological reaction can be reproduced in invertebrates, and also provided that the experimental conditions include the frequent repetition of the tests and the use of large numbers of animals.

Observations on animals of the phyla Chordata and Echinodermata, immunized with the tissue proteins of molluscs, gave negative results in all series of our experiments.

We paid special attention to the study of the immunological reaction of the Mollusca after administration of proteins from the Chordata, i.e., animals with a more complex biological organization. We immunized altogether 63 molluscs with extracts of tissues of the Chordata. The results of these experiments showed that the celomic fluid of molluscs, immunized with tissue proteins of the Chordata, gave a positive precipitation test with the proteins of these animals in the majority of cases investigated – in 48 of 63, and at times between the 10th and 29th days. It was also found that this reaction was clearest in cases when the antigen was prepared in sea water without the addition of physiological saline and when the tissue extract was given to the animal by direct injection from a syringe and not in capillary tubes.

Occasionally the celomic fluid of control (unimmunized) molluscs also gave a positive precipitation test with tissue proteins of the Chordata. In these cases, however, the precipitate was formed only 40-60 min after

setting up the test, and moreover the precipitation ring had a tendency to be displaced upward—towards the celomic fluid. So far as the other control tests were concerned—the reaction between the tissue proteins of the Chordata and the celomic fluid of the Mollusca, immunized with the tissue proteins of other Mollusca or of animals of another phylum—in all these cases they were negative.

The preliminary findings obtained in this research led us to conclude that the experiments in which Mollusca were immunized with tissue proteins of the Chordata must be repeated.

For preparation of the "antigen" the tissues of three species of ascidia were used: Styela rustica, Molgula retortiformis and Boltenia echinata (10-20 animals of each species). The molluscs were immunized by means of injection of tissue extract with a syringe (0.3 ml at each injection).

The response reaction of the molluscs was tested at various times—from one to seven days after the last injection of tissue extract.

The experimental animals were divided into four groups with 40 animals in each. The animals of the first group were immunized once. Half the total number of animals in the group were sacrificed and tested 24 hr after immunization and the other half 7 days after immunization. The animals of the second group were immunized three days in succession and investigated at the same intervals (24 hr and 7 days after the last injection of antigen). The animals of the third group were immunized five days in succession and also investigated after two intervals. The animals of the fourth group were immunized per os: they were kept in an aquarium with sea water, to which was added every day a mixture of minced tissues of ascidia of the three species mentioned above (100 mg of tissue to 5 liters of sea water). The medium was enriched with oxygen by the periodic pumping of air into the "ascidium" medium. One part of these animals was investigated immediately after continuous oral immunization for four days, and the rest 24 hours after supplementary immunization of the animals by injection of antigen from a syringe.

In order to study the immunological response reaction in the experimental molluscs, at suitable times the celomic fluid was extracted and investigated by the ring-precipitation test with "antigens" prepared from ascidia tissues. The celomic fluid and "antigen" were used in the test undiluted and in dilutions of 1:10, 1:100 and 1:1000.

The results of the investigation of the "antibodies" in the celomic fluid of the immunized molluscs are summarized in the table.

It will be seen from the results described that the ring-precipitation test was positive in by no means every case. In not one series of experiments when the celomic fluid of the experimental molluscs was tested 24 hr after immunization of the animals could the presence of precipitins be demonstrated in this fluid. The precipitation test was positive only in those cases in which the celomic fluid of the immunized molluscs was tested 7-12 days after the first injection of antigen. Oral immunization of the molluscs did not give positive results when the celomic fluid of the animals was tested on either the first or seventh day after immunization.

It can thus be concluded from the results of these experiments that invertebrate animals of the phylum Mollusca react to injections of foreign protein (tissue protein of invertebrates of the phylum Chordata, i.e., animals at a higher level in the scale of biological organization) by the production of precipitins, which can only be demonstrated after a certain time has elapsed since immunization of the animals. The number of injections of "antigen," as the experiments showed, did not play a decisive part in the formation of "antibodies" in the experimental animals. Consequently, the formation of precipitins (the possibility of detection of precipitins in the celomic fluid) in the experimental animals depends not so much on the dose of antigen injected as on the time elapsing between the day of immunization and the day of investigation of the celomic fluid.

On account of the short duration of our expedition we were unable to find out how long the precipitins remained detectable in the celomic fluid of the immunized molluscs. We can only state that the reproducibility of the findings affords convincing evidence of a regular pattern, the nature of which cannot yet be explained. We also find it difficult to give an answer to the question of whether the substances formed in the organism of molluscs in response to the injection of tissue proteins of Chordata are true antigens, and what is the degree of specificity of these substances. In order to answer these questions further experiments are necessary, using, in particular, methods such as the adsorption of antibodies, and also immunochemical investigations. We are continuing to study this problem.

SUMMARY

The authors discuss the possibility of immune antibody production by marine invertebrates. Molluscs were immunized by extracts of Chordata (Styela rustica, Molgula retortiformis, Boltenia echinata), animals of higher organization than the molluscs. It was shown with the aid of the ring precipitation test that the corresponding antibodies appear in the celomic fluid of experimental animals 7-12 days after the completion of immunization. No appearance of antibodies was noted when more highly organized animals were immunized with the tissue of animals with a lower level of organization.

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