THE IMMUNE RESPONSE OF THE ANTARCTIC TELEOST *Notothenia rossii* TO THE BACTERIOPHAGE *MS2*

By JULIAN G. O'NEILL*

ABSTRACT. The primary and secondary humoral immune responses to single intraperitoneal inoculations of *MS2* bacteriophage were followed in *Notothenia rossii*, maintained at 2°C in experimental aquaria. All the specimens examined were immuno-competent and produced IgM-like neutralization antibody. The antibody titre curves were typical of responses to viral immunogen observed in higher vertebrates but the clearance of bacteriophage and the latent, or inductive, phase of the primary response were prolonged. The secondary response indicated immune memory with a reduced latent phase, followed by enhanced and maintained antibody titres. Adjuvants were found to have little effect on antibody titre but they did enhance the clearance of the bacteriophage from the sera.

THE examination of the immune response in the poikilothermic teleosts has centred on temperate and warm-water species. Those species examined have been eurythermal and have been found to have optimum temperature ranges below which the immune response may be retarded or is absent (Snieszko, 1969). When maintained at low temperatures, below 9° to 12°C, certain eurythermal species, *Anguilla japonica* (Muroga and Egusa, 1969), *Cyprinus carpio* (Avtalion and others, 1973) and *Salmo trutta* (Ingram and Alexander, 1977), were found not to produce a humoral immune response. On the other hand, some workers have noted immune competence at temperatures below the species optima, though at these lower temperatures an increase in the induction or latent period of the primary immune response was observed (Nybelin, 1968; Klontz, 1972). Even at 2°C, Harris (1973) was able to induce antibody synthesis in *Leuciscus leuciscus* after an induction period of 20–30 d.

The study of the humoral immune response of a cold-water and highly stenothermal teleost, which has been adapted to its thermal environment over a long period of evolutionary time, would extend the knowledge of immunity at low temperatures, previously restricted to eurythermal species. Such an opportunity was made available with the present study of the immune response of the Antarctic teleost *Notothenia rossii*, specimens of which were held in the marine aquarium of the British Antarctic Survey in Cambridge.

MATERIALS AND METHODS

Experimental fish

Specimens of immature "fjord phase", 3+ to 4+ year and 58–69 g, *Notothenia rossii* Fischer (Norman, 1938), caught on the continental shelf of South Georgia, South Atlantic, were maintained by the British Antarctic Survey, Cambridge. The experimental fish were held at 1.0±0.3°C in the Survey's re-circulating sea-water aquaria under a continuous low-light regime. Individual fish were separated by Perspex partitions in order to stop cannibalism observed in this species. The fish were fed chopped Antarctic "krill", *Euphausia superba*, and squid every 2 days.

Immunogen

The bacteriophage *MS2* (picornovirus, group 1 RNA phage) was grown using the Petri-plate and "soft agar overlay" method of Eisenstark (1967) with *Escherichia coli* K12 (National Collection of Industrial Bacteria, Aberdeen, No. 10235) as the host. The bacteriophage was isolated from the bacterium by ultrasonic disruption (20 kHz at 6 nm) followed by one low-speed (20 min × 3 000 *g*) and two high-speed (120 min × 40 000 *g*) centrifugations at 4°C. The bacteriophage pellets were de-aggregated in sterile saline (120 mM NaCl, 4 mM KCl, 3 mM $CaCl_2$, 1 mM $MgSO_4$ and 2 mM $NaHCO_3$ at pH 7.6) using ultrasonic disruption and stored at

* Department of Life Sciences, Trent Polytechnic, Burton Street, Nottingham NG1 4BU.

1°C. Similar techniques were used to culture the bacteriophages *Qβ*, *X174* and *P22* using their host bacteria, respectively *E. coli* K12, *E. coli* C and *Salmonella typhimurium*, for the assay of neutralization specificity of the fish sera.

Immunization and blood sampling

6 weeks after the introduction of the *N. rossii* into the aquaria, three groups of five fish were intraperitoneally inoculated with 1.95×10^9 plaque-forming units (PFU) of *MS2* in 0.1 cm^3 of sterile saline, Freund's incomplete adjuvant (FIA) or Freund's complete adjuvant (FCA). The FIA and FCA were obtained from Difco Laboratories, Michigan. A control group of five fish was intraperitoneally inoculated with 0.1 cm^3 sterile saline. A second inoculum of 1.95×10^9 PFU *MS2* in saline was administered after the peak of antibody activity had been observed in each group, and the control fish were again inoculated with sterile saline. Blood samples of not more than 0.05 cm^3 were taken by caudal venipuncture at 14 d intervals and, after the samples had clotted overnight at 1°C, the sera were assayed for live *MS2* and neutralization antibody activity. Residual sera were stored at −20°C. The fish were gently netted and anaesthetized in 1 g : 200 dm^3 solution of tricaine methanesulphonate (MS222, Sandoz, Basle) in sea-water, at 2°C, before all inoculation and venipuncture procedures.

Assay of neutralization antibody and live bacteriophage

A semi-micro viral plaque neutralization assay procedure was modified from the Petri-plate methods of Adams (1959), and the neutralization antibody titre was described as the dose of serum required to produce 50% inactivation of the bacteriophage (SD_{50}), as suggested by Stashak and others (1970). Live bacteriophage in the sera could also be detected and titred by the same method by omitting the stage at which the replicate two-fold dilutions in saline were incubated with a known number of viral particles. The full procedure has been described elsewhere (O'Neill, 1979).

Neutralization specificity

Sera of known anti-*MS2* activity were assayed for their neutralization activity against the bacteriophages *Qβ*, *X174* and *P22*.

2-Merocaptoethanol (2-ME) sensitivity

Antibody-rich sera were added in equal volume to 0.1 cm^3 sterile saline containing 0.2 M 2-ME. After incubation at 4°C for 24 h, the samples were acidified with 0.01 cm^3 4M HCl and the 2-ME was removed by dialysis against two changes of sterile saline for 6 h and 24 h at 4°C. Control sera were treated in a similar manner without the addition of 2-ME. The treated sera were then assayed for bacteriophage neutralization activity.

Gel filtration

Antibody-rich sera were fractionated on Sephadex G-200 (Pharmacia) using an ascending 50×1.5 cm vertical column with a buffer head of 10 cm. Elution of the sera was performed at 4°C using 0.1 M Tris-HCl with 0.2 M NaCl buffer, pH 8.0, at a flow rate of 8.6 $cm^3\ h^{-1}$ and the fractions were collected at 15 min intervals using an automatic fraction collector (Shandon-Jeffs). The fractions were examined spectrophotometrically at 280 nm and for bacteriophage neutralization activity. The exclusion and inclusion volumes of the gel were determined using 0.1% blue dextran and 0.1% potassium dichromate. Urease (molecular weight 280 000 Daltons) was used as a marker protein.

RESULTS

Bacteriophage clearance

Live *MS2* bacteriophage, from the primary intraperitoneal inocula, was found to enter the sera of *N. rossii* and was not cleared for a period of 28–70 d, depending on the form of the inocula. The bacteriophage clearance was enhanced by the addition of adjuvant to the inocula, the FIA inocula being cleared within 42–56 d and the FCA inocula within 28–42 d, in comparison with the 56–70 d required to clear the bacteriophage-in-saline inocula (Fig. 1). All the fish in the three experimental groups demonstrated complete clearance of a secondary challenge of live *MS2* from the sera within 14 d of the inoculation.

Humoral neutralization antibody

All the specimens of *N. rossii* challenged with *MS2* were found to be immuno-competent and capable of producing high levels of viral neutralization activity in the sera. No neutralization activity was detected in the sera of *N. rossii* prior to immunization or subsequently in the sera of fish challenged with sterile saline. The immune sera demonstrated specific neutralization of *MS2* bacteriophage and this activity was totally eliminated by 2-ME treatment. Further, the neutralization activity of the sera from fish receiving a second challenge was eluted from G-200

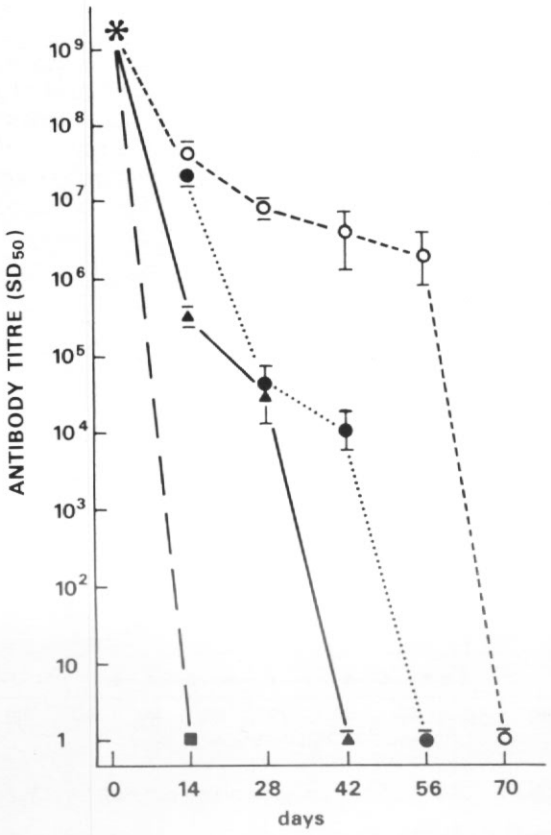

Fig. 1. The clearance of *MS2* from the blood of *N. rossii* at 2°C and the effect of adjuvants. Primary inocula of $1.95\times10^9\pm7.2\times10^7$ PFU *MS2* in saline (○), FIA (●) and FCA (▲) were administered on day 0. A secondary challenge of 10^9 PFU *MS2* in saline was cleared within 14 d in all groups (■). Means of live *MS2* titre values from five fish are plotted ±SE bars. Maximum *MS2* titre if all the inoculum were found in the sera (✱).

Sephadex in the heavy molecular weight (HMW) fraction before the urease marker (Fig. 2). These results indicate that the humoral neutralization activity was produced by an IgM-like antibody.

On clearance of the primary challenge of *MS2* bacteriophage from the sera, the humoral neutralization antibody titres were found to rise quickly and reached a peak from days 98 to 112 after challenge (Fig. 3a). The latter peaks of antibody activity demonstrated no significant difference between the saline and adjuvant groups. A rapid fall in antibody titre followed the peak, characteristic of a primary immune response to a viral immunogen.

The second challenge of *MS2* bacteriophage in saline resulted in an immediate rise in neutralization antibody titres, which reached a peak within 14–28 d (Fig. 3b). The peak antibody titres of the saline and FCA-inoculated groups were significantly greater ($P<0.05$) than the peak titres observed in these two groups during the primary response. In the subsequent response, the antibody titres did not fall to the extent observed in the primary response and high levels of antibody activity were maintained.

From day 84 after the primary inoculation the FIA-*MS2* group was reduced to one specimen due to accidental mortality. Although the response of only one fish was followed, the pattern of the response closely followed that of the two other *MS2*-inoculated groups.

Discussion

At a temperature of 2°C, all the specimens of *N. rossii* challenged with the bacteriophage *MS2* were found to be immuno-competent and demonstrated active production of a specific HMW neutralization antibody. These results contrast with those observed for other teleost species (Muroga and Egusa, 1969; Avtalion and others, 1973; Ingram and Alexander, 1977), in which low temperatures were found to inhibit the release of humoral antibody. However, the results do support the proposal by Harris (1973) that teleosts are capable of producing an

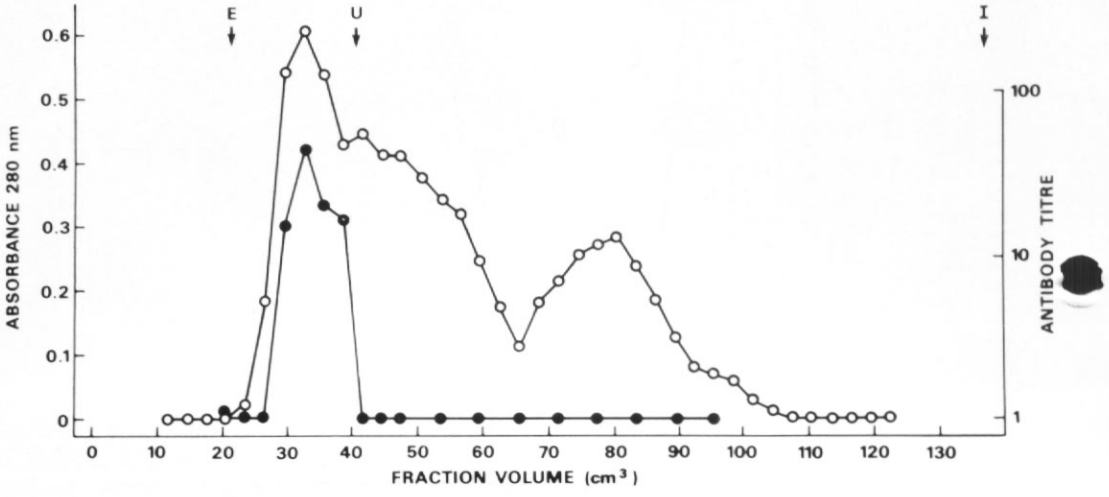

Fig. 2. Gel filtration on Sephadex G-200 of 0.75 cm³ pooled antibody-rich sera from *N. rossii* after secondary *MS2* challenge.
○—○ Protein measurement at 280 nm.
●—● *MS2* neutralization antibody titre.
E Gel exclusion volume.
I Gel inclusion volume.
U Elution of urease (280 000 Daltons).

immune response at low temperatures though with an increased induction, or latent, period before antibody is released.

The time course of *MS2* clearance from the sera of *N. rossii* on primary challenge, and the subsequent induction of neutralization antibody, was found to be longer than that observed by Harris (1973), when he challenged *L. leuciscus*, held at 2°C, with human erythrocytes. However, the elimination of an immunogen from the sera may be dependent on the size and the opsinization by antibody of the immunogen. Thus a small particle, like the bacteriophage *MS2*, would be expected to be cleared from the sera at a later time than a larger particle and would be dependent on opsinization before it was eliminated (Hájek, 1970). The slow clearance of the bacteriophage may also be attributed to the depression of bodily functions observed in teleosts at low temperatures. Feeding slows (Keast, 1968), the processes of digestion, metabolism and growth decrease (Ouchi, 1969). Cellular multiplication slows and direct effects on the replication of antibody-producing cells have been demonstrated in teleosts (Ortiz-Muniz and Sigel, 1971). The inflammatory and cell-mediated immune processes have also been found to slow down (Finn and Nielson, 1971; McQueen and others, 1973; Ferguson, 1975; Roberts, 1975). However, at low temperatures the bodily functions of teleosts are not totally inhibited, for acclimation and adaptation of enzymatic processes do occur with temperature variations (Hazel and Prosser, 1974), and this may also be extended to the mechanisms of antibody synthesis. Indeed, a rapid release of antibody was indicated by the final clearance of *MS2* and the rise in neutralization antibody titres.

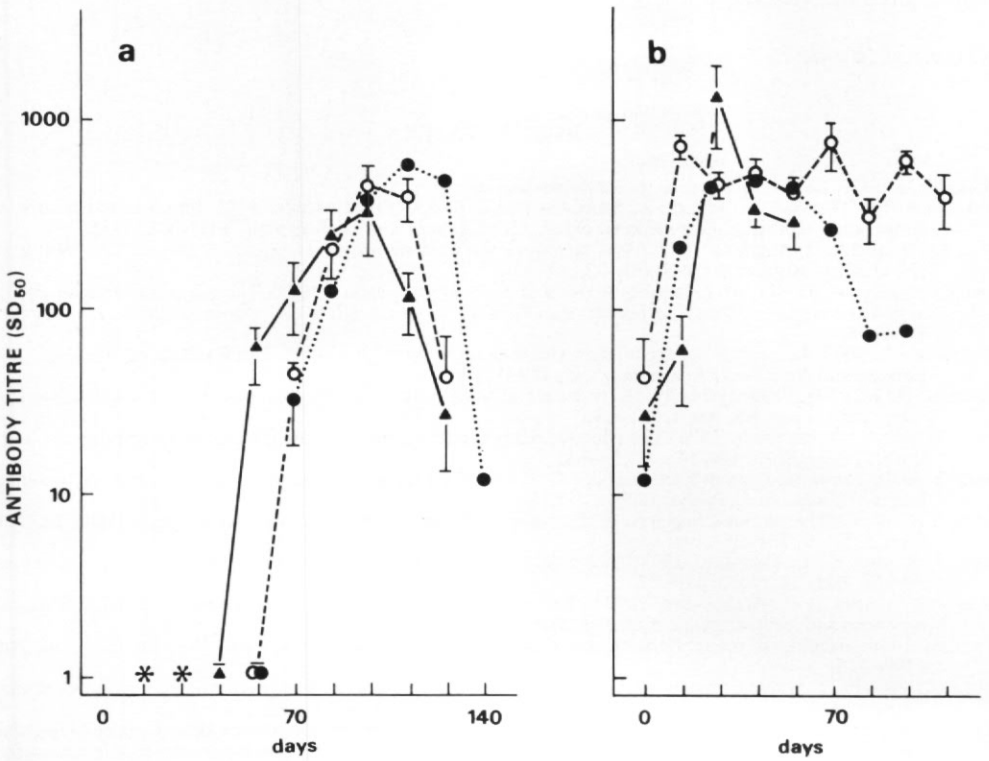

Fig. 3. The neutralization antibody response of *N. rossii* on (a) primary challenge on day 0 with 1.95×10^9 PFU *MS2* in saline (○), FIA (●) and FCA (▲); (b) secondary challenge with 1.95×10^9 PFU *MS2* in saline, administered on the last day of the primary response plotted (day 0 of secondary response). Mean antibody-titre values are plotted ±2SE. Live *MS2* in the sera (✱).

The antibody-titre curves produced in response to both primary and secondary challenges of *MS2* bacteriophage were characteristic of those described for mammals when challenged with viral particles (Burns and Allison, 1975). In comparison to the peak and fall in antibody titre of the primary response, the response to the second challenge of *MS2* demonstrated a classical anamnestic response, in which the antibody titres remained elevated. This, in conjunction with the enhanced clearance of a secondary *MS2* challenge, indicates that the immune memory demonstrated by *N. rossii* was independent of the low temperature.

The addition of FIA and FCA to the bacteriophage inoculum had no apparent effect on the levels of neutralization antibody in *N. rossii*. However, these adjuvants were found to enhance the clearance of the primary bacteriophage challenge. The observed enhancement by FIA may have been produced by the early stimulation of antibody production, possibly, as in mammals, by an enhanced expansion of B-cell clones (Civin and others, 1976), whereas the additional enhancement produced by the FCA may be linked in mammals to an expansion of T-cell clones and the stimulation of macrophages (Taussig, 1974).

ACKNOWLEDGEMENTS

I wish to thank the Director, British Antarctic Survey, for making available fish and aquarium facilities for this study, and the Head of the Department of Life Sciences, Trent Polytechnic, for laboratory facilities. I am grateful to Drs R. B. Heywood, N. J. Walesby and N. A. A. Macfarlane for their advice and interest, and to D. G. Bone for looking after the fish. The work was supported by NERC grant GR3/3088.

MS received 12 July 1979

REFERENCES

ADAMS, M. H. 1959. *Bacteriophages*. New York, Interscience.

AVTALION, R. R., WOJDANI, A., MALIK, Z., SHAHRABANI, R. *and* M. DUCZYMINER. 1973. Influence of environmental temperature on the immune response in fish. *Curr. Top. Microbiol. Immunol.*, **61,** No. 2, 1–35.

BURNS, W. H. *and* A. C. ALLISON. 1975. Virus infections and immune responses. (*In* SELA, M., *ed. The antigens. Vol. III*. London, Academic Press, 439–574.)

CIVIN, C. I., LEVINE, H. B., WILLIAMSON, A. R. *and* S. F. SCHLOSSMAN. 1976. The effects of antigen dose and adjuvant on the antibody response; amplification of restricted B cell clones. *J. Immun.*, **116,** No. 5, 1400–06.

EISENSTARK, A. 1967. Bacteriophage techniques. (*In* MARAMOROSCH, K. *and* H. KOPROWSKI, *ed. Virology. Vol. I*. London and New York, Academic Press, 449–524.)

FERGUSON, H. W. 1975. Phagocytosis by the endocardial lining cells of the atrium of plaice (*Pleuronectes platessa*). *J. comp. Path. Ther.*, **85,** No. 4, 561–69.

FINN, J. P. *and* N. O. NIELSON. 1971. The effect of temperature variation on the inflammatory response of rainbow trout. *J. Path. Bact.*, **105,** No. 4, 257–68.

HÁJEK, P. 1970. The elimination of bacteriophage ØX174 and T2 from the circulating blood of newborn precolostral pigs. *Folia microbiol., Praha,* **15,** No. 2, 125–28.

HARRIS, J. E. 1973. The immune response of dace *Leuciscus leuciscus* (L.) to injected antigenic materials. *J. Fish Biol.*, **5,** No. 2, 261–76.

HAZEL, J. R. *and* C. L. PROSSER. 1974. Molecular mechanisms of temperature compensation in poikilotherms. *Physiol. Rev.*, **54,** No. 3, 620–77.

INGRAM, G. A. *and* J. B. ALEXANDER. 1977. The primary immune response of brown trout (*Salmo trutta*) to injections with cellular antigens. *J. Fish Biol.*, **10,** No. 1, 63–72.

KEAST, A. 1968. Feeding of some Great Lakes fishes at low temperatures. *J. Fish. Res. Bd Can.*, **25,** No. 6, 1199–218.

KLONTZ, G. W. 1972. Haematological techniques and the immune response in rainbow trout. (*In* MAWDESLEY-THOMAS, L. E., *ed. Diseases of fish*. London and New York, Academic Press, 89–99.)

MCQUEEN, A., MACKENZIE, K., ROBERTS, R. J. *and* H. YOUNGE. 1973. Studies on the skin of plaice (*Pleuronectes platessa,* L.). III. The effect of temperature on the inflammatory response to the metacercariae of *Cryptocotyle lingua* (Crèplin 1825) (Digenea ; Heterophyidae). *J. Fish Biol.*, **5,** No. 2, 241–48.

MUROGA, K. *and* S. EGUSA. 1969. Immune response of the Japanese eel to *Vibrio anguillarium*—I. Effects of temperature on agglutinating antibody production in starved eels. *Bull. Jap. Soc. scient. Fish.*, **35,** No. 9, 868–78.

NORMAN, J. R. 1938. Coast fishes, Part III. The Antarctic zone. *'Discovery' Rep.*, **18,** 1–104.

NYBELIN, O. 1968. The influence of temperature on the formation of agglutinins in fish. *Bull. Off. int. Epizoot.*, **69,** Nos. 9–10, 1353–55.

O'NEILL, J. G. 1979. The immune response of the brown trout (*Salmo trutta*, L.) to *MS2* bacteriophage: immunogen concentration and adjuvants. *J. Fish Biol.*, **15,** No. 2, 237–48.

ORTIZ-MUNIZ, G. *and* M. M. SIGEL. 1971. Antibody synthesis in lymphoid organs of two marine teleosts. *J. Reticuloendothel. Soc.*, **9,** No. 1, 42–52.

OUCHI, K. 1969. Effects of water temperature on the scale growth and width of the ridge distance in goldfish. *Bull. Jap. Soc. scient. Fish.*, **35,** No. 1, 25–31.

ROBERTS, R. J. 1975. The effects of temperature on diseases and their histopathological manifestations in fish. (*In* RIBELIN, W. E. *and* G. MIGAKI, *ed. The pathology of fishes*. Madison, Wisconsin, University of Wisconsin Press, 477–96.)

SNIESZKO, S. F. 1969. Cold-blooded vertebrate immunity to Metazoa. (*In* JACKSON, G. J., HERMAN, R. *and* I. SINGER, *ed. Immunity to parasitic animals. Vol. I.* New York, Appleton-Century Crofts, 267–75.)

STASHAK, P. W., BAKER, P. J. *and* B. S. ROBERSON. 1970. The serum antibody response to bacteriophage ØX174 in germ-free and conventionally reared mice. I. Assay of neutralisation antibody by a 50 per cent neutralisation method. *Immunology*, **18,** No. 2, 295–305.

TAUSSIG, M. J. 1974. The effect of Freund's complete adjuvant on the specificity of the immune response, and its relationship to antigenic competition. *Cell. Immunol.*, **11,** No. 3, 484–92.