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PRESENCE OF ANTIGEN SENSITIZED LEUKOCYTES IN CARP (CYPRINUS CARPIO L) FOLLOWING BATH IMMUNIZATION AGAINST FLEXIBACTER COLUMNARIS

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ABSTRACT

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Bath immunization of carp *(Cyprinus carpio* L*)* resulted in protection of fish at natural challenge. Stimulation of leukocytes derived from thymus, spleen, anterior kidney and mid-kidney of fish immunized with *Flexibacter columnaris* bacterin revealed the presence of antigen sensitized cells in all lymphoid tissues except the anterior kidney. After 28 days a response was obtained in thymus and spleen leukocyte cultures.

INTRODUCTION

Columnaris disease can infect a wide variety of fish species e.g. salmonids, catfish, eel, carp and aquarium fish (for reviews see Davis, 1973; Roberts, 1978). The losses caused by this disease are considerable and have been reported from North-America, Great Britain, Japan, The Netherlands, New Zealand, South Africa and Korea. In the Netherlands carp culture in heated effluent water of a power plant suffered seriously from columnaris disease. Therefore, immunization methods to reduce the susceptibility of carp were tested.

One of the most promising methods of large scale immunization of fish is the bath technique (Amend & Fender, 1976). Several papers on bath immunization against bacterial diseases have already been published (Gould et al., 1978 and Schachte, 1978). This procedure enables the fish to withstand a challenge with a pathogen under laboratory conditions (Egidius and Andersen, 1979; Fryer et al., 1978). Bath immunization of carp resulted in negligible titers of agglutinating antibodies in the serum as was found in our laboratory after immersion of carp in *Flexibacter columnaris* bacterin (Teunissen et al., unpublished) and *Vibrio anguillarum* bacterin[1]. These results indicate that alternative immunological

[1]Hivax®, Tavolek, Titusville, New Yersey, USA

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parameters for the assessment of immunity in bath-immunized fish should be explored.

In that case one can chose from two methods which have been developed for carp: the haemolytic plaque assay (Rijkers et al., 1980) and the leukocyte stimulation assay (Liewes et al., 1982). The latter method was adopted in our experiments. Short term in vitro stimulation of Teleost leukocytes has been reported in several papers (Chilmonczyk, 1978, Cuchens and Clem, 1977 and Ettlinger et al., 1976), but all in relation to the phylogeny and development of the immune system. As a new approach, this technique was employed to assess the acquired immunity in fish following immunization. In this paper, the results of stimulation of carp leukocyte cultures with *Flexibacter columnaris* antigen, and the location of sensitized lymphocytes in different lymphoid organs will be presented.

MATERIALS AND METHODS

Preparation of *Flexibacter columnaris* bacterin

The bacterin of *Flexibacter columnaris* was prepared from the strains M 15 and M 17 (Bootsma and Clerx, 1976). These strains were isolated from outbreaks of Columnaris disease in The Netherlands. The bacteria were cultured in a newly developed culture medium, which resulted in significantly faster growth rates compared with those obtained in previously described *Flexibacter* culture media (Bootsma and Clerx, 1976; Chase, 1965). This new medium consisted of 0.45% casitone[1]; 0.45% yeast extract[1] and 1.0% gelatine[1] dissolved in 30% Earle's salt solution (v/v). The latter solution contained 2.04 g/l NaCl; 0.12 g/l KCl; 0.03 g/l K_2HPO_4; 0.06 g/l $MgSO_4.7H_2O$; 0.04 g/l $CaCl_2.2H_2O$ and 0.015 g/l Na-acetate. The medium was adjusted to pH 7.5 with 1N NaOH.

The use of 30% Earle's salt solution was introduced by Chase (1965) who noted that this balanced salt solution reduced the formation of cell aggregates in liquid cultures. The formation of cell aggregates generally occurs in *Flexibacter columnaris* cultures grown in the liquid basal medium described by Bootsma and Clerx (1976) and Bootsma (1976).

The bacterin was produced as follows: Two liters of culture media were continuously agitated with a magnetic follower for 60 - 70 h. at room temperature. A cell concentration of 1 - 5 x 10^9 cells ml^{-1} was achieved after this incubation period. Finally the cells were disrupted by freezing the culture at -20°C followed by thawing. This procedure was carried out three times in total. The bacterin was inactivated by maintaining the culture at 60°C for 30 min. then diluted with distilled water to give a final concentration of 1 x 10^8 (desintegrated) cells ml^{-1}.

[1] Difco laboratories

Bath immunization procedures

Prior to immersion in the diluted bacterin the carp were immersed in a hyperosmotic solution of NaCl (2% w/v). The carp were immersed for 5 min. in both baths, which were aerated during the treatment.

Fish

One summer old carp *(Cyprinus carpio L)*, male and female, with a weight of 5 to 60 g were used in the experiments. The fish were acclimatised for two weeks prior to the experimental period in aerated aquaria through which running tap water (23 - 25°C) flowed. The carp were fed a commercially available pelleted trout feed[1] 4 times a day. Scaled carp of approximately 5 g were used in large scale bath immunization experiments.

Mitogens and antigens

Phytohemagglutinin (PHA-P)[2](Lot No. 640551); Concanavalin A (ConA)[3] (grade 4) and lyophillized lipopolysaccharide of *Escherichia coli* (LPS 055 - B5)[2] (Lot no. 63093) were used in this experiment.

The *Flexibacter columnaris* antigen employed in the leukocyte stimulation micro-assays was prepared by filter sterilization of the bacterin (0.45 μ disposable Millipore filter; concentration 1 x 10^8 desintegrated cells/ml). All mitogens were either diluted in diluting medium (Liewes et al., 1982) or in distilled water according to the manufacturer's instructions. The final mitogen concentrations in the wells were: PHA-P 10 μg, for ConA 0.1 μg and LPS 35 μg. These were optimal concentrations for carp leukocyte stimulation as determined in previous experiments (Liewes et al., 1982).

Culture techniques

The media and solutions as well as the leukocyte isolation procedures and leukocyte stimulation techniques are described in Liewes et al. (1982). Harvesting procedures and liquid scintillation counting were performed according to Van Dam et al. (1978).

RESULTS

The presence of antigen sensitized leukocytes following bath immunization was tested after 14, 28 and 56 days in leukocyte cultures derived from thymus, anterior kidney, spleen and mid-kidney. Stimulations were performed with *Flexibacter columnaris* antigen (dilutions 1:10; 1:100 and 1:1000). In addition the leukocyte suspensions were stimulated with PHA-P, ConA and LPS.

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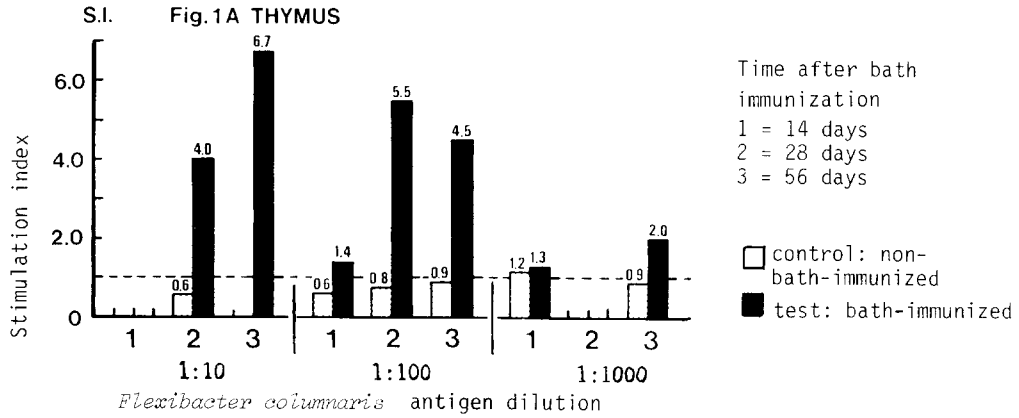
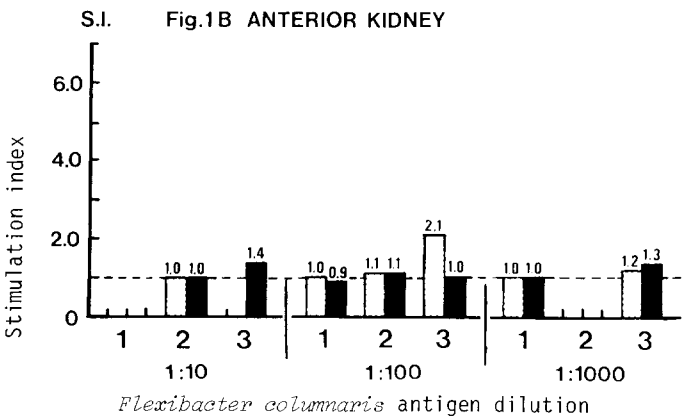
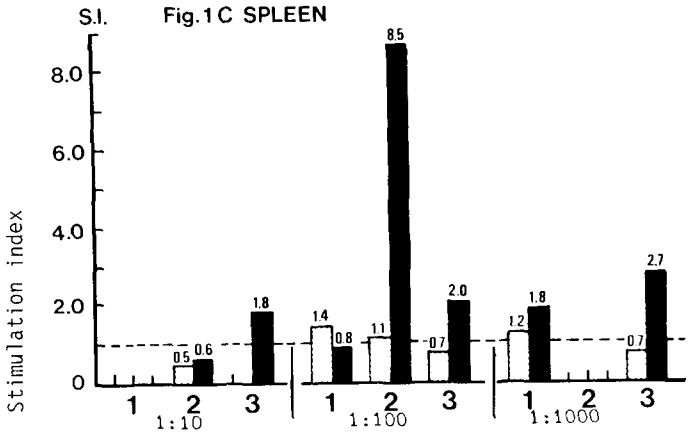
[1]Trouvit®, Trouw en Co. Putten, The Netherlands
[2]Difco Laboratories
[3]Sigma Chemical Company Lot. no. 58C - 7336

A slight response to *Flexibacter columnaris* antigen was observed in leukocyte cultures from the thymus antigen dilution (1:100) and spleen (dilution 1:1000) 14 days after bath immunization. At 28 and 56 days after bath immunization leukocyte cultures from thymus, spleen and mid-kidney responded considerably better to *Flexibacter columnaris* antigen. Good stimulation indices were obtained with spleen (1:100) and mid-kidney (1:100) leukocyte cultures. In addition stimulation indices of 4.0 - 6.7 were measured in thymus leukocyte cultures using *Flexibacter columnaris* antigen dilutions of 1:10 and 1:100 (see fig. 1 A-D). No response was observed to any of the antigen dilutions in the anterior kidney leukocyte cultures.

Figure 1: Results of the leukocyte stimulations with *Flexibacter columnaris* antigen with bath immunized carp. (The results shown were obtained with 3 carps from each group. All tests were done in triplicate.)







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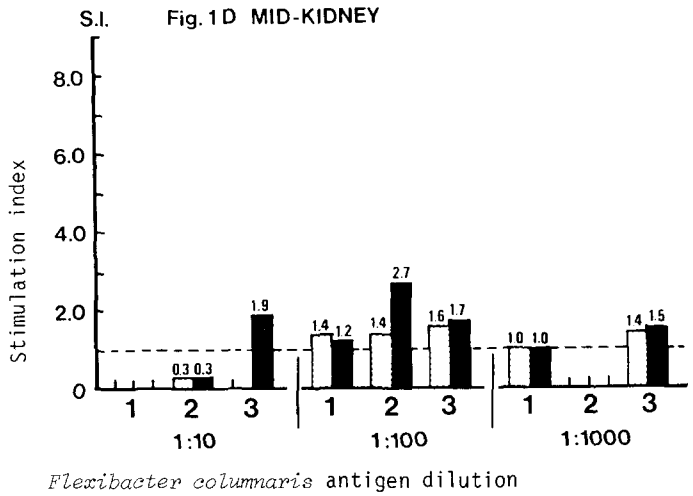

The stimulation patterns to PHA-P, LPS and ConA in the immunized fish were not significantly different from the control group (results not shown). An increased response to LPS stimulation was observed only in the thymus 14, 28 and 56 days after immunization. A slight decrease in LPS stimulation was observed in the anterior kidney leukocyte cultures of the bath immunized fish 28 days after immunization. A somewhat elevated response to PHA-P in the spleen and thymus leukocyte cultures was observed 56 days after immunization. No changes in the response to ConA in the leukocyte cultures of the bath immunized fish were obtained.

DISCUSSION

The application of the leukocyte stimulation assay to assess the acquired immunity in carp proved to be a reliable technique. The results indicated the presence of sensitized cells in thymus, spleen and mid-kidney. Following bath immunization the first response to the antigen was located in the thymus and the spleen 14 days after the immersion treatment, but the SI values were however still low. Twenty-eight days after bath immunization an increased stimulation index (SI) was observed in the thymus leukocyte cultures, which had even increased further after 56 days. In the spleen cultures an increased SI was noted 28 days after immunization, but it decreased after 56 days. Although a response was observed in the mid-kidney leukocyte cultures after 28 days, no stimulation was recorded after 56 days. No response to *Flexibacter columnaris* antigen was observed in anterior kidney leukocyte cultures of the carp. Although negligible agglutinating antibody titers were observed in bath immunized carp, specific sensitized leukocytes could be found, which indicate that the immunologic memory of carp can occur without the presence of significant agglutinating antibody titers in the serum. In contrast higher agglutinating antibody titers can be obtained in carp and other fish when the antigen is injected intra-peritoneally. The question arises whether a different route of antigen administration also results in a different type of immunologic response. High responses in the carp spleen leukocyte cultures were measured 14 days after an intra-peritoneal injection of *Flexibacter columnaris* bacterin with complete Freuds adjuvant, while no response was recorded in the leukocyte cultures derived from the other lymphoid organs, including the thymus (Liewes, unpublished results). Whether the slow response to the antigen after bath immunization was caused by local T cell-like mediated responses could not be established. Local responses to antigens have however been reported for some fishes e.g. in the gut following oral immunization in plaice (Fletcher and White, 1973). In carp no specific agglutinating or precipitating antibody titers could be found in the skin mucus following bath immunization (Teunissen, pers. comm.). The possibility of local reactions of lymphoid cells in the skin and the gut should be investigated further as in carp large numbers of lymphocytes and PAS-positive granulocytes can be found in the skin (Liewes, 1977) and gut epithelium (Weinberg, 1975; Davina et al., 1979).

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