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THE IMMUNE RESPONSES OF CARP, CYPRINUS CARPIO L., FOLLOWING DIRECT IMMERSION IMMUNIZATION

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THE IMMUNE RESPONSES OF CARP, CYPRINUS CARPIO L., FOLLOWING DIRECT IMMERSION IMMUNIZATION

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The immune responses of carp, Cyprinus carpio L., following direct immersion immunization.

by Alan Foster Bridges

Abstract.

The investigations presented in this thesis include studies on a) the immune responses of carp following direct immersion immunization and subsequent intraperitoneal (i.p.) challenge, b) the uptake and accumulation in carp of a direct immersion vaccine and c) phagocytic uptake by carp peritoneal exudate cells (PECs).

To assess the cell-mediated immune response of carp, a micro chemotaxis technique was developed, measuring the production of chemotactic factor-like activity in supernatants from incubations of pronephric cells with antigen.

In no case were serum antibody titres or a cell-mediated immune response detectable after immersions alone in antigen. It was found that an i.p. challenge of antigen in adjuvant, subsequent to the immersions, was needed to stimulate a measurable response, with effective priming immersions stimulating a secondary response to the i.p. challenge.

It was found that the opsonization of both soluble and particulate immersion vaccines with immune carp serum significantly increased the immunological memory for both the humoral and the cell-mediated immune responses following immersion. Opsonization of the vaccines with normal serum, however, had no detectable effect. The cell-mediated immune responses following immersion were only measured in immunologically mature carp, but the humoral immune responses were measured in both immunologically mature and immature carp, which were 4 weeks old at the beginning of the experiments. Using the bacterial *Aeromonas salmonicida* antigen, all the responses measured post-immersion were found to be positive in both immunologically mature and immature carp. However, with the T-dependent antigen, human gamma globulin (HGG), the immune responses post-immersion were found to be positive only in the immunologically mature fish, with immersion of the immature carp in HGG-coated latex particles opsonized with immune serum producing a tolerizing effect on the humoral immune response.

There was no detectable uptake of a non-opsonized *A.salmonicida* vaccine in normal carp when immersed in a bath of the vaccine. However, if the vaccine was opsonized with immune carp serum, uptake and accumulation of the vaccine was detectable, mostly accumulating in the internal lymphoid organs. Uptake of the non-opsonized vaccine was, however, also found when the recipient carp had been previously immunized against *A.salmonicida*, by immersion. The phagocytic uptake of particles by carp PECs was also found to be enhanced by opsonization of the particles with immune carp serum, this effect being partially reduced by decomplementation of the opsonizing serum. Opsonization of particles with normal serum was found to have no effect on phagocytic uptake.

Immersions in several different sizes of latex particles (from 0.05μm to 15μm) coated with HGG were found to stimulate greater humoral immunological memory than immersions in soluble HGG. This was not the case for memory for the cell-mediated response, where immersions in latex particle-bound HGG were no more stimulatory than immersions in soluble HGG. Carp PECs were found to be able to ingest 0.8μm and 3.0μm diameter particles but uptake of 15μm diameter particles was not observed.

The specificity of the humoral immune response after direct immersion immunization was found to be high with no cross-reacivity with any of the other antigens used. The cell-mediated immune response following direct immersion immunization was found to be slightly less specific; cross-reactivity between HGG and chicken gamma globulin was detected, although the other antigens used showed no cross-reactivity.

DECLARATION

This work has not been accepted for any other degree and is not concurrently being submitted for any other degree.

This is to certify that the work here submitted was carried out by the candidate himself. Due acknowledgement has been made of any assistance received.

Signed 

(Candidate)

Signed 

(Supervisor)

ABBREVIATIONS

A.salmonicida	*Aeromonas salmonicida*
°C	degrees centigrade
FIA	Freund's incomplete adjuvant
FCA	Freund's complete adjuvant
HGG	human gamma globulin
CGG	chicken gamma globulin
KLH	keyhole limpet haemocyanin
BSA	bovine serum albumin
HGG-Latex	HGG-coated latex particles
i.p.	intraperitoneal
SRBC	sheep red blood cells
g	gram
mg/dm3	milligrams per litre
PECs	peritoneal exudate cells
mins.	minutes
hrs.	hours
Ig	immunoglobulin
MIF	migration inhibition factor
MAF	macrophage activation factor
MCCs	melano-macrophage centres
cpm	counts per minute

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CHAPTER 1

Title

General Introduction

The lymphoid system of fish is, in some ways, very similar to that of mammals. Teleost fish possess both primary and secondary lymphoid organs and also gut-associated lymphoid tissue. The major lymphoid organs found in teleost fish are the spleen, the kidneys and the thymus, whilst lymph nodes are lacking and there is little, if any, bone marrow (Secombes, 1981; Lamers, 1985; Ellis, 1988). The thymus is a paired bilateral organ, found beneath the pharyngeal epithelium, dorso-laterally in the gill chambers, and is composed mainly of developing lymphocytes (Ellis, 1988). In fish, as in mammals, it is considered a central primary lymphoid organ, where a pool of virgin lymphocytes is produced, which then can migrate to the secondary lymphoid organs and the periphery (Ellis, 1978; Secombes, 1981; Lamers, 1985; Ellis, 1988). The thymus does not participate in antibody production (Ellis, 1988) although plaque-forming cells have been detected in the teleost thymus (Sailendri & Muthukkaruppan, 1975; McCumber *et al.*, 1982) and immunoglobulin has been described on certain thymocytes (Ellis, 1977a). No antigen trapping or processing has been found in the thymus (Ellis *et al.*, 1976; Ellis, 1980; Secombes & Manning, 1980; Secombes, 1981).

The major secondary lymphoid organs found in teleost fish are the kidneys and the spleen. The spleen is a haemopoetic organ which contains lymphoid tissue within it. This splenic lymphoid tissue, unlike that in mammals, does not contain distinct white pulp compartments or germinal centres, but is more diffuse (Lamers, 1985). The kidneys, divided into the pronephros and opisthonephros (head and trunk kidneys) are extremely important lymphoid organs in fish. The lymphoid tissue within the pro- and opisthonephros has similar morphology, but as an organ, the pronephros seems more specialised towards its

immunological function than the opisthonephros, which still retains its secretory function (Lamers, 1985). An overall view seems to be that the kidneys in fish function as both a primary and secondary lymphoid organ, with both the development of stem cells into mature leukocytes and the trapping, processing and response to antigen all occuring within them (Lamers, 1985). Antibody-producing and antigen-binding cells can be found within both the kidneys and the spleen (Secombes, 1981; Secombes *et al.*, 1982a; Lamers, 1985; Ellis, 1988), although the spleen does contain fewer lymphoid cells than the kidneys (Ellis, 1988).

The leukocytes of fish show distinct morphological similarities to those in birds and mammals, comprising lymphocytes, plasma cells, mononuclear phagocytes and granulocytes (Lehmann & Sturenberg, 1975, 1981; Ellis, 1976; Davina *et al.*, 1980). Ellis (1977b) reviewed the morphology and function of fish leukocytes and described considerable variation among fishes. The presence of both T and B-lymphocyte types in fish has also been demonstrated (De Luca *et al.*, 1983; Sizemore *et al.*, 1984) by monoclonal antibody studies.

One cell type which seems to be conspicuous in the lymphoid organs of most teleost fish is the melano-macrophage (Agius, 1985). These are not, however, restricted to lymphoid organs and appear at sites around the body where foreign substances may be encountered (Lamers, 1985). In the lymphoid tissues of fish, these melano-macrophages can be found both singly and in accumulations, called melano-macrophage centres (MMCs), which are proposed to represent primitive germinal centres (Ellis & de Sousa, 1974; Lamers, 1985). Injected material is known to localise in both the spleen and the kidneys (Secombes, 1981; Lamers, 1985), being eventually concentrated within the MMCs (Lamers & Pilarczyk, 1982) where it can be retained for up to 12 months or more

(Lamers, 1985).

Fish also possess epithelial lymphoid tissue, which is most apparent in the intestine (Rombout *et al.*, 1986; Hart *et al.*, 1988; Doggett, 1989; Rombout, Bot, & Taverne-Thiele, 1989; Rombout, van den Berg, van den Berg, Witte & Egberts, 1989). The alimentary tract is known to contain a number of different types of leukocyte (Davina *et al.* 1980, 1982; Rombout, Bot & Taverne-Thiele, 1989), which are found mainly as a diffuse cell population in the epithelium and lamina propria, with only occasional discrete lymphoid accumulations (Doggett, 1989). Antigen administered into the intestine is known to be absorbed, mostly in the 2nd gut segment (hindgut), by enterocytes, epithelial macrophages and intraepithelial lymphoid cells and macrophages and seems to be associated with the cell membranes of these macrophages as if being presented to lymphoid cells (Georgopoulou *et al.*, 1985, 1988; Georgopoulou & Vernier, 1986; Lamers, 1985; Rombout et al., 1985; Rombout *et al.*, 1986; McLean & Ash, 1986, 1987; Doggett, 1989).

Using this knowledge of the lymphoid system of teleost fish, there has been much interest, in recent years, in the search for effective and convenient ways to vaccinate fish without the need for injection or for administration in the food. In 1976, Amend & Fender published a study which described the uptake of bovine serum albumin (BSA) in rainbow trout from hyperosmotic solutions, and also from a normal bath containing BSA after just having been immersed in a hyperosmotic salt solution (hyperosmotic dip), and suggested that this might be a model for vaccinating fish.

The hyperosmotic dip appears to increase the subsequent uptake of the antigen. Unfortunately, it was soon discovered that the osmoregulatory stress imposed on the fish during the hyperosmotic dip was not good for

fish health (Giorgetti *et al.*, 1981), and that it is likely that gill damage will result (Bowers & Alexander, 1981).

However, subsequent reports of the protection of fish from various diseases using hyperosmotically administered vaccines were being published (Antipa & Amend, 1977; Croy & Amend, 1977). It was then reported that omitting the hyperosmotic bath stage and simply immersing the fish in a bath of the vaccine gave good protection (Egidius & Anderson, 1979), and this method became known as direct immersion vaccination.

Certain workers have also tried applying vaccines via a spray onto the fishes body surface (Gould *et al.*, 1978; Itami & Kusuda, 1978; Hockney, 1984) but results have shown that administration of a vaccine by direct immersion is more effective than administration by spray (Johnson & Amend, 1983). General reviews of various fish vaccination procedures, either in commercial use or under development for such use have been written by Ellis (1988) and Lillehaug (1989).

It is now well established that direct immersion vaccination of fish is effective (Gould *et al.*, 1979; Newman & Majnarich, 1982; Johnson *et al.*, 1982a, 1982b; Johnson & Amend, 1983), and that a good memory response is given by this method of vaccination (Lamers et al., 1985; Tatner & Horne, 1985). This method is also widely used in commercial aquaculture, especially when large numbers of relatively small fish need to be immunized. What is not totally established, however, is the portal of entry of the antigen into the fish and the mechanisms involved in the stimulation of the immune system after direct immersion vaccination.

Many factors seem to affect the efficacy of direct immersion vaccination including concentration, length of immersion time and the temperature of the vaccine bath (Tatner & Horne, 1983). The age of the fish being

vaccinated also has an effect. It has been shown that multiple immersions of 4 week old carp in a bath of human gamma globulin (HGG)-coated latex particles induces immunological tolerance to HGG instead of the protective immune response which would have been induced in older fish (Mughal, 1984). This phenomenon of tolerance induction in very young fish is not just seen with the direct immersion route of vaccination. Secombes (1981), van Loon *et al.* (1981) and Mughal (1984) have all found that fish of 4 weeks of age or less, immunized by intraperitoneal (i.p.) injection with either HGG or sheep red blood cells (SRBC) showed immunological tolerance to a subsequent i.p. challenge with the antigen.

Another influencing factor to be considered in the efficacy of direct immersion vaccination is the physical form of the antigen, with the uptake of particulate antigens being greater than that of the corresponding soluble antigen (Smith, 1982). Mughal, Farley-Ewans and Manning, (1986) also showed that immature carp respond differently to an i.p. challenge of HGG when previously primed by direct immersion with soluble HGG compared to when primed with latex particle-bound HGG. This study continues the investigations on this theme by looking at the humoral immune response to an i.p. challenge of HGG following priming by direct immersion with a large range of sizes of latex particle-bound HGG (from 0.05μm to 15μm) as well as soluble HGG (chapter 3). To correlate with this, the phagocytic uptake by carp peritoneal exudate cells of these different sizes of latex particles was also studied (chapter 5).

In the direct immersion experiments presented here, each immersion lasted for 10 mins. It has been shown (Tatner, 1987) that unless the vaccine bath is very dilute, lengthening the immersion time will not result

in greater uptake of the vaccine, with maximum uptake occuring within 5-10 seconds (Tatner & Horne, 1983; Johnson *et al.*, 1982). It seems likely, however, that this may not be the case in all circumstances as Johnson & Amend (1984) found that at least a 5 minute immersion was needed in their vaccine to confer protection to salmon against *Aeromonas salmonicida*. A ten minute immersion time was, therefore, chosen in preference to a very short immersion time, to ensure that a long enough exposure to the antigen was given. This was also considered to be less stressful to the fish than a very short immersion time.

The investigations presented here also look into the question of whether opsonization of a direct immersion vaccine alters the subsequent immune response of a fish to that vaccine. Opsonization being defined as the deposition of opsonins (e.g. antibody and certain complement factors) on the antigen thus facilitating its phagocytosis (Roitt *et al.*, 1985). It has already been established that i.p. administration of an opsonized antigen gives greater trapping in the pronephros and higher subsequent antibody titres than when the corresponding non-opsonized antigen is used (Secombes *et al.*, 1982a; Secombes & Resink, 1984), but any effects of opsonization on direct immersion vaccination have not, as yet, been demonstrated.

There are two main types of immune response to be considered, following an antigenic challenge, these being the humoral and the cell-mediated immune responses. The humoral immune response is often important in the protective response of a fish to a challenge of virulent organisms, and the transfer of plasma from an immune fish to a naive fish can be shown to confer immunity in certain circumstances (Viele *et al.*, 1980). However, it is known that the teleost immune response to certain antigens is composed almost entirely of a cell-mediated

response with almost no humoral response at all (Mohan, 1977). It is also well known that the presence of circulating antibodies in the blood does not necessarily indicate protection to a virulent challenge (Smith *et al.*, 1980; Aoki *et al.*, 1984; Ward *et al.*, 1984). Michel & Faivre (1982) even found high serum antibody titres in trout artificially immunized against A.salmonicida but not in fish which had survived an injected challenge of virulent organisms.

This evidence suggests that the cell-mediated immune response is often a major factor in the protection of fish to various diseases. Despite this, a large majority of the published research into the immune response of fish has looked only at the humoral immune response, the cell-mediated immune response only fairly recently being investigated.

Quantitative In vitro tests in use, to assay cell-mediated immunity in fish, have so far been limited almost exclusively to the migration inhibition test (Mohan, 1977; Jayaraman *et al.*, 1979; Smith *et al.*, 1980; Secombes, 1981; Blazer *et al.*, 1984; Bennett & Wolke, 1987; Song *et al.*, 1989). A small amount of work has been done in this laboratory (Howell, 1987), looking at the production of a chemotactic factor by pronephric cells from immunized carp on subsequent in vitro stimulation with the immunizing antigen, the chemotactic factor being assayed using the migration of carp pronephric cells in a Boyden chamber.

The work presented here has attempted to improve upon this technique to develop it into a viable tool for the experimental assessment of cell-mediated immunity in fish and to use it to assess the cell-mediated immune response to antigens administered by both direct immersion and i.p. injection (chapter 4).

These studies also approach the question of the specificity of the immune response (both humoral (chapter 3) and cell-mediated (chapter

4)) in fish, which has, in the past, attracted little attention from researchers. When studying the mechanisms involved in the immune response it is, of course, important to know whether an apparent immune response to an antigen to which a fish has been immunized is actually antigen-specific or merely a non-specific response. Secombes (1981) and Espelio *et al.* (1987) have shown good specificity of the immune response of salmonids to injected antigens, but the only work showing any specificity of the immune response to direct immersion vaccination has been by Gould *et al.* (1979) who showed that direct immersion immunization of salmon showed strain specificity. The investigations presented here continue on this line, studying the cross-reactivity of the immune response to both a T-independent and various T-dependent antigens.

This thesis also reports a study looking at the uptake and accumulation of a radiolabelled *Aeromonas salmonicida* bacterin after a single immersion in a bath of the vaccine (chapter 5), and the effects of opsonization of the vaccine and the immune status of the recipient fish. It is clearly important to understand the physical movement of antigen, administered by direct immersion, into and within the recipient fish before the full picture of the immune response, both humoral and cell-mediated is understood.

CHAPTER 2

Title

General materials and methods

GENERAL MATERIALS AND METHODS

Fish

The fish used in these experiments were all mirror carp (*Cyprinus carpio* L.) obtained from either Munton and Fisons (Stowmarket, Suffolk), Sunridge Nurseries (Yealmpton, Devon) or Ludbrook trout farm (Ermington, Devon). For the studies into the humoral response to direct immersion immunization, fish obtained from Munton and Fisons were used, aged either 5-9 months or 4 weeks at the start of the experiments.

For the studies on the uptake and accumulation of radiolabelled Aeromonas salmonicida bacterin, carp from Munton and Fisons were used, aged between 5-7 months at the start of the experiments. For the studies into the phagocytosis of latex particles, carp of two years of age were used, obtained from Ludbrook trout farm.

For the studies into the cell-mediated immune response to direct immersion immunisation, carp of between 5-9 months old were used, obtained from Sunridge Nurseries.

Adult carp which had been maintained for several years in the laboratory, weighing between 0.5 and 1.5 kg., were kept in a large holding tank and were used as a source of normal carp serum. Similar fish were used to raise anti-HGG and anti-*A.salmonicida* antibodies.

All fish were kept in tanks containing dechlorinated standing tap water at room temperature ($20\pm2^{o}C$)(see Fig.1). Undergravel or external power driven filters were used for all tanks and 50% water changes were carried out at 2 week intervals, except for the 4 week old carp which, being more sensitive to poor water quality, received 50% water changes every 7 days

throughout the duration of the experiments.

Fish under the age of two months were fed twice daily on Promin tropical fish food (fine)(Promin Ltd., Winchester, U.K.). Fish of between 2-3 months were fed twice daily on Promin tropical fish food (medium)(Promin Ltd., Winchester, U.K.). Fish of between 3 months and 2 years of age were fed once daily on number 4 salmon crumb (EWOS, Scotland). The large stock carp used for serum collection and raising antibodies were fed once daily on high density number 7 trout pellets (EWOS, Scotland).

Anaesthesia

Benzocaine (ethyl amino-p-benzoate) (Sigma,U.S.A.) at a concentration of 40 mg/dm^3 (previously dissolved in absolute alcohol (10% w/v)) was used to anaesthetize the fish. To kill fish, a concentration of 80 mg/dm^3 of benzocaine was used. The anaesthetic was made up in standing tap water.

ANTIGENS AND DOSES

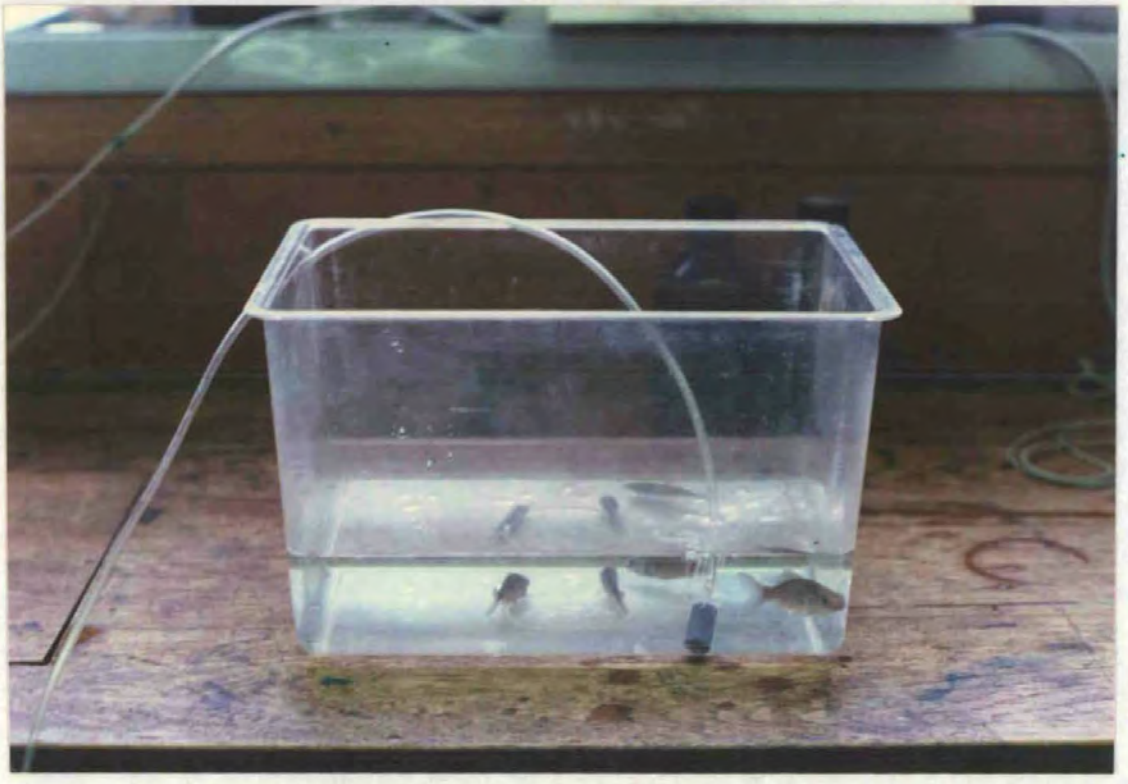
Two types of antigens were used ; soluble protein antigens (either in soluble form or latex particle-borne), all of which are murine thymus dependent antigens, and a particulate antigen, formalin-killed *Aeromonas salmonicida* cells. *A.salmonicida* is the causative agent of furunculosis in fish and is a thymus independent antigen in *Xenopus laevis* (Manning and Jurd, 1981).

Fig.1. **Maintenance of carp. (x1/6.4)**

Tanks set up to maintain carp, of up to 50g. in weight. Undergravel filters utilizing air-lifts were used for the removal of waste products.

Fig.2. **Apparatus for direct immersion of carp in antigen. (x1/3.7)**

Carp were immersed for 10 minutes in a 5 litre immersion bath containing antigen.



A. Soluble protein antigens

The soluble protein antigens used were:

Human gamma globulin (HGG), Cohn fraction II (Koch-Light laboratories, Berks., England).

Chicken gamma globulin (CGG)(Koch-Light laboratories, Berks., England).

Keyhole limpet haemocyanin (KLH)(Calbiochem, U.S.A.).

i) Native antigen.

For most of these experiments the antigen was administered in unaltered form, either with or without Freund's complete adjuvant (FCA)(Difco, Detroit, U.S.A.).

For administration by intraperitoneal injection, the antigen was prepared by dissolving 5mg/cm^3 antigen in 0.85% saline, then diluting this to 2.5mg/cm^3 by emulsifying with an equal volume of FCA. This emulsion was then administered at either 0.01cm^3/g body weight (25ug antigen per gram body weight) in fish of under 30g in weight or at a rate of 0.001cm^3/g body weight for raising anti-HGG antibodies in large fish of >500g in weight. As a control injection to this, 0.85% saline was emulsified with an equal volume of FCA and administered at the same dose.

For administration of soluble HGG by direct immersion, a bath of standing tap water containing HGG at a concentration of 9.8mg/dm^3 was used. As a control immersion to this, a bath of standing tap water containing no HGG was used.

ii) Antigen on latex particles

Latex particles of diameters between 0.05µm and 15µm (Sigma, U.S.A. or Polysciences, Warrington, U.K.) were coated with antigen, based on the method outlined in Hudson and Hay (1980). 1cm^3 of a 2.5% (w/v) suspension of latex particles was washed twice, for 4 mins. at high speed on an MSE microcentaur centrifuge, in a glycine saline buffer (GSB)(see Formulae). The particles were then incubated at room temperature with 1cm^3 of a solution of the antigen (30mg/cm^3 in GSB) for 30 minutes. After washing twice with PBS pH7.2 (see Formulae), the particles were suspended to 5% (w/v) in PBS pH7.2 and stored as a stock suspension at 4^{o}C for no longer than 24 hours, if to be used in the preparation of supernatants for the microchemotaxis assay, and for no longer than 7 days if to be administered to fish by direct immersion.

For HGG-coating of the latex particles, the supernatant from the incubation of the particles with HGG was assayed for residual HGG using a Unicam SP1800 spectrophotometer, to determine the amount of HGG adsorbed onto the particles. This was found to be within the range of 9.8$\pm$1.6mg per 10mg latex for all the sizes of latex particles used, despite the much greater surface area to mass ratio of the smaller particles. This suggests that the antigen concentration, not the available surface area of latex, was the limiting factor on the amount of antigen being adsorbed onto the particles.

When these antigen-coated latex particles were being administered to fish, the direct immersion method was always used, with the direct immersion bath consisting of 5 litres of standing tap water containing 2cm^3 of the stock suspension of antigen-coated latex particles. A corresponding

control bath would contain no antigen-coated latex particles. When different sizes of latex particles were being compared, the same concentration (w/v) of particles was used for all particle sizes.

iii) Opsonized antigen and immune complexes

Soluble and latex particle-bound HGG was also administered to fish, by direct immersion, in an opsonized or immune complexed form. When in this form, the soluble HGG or HGG-coated latex particles were administered at the same dose (in terms of mg HGG per dm^3 of immersion bath) as the corresponding non-opsonized forms.

To opsonize the HGG-coated latex particles, $0.3cm^3$ of either;

a) Fresh normal carp serum

or b) Anti-HGG hyperimmune carp antiserum (see Raising Antisera), either fresh or decomplemented by heat inactivation at 47^oC for 20 minutes (Ourth & Wilson, 1982; Sakai, 1981)

was added to $1cm^3$ of a 2.5% (w/v) suspension of these coated particles and incubated for 30 minutes at room temperature. The particles were then washed twice with a phosphate buffered saline (PBS) (pH7.2)(see Formulae) and stored for not more than 1 week in PBS pH7.2 at 4^oC.

To complex the soluble HGG with antibody, a method based on that of White et al. (1975) was used. Anti-HGG hyperimmune carp serum was obtained by hyper-immunizing carp with HGG in FCA (see Raising antisera). The optimal concentration of antigen for use with this antiserum was determined using the interfacial ring test (Campbell et al., 1963). The remaining antisera was then mixed with an equal volume of the antigen

(at optimal concentration) and left for 2 hours at room temperature, then overnight at 4^{O}C. The immune complexes had precipitated to the bottom of the tube and were recovered by centrifugation at 250g for 5 mins., discarding the supernatant. The complexes were then washed in 0.85% saline, then dissolved in an antigen excess using a ten-fold concentration of the antigen solution to that used at optimal concentration. This solution was stored for no more than 7 days at 4^{O}C before use. These immune complexes were administered , by direct immersion, at a dose of 9.8mg of HGG (including both that complexed to antibody and the HGG used to dissolve the complexes) per litre of immersion bath.

B. ***Aeromonas salmonicida*** **bacterin**

Aeromonas salmonicida bacteria of strain 25/77 were maintained in liquid nitrogen. After removal from liquid nitrogen they were grown on Tryptone soy agar (TSA)(London Analytical & Bacteriological Media Ltd.,England), then inoculated into 10cm^{3} of Tryptone soy broth (TSB)(London Analytical & Bacteriological Media Ltd.,England) and incubated at 25^{O}C for 18 hours. Twenty 250cm^{3} flasks, each containing 100cm^{3} TSB, were inoculated with 1cm^{3} of the 18 hour bacterial culture and incubated for a further 48 hours at 25^{O}C, unshaken. The cells were then formalin-killed (formalinized with 0.6% formalin solution) by the addition of 1.5cm^{3} of 40% formaldehyde solution (BDH Chemicals Ltd., U.K.) to each flask and stored at 4^{O}C for 24 hours. A loopful of this formalinized culture from each flask was then plated onto a TSA plate and left at 25^{O}C for 48 hours. No growth on the plate confirming that the formalinized culture was sterile.

The killed bacterial culture was then centrifuged at 4000g for 5 minutes to recover the bacterial cells. The cells were then washed thrice with sterile 0.85% saline at 4000g for 5 minutes and the pellet was resuspended in saline to a concentration of $3x10^{10}$cells/cm^3 and stored at $4^{O}C$ ready for use.

The *A.salmonicida* bacterin was administered in two forms ;

i) Native antigen

Normal unaltered *A.salmonicida* bacterin was administered to fish by direct immersion in a bath consisting of standing tap water containing $2x10^9$ bacterin cells/dm^3. A corresponding control bath would contain no bacterin cells.

If to be administered by intraperitoneal injection, a suspension of the A.salmonicida bacterin was made up in 0.85% saline ($2x10^{10}$ cells/cm^3) and emulsified in an equal volume of FCA. This emulsion was administered at a dose rate of 0.01cm^3/g body weight for fish of under 30g in weight, and at a rate of 0.001cm^3/g body weight for raising anti-A.salmonicida antibodies in large fish of >500g in weight. A corresponding control injection consisted of 0.85% saline emulsified in an equal volume of FCA and and was administered at the same rate.

ii) Opsonized antigen

Opsonized *A.salmonicid*a bacterin was administered to fish, by direct immersion, using the same dose as for the corresponding non-opsonized bacterin.

To opsonize the *A.salmonicida* bacterin, 0.3cm^3 of anti-*A.salmonicida*

hyperimmune carp serum was added to 1cm^3 of a 3x10^{9}cells/cm^3 suspension of the bacterin in PBS pH7.2. This was incubated at room temperature for 30 minutes and then centrifuged at high speed in an MSE microcentaur centrifuge for 2 minutes to pellet down the cells. The cells were then washed twice in PBS pH7.2, and resuspended to 1cm^3 in PBS pH7.2 and stored at 4^{o}C for no longer than 1 week before use.

Observation under the microscope showed no obvious clumping or agglutination in the opsonized antigen preparations, either in soluble or particle-bound HGG or in *A. salmonicida* bacterin.

ANTIGEN ADMINISTRATION

There were two methods used, to administer antigen to the fish ;

A. Intraperitoneal injection

Injections of antigen in saline/FCA emulsions were made aseptically with a 1cm^3 plastic syringe (Sabre international products Ltd., Reading, U.K.)fitted with a 23 gauge needle (Sabre international products Ltd., Reading, U.K.) in all fish except those injected at 10 weeks of age, where a 25 gauge needle (Sabre international products Ltd., Reading, U.K.) was used.

The injections were made by inserting the needle through the skin into the abdominal cavity from a ventro-lateral position. All injections were made at a dose rate of 0.01cm^3/g body weight in fish of under 30g in weight and at a rate of 0.001cm^3/g body weight for large fish of >500g in weight. These large fish of >500g in weight were anaesthetised in benzocaine prior to injection. All other fish were injected without anaesthesia.

B. **Direct immersion**

Using this method, antigens were administered by allowing the fish to swim in a bath of the antigen, either soluble or particulate. Fish were immersed for 10 minutes in a 5 litre bath of the antigen in standing tap water (see Fig.2). After the immersion in antigen, fish were removed to a bath containing clean standing tap water for 20 minutes, to wash off any external antigen, before being returned to their original tanks.

Collection of blood and preparation of sera

A. **Blood collection**

Adult fish were bled aseptically with anaesthesia through the dorsal blood sinus situated beneath the vertebral column in the caudal region. A 21 gauge needle (Sabre international products Ltd., Reading, U.K.) was inserted just behind the anal fin, the sinus was carefully located, punctured, and blood drawn into the syringe.

Fish of 30g. or less were killed in a bath of benzocaine, wiped dry with a paper towel and the caudal peduncle was severed with a clean razor blade.The blood was then allowed to drip into a 1.5cm^3 microcapped centrifuge tube (Hughes and Hughes, U.K.).

B. **Preparation of sera**

The blood of adult fish was placed in 10cm^3 capped centrifuge tubes (Sterilin, U.K.) and that from smaller fish in microcentrifuge tubes. The blood was left to clot for 1 hour at room temperature ;then left overnight at 4^{o}C for clot retraction. The 10cm^3 centrifuge tubes were then centrifuged at 500g for 5 minutes, and the microcentrifuge tubes were centrifuged in

an MSE microcentaur centrifuge at high speed for 2 minutes. The serum was then removed and, if to be used for serum diluent in passive haemagglutination tests, was aliquoted in 0.5cm^3 amounts in microcentrifuge tubes and stored at -20^{O}C. Test sera were always used fresh as freezing or storage for any longer than absolutely neccesary is known to reduce antibody levels (Rio & Recco,1971).

Raising antisera

Adult carp of between 0.5 and 1.5 kg. were hyperimmunised with either HGG in 0.85% saline (5mg/cm3) or A.salmonicida bacterin in 0.85% saline (2x10^{10} formalin-killed cells/cm^3) in 0.85% saline via intraperitoneal injections at six week intervals. The initial challenge being the same dose of antigen but emulsified in equal volumes of 0.85% saline and FCA. All the injections were administered at a rate of 1cm^3/kg body weight.

At a minimum of 12 weeks after the initial challenge, these fish were bled to obtain the serum containing either anti-HGG antibodies or anti-*A.salmonicida* antibodies.

FORMULAE

i) **Phosphate Buffered Saline pH 7.2**

NaCl - 8.50g.
$Na_2HPO_4.12H_2O$ - 2.697g.
KH_2PO_4 - 0.20g.

Dissolve in double distilled water and make up to 1 litre. Adjust pH to 7.2 with HCl or NaOH.

ii) **Phosphate Buffered Saline pH 6.4**

0.15M KH_2PO_4 - $67.8cm^3$
0.15M Na_2HPO_4 - $32.2cm^3$
0.85% saline - $100cm^3$

Mix together and adjust to pH 6.4 with HCL or NaOH.

iii) **Glycine Saline Buffer pH 8.2**

Glycine - 1.51g.
NaOH - 0.075g.
NaCl - 1.836g.

Dissolve in double distilled water and make up to a litre. Adjust pH to 8.2 with HCL or NaOH.

iv) **Citric Acid / Disodium Hydrogen Phosphate Buffer pH 5.75**

0.1M citric acid - $85cm^3$
0.2M Na_2HPO_4 - $115cm^3$

Make up to 1 litre with double distilled water. Adjust pH to 5.75 with HCL or NaOH.

v) **Shieh & Reddy Medium**

$MgSO_4.7H_2O$ - $500mg/dm^3$
$K_2HPO_4.3H_2O$ - $1g/dm^3$
NaCl - $5g/dm^3$
$FeSO_4.7H_2O$ - $10mg/dm^3$
DL-alanine - $500mg/dm^3$
Arginine - $1g/dm^3$
L-methionine - $100mg/dm^3$
L-cysteine.HCL - $10mg/dm^3$
DL-serine - $100mg/dm^3$
DL-isoleucine - $50mg/dm^3$
Na-glutamate - $100mg/dm^3$

Make up in double distilled water.

CHAPTER 3

Title

Serum humoral immune responses of carp, *Cyprinus carpio* L., following direct immersion in antigen.

INTRODUCTION

This chapter describes certain studies on the humoral immune response following priming by direct immersion vaccination. Although some workers have found slightly elevated serum antibody titres after multiple direct immersions in antigen (Watson, 1984; Lamers & Pilarczyk, 1985; Lamers *et al.*, 1985), others have found that an intraperitoneal (i.p.) challenge of the antigen, usually administered in adjuvant, is necessary before a measurable humoral immune response can be detected (Mughal, 1984; Manning *et al.*, 1989), with no humoral immune response detectable after immersions alone in antigen. In this laboratory, using HGG and *Aeromonas salmonicida* bacterin as antigens, it has been generally found that priming alone, by multiple immersions in antigen, does not give detectable serum antibody titres in young fish and that a secondary challenge by i.p. injection, of antigen in FCA, is needed before elevated antibody titres are found. If the priming, by direct immersion, has been effective then an increased secondary humoral immune response will be observed following the i.p. challenge, compared to the normal primary response which follows an i.p. challenge of the antigen in FCA, when no prior immersions in antigen have been administered.

The humoral immune response, detected by elevated serum antigen-specific antibody titres, following either i.p. or intramuscular (i.m.) injection of antigen is well known (Di Conza & Halliday, 1971; Bradshaw *et al.*, 1971). It is suggested that the pronephros is the organ which may be largely the origin of this response, in teleost fish. Smith *et al.* (1967) found antibody-forming cells in the pronephros and Ferren (1967) observed that splenectomy of fish fails to significantly depress the humoral immune

response. The presence in the pronephros, of all the cellular elements essential for antigen trapping, processing and presentation have also been shown (Agius, 1981). It is also known that the proliferative reponse in the pronephros, following an i.p. challenge of HGG in FCA, peaks at 3 weeks post-challenge (Secombes *et al.*, 1982b), which corresponds much more closely to the time scale of a humoral immune response than to that of a cell-mediated response (see results presented in this chapter and chapter 4).

In teleost fish, IgM is the only firmly identified class of immunoglobulin, and is found as a tetramer (Shelton & Smith, 1970; Dorson, 1981) although there is some evidence of monomeric Ig in some fish species (Clem & McLean, 1975).

Studies on protective immunity show that booster immersions in a vaccine can give increased protection against live challenge with the pathogen (Tatner & Horne, 1985) and that these immersions can be as near to each other as 4 days (Johnson & Amend, 1984) or as far apart as 8 months (Lamers *et al.*, 1985) to elicit a stimulating secondary response to the initial immersion.

The exact antibody response following exposure to external antigens, by either direct immersion vaccination, oral or anal administration, is not totally understood and what has been discussed so far is the serum antibody response. It is known that the antibody response can be comprised of both a serum response and a secretory response. Various substances with bactericidal or agglutinating activities have been described in the mucus of fish (Ingram, 1980) and antibodies have been shown in both skin mucus (Bradshaw *et al.*, 1971) and gut mucus (Rombout *et al.*, 1986). Antibody-forming cells have also been found in the

cutaneous dermis and epidermis of fish (St.Louis-Cormier *et al.*, 1984; Peleteiro & Richards, 1988) supporting the theory of a seperate secretory immune system. How much this secretory immune response is linked to the serum humoral immune response is unsure. Lobb & Clem (1981) showed that immunoglobulin found in cutaneous mucus and bile was not due to transudation or active transport of serum antibodies, and there is suggestive evidence that secretory immunoglobulin (Ig) may possibly be a different class of Ig to that found in the serum (Rombout, Bot & Taverne-Thiele, 1989). However, our understanding of the secretory immune response still remains unclear, as some workers have found that an i.p. or i.m. challenge of antigen has stimulated an increase in mucosal antibody titres (Ourth, 1980; St.Louis-Cormier *et al.*, 1984) whereas Rombout, van den Berg, van den Berg, Witte & Egberts (1989) found that an i.m. challenge of ferritin or *Vibrio* bacterin induced no production of mucosal antibodies, only an increase in serum antibody titres. Differing results have also been found after exposure to external antigens (i.e. direct immersion, oral or anal immunization), some workers finding increased mucosal antibody titres, but not serum titres (Lobb, 1987), others finding increases in both the serum and mucosal antibody titres (Rombout, van den Berg, van den Berg, Witte & Egberts, 1989). Watson (1984) and Robohm (1986) found interesting evidence that the normal rate of water swallowing during direct immersion vaccination would allow enough antigen into the gut to account for a protective immune response and, as has already been discussed (see General Introduction), antigen administered into the gut is known to be taken up and processed by intestinal lymphoid cells (Lamers, 1985; Rombout *et al.*, 1986; Hart *et al.*, 1988; Doggett, 1989). Therefore, the exact mechanisms of antibody

response following external antigenic exposure and the roles of the secretory and serum antibody responses are, as yet, incompletely understood.

The presence of antibody in the mucus of fish raises the question of whether secretory antigen-specific antibodies have a role to play in the response to direct immersion vaccination. Does the binding of an external antigen by secretory antibody possibly enhance the uptake or response to that antigen? Work presented in this chapter looks at this possibility, by comparing the serum antibody response following exposure to normal, unaltered antigens with the response following exposure to the corresponding opsonized antigens. It has already been shown that the i.p. injection of immune complexed antigen gives a better immune response and memory than the injection of the antigen alone (Secombes & Resink, 1984), however, any effects of opsonization on direct immersion vaccination have yet to be demonstrated.

Results are also presented in this chapter looking at the effects of opsonization on direct immersion immunization in immunologically immature (4 week old) carp. It is known that very young fish respond differently to antigenic exposure, both by direct immersion and injection, compared to more immunologically mature fish. There also seems to be a marked difference in the response of very young fish to T-dependent and T-independent antigens. Secombes (1981) found that 21 day old trout fry could mount a positive antibody response to injected *Aeromonas salmonicida* bacterin and Manning *et al.* (1989) found that direct immersion of 4 week old carp in an *A.salmonicida* bacterin induced a secondary response to a later i.p. challenge. However, the experiments of Secombes (1981), van Loon *et al.* (1981) and Mughal *et al.* (1986)

indicate that early exposure of young carp and trout to T-dependent antigens, such as HGG or sheep erythrocytes, can result in immunological tolerance when the fish are challenged with an injection of the same antigen at an age when fish are capable of giving a positive humoral antibody response to a primary challenge.

The effects of vaccine particle size on the efficacy of direct immersion vaccination are also studied in this chapter. Smith (1982) found greater uptake of bovine serum albumin (BSA), from an immersion bath, when bound to latex particles compared to when it was administered in its soluble form. Mughal (1984) also found that immature carp responded differently to HGG, administered by direct immersion, when it was bound to latex particles compared to when in its soluble form. The results presented here describe the response of carp to direct immersion in HGG in either its soluble form or bound to a range of sizes of latex particles, from 0.05µm to 15µm, is studied. This is also compared to the phagocytosis by carp peritoneal exudate cells of these different sizes of particles (see chapter 5).

Lastly, a study into the specificity of the humoral immune response to priming by direct immersion was carried out. The cross-reactivity between the responses to two T-dependent antigens was studied, as was the cross-reactivity between the response to a T-dependent antigen and a T-independent antigen.

MATERIALS AND METHODS

Immunization, challenge and testing regime for the response to direct immersion vaccines

Fish were directly immersed three times, for ten minutes each, at weekly intervals in either a vaccine bath or a control bath, with no additions (see Fig.3). Four weeks after the final immersion, the fish were given an intraperitoneal (i.p.) challenge injection. The challenge injection was either saline in Freund's complete adjuvant (FCA), for a control challenge, or antigen in saline in FCA. The antigen in saline being either human gamma globulin (HGG), keyhole limpet haemocyanin (KLH) or formalin-killed *Aeromonas salmonicida* bacterin. Five weeks (unless stated otherwise) after this challenge injection, the fish were sacrificed and the serum was assayed for either specific anti-*A.salmonicida* antibodies, using direct bacterial agglutination or for specific anti-HGG, anti-KLH or anti-chicken gamma globulin (CGG) antibodies using the passive haemagglutination technique. The antibody titres obtained were then analysed using ANOVA and the t-test corrected for small samples (McGhee, 1985).

It was chosen to assay the serum for antigen-specific antibodies at 5 weeks post-challenge as results from these investigations had shown that carp undergoing this immunization and challenge regime with HGG, although producing specific antibodies by 4 weeks post-challenge, did not reach peak titres until 6-8 weeks post-challenge (see Fig.6). Assaying at 5 weeks post-challenge was therefore chosen to detect any acceleration in

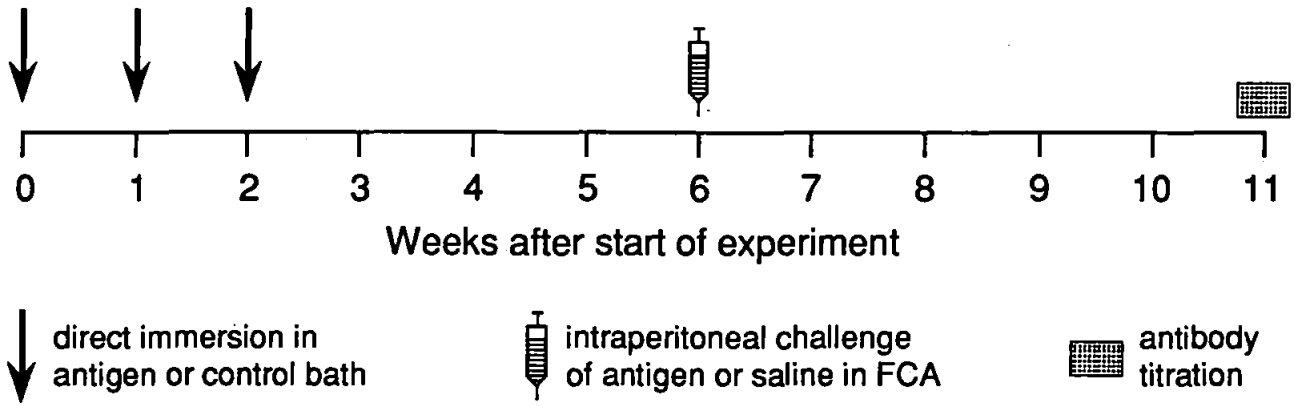


Figure 3

Protocol for direct immersion in antigen, subsequent intraperitoneal challenge and antibody titration.

the response.

Direct agglutination of the killed *A.salmonicida* bacterin

Antibody titres against *A.salmoncida* were tested using standard microtitre procedures (Antipa & Amend, 1977). In this test, which was carried out in 96 well microtitre plates (Richardsons Ltd., Leicester, U.K.), 50µl of 0.85% saline was added to each well being used. 50µl of each test serum (always used fresh) was then serially diluted in two fold steps through three rows of a microtitre plate (36 wells). The serum from a control fish was also tested.

50µl of 10^9cells/cm^3 saline of *A.salmonicida* bacterin were then added to each well. The plate was then gently agitated and sealed with sellotape. It was kept at room temperature for 3 hours and then overnight at 4^oC before reading the plate. The end point was considered as the last well showing any agglutination (see Fig.4).

Passive haemagglutination

Serum antibody levels to soluble protein antigens were titrated using the passive haemagglutination technique, based on that described by Stavitsky (1954), employing tanned sheep erythrocytes.

The sera to be tested using this technique were always used fresh. Naturally occuring agglutinins were removed by absorbing the serum for 45 minutes with an equal volume of packed sheep red blood cells (SRBC)(Tissue Culture Services Ltd., Claydon, U.K.), which had been thrice washed with PBS pH7.2 (see Formulae) at 500g for 5 minutes.

Fig.4. Antibody titration (bacterial agglutination). (x1.15) Bacterial cells pellet down to the bottom of the well unless sufficient antibody is present to agglutinate them. The endpoint is shown by the last well of a dilution series to show any agglutination. Agglutination is seen in all the wells of rows A & D, well no.1 of row B and wells 1-8 of row E.

Rows A-C = 50µl of test serum 1, serially diluted (-log 2), + 50µl of *Aeromonas salmonicida* bacterin suspension per well. Endpoint = well no. 13.

Rows D-F = 50µl of test serum 2, serially diluted (-log 2), + 50µl of *Aeromonas salmonicida* bacterin suspension per well. Endpoint = well no. 8.

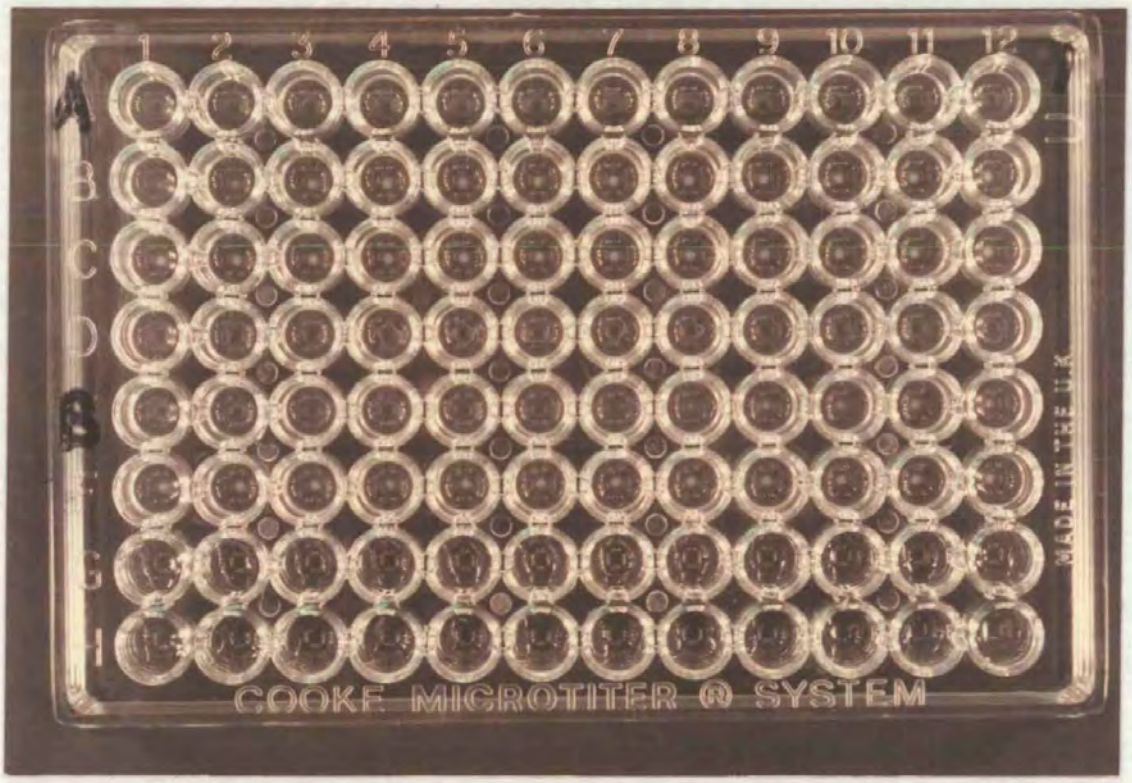
Fig.5. Antibody titration (passive haemagglutination). (x1.15) SRBC pellet down to the bottom of the well unless sufficient antibody is present to agglutinate them. The endpoint is shown by the last well of a dilution series to show any agglutination. Agglutination is seen in all the wells of rows A,B,E and F, the first 2 wells of row C, the first 6 wells of row D, and the first 4 wells of row H.

Rows A-C = 50µl of test serum 1, serially diluted (-log 2), + 50µl of SRBC coated with HGG per well. Endpoint = well no. 29.

Row D = 50µl of test serum 1, serially diluted (-log 2), + 50µl of uncoated SRBC per well. Endpoint = well no. 6.

Rows E-G = 50µl of test serum 2, serially diluted (-log 2), + 50µl of SRBC coated with HGG per well. Endpoint = well no. 24.

Row H = 50µl of test serum 2, serially diluted (-log 2), + 50µl of uncoated SRBC per well. Endpoint = well no. 4.


1 2 3 4 5 6 7 8 9 10 11 12
A
B
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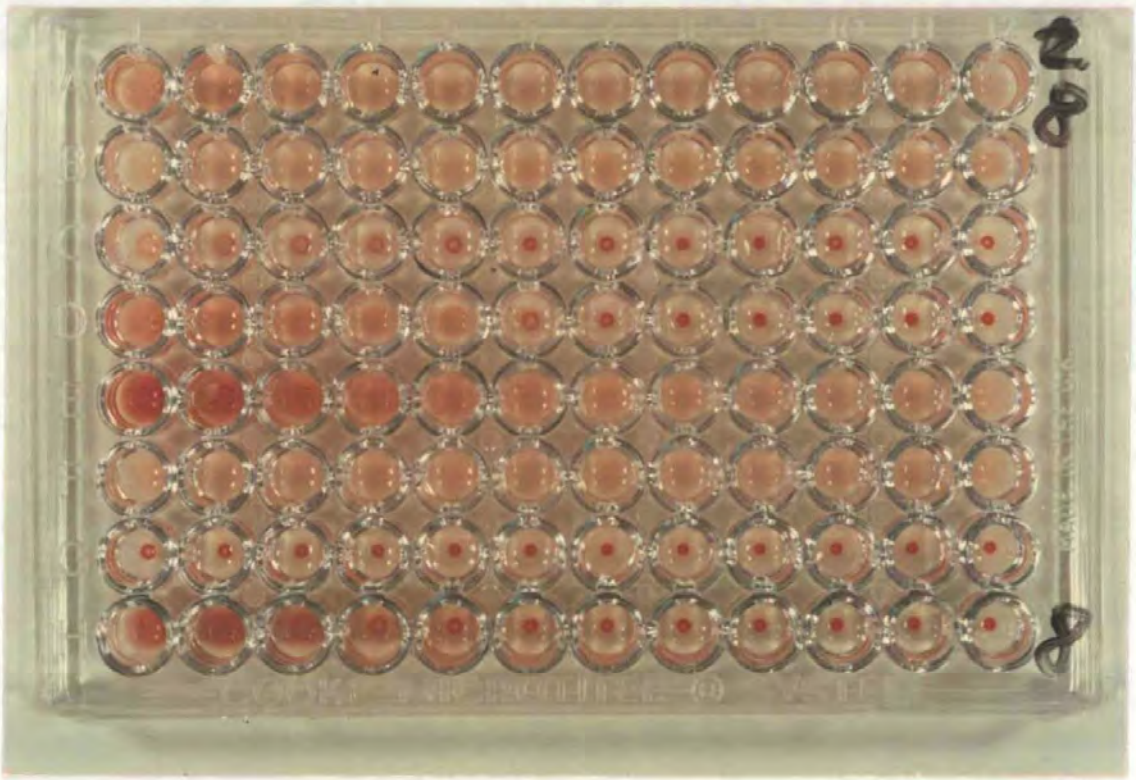


For passive haemagglutination, thrice washed SRBC were suspended to 2.5% (v/v) in PBS pH 7.2, then mixed with an equal volume of 0.005% (w/v) tannic acid (Sigma, U.S.A.) in 0.85% saline and incubated at 37^{O}C for 15 minutes. The cells were then washed in PBS pH 7.2 at 500g for 5 minutes and resuspended to 1.25% (v/v) in PBS pH6.4 (see Formulae). These tanned cells were then either coated with the appropriate antigen (HGG, KLH or CGG) or left uncoated as control cells.

To coat with antigen, 8cm^3 of the 1.25% (v/v) suspension of tanned SRBC in PBS pH6.4 was mixed with 2cm^3 of PBS pH7.2 containing the protein antigen at a concentration of 1 mg/cm^3. For the control cells, 2cm^3 of normal PBS pH7.2 was added instead of the antigen solution. The mixture was then left at room temperature for 15 minutes.

The cells were then washed in PBS pH7.2 (500g for 5 minutes) and resuspended to 0.25% in serum diluent. The serum diluent, which served to stabilize the tanned cells and prevent spontaneous agglutination, consisted of 1% normal, pooled, heat-inactivated (47^{O}C for 20 minutes) carp serum in PBS pH7.2. The normal serum used to make up this diluent had been absorbed with SRBC to remove any non-specific agglutinating activity.

The antibody titration was made in 96 well microtitre plates (Richardsons Ltd., Leicester, U.K.). 50µl of serum diluent was added to each well. The serum sample was then serially diluted in two-fold dilutions along 3 rows (36 wells) and 50µl of antigen-coated SRBC was added to each well. In a fourth row, similar two-fold dilutions were made, but this time 50µl of uncoated SRBC were added to each well.

Other controls were set up in a similar manner, including those using sera from normal (non-immunized) fish and those using serum diluent

alone. The plates were then covered and left for 3 hours at room temperature and then overnight at 4^{O}C. Plates were read the following day and the last well to show any agglutination was taken as the endpoint (see Fig.5). The specific antibody titre against the antigen in question was taken as the number of wells of agglutination in the dilution series containing antigen-coated SRBC minus the number of wells of agglutination in the dilution series containing the uncoated SRBC.

EXPERIMENTAL DESIGN

There were four areas of study undertaken, investigating the humoral immune respose of carp to direct immersion vaccination. Unless otherwise stated, the fish were between 5 and 9 months old at the beginning of the experiments and were considered to be immunologically mature.

1) a) Investigations as to whether serum antibody levels could be detected after direct immersions in antigen, with no intraperitoneal (i.p.) challenge.

   b) Investigations into the time-course of the humoral immune response following direct immersion in antigen with a subsequent i.p. challenge.

2) a) Investigations into the effects of the size of a direct immersion vaccine on the humoral immune response.

Comparing soluble antigen with antigen bound to different sizes of latex particle.

b) Investigations into the effects of opsonization of direct immersion vaccines on the humoral immune response.

3) Investigations into the effects of opsonization of direct immersion vaccines on the humoral immune response of immunologically immature carp (4 weeks old at the beginning of the experiments).

4) Investigations into the specificity of the humoral immune response to direct immersion vaccines.

SECTION 1

1. a) Six fish were directly immersed 5 times, at monthly intervals in HGG-coated 0.8µm latex particles. Another group of six fish received control immersions. Four weeks after the final immersion, the fish were sacrificed and the serum anti-HGG antibody titres were measured using the passive haemagglutination technique.

1. b) Fish were given three, weekly, immersions in either HGG-coated 0.8µm latex particles or a control bath. Four weeks after the final

immersion, the fish received an i.p. challenge of either HGG in FCA or saline in FCA. At 2, 4, 6 and 8 weeks post-challenge, fish were sacrificed and their serum anti-HGG antibody titres were measured using the passive haemagglutination technique. There were 6 fish per experimental group.

SECTION 2

2.a) Fish were given three, weekly, immersions in either:-

i) A control bath (12 fish)

ii) Soluble HGG (12 fish)

iii) HGG-coated 0.05µm latex particles (6 fish)

iv) HGG-coated 0.2µm latex particles (6 fish)

v) HGG-coated 0.8µm latex particles (12 fish)

vi) HGG-coated 3.0µm latex particles (6 fish)

vii) HGG-coated 15.0µm latex particles (6 fish)

Four weeks after the final immersion the fish received an i.p. challenge of HGG in FCA. Five weeks post-challenge the fish were sacrificed and their serum anti-HGG antibody titres were measured using the passive haemagglutination technique.

2. b) i) Fish were given three, weekly, immersions in either:-

i) A control bath (12 fish)

ii) Soluble HGG (12 fish)

iii) Soluble HGG opsonized with immune serum (6 fish)

iv) HGG-coated 0.8µm latex particles (12 fish)

v) HGG-coated 0.8µm latex particles opsonized with normal

carp serum (6 fish)

vi) HGG-coated 0.8µm latex particles opsonized with immune carp serum (12 fish)

Four weeks after the final immersion the fish were given an HGG in FCA i.p. challenge. Five weeks post-challenge the fish were sacrificed and their serum anti-HGG antibody titres were measured using the passive haemagglutination technique.

2. b) ii) Fish were given three, weekly, immersions in either:-

i) A control bath (16 fish)

ii) *A.salmonicida* bacterin (16 fish)

iii) *A.salmonicida* bacterin opsonized with immune carp serum (16 fish)

Four weeks after the final immersion half the fish from each group received an i.p. challenge of saline in FCA and the other half received an intraperitoneal challenge of bacterin in FCA. Five weeks post-challenge the fish were sacrificed and their serum anti-*A.salmonicida* antibody titres were measured using the direct bacterial agglutination technique.

SECTION 3

a) Fish of 4 weeks of age were given three, weekly, immersions in either:-

i) A control bath (40 fish)

ii) HGG-coated 0.8µm latex particles (40 fish)

iii) HGG-coated 0.8µm latex particles opsonized with normal carp serum (40 fish)

iv) HGG-coated 0.8μm latex particles opsonized with immune carp serum (40 fish)

Four weeks after the final immersion the fish were given an i.p. challenge of HGG in FCA. Five weeks post-challenge the fish were sacrificed and their serum anti-HGG antibody titres were measured using the passive haemagglutination technique. Due to the small size of the fish, each serum sample used in the assay was pooled from the blood of 5-11 fish. There were 4 serum samples assayed from each experimental group.

b) Fish of 4 weeks of age were given three, weekly, immersions in either:-

i) A control bath (40 fish)

ii) *A.salmonicida* bacterin (40 fish)

iii) *A.salmonicida* bacterin opsonized with normal carp serum (40 fish)

iv) *A.salmonicida* bacterin opsonized with immune carp serum (40 fish)

Four weeks after the final immersion the fish received an i.p. challenge of bacterin in FCA. Five weeks post-challenge the fish were sacrificed and their serum anti-*A.salmonicida* antibody titres were measured using the direct bacterial agglutination technique. Due to the small size of the fish, each serum sample used in the assay was pooled from the blood of 5-10 fish. There were 4 serum samples assayed from each experimental group.

SECTION 4

a) Fish were given three, weekly, immersions in either a control bath or

HGG-coated 0.8µm latex particles. Four weeks after the final immersion, half the fish from each group received an i.p. challenge of HGG in FCA, and the other half received an i.p. challenge of KLH in FCA. Five weeks post-challenge the fish were sacrificed and the serum from the fish which had received an HGG in FCA challenge was assayed for anti-HGG antibodies, and the serum from the fish which had received a KLH in FCA challenge was assayed for anti-KLH antibodies. The passive haemagglutination technique was used. There were 6 fish in each experimental group.

b) Fish were given three, weekly, immersions in either:-

i) A control bath (10 fish)

ii) HGG-coated 0.8µm latex particles (10 fish)

iii) *A.salmonicida* bacterin (10 fish)

Four weeks after the final immersion, half the fish from each group received an HGG in FCA i.p. challenge and the other half received a bacterin in FCA i.p. challenge. Five weeks post-challenge the fish were sacrificed and those which had received an HGG in FCA challenge had their sera assayed for anti-HGG antibodies using the passive haemagglutination technique. The fish which had received a bacterin in FCA challenge had their sera assayed for anti-*A.salmonicida* antibodies using the direct bacterial agglutination technique.

c) Fish were given three, weekly, immersions in either a control bath or HGG-coated 0.8µm latex particles. Four weeks after the final immersion the fish received an HGG in FCA i.p. challenge. Five weeks post-challenge the fish were sacrificed and their sera were assayed for anti-

HGG antibodies and for anti-CGG antibodies, using the passive haemagglutination technique.

RESULTS

SECTION 1

Detection of serum antibodies after direct immersion in HGG-coated latex particles

One group of fish received multiple immersions in HGG-coated latex particles (HGG-Latex), whilst another group received immersions in a control bath.

Four weeks after the final immersion no anti-HGG antibodies could be detected in the serum from either the control fish or from the fish which had undergone the multiple immersions in HGG-Latex.

Time-course of the humoral immune response after direct immersion in HGG-coated latex particles with a subsequent intraperitoneal challenge

Fish were given multiple immersions in either HGG-Latex or a control bath, with a subsequent i.p. challenge of either HGG in FCA or saline in FCA. The serum from these fish was then assayed for anti-HGG antibodies at 2,4,6 and 8 weeks post-challenge. The results from this experiment are expressed in Fig.6.

The fish which had received multiple immersions in HGG-Latex, with a subsequent intraperitoneal (i.p.) HGG in FCA challenge, showed detectable levels of serum anti-HGG antibodies at 2 and 4 weeks post-challenge but the titres did not peak until 6 weeks post-challenge and were at the same level at 8 weeks post-challenge.

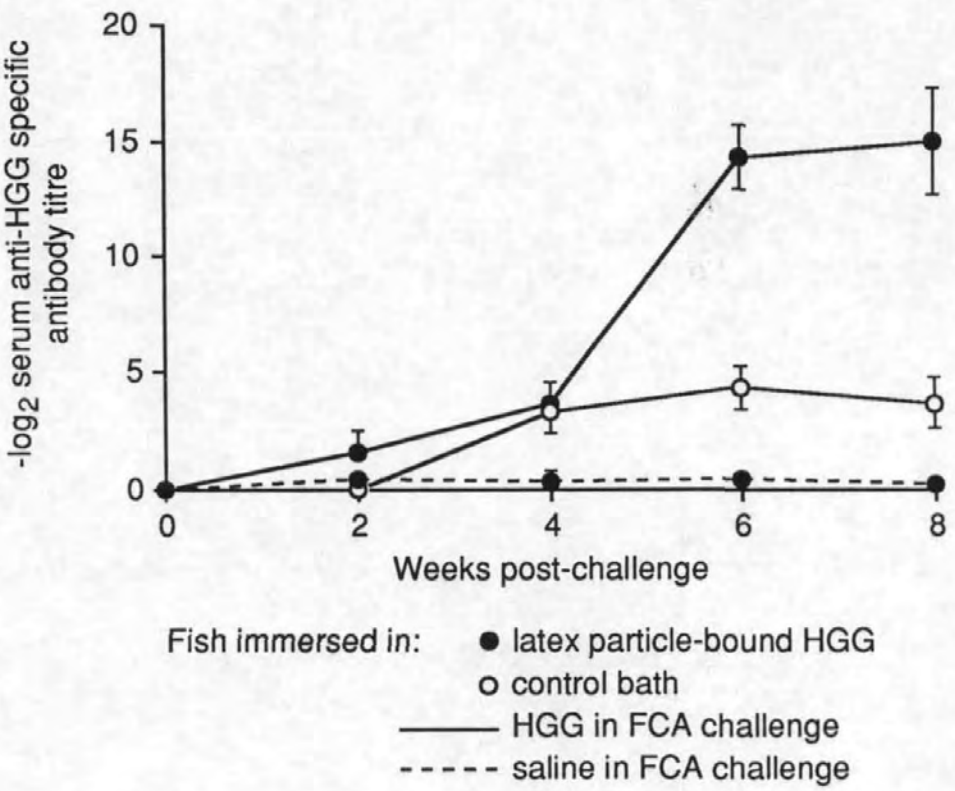


Figure 6

Serum antibody production following multiple immersions in either latex particle-bound HGG or a control bath with a subsequent intraperitoneal challenge. Fish killed at different times post-challenge.
Mean ± SE.

The fish which received multiple immersions in HGG-Latex, but with a subsequent i.p. challenge of saline in FCA showed no significantly increased levels of anti-HGG serum antibodies at either 2,4,6 or 8 weeks post-challenge.

The fish which received immersions in HGG-Latex, with a subsequent HGG in FCA i.p. challenge did not have significantly greater serum anti-HGG antibody titres at 2 weeks post-challenge than the fish which received the same immersions but had a saline in FCA i.p. challenge. At 4 weeks post-challenge, however, of the fish which had been immersed in HGG-Latex, those which had received an HGG in FCA challenge had significantly ($p<0.05$) higher anti-HGG serum antibody titres than those which had received a saline in FCA challenge. At 6 and 8 weeks post-challenge the difference was even greater ($p<0.001$).

The fish which received the control immersions with a subsequent i.p. challenge of HGG in FCA showed no detectable serum anti-HGG antibodies until 4 weeks post-challenge. No significant further increase in the serum antibody titres of these fish was observed at either 6 or 8 weeks post-challenge.

Of the fish which received an HGG in FCA i.p. challenge, those which had received prior immersions in HGG-Latex showed significantly greater ($p<0.001$) anti-HGG serum antibody titres at 6 weeks post-challenge. At 8 weeks post-challenge the difference was significant at $p<0.01$.

SECTION 2

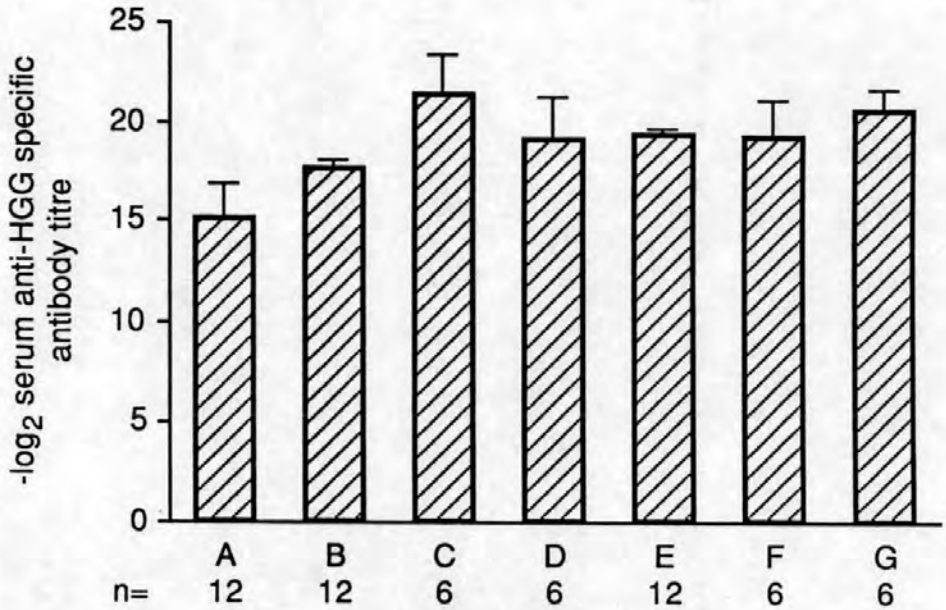
Effects of particle-size on direct immersion vaccination

Fish were given multiple immersions in either a control bath, soluble HGG or one of a range of different sized HGG-coated latex particles (HGG-Latex), with a subsequent HGG in FCA i.p. challenge. The sera from these fish were then assayed for anti-HGG antibodies at 5 weeks post-challenge. The results for this experiment are expressed in Fig.7.

Those which received prior immersions in soluble HGG had slightly greater anti-HGG serum antibody titres than those which had only received prior immersions in a control bath, but the difference was not statistically significant.

Those fish which received prior immersions in HGG-Latex of all the sizes used had significantly greater ($p<0.05$) serum anti-HGG antibody titres than those which had only received prior immersions in a control bath.

When analysed using the ANOVA test, the difference between the response to the soluble HGG immersions compared to the reponse to all the HGG-Latex immersions together was significant at $p<0.02$. Using the same statistical method, the *difference* between the response to all the HGG-Latex immersions together compared to the response to the control bath was significant at $p<0.05$.



Fish immersed in:
A = control bath
B = soluble HGG
C = HGG-coated 0.05µm latex particles
D = HGG-coated 0.2µm latex particles
E = HGG-coated 0.8µm latex particles
F = HGG-coated 3.0µm latex particles
G = HGG-coated 15.0µm latex particles

Figure 7

Serum antibody production following multiple immersions in different sizes of HGG-coated latex particles, soluble HGG or a control bath, with a subsequent HGG in FCA intraperitoneal challenge.
Mean ± SE.

Effects of opsonization on direct immersion immunization with HGG, both soluble and latex particle-borne

Fish were given multiple immersions in either:-

a) A control bath

b) Soluble HGG

c) Soluble HGG opsonized with immune carp serum

d) 0.8μm HGG-Latex

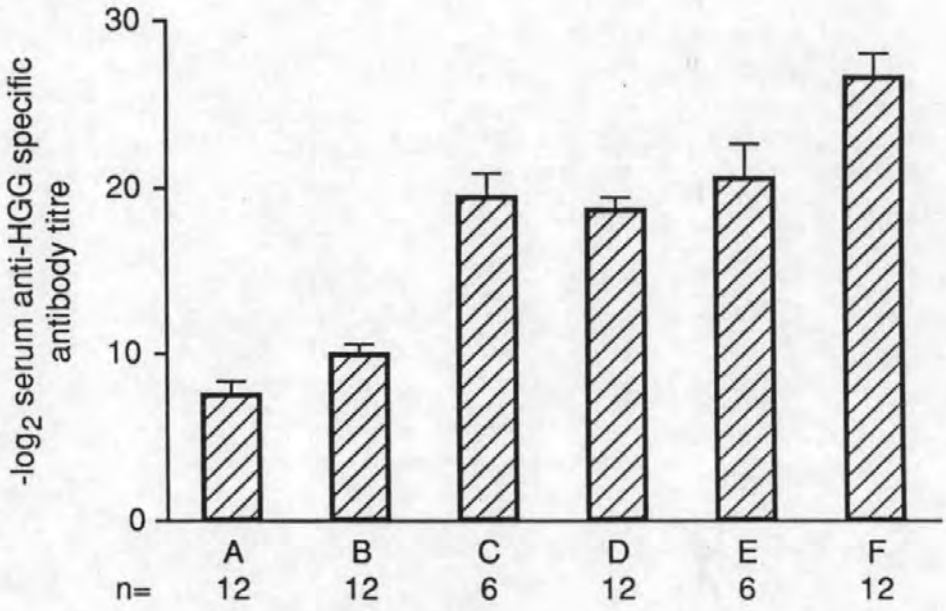
e) 0.8μm HGG-Latex opsonized with normal carp serum

f) 0.8μm HGG-Latex opsonized with immune carp serum

These fish then received a subsequent i.p. challenge of HGG in FCA, and their sera were assayed for anti-HGG antibodies at 5 weeks post-challenge. The results for this experiment are expressed in Fig.8.

Those fish which received immersions in soluble HGG had significantly greater ($p<0.05$) anti-HGG serum antibody titres than those which had received immersions in a control bath. Those fish which had received immersions in the soluble HGG opsonized with immune carp serum had significantly greater ($p<0.001$) anti-HGG antibody titres than those which had been immersed in the non-opsonized soluble HGG.

Fish which had been immersed in HGG-Latex had significantly greater ($p<0.001$) anti-HGG antibody titres than those which had been immersed in either a control bath or soluble HGG. Fish which had been immersed in HGG-Latex opsonized with normal carp serum had significantly greater



Fish immersed in: A = control bath
B = soluble HGG
C = soluble HGG opsonized with immune serum
D = HGG-coated 0.8µm latex particles
E = HGG-coated 0.8µm latex particles opsonized with normal serum
F = HGG-coated 0.8µm latex particles opsonized with immune serum

Figure 8

Serum antibody production following multiple immersions in soluble or latex particle-bound HGG, opsonized or non-opsonized, with a subsequent intraperitoneal HGG in FCA challenge.
Mean ± SE.

($p<0.001$) anti-HGG antibody titres than those immersed in either a control bath or soluble HGG but did not have significantly greater anti-HGG antibody titres than those which had been immersed in the non-opsonized HGG-Latex. However, those fish which had received immersions in the HGG-coated latex particles opsonized with immune carp serum had significantly greater anti-HGG antibody titres than those immersed in either a control bath ($p<0.001$), soluble HGG ($p<0.001$), soluble HGG opsonized with immune serum ($p<0.01$), HGG-coated latex particles ($p<0.001$) or HGG-coated latex particles opsonized with normal carp serum ($p<0.05$).

Effects of opsonization on direct immersion immunization with *A.salmonicida* bacterin

Fish were directly immersed in either a control bath, *A.salmonicida* bacterin or the bacterin opsonized with immune carp serum, with a subsequent i.p. challenge of either bacterin in FCA or saline in FCA. The sera from these fish were then assayed for anti-*A.salmonicida* antibodies at 5 weeks post-challenge. The results for this experiment are expressed in Fig.9.

All the fish which received a saline in FCA challenge showed much lower anti-*A.salmonicida* serum antibody levels than those which received a bacterin in FCA challenge.

Of those fish which received a bacterin in FCA challenge, those which had been previously immersed in the opsonized bacterin had significantly greater anti-*A.salmonicida* serum antibody titres than those which had been immersed in either the normal bacterin ($p<0.05$) or the control bath

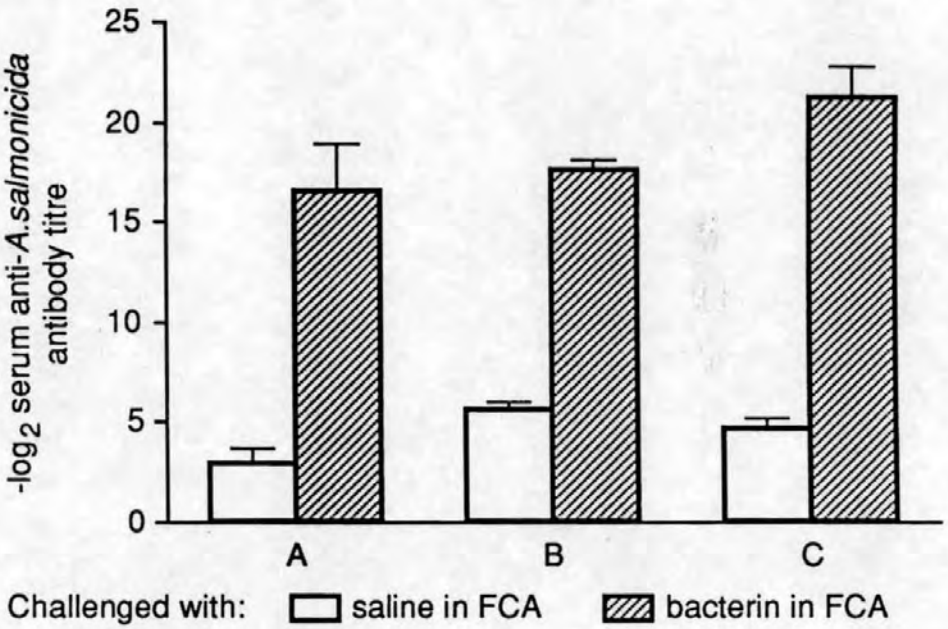


Figure 9

Serum antibody production following multiple immersions in an *A.salmonicida* bacterin, either opsonized or non-opsonized, or in a control bath, with a subsequent intraperitoneal challenge.
Mean ± SE, n=8.

($p<0.01$). There was no significant difference between the antibody titres of the fish which had received immersions in the normal bacterin, with a subsequent bacterin in FCA challenge and those which had received immersions in a control bath with the same i.p. challenge.

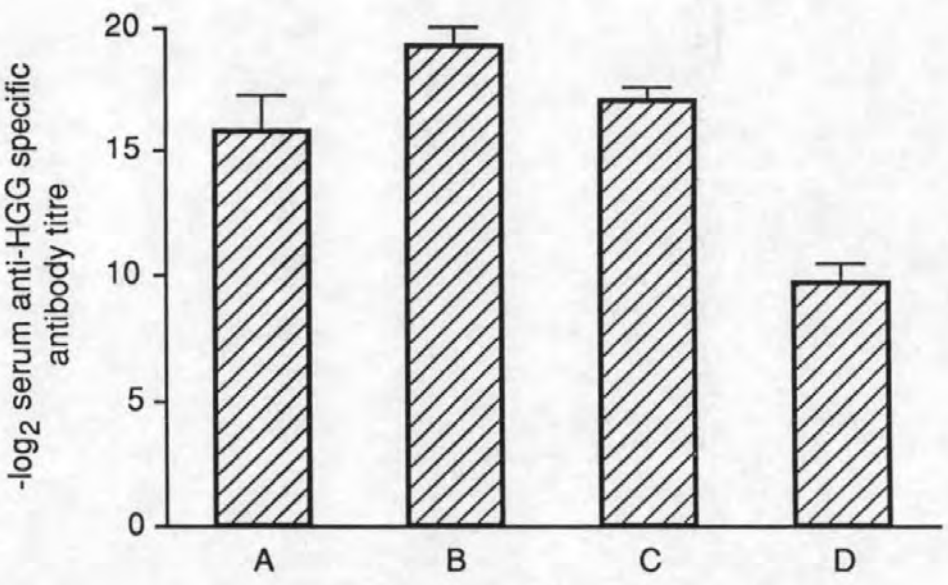
SECTION 3

Effects of opsonization on direct immersion in particle-borne HGG in immunologically immature fish

Four week old fish were given multiple immersions in either a control bath, HGG-Latex, HGG-Latex opsonized with normal carp serum or HGG-Latex opsonized with immune carp serum. These fish then received an i.p. challenge of HGG in FCA and their sera were assayed for anti-HGG antibodies at 5 weeks post-challenge. The results from this experiment are expressed in Fig.10.

The fish which received immersions in HGG-Latex and the fish which had immersions in HGG-Latex opsonized with normal carp serum had greater anti-HGG antibody titres than those fish immersed in a control bath, but not statistically significantly so. The fish which had received immersions in HGG-Latex did, however, have significantly greater ($p<0.05$) anti-HGG antibody titres than those which had been immersed in the HGG-Latex opsonized with normal carp serum.

The fish which had been immersed in HGG-Latex opsonized with immune carp serum had significantly lower anti-HGG antibody titres than those which had been immersed in a control bath ($p<0.02$), HGG-Latex ($p<0.001$) or HGG-Latex opsonized with normal carp serum ($p<0.001$).



Fish immersed in:
A = control bath
B = HGG-coated 0.8μm latex particles
C = HGG-coated 0.8μm latex particles opsonized with normal serum
D = HGG-coated 0.8μm latex particles opsonized with immune serum

Figure 10

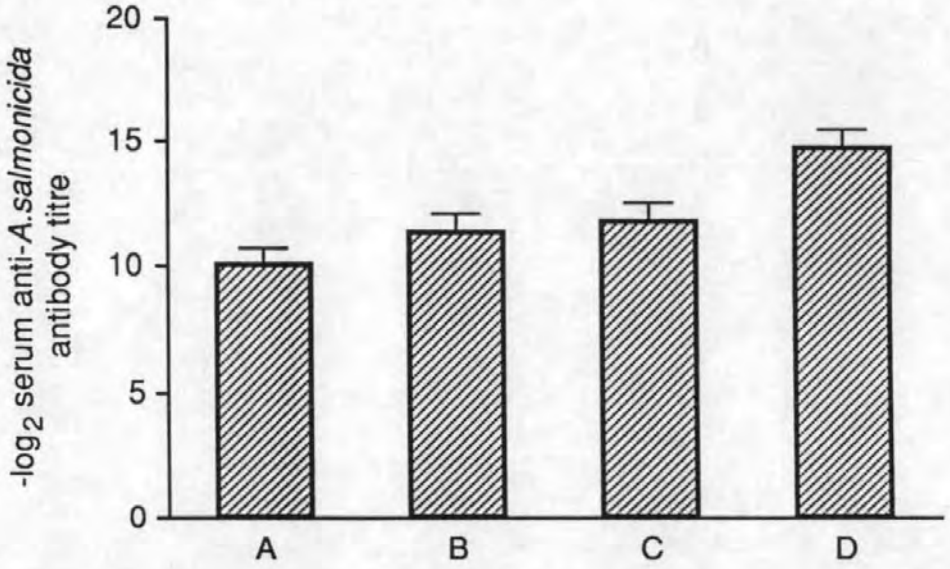
Serum antibody production following multiple immersions in HGG-covered latex particles, either opsonized or non-opsonized, or a control bath, with a subsequent intraperitoneal HGG in FCA challenge. Initial immersion was carried out on carp of 4 weeks of age.
Mean ± SE, n=4.

Effects of opsonization on direct immersion in A.salmonicida bacterin in immunologically immature fish

Four week old fish were given multiple immersions in either a control bath, *A.salmonicida* bacterin, the bacterin opsonized with normal carp serum or the bacterin opsonized with immune carp serum. They then received a bacterin in FCA i.p. challenge and their sera were assayed for anti-*A.salmonicida* antibodies 5 weeks post-challenge. The results from this experiment are expressed in Fig.11.

Those fish which had been immersed in the *A.salmonicida* bacterin and those which had been immersed in the bacterin opsonized with normal carp serum had slightly greater serum anti-*A.salmonicida* antibody titres than those which had received immersions in a control bath but the difference was not statistically significant.

The fish which had received immersions in the bacterin opsonized with immune carp serum had significantly greater ($p<0.01$) *anti-A.salmonicida* antibody titres than those which had received any of the other immersions.



Fish immersed in: A = control bath
B = *A. salmonicida* bacterin
C = *A. salmonicida* bacterin opsonized with normal serum
D = *A. salmonicida* bacterin opsonized with immune serum

Figure 11

Serum antibody production following multiple immersions in *A. salmonicida* bacterin, either opsonized or non-opsonized, or a control bath, with a subsequent intraperitoneal challenge of bacterin in FCA. Initial immersion was carried out on carp of 4 weeks of age.
Mean ± SE, n=4.

SECTION 4

Specificity of the humoral immune response to direct immersions in particle-borne HGG : cross-specificity to KLH

Fish were given multiple immersions in either a control bath or HGG-Latex, with a subsequent i.p. challenge of either KLH in FCA or HGG in FCA. Five weeks post-challenge the fish were sacrificed and the sera from those which had received an HGG in FCA challenge were assayed for anti-HGG antibodies. The sera from those fish which had received a KLH in FCA challenge were assayed for anti-KLH antibodies. The results from this experiment are expressed in Fig.12.

Of the fish which received an HGG in FCA challenge, those which had been previously immersed in HGG-Latex had significantly greater ($p<0.01$) anti-HGG antibody titres than those fish which had only received control bath immersions.

In none of the sera from the fish which received a KLH in FCA challenge, whether previously immersed in HGG-Latex or not, could any anti-KLH antibodies be detected.

Specificity of the humoral immune response to direct immersion in particle-borne HGG and *A.salmonicida* bacterin : cross-specificity between the two antigens

Fish were given multiple immersions in either a control bath, HGG-Latex or *A.salmonicida* bacterin. Fish from each group then received either an HGG in FCA or a bacterin in FCA i.p. challenge. Five weeks post-

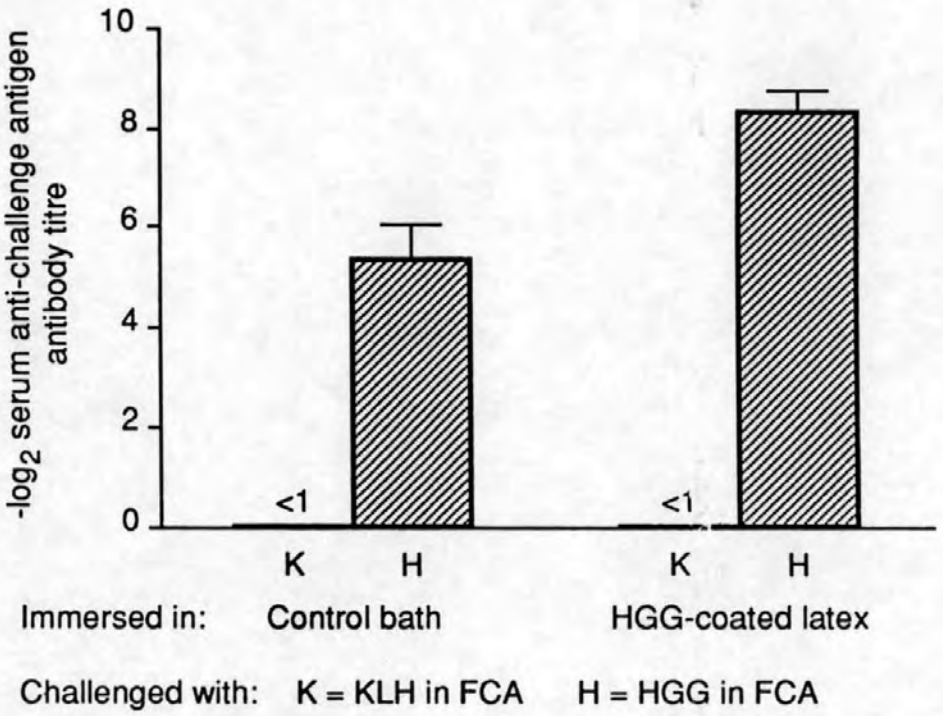


Figure 12

Serum antibody production following multiple immersions in HGG-coated 0.8µm latex particles or a control bath, with a subsequent intraperitoneal challenge of either HGG in FCA or KLH in FCA.
Mean ± SE, n=6.

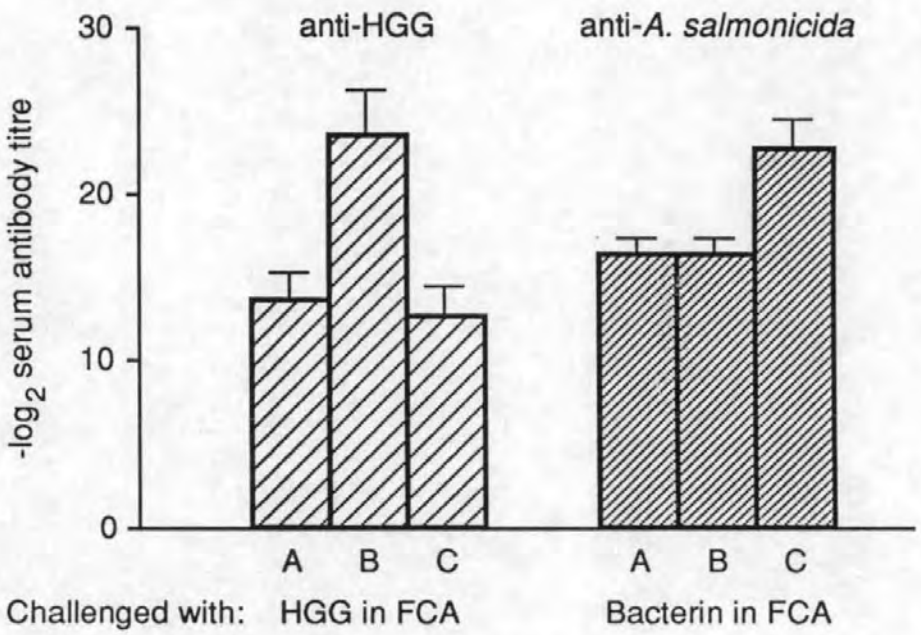
challenge the fish were sacrificed and their sera were assayed for antibodies towards the antigen they had been intraperitoneally challenged with. The results from this experiment are expressed in Fig.13.

Of those fish which received an HGG in FCA challenge, those which had been previously immersed in HGG-Latex had significantly greater anti-HGG serum antibody titres than those which had been immersed in either a control bath ($p<0.02$) or in *A.salmonicida* bacterin ($p<0.01$). Of the fish which had received an HGG in FCA challenge, there was no significant difference in the anti-HGG antibody titres between those which had been immersed in a control bath and those which had been immersed in *A.salmonicida* bacterin.

Of the fish which received an *A.salmonicida* bacterin in FCA challenge, those which had previously been immersed in the bacterin had significantly greater anti-*A.salmonicida* antibody titres than those which had been immersed in either a control bath ($p<0.02$) or in HGG-Latex ($p<0.02$). Of the fish which received a bacterin in FCA challenge, there was no significant difference in the anti-*A.salmonicida* antibody titres between those which had been previously immersed in a control bath and those which had been immersed in HGG-Latex.

Cross-reactivity of antibodies raised by an intraperitoneal challenge of HGG in FCA

Fish were given multiple immersions in either a control bath or in HGG-Latex. The fish then received an i.p. challenge of HGG in FCA. Five weeks post-challenge, the sera of these fish were assayed for anti-HGG and anti-CGG activity. The results from this experiment are expressed in Fig.14.



Immersed in:

A = control bath
B = HGG-coated 0.8µm latex particles
C = *A. salmonicida* bacterin

Figure 13

Serum antibody production following multiple immersions in either *A. salmonicida* bacterin, HGG-coated latex partricles or a control bath, with a subsequent intraperitoneal challenge of either HGG or bacterin in FCA.
Mean ± SE, n=5.

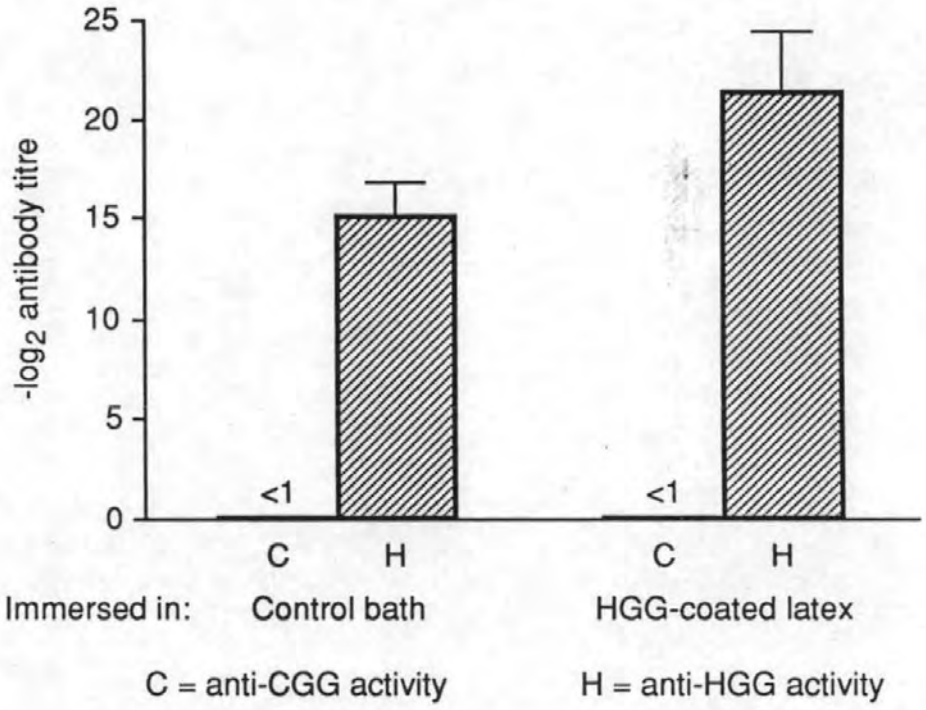


Figure 14

Cross-reactivity of antibodies raised by intraperitoneal challenge with HGG in FCA either with or without previous multiple immersions in HGG-coated 0.8µm latex particles. Sera assayed for anti-HGG and anti-CGG activity. Mean ± SE, n=5.

Anti-HGG activity was detected in the sera of fish from both groups, with greater levels in the fish which had been immersed in HGG-Latex. However, anti-CGG activity could not be detected in any of the sera.

DISCUSSION

These results document certain aspects of the humoral immune response following direct immersion immunization in carp. Initially it had to be established whether increased antibody titres could be detected after multiple immersions alone in antigen. These results agreed with those of Mughal (1984) and Manning *et al.* (1989) who found that no antigen-specific serum antibody could be detected after multiple immersions alone in antigen. However, this disagrees with the findings of Watson (1984) and Lamers *et al.* (1985) who did find serum antibody responses after multiple immersions in antigen. This may possibly be due to the antigens in use and experimental conditions in each of these investigations. However, it has also been found in this laboratory that different batches of fish from different sources, and even different spawnings from the same source, can react very differently to the same antigen, some batches being almost unresponsive to an antigen whereas others can give a good response. There are likely to be multiple factors involved in the immune response of fish which we are very far from understanding. In general, however, previous work in this laboratory on young fish (Mughal, 1984; Mughal *et al.*, 1986; Manning *et al.*, 1989), using human gamma globulin (HGG) and *Aeromonas salmonicida* bacterin as antigens, has found no measurable serum antibody response following multiple direct immersions alone in antigen (even following up to 10 weekly immersions). The method of priming with multiple immersions and measuring the humoral response to a subsequent i.p. challenge of antigen in Freund's complete adjuvant (FCA) was, therefore, chosen, as used by the above mentioned previous workers in this laboratory.

To determine the optimum time to sample the serum after i.p. challenge, the time-course of the antibody response following challenge was studied. The primary response to an i.p. challenge seemed to have peaked by 4 weeks post-challenge and remained at the same level for at least the next 4 weeks. If the fish had received prior multiple immersions in antigen coated onto latex particles then a secondary response to the i.p. challenge, greater than the primary response, was measurable at 4 weeks post-challenge, but did not reach its peak until 6 weeks post-challenge. It was therefore chosen to sample the serum at 5 weeks post-challenge to ensure good antibody titres but also to detect any acceleration in the response. This differed slightly from the experiments of Mughal (1984) who, at the earliest, sampled at 5-6 weeks post-challenge and, at the latest, at 13 weeks post-challenge.

Agreeing with the findings of Secombes (1981), Mughal (1984), Watson (1984) and Mughal *et al.* (1986) who found that carp, by 2-3 months of age could give a good positive humoral immune response to an i.p. injection of HGG or *A.salmonicida* bacterin in FCA, this study found good responses to both of these antigens when injected with FCA. The experiments presented here also found that prior multiple immersions in either soluble HGG, latex particle-bound HGG or *A.salmonicida* baterin in carp of 3 months or more in age induced a secondary humoral immune response to a subsequent i.p. challenge of the same antigen in FCA.

This study found, in agreement with Mughal (1984) and Mughal et al. (1986) that direct immersion in a bath of a soluble HGG coated onto latex particles gives a differing immune response to that following immersion in soluble HGG alone. This also correlates well with the findings of Smith (1982) who found far greater uptake of bovine serum albumin from an

immersion bath when coated onto latex particles compared to uptake of the soluble antigen alone. The investigations presented here, apart from finding greater stimulation of the immune system to HGG by immersion in latex-bound HGG compared to soluble HGG, also looked at the response to HGG coated onto different sizes of latex particle. No difference could be seen between the responses to immersions in any of the particle sizes used (from 0.05µm to 15µm), although immersions in all the different sizes of particle-bound HGG showed greater priming than that of soluble HGG.

Experiments presented here also show the effects of opsonization of antigens on the immune response to multiple immersions in these preparations. It has already been shown (Secombes & Resink, 1984) that the opsonization, with immune serum, of HGG increases the humoral immune response to it, when injected intraperitoneally. This study also now shows that the opsonization of direct immersion vaccines affects the immune response to these vaccines. Multiple immersions of immunologically mature carp in either soluble HGG, latex particle-bound HGG or *A.salmonicida* bacterin opsonized with immune carp serum were found to significantly increase the humoral immune response to a subsequent i.p. challenge of the same antigen (non-opsonized) in FCA, compared to when priming immersions in the corresponding non-opsonized antigens were given. Multiple immersions in latex-bound HGG opsonized with normal serum instead of immune serum, however, did not have the same effect, and stimulated a secondary response to a subsequent i.p. challenge of HGG in FCA no greater than that stimulated by prior immersions in normal non-opsonized latex-bound HGG. Interestingly, it can be shown that short term protection against *Edwardsiella tarda* can be conferred to glass eels by immersion in a bath

containing hyperimmune sera raised against *E.tarda* (Salati *et al.*, 1989). This evidence, plus the findings, described here, showing that the opsonization of direct immersion vaccines with immune serum can increase the efficacy of vaccination, suggests the possibility of some mechanism which specifically enhances the uptake of antibodies, either free or in immune complexes, from the external aqueous environment. The exact nature of any such mechanism is, of course, a matter for future research.

The effects of multiple immersions of immature (4 week old) carp in opsonized vaccines were different to those observed in the older fish. Firstly it must be remembered that it is already known that the immune systems of very young fish respond differently to antigenic exposure, especially to exposure to thymus-dependent antigens. Secombes (1981) found that although juvenile trout could respond in a positive way to *A.salmonicida* bacterin administered intraperitoneally at 3 weeks of age, injection of HGG induced tolerance when administered within the first month of life. Mughal (1984) and Mughal *et al.* (1986) also found that although injection at 4 weeks of age, or 5 multiple immersions, starting at 4 weeks of age, of *A.salmonicida* bacterin induced a positive secondary immune response to an i.p. challenge of the same antigen in FCA at 10 weeks of age, an i.p. injection of HGG at 4 weeks of age induced tolerance to a subsequent i.p. challenge of HGG in FCA at 10 weeks of age. It was also shown that if carp received 5 weekly immersions in soluble HGG, starting at 4 weeks of age, a primary response was seen in these fish following an i.p. challenge of HGG in FCA at 10 weeks of age, the prior immersions seemimgly having no effect. Multiple immersions in latex particle-bound HGG, starting at 4 weeks of age were observed to

induce tolerance to a subsequent i.p. challenge of HGG in FCA, although this immunosupressive effect was only seen when at least 10 multiple immersions were administered, with only 5 immersions having no significant effect. This agrees with the findings of Wolf and Markiw (1982) who showed that a greater number of immersions can affect the outcome of immunization.

This tolerogenic effect of HGG in immature carp, when administered by injection, or by a large number of immersions in latex-bound HGG plus the unresponsiveness of immature carp to immersions in soluble HGG suggests that the more efficient the delivery into the fish, the more likely it is that a thymus-dependant antigen, like HGG, will induce tolerance in immature carp.

Returning to the findings of the investigations presented here, it was found that 3 weekly immersions of 4 week old carp in latex-bound HGG or latex-bound HGG opsonized in normal carp serum had no significant effect on the response to an i.p.challenge of HGG in FCA at 10 weeks of age. This corresponds with the findings of Mughal (1984) who found that at least 10 weekly immersions in latex-bound HGG were needed to induce a tolerogenic effect to a subsequent i.p. challenge of HGG in FCA. The present findings, however, showed that 3 weekly immersions of 4 week old carp in latex-bound HGG opsonized with immune carp serum did induce tolerance to an i.p. challenge of HGG in FCA at 10 weeks of age. This suggests that opsonizing latex-bound HGG with immune carp serum might increase the efficiency of the delivery of HGG into the fish.

These experiments also looked at the effects of immersing 4 week old carp in *A.salmonicida* bacterin, both opsonized and non-opsonized. It was found that 3 weekly immersions of 4 week old carp in both normal *A.salmonicida* bacterin and *A.salmonicida* bacterin opsonized with normal

carp serum had no effect on the response to a subsequent i.p. challenge of the bacterin in FCA at 10 weeks of age. Mughal (1984) found that multiple immersions of 4 week old carp in *A.salmonicida* bacterin did induce a positive secondary response to an i.p. challenge of bacterin in FCA at 10 weeks, but used at least 5 weekly immersions, compared to only 3 immersions used in these experiments. Therefore, the lack of response in these experiments may well have been due to the lesser number of immersions used. These experiments, however, did find that 3 weekly immersions of 4 week old carp in *A.salmonicida* bacterin opsonized with immune carp serum stimulated a positive secondary response to a subsequent i.p. challenge of bacterin in FCA at 10 weeks of age, again suggesting that the opsonization of immersion vaccines with immune serum increases the efficiency of delivery of the vaccine into the fish. As with previous findings from this laboratory these results highlight the difference in the response of young fish to thymus-independent vaccines as compared to thymus-dependent antigens.

Finally, these experiments found that the specificity of the immunological memory following multiple direct immersions in antigen was high, with no cross-reactivity between antigens being observed. This agrees with the findings of Gould *et al.* (1979) who found high immune specificity when vaccinating salmon with 2 different types of a *Vibrio anguillarum* bacterin, with no protection to type I given by immersion in type II, and vica-versa, no protecion to type II given by immersion in type I. Espelid *et al.* (1987) also found the specificity of the antibody response of salmon to the fish pathogen, *Vibrio salmonicida*, to be high, with 90% of responding B-cells recognising just one antigenic determinant on the bacterial surface. These experiments also looked at the specificity of carp antibodies raised

against HGG. These antibodies showed no measurable affinity for a related molecule, chicken gamma globulin.

CHAPTER 4

Title

Cell-mediated immune responses of carp, *Cyprinus carpio* L., following direct immersion in antigen.

INTRODUCTION

Lymphokines are substances, produced by lymphocytes under certain circumstances, which have pharmacological effects often associated with the inflammatory response (Ellis, 1977a). Over a hundred different biological activities have been ascribed to lymphokines produced by activated lymphocytes (Cohen *et al.*, 1979) but, as yet, relatively few of these can be attributed to single molecules (Roitt *et al.*, 1985). The biological effects of these soluble lymphocyte products include migration inhibition, induction of aggregation of macrophages, stimulation of phagocytosis, macrophage activation, stimulation of DNA synthesis in certain cells, interferon-like properties, stimulation of histamine release from platelets and chemotactic properties, amongst many others (Pick & Turk, 1972). The broader term "cytokine" is now frequently used so as to include substances secreted by other cells (e.g. interleukin-1 produced by macrophages).

This chapter is concerned with the chemotactic cytokines (chemotactic factors) produced by stimulated leukocytes. The *in vivo* action of these chemotactic factors can be readily shown by the active influx of inflammatory cells into an inflammmatory lesion. Rebuck & Crowley (1955) described a skin window technique, which displayed this active migration of cells into a lesion. It involved scraping away the outer skin layers and placing a glass cover slip over the lesion. Macrophages and granulocytes entering the lesion could then be seen, and would adhere to the cover slip, which could then be removed and stained. Other methods have also been used to study acute inflammatory reactions in the skin, these include the radiolabelling of cells to track their infiltration into a

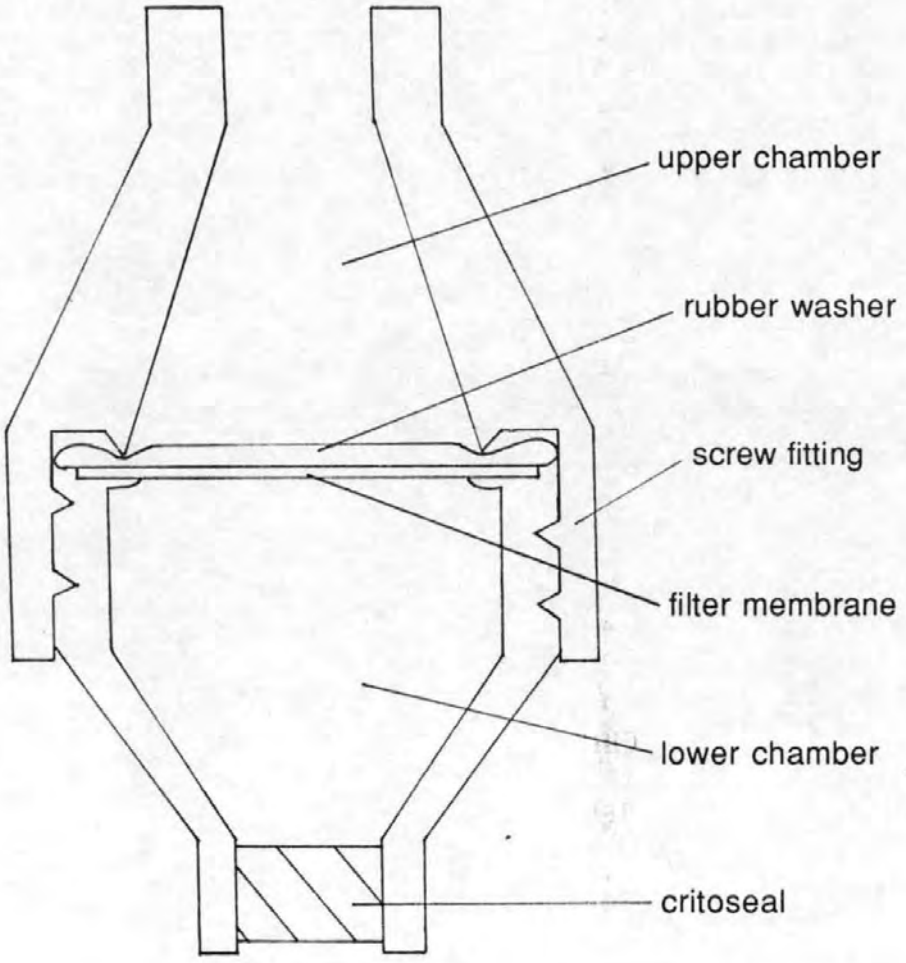
lesion (Issekutz, 1983).

Techniques have also been developed which measure the chemotactic properties of substances. These include the under agarose migration technique (Nelson *et al.*, 1975) where an attractant is placed in one well, made in an agarose gel, and a suspension of target cells, usually granulocytes or macrophages, is placed in another. The attractant under test will diffuse outwards through the gel and if it is chemotactic, the target cells will be seen to migrate towards the other well, up the concentration gradient, more than in any other direction. One advantage of this technique is that it is quite obvious whether a test substance is chemotactic , inducing active migration towards itself, or merely chemokinetic, inducing an increase in the random migration of the cells (Repo *et al.*, 1978).

Perhaps the most used, and quantifiable, technique for measuring the chemotactic acivity of a substance is the chemotaxis assay involving cell migration through a filter membrane. Boyden (1962) developed this technique and developed an apparatus which consisted of a lower well containing the test attractant. A filter membrane was then placed over this lower well, and an upper well was placed on top of this, and filled with a suspension of the target cells (see Fig.15). If the test solution was chemotactic to the target cells then the cells would actively migrate through the membrane towards the attractant. Many substances have been found, in the mammalian system, to be chemotactic for cells involved in the inflammatory response. These include tuberculoprotein (Boyden, 1962), bacterial culture filtrates (Keller & Sorkin, 1967a; Horwitz & Garrett, 1971; Keller *et al.*, 1973), certain components of the complement system (Ward, 1974) especially C5a which seems to be the most chemotactic of

Fig.15. Basic design of a simple single well chemotactic chamber (Howell, 1984).

The chamber consists of an upper and a lower section, which can be screwed together. When the filter membrane and the rubber washer are in position, a watertight seal is made, separating the upper and lower chambers. The chemoattractant is placed in the lower chamber and the target cells, suspended in cell culture medium, are placed in the upper chamber.



these (Ward et al., 1965; Ward & Newman, 1969; Gigli & Austen, 1971; Gallin & Rosenthal, 1974) and, of course, chemotactic factors produced by lymphocytes (Ward *et al.*, 1969; Snyderman *et al.*, 1972).

Many different types of mammalian cell are known to be able to respond to chemotactic stimuli. These include macrophages (Keller & Sorkin, 1967b; Ward *et al.*, 1970), neutrophils (Ward *et al.*, 1970; *Ward et al.*, 1971; Keller *et al.*, 1980), lymphocytes (Ward *et al.*, 1971), basophils (Kay & Austen, 1972), eosinophils (Cohen & Ward, 1971; Keller *et al.*, 1980) and fibroblasts (Postlethwaite *et al.*, 1976). Since Boyden developed his original apparatus in 1962, several variations and alterations have been tried, in attempts to acheive greater sensitivity and accuracy in the technique. The size of the pores, through which the cells must pass is obviously a critical factor. Keller & Sorkin (1967b) investigated this and found that, for rabbit cells, the pores needed only to be 3μm in diameter to allow granulocytes to pass through the membrane, but for mononuclear cells, 8μm diameter pores were needed.

The usual technique for quantifying the number of cells which have passed through a membrane is to stain the membrane and count the number of cells on its underside (the attractant side). However, many workers have found that once certain cells have passed through the membrane, they simply drop off (Zigmond & Hirsch, 1973), although others have found little detachment (Gallin *et al.*, 1973). Later work discovered that it was probably the type of cell which determined its adherant qualities to the membrane. Both Jungi (1975) and Keller *et al.* (1980) finding that neutrophils were far less likely to drop off the filter than eosinophils. Researchers have also tried modifications to the apparatus involving the addition of an impermeable lower filter to catch any cells

which might drop off the upper filter, once having passed through (Keller *et al.*, 1980), and counting the cells on both the underside of the upper filter and those on the lower impermeable filter. Another modification was tried by Baum *et al.* (1971) who used a cytospin to concentrate the cells onto a smaller area of the filter before the migration began.

The original apparatus of Boyden (1962) consisted of only one well, with a single filter. Great advances have been made in the reproducibilty and ease of use of the technique since then by the introduction of multiwell chambers. Swanson (1977) designed a 30 well chamber, which meant that numbers of replicates could be easily done using the same target cell population. Unfortunately, however, this apparatus still involved a single filter for each well. Three years later, however, a 48-well chamber assembly was developed (Falk *et al.*, 1980; Harvath *et al.*, 1980), which needed only 25µl of attractant per well, instead of the minimum of 0.25cm^3 needed in earlier chambers. This chamber also only utilized one large filter membrane for all of the wells, thus drastically reducing filter manipulation and staining time. This filter was also different in other ways from the earlier filters, which were between 100-150µm thick and were made of cellulose acetate. These new filters, made by Nucleopore, were only 10µm thick, thus greatly reducing the experimental time needed to allow the cells to pass through the membrane. The new filters were also different in that they were made of polyvinylpyrrolidone-free polycarbonate. Harvath *et al.* (1980) found that this composition of the filter meant that cells were far less likely to drop off once having passed through, being more adherant to this type of membrane, again adding to the accuracy of the technique. The use of this multiwell chemotaxis chamber to assay chemotaxis was called the micro chemotaxis assay,

because of the small amounts of attractant and low numbers of cells needed. Gee (1984) when reviewing chemotaxis assays concluded that the use of polycarbonate filters in a multiwell assembly was a considerable improvement on previous techniques.

One of the major applications of chemotaxis assays has been to study the effects of lymphocyte-derived factors on the migration of certain other types of cell, usually granulocytes or mononuclear cells. It is known that when stimulated with a mitogen, mammalian lymphocytes produce factors into the supernatant which have certain biological activities, including cell growth promotion (Spiess & Rosenberg, 1981; *Wu et al.*, 1982), antiviral activity (Epstein *et al.*, 1974) and chemotactic properties (Altman *et al.*, 1973; Altman & Kirchner, 1974; Altman *et al.*, 1975). Perhaps of more importance have been the findings of workers looking at the cytokine production of antigen-sensitized lymphocytes on stimulation, *in vitro*, with the sensitizing antigen. Migration inhibition factor (Thor *et al.*, 1968; Pick *et al.*, 1972; Tsai *et al.*, 1974), macrophage activation factor (MAF) (Meltzer, 1981) and chemotactic factor (Ward *et al.*, 1969; Ward *et al.*, 1970; Altman & Kirchner, 1974) have all been detected after antigen stimulation of mammalian antigen-sensitized lymphocytes. There is also a very good link between the production of these cytokines after antigen stimulation and cell-mediated immunity of the animal donor of the lymphocytes towards the same antigen. George & Vaughan (1962), Soberg & Bendixen (1967), Thor *et al.* (1968) and Altman *et al.* (1973) have all found that lymphocytes, taken from patients showing hypersensitivity to skin tests with a certain antigen, will produce chemotactic factors and/or migration inhibition factor (MIF) on *in vitro* stimulation with the antigen, whereas the lymphocytes from non-sensitive

patients will not.

The species-specificity of these cytokines is a matter of some disagreement. Many workers finding chemotactic factors and MIF acting on cells from other species (Thor *et al.*, 1968; Ward *et al.*, 1969; Tsai *et al.*, 1974), whereas others finding no cross-species activity (Altman & Kirchner, 1974). Bloom (1971) even found that guinea pig MIF is not active on rabbit macrophages but that guinea pig chemotactic factor is.

Cell-mediated immunity and cytokine production has also been studied in fish, although not, as yet, to quite the lengths found in mammalian research. The influx of inflammatory cells into inflammatory lesions in fish has been shown by many workers (Jakowska & Nigrelli, 1953; Klontz *et al.*, 1966; Finn & Nielson, 1971; Joy & Jones, 1973; Roberts *et al.*, 1973), indicating the presence of chemotactic substances, attracting these cells. The cell-mediated immune system is also known to be very active in fish, as in mammals. The rejection of skin allografts is well documented in fish (Manning *et al.*, 1982; Tatner & Manning, 1983), and is also known to have good memory (Botham *et al.*, 1980). Bartos & Summer (1981) showed cell-mediated immunity in trout by demonstrating skin hypersensitivity to mycobacteria in previously immunized fish. Baba *et al.* (1988) also found that the transfer of non-adherent pronephric cells from fish immunized against *Aeromonas hydrophila* to normal fish, confered protection to the recipient fish to a live virulent challenge 3 days later, thus demonstrating the transfer of immunity by cells only. As in mammals, many substances are known to be chemotactic for fish inflammatory cells, including glycogen (MacArthur *et al.*, 1985), bacterial culture supernatants (Nash *et al.*, 1986) and lymphocyte-derived chemotactic factors (Howell, 1987). There is also evidence that certain activated complement factors

are also chemotactic in fish (Griffin, 1984; Obenauf & Hyder-Smith, 1985) and C5a (the most chemotactic complement component in mammals) has been isolated and purified from trout serum (Nonaka, Natsuume-Sakai & Takahashi, 1981; Nonaka, Yamaguchi, Natsuume-Sakai & Takahashi, 1981).

Work has also been done, in fish, investigating the production of cytokines by leukocytes during mitogen stimulation. When stimulated with mitogens such as phytohaemagglutinin and concanavalin A, fish leukocytes have been shown to produce many biologically active products, including MAF (Smith & Braun-Nesje, 1982; Secombes, 1987), MIF (Secombes, 1981; Howell, 1984), an interleukin 2-like factor (Caspi & Avtalion, 1984; Grondel & Harmsen, 1984), an interferon-like factor (Graham & Secombes, 1990) and chemotactic factor (Howell, 1987).

Research has also been undertaken on cytokine production from antigen-sensitized fish leukocytes on stimulation in vitro with the sensitizing antigen. Jayaraman et al. (1979), Secombes (1981), Blazer *et al.* (1984), Howell (1984) and Song *et al.* (1989) all found the production of MIF when incubating previously antigen-sensitized leukocytes with the antigen, *in vitro*. Smith *et al.* (1980) also found that, as in mammals, MIF production by fish leukocytes, on in vitro stimulation with a bacterial antigen, was consistent with protection to a live challenge of the bacteria, indicating that assaying for antigen-stimulated cytokine release might be a good test of a fish's cell-mediated immunity to that antigen.

Secombes (1981) found the production of a chemokinetic factor by antigen-sensitized carp pronephric cells on *in vitro* stimulation with the sensitizing antigen, using the MIF test. Only a very small amount of preliminary work, however, has been published concerning the production

of a chemotactic factor by the stimulation of antigen-sensitized fish leukocytes, *in vitro*, with the sensitizing antigen. Howell (1987) found some chemotactic factor-like activity in the supernatants of incubations of leukocytes from carp, injected with human gamma globulin (HGG) 7 or 14 days earlier, when incubated for 48 hours with HGG. This chemotactic activity was measured using the single chamber Boyden-type apparatus.

The investigations in this chapter describe the development of an assay to measure the production of chemotactic factor by antigen-stimulated pronephric cells (which will contain approximately 50-70% lymphocytes (Secombes, 1981; Bayne, 1986)), utilizing the micro chemotaxis assay. Using this as a measure of the cell-mediated immunity of a fish to an antigen, it is intended to define certain aspects of the cell-mediated immune response of fish, including the time-course and the specificity of the response. This chapter also presents results, using this technique, assessing the effects of opsonization and particle size of a direct immersion vaccine on the stimulation of the cell-mediated immune response after immersion.

MATERIALS AND METHODS

THE MICRO CHEMOTAXIS ASSAY

As the production of a chemotactic factor, or factors, by antigen-sensitized carp pronephric cells during in vitro exposure to antigen had not been investigated before, studies were undertaken to discover the optimal conditions for the *in vitro* production of, and subsequent measurement of, a chemotactic cytokine.

Supernatants from incubations of normal pronephric cells with medium only were found to have greater chemotactic activity than supernatants from incubations of medium alone. It was not known whether this greater activity was due to a chemotactic cytokine in the supernatant or merely due to the presence of other cellular chemotactic products or debris. However, when the chemotactic activity of a supernatant from an incubation of cells with an antigen was found to be greater than that of supernatants from incubations of pronephric cells with medium alone, this was taken to indicate the production of some chemotactic factor-like cytokine, or cytokines, being stimulated by the antigen.

These studies and the exact conditions used in the assay are described below.

Intraperitoneal challenge

Fistly it needed to be established whether carp pronephric cells could be sensitized, by an intraperitoneal (i.p.) injection of human gamma globulin (HGG) in Freund's complete adjuvant (FCA), to produce a chemotactic

factor, or factors, in response to a subsequent in vitro exposure to HGG. If this happened to be the case then the time-course of this sensitization reponse would also need to be known, to be able to sample at the time of maximum response.

To do this, fish were intraperitoneally injected with HGG in FCA at either 1 day, 2 days, 4 days, 1 week, 2 weeks or 3 weeks before being sacrificed. Pronephric cells from these fish and normal fish were then removed and incubated with or without HGG-coated latex particles (HGG-Latex) and the supernatants from these incubations were assayed for chemotactic activity (as described later).

As can be seen in Fig.16 there was a significant ($p<0.01$) response at 1 day post-challenge but the maximal reponse was seen at 2 days post-challenge ($p<0.001$). At 4 days post-challenge the response was still detectable ($p<0.01$) but by 1 week post-challenge there was no detectable response.

It was therefore decided, in subsequent experiments, to sacrifice fish at 2 days post-challenge to detect the maximal response to the i.p. injection.

Pronephric cell preparations

Fish were lethally anaesthetised in benzocaine, their tails were severed at the caudal peduncle to bleed the fish and reduce the amount of blood in the pronephros. The pronephroi were then removed and homogenised in $1cm^3$ of RPMI 1640 cell culture medium (Gibco Ltd., Scotland). All cell culture media in which the cells were suspended were at room temperature, as previous unpublished work in this laboratory has shown that carp cells exposed to a cold temperature shock do not proliferate in

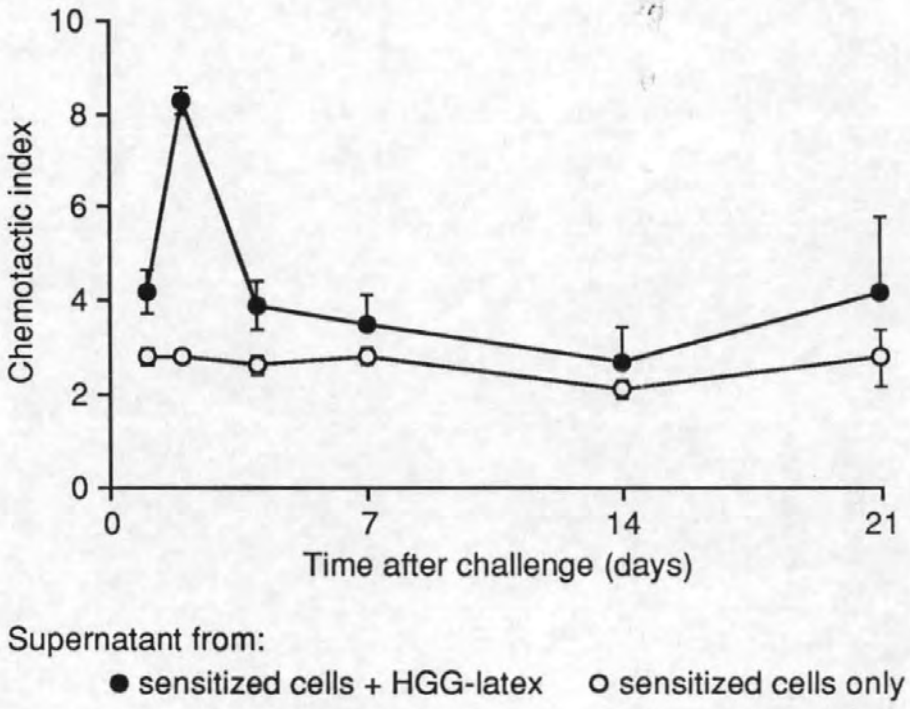


Figure 16

Production of chemotactic factor by pronephric cells from HGG in FCA injected fish, when incubated with or without HGG-coated latex particles, at different times after intraperitoneal challenge.
Mean ± SE, n=4.

response to mitogens as well as cells which have been kept constantly at room temperature. This $1cm^3$ of cell suspension was then added to another $3cm^3$ of RPMI 1640 and centrifuged at 400g for 4 minutes at 20^oC. The supernatant was discarded and the cells were washed again in RPMI 1640 a further 2 times. The cell suspension was then counted using a haemocytometer and if the cells were to be incubated, the suspension was adjusted to $5x10^7$cells/cm^3 in RPMI 1640. If the cells were to be used as target cells in the micro chemotaxis assay then the suspension was adjusted to $1x10^7$cells/cm^3. In either case the cells were used immediately. The target cells were obtained from normal, non-immunized fish.

Incubation of cells with antigen

To each of two $10cm^3$ capped plastic centrifuge tubes (Sterilin, U.K.) was added $0.2cm^3$ of each $5x10^7$cells/cm^3 cell suspension in RPMI 1640. To one of these tubes was added another $0.2cm^3$ of RPMI 1640, to the other was added $0.2cm^3$ of RPMI 1640 containing either antigen-coated 3.0µm latex particles ($10^9/cm^3$) or formalin-killed *Aeromonas salmonicida* bacterin ($2x10^9$cells/cm^3). Controls were also set up for each experiment with $0.2cm^3$ of RPMI 1640 being initially added to the tube instead of a cell suspension. 20ul of heat-inactivated normal carp serum was then added to every tube to give a concentration of 5% carp serum.

A pilot experiment was run to discover the kinetics of the antigen-stimulated production of chemotactic factor in vitro (see Table.1). Normal fish and fish challenged i.p. with HGG in FCA, 4 days previously, were sacrificed and their pronephric cells were incubated with or without HGG-Latex. These incubations were shaken and then centrifuged at 500g for 5

TABLE 1

Chemotactic activity of supernatants from incubations of pronephric cells from normal fish, and fish injected with HGG in FCA, when incubated with or without HGG-coated latex particles for different lengths of time.
Results are expressed as cells/field of view (mean±S.E., n=3)

	Incubation time		
Supernatant from	12 hours	18 hours	30 hours
Medium only	0.7±0.2	2.1±0.2	1.3±0.3
Medium + HGG-Latex	0.5±0.1	2.2±0.1	25.3±3.5
Normal cells only	2.6±0.4	8.6±0.1	59.9±5.3
Normal cells + HGG-Latex	2.7±0.2	9.2±0.4	83.2±9.3
Sensitized cells only	3.0±0.3	7.0±1.0	199±99
Sensitized cells + HGG-Latex	4.7±2.0	13.0±3.5	215±108

minutes at 20°C and the supernatants tested for chemotactic activity using the micro chemotaxis assay at 12 hours, 18 hours and 30 hours after the start of the incubation. The antigen-stimulated chemotactic activity appeared to be greater at 18 hours than 12 hours and by 30 hours the chemotactic activity was very high in all the incubations containing cells, whether they contained HGG-Latex or not. This was probably due to the multiple shakings and centrifugations the cells had to endure by this time causing many cells to rupture and die leaving a lot of highly chemotactic waste in the supernatant. However, from these results it was chosen to incubate the cells for 18 hours before taking supernatant samples.

Supernatant preparation

At the end of the incubation the tubes were centrifuged at 500g for 5 minutes and 0.1cm^3 of the each supernatant was removed. If the incubation had included the addition of A.salmonicida bacterin, the supernatant was further centrifuged at high speed in an MSE microcentrifuge for 5 minutes to pellet down any remaining bacterin cells.

Migration of target cells towards supernatants

The supernatants were added to the lower wells of the micro chemotaxis chamber. Approximately 28µl of supernatant was required to fill each lower well to the required level. A slightly convex meniscus was needed so that when the filter was applied, no air bubbles were trapped. Three replicates of each supernatant were used giving a possible maximum of running 16 supernatants at one time with the same set of target cells in the

48 well apparatus.

Once the lower wells had been filled with the supernatants being tested, a polyvinylpyrrolidone-free filter membrane with 3μm diameter holes (Nucleopore corporation filtration products, U.S.A.) was lowered gently, shiny side up, onto the supernatant-filled lower wells carefully trying not to trap any air bubbles. The silicon gasket was then lowered over the top of the filter, followed by the top plate, containing the upper well chambers. The whole assembly was then secured with the 6 knurled nuts and a glass slide was placed over the top of the upper wells and the apparatus was left to stand to acheive thermal equilibrium (photographs of the micro chemotaxis chamber and accesories can be seen in Figs.17 and 18). After approximately 30 minutes the target carp pronephric cells (0.45μl of $1x10^7$cells/cm^3) were added to the upper wells.

To determine the optimum time of migration to see the greatest difference between migration towards antigen-stimulated chemotactic factors in the lower well and random migration through the membrane, an experiment was set up looking at the migration towards antigen-stimulated sensitised cell supernatants using migration times of 30 and 60 minutes (see Fig.19).

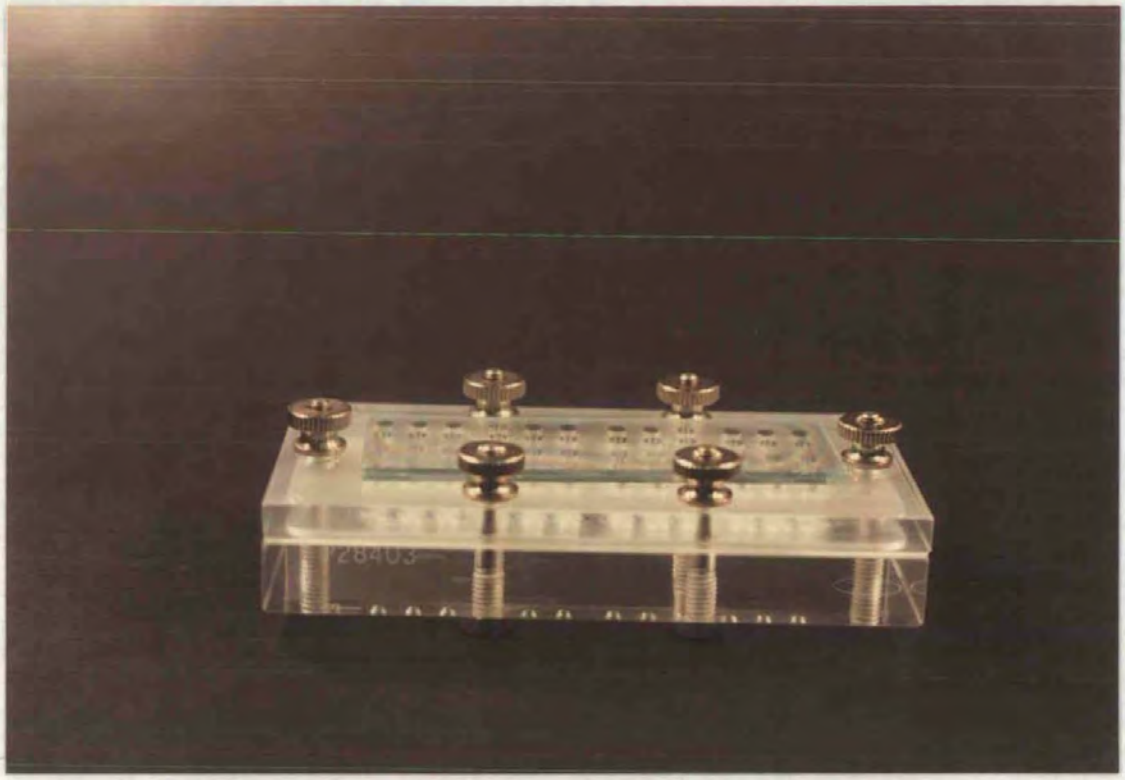
At 30 minutes migration time, there was a significantly greater ($p<0.01$) difference between migration of target cells through the membrane to supernatants from cells + HGG-Latex compared to supernatants from cells only, than at 60 minutes migration time. 30 minutes migration time was therefore chosen for all subsequent experiments.

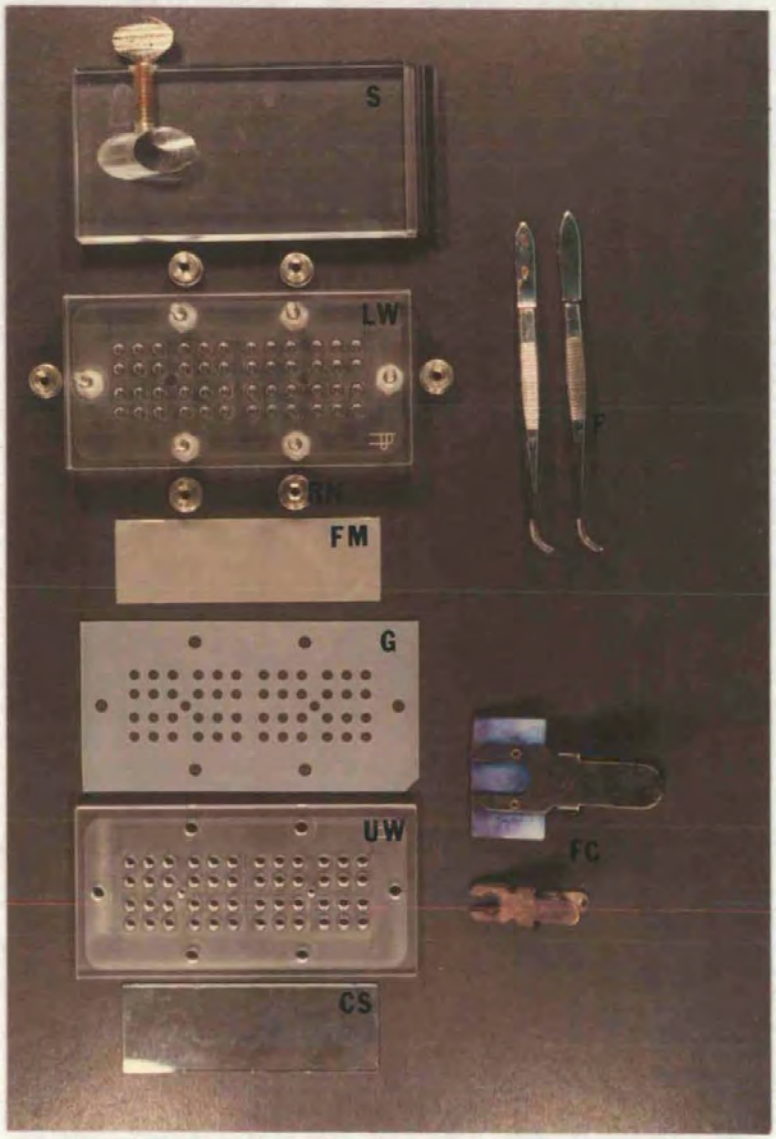
At the end of the alloted migration time the apparatus was disassembled and the upper side of the filter membrane was cleaned of cells by grasping the membrane at each end with a metal clip, dipping the upper side of the membrane in 0.85% saline and then passing the membrane

Fig.17. Microchemotaxis chamber (assembled). (x0.85)

Fig.18. Microchemotaxis chamber and accessories (disassembled). (x0.45)

S = scraper to clean the upper side of the filter .
LW = lower well assembly.
RN = retaining nuts.
FM = filter membrane.
G = gasket.
UW = upper well assembly.
CS = cover slip.
F = forceps.
FC = filter clamps.

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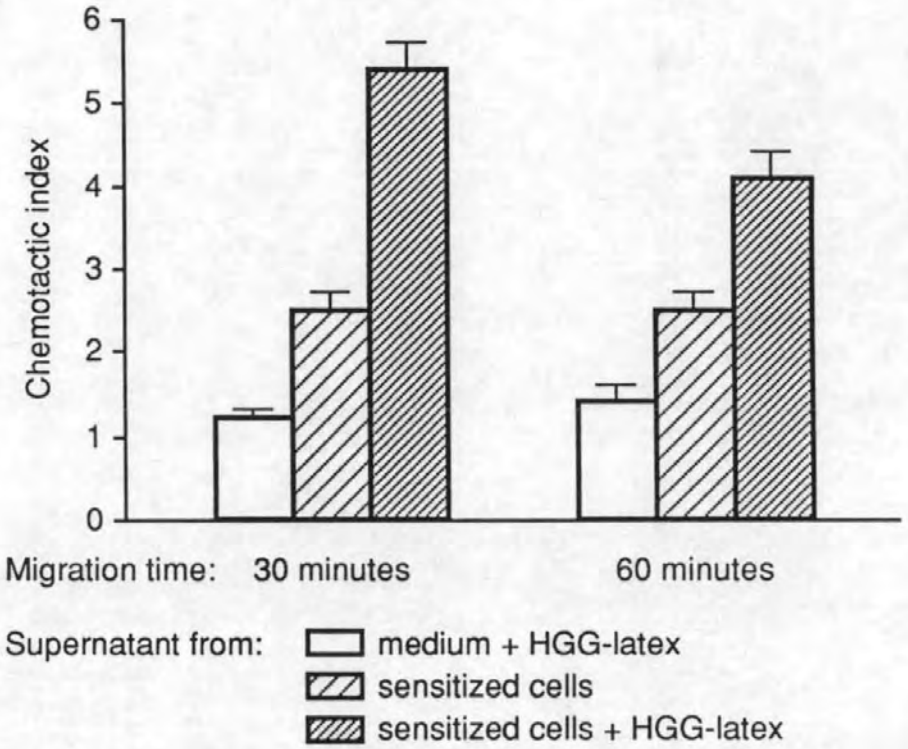


Figure 19

Migration of normal pronephric cells over 30 and 60 minutes to chemotactic substances produced by pronephric cells from HGG in FCA injected fish, when incubated with or without HGG-coated latex particles.
Mean ± SE, n=3.

over a rubber scraper (Nucleopore corporation filtration products, U.S.A.). The scraping operation was repeated twice and then the filter membrane was fixed in absolute methanol for 10 minutes.

Staining the filter membrane and quantitation of chemotaxis.

After fixation in methanol, for 5 minutes, the membrane was immersed in a citric acid/Na_2HPO_4 buffer (see Formulae) for 5 minutes and then transfered to Giemsa stain (BDH Chemicals Ltd., Poole, U.K.) mixed 1:5 with the buffer for 15 minutes. The membrane was then rinsed well in the buffer and wet mounted, lower side up, in the buffer on a glass slide. Cover slips were placed on top of the membrane and it was then observed under the light microscope at 400x magnification (see Fig.20).

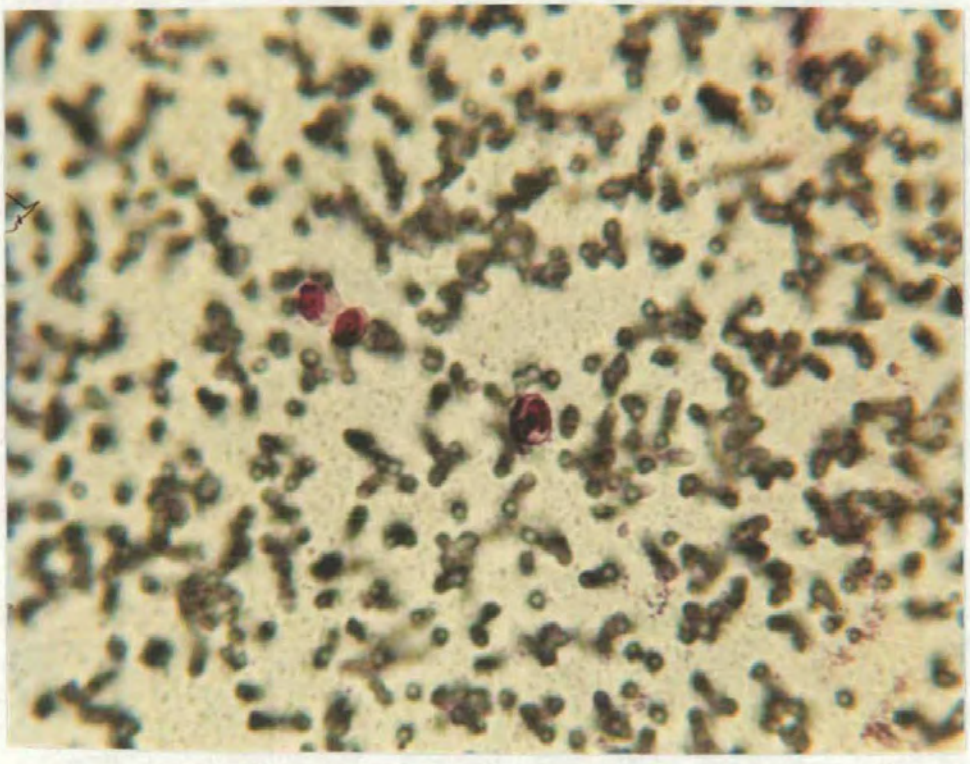
For each well, the cells per field of view were counted for 6 random fields of view. In some experiments the migration of the cells through the filter was expressed as cells per field of view. In the majority of experiments, however, the migration of target cells towards a particular supernatant was expressed as a chemotactic index, calculated as :-

$$\text{Chemotactic index} = \frac{\text{Migration towards test supernatant}}{\text{Migration towards RPMI 1640 plus 5\% carp serum}}$$

with the migration of any batch of target cells towards RPMI 1640 plus 5% carp serum being defined as 1.

Fig.20. Migration of carp pronephric cells. (x500)

Photograph shows 3 carp pronephric cells which have migrated through a filter membrane with 3μm diameter pores.



EXPERIMENTAL DESIGN

1) Cell-mediated immune response to HGG following intraperitoneal injection of HGG in FCA

Nineteen fish were given an i.p. injection of HGG in FCA. Between 2 and 4 days later, these fish and 12 normal fish were sacrificed and their pronephric cells were incubated with or without HGG-Latex. The supernatants from these incubations were then assayed for chemotactic activity using the micro chemotaxis assay.

2) Cell-mediated immune response to HGG following intraperitoneal injection of saline in Freund's complete adjuvant

As a control to experiment 1, four fish were given an i.p. injection of saline in FCA. Two days later, these fish and 4 normal fish were sacrificed and their pronephric cells were incubated with or without HGG-Latex. The supernatants from these incubations were then assayed for chemotactic activity using the micro chemotaxis assay.

3) Cell-mediated immune response following immersions in antigen (HGG-Latex)

Five fish were given 3, weekly, immersions in a 0.8μm HGG-Latex bath. Four weeks after the last of these 3 immersions, the fish were given another immersion in HGG-Latex (see Fig.21). Two days after this final

immersion, these fish and 5 normal fish were sacrificed and their pronephric cells were incubated with or without HGG-Latex. The supernatants from these incubations were assayed for chemotactic activity using the micro chemotaxis assay.

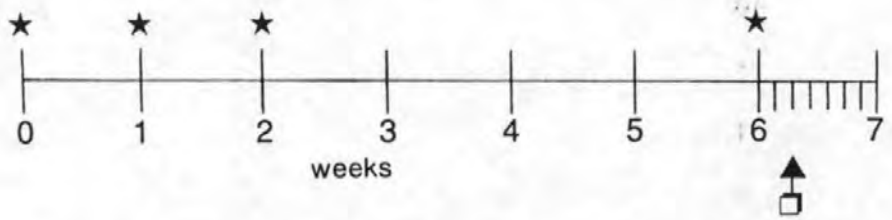
4) Cell-mediated immune response to *Aeromonas salmonicida* following an intraperitoneal injection of *A.salmonicida* bacterin in FCA

Five fish were given an i.p. challenge of formalin-killed *A.salmonicida* bacterin in FCA. Two days later, these fish and 5 normal fish were sacrificed and their pronephric cells were incubated with or without *A.salmonicida* bacterin. the supernatants from these incubations were then assayed for chemotactic activity using the micro chemotaxis assay.

5) The antigen-specificity of the cell-mediated immune response to an intraperitoneal injection of HGG in FCA

Fish were given an i.p. injection of HGG in FCA. Two days later, these fish and normal fish were sacrificed and their pronephric cells were incubated with or without HGG-Latex, keyhole limpet haemocyanin (KLH)-Latex, chicken gamma globulin (CGG)-Latex or *A.salmonicida* bacterin (see Fig.28 for numbers of fish, and Fig.22 for experimental protocol). The supernatants from these incubations were then assayed for chemotactic activity using the micro chemotaxis assay.

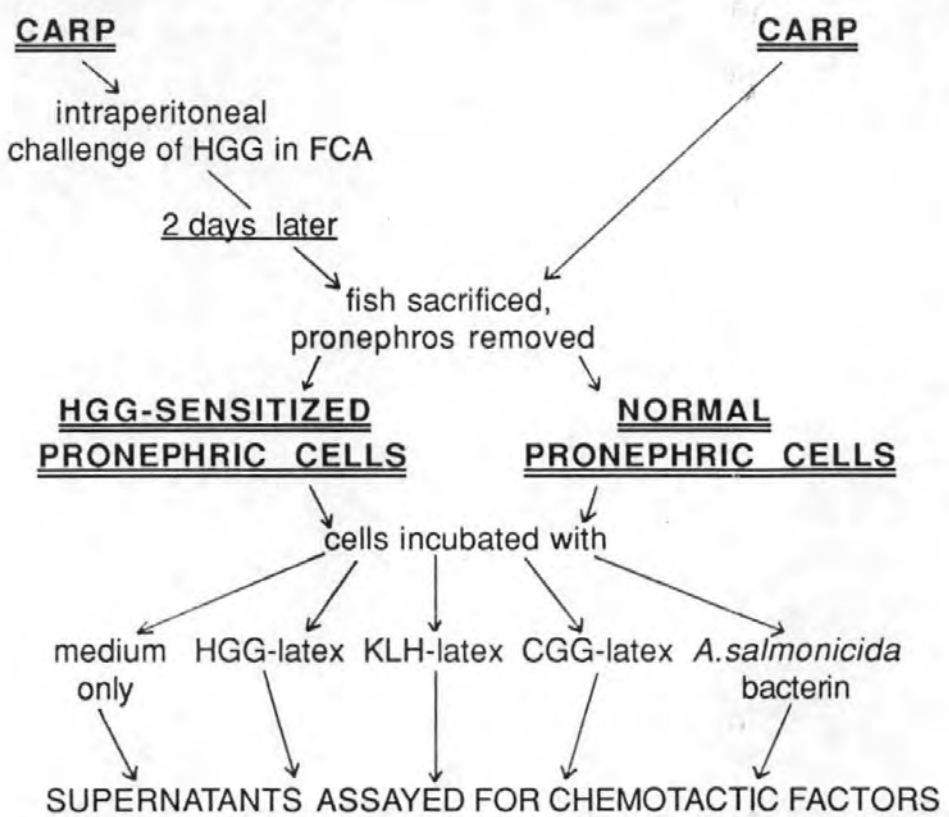
Fig.21. Experimental protocol for direct immersion in HGG-coated latex particles and subsequent assessment of cell-mediated immunity to HGG.



★ = direct immersion in bath containing HGG-coated latex particles.

❒ = pronephros removed, cells incubated with or without HGG-coated latex particles, and the supernatants assayed for chemotactic factors.

Fig.22 Specificity of the cell-mediated immune response to an intraperitoneal challenge of HGG in FCA.



6) Migration inhibiting activity of supernatants from HGG-sensitized cells incubated with HGG-Latex

Four fish were given an i.p. injection of HGG in FCA. Two days later, the fish were sacrificed and their pronephric cells were incubated with or without HGG-Latex. The migration of normal carp pronephric cells in response to these supernatants was studied using the micro chemotaxis chamber. To study the migration of cells towards a supernatant, the supernatant was placed in the lower well and the migration of cells, suspended in RPMI 1640 in the upper well, through the membrane to the supernatant in the lower well was measured. To study the migration of cells when suspended in the supernatant, RPMI 1640 was placed in the lower well and the migration of cells, suspended in the supernatant, in the upper well, through the membrane to the RPMI 1640 in the lower well was measured.

7) The effects of opsonization and vaccine particle size on the cell-mediated response to direct immersion immunization with HGG

To study the effects of particle size and opsonization on the cell-mediated immune response to direct immersion immunization with HGG, it was found (see later) that no cell-mediated immune response to HGG could be detected after immersions only in the antigen. An experimental protocol was therefore devised to investigate whether prior immersions in the antigen would give a greater cell-mediated immune response to an i.p. challenge of HGG in FCA, 4 weeks after the final immersion (see Fig.23).

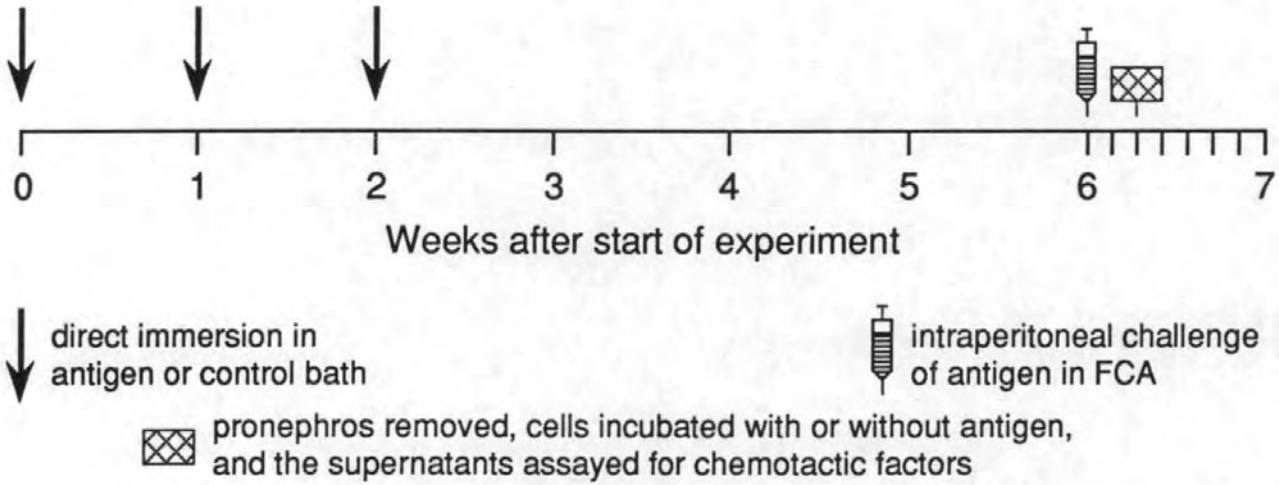


Figure 23

Protocol for direct immersion in antigen, subsequent intraperitoneal challenge and assessment of cell-mediated immunity to the antigen.

Fish were given 3, weekly, immersions in either:-

i) A control bath (5 fish)

ii) Soluble HGG (5 fish)

iii) HGG-coated 0.8µm latex particles (5 fish)

iv) HGG-coated 0.8µm latex particles opsonized with normal carp serum (5 fish)

v) HGG-coated 0.8µm latex particles opsonized with immune carp serum (5 fish)

Four weeks after the final immersion the fish were given an i.p. challenge of HGG in FCA. Two days post-challenge the fish were sacrificed and their pronephric cells were incubated with or without HGG-Latex. The supernatants from these incubations were assayed for chemotactic activity using the micro chemotaxis assay.

RESULTS

1) Cell-mediated immune response to HGG following intraperitoneal injection with HGG in FCA

Supernatants from incubations of HGG-coated latex particles (HGG-Latex) with pronephric cells from normal fish showed no greater chemotactic activity than supernatants from incubations of cells only. However, supernatants from incubations of HGG-Latex with pronephric cells from fish which had received an i.p. injection of HGG on FCA, 2-4 days earlier, showed significant levels ($p<0.05$) of chemotactic factor production (see Fig.24), whilst the chemotactic activity of supernatants from incubations of these cells with medium only was no greater than that of supernatants from incubations of normal cells with medium only. The presence of HGG-Latex in the incubation medium, with no cells present, gave no increase in chemotactic activity to the supernatant.

2) Cell-mediated immune response to HGG following intraperitoneal injection of saline in Freund's complete adjuvant

Supernatants from incubations of HGG-Latex with pronephric cells from both normal fish and fish which had been injected with saline in FCA, 2 days earlier, showed no greater chemotactic activity than supernatants from incubations of the cells with medium only (see Fig.25). The presence of HGG-Latex in the incubation medium, with no cells present, gave no significant increase in chemotactic activity to the supernatant.

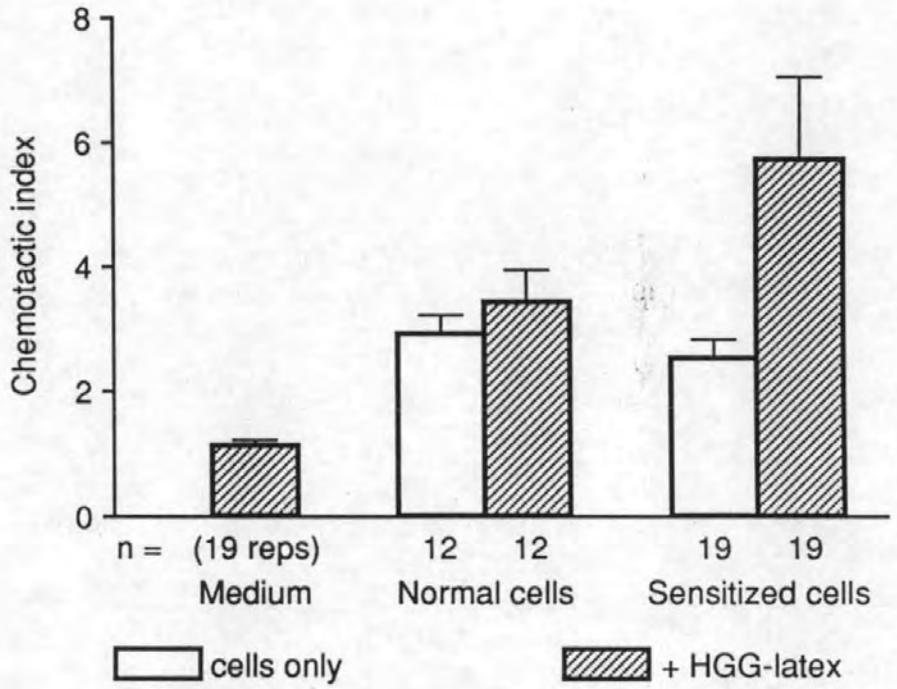


Figure 24

Production of chemotactic factor by normal and HGG-sensitized pronephric cells, when incubated with or without HGG-coated latex particles. The sensitized cells came from fish intraperitoneally challenged with HGG in FCA, 2-4 days previously.
Mean ± SE.

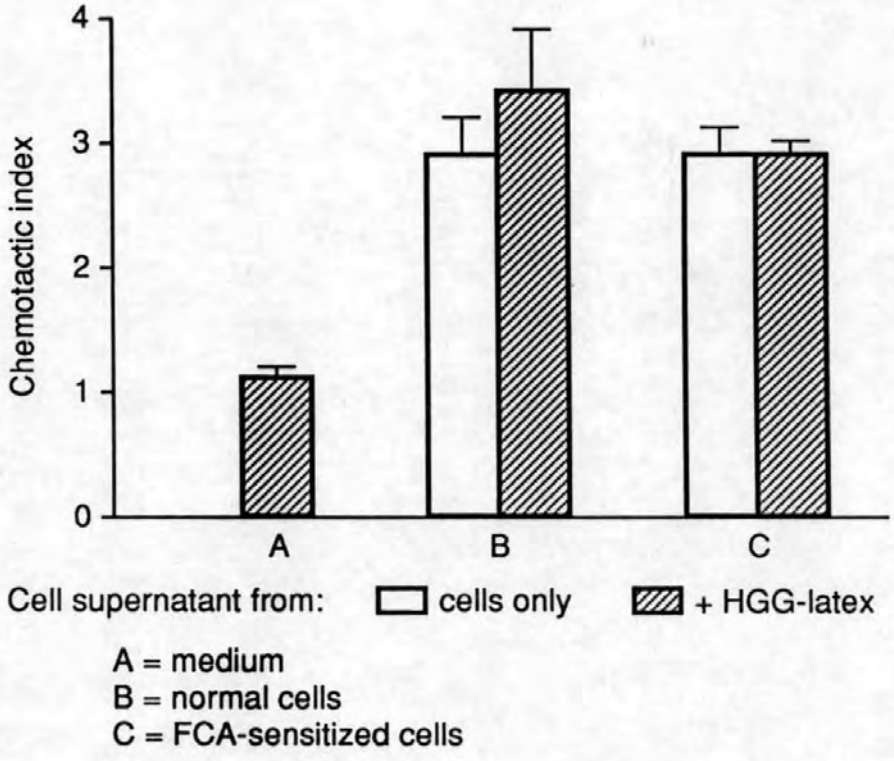


Figure 25

Production of chemotactic factor by pronephric cells from normal fish and fish injected with FCA, when incubated with or without HGG-coated latex particles.
Mean ± SE, n=4.

3) Cell-mediated immune response followng immersions only in HGG-Latex

Supernatants from incubations of HGG-Latex with pronephric cells from both normal fish and fish which had undergone multiple immersions in HGG-Latex, showed no greater chemotactic activity than supernatants from incubations of the cells with medium only (see Fig.26). The presence of HGG-Latex in the incubation medium, with no cells present, gave no significant increase in chemotactic activity to the supernatant.

4) Cell-mediated immune response to *Aeromonas salmonicida* following intraperitoneal injection of *A.salmonicida* bacterin in FCA

Pronephric cells from both normal fish and fish which had received an i.p. injection of *A.salmonicida* bacterin in FCA, 2 days earlier, showed significant levels ($p<0.001$) of chemotactic factor production when incubated with *A.salmonicida* bacterin, whilst showing no chemotactic factor production when incubated without the bacterin (see Fig.27). However, the chemotactic factor production by the cells from fish previously injected with *A.salmonicida* bacterin in FCA, when incubated with bacterin, was significantly greater ($p<0.001$) than that shown by the normal cells when incubated with bacterin. The presence of *A.salmonicida* bacterin in the incubation medium, with no cells present, gave no increased chemotactic activity to the supernatant.

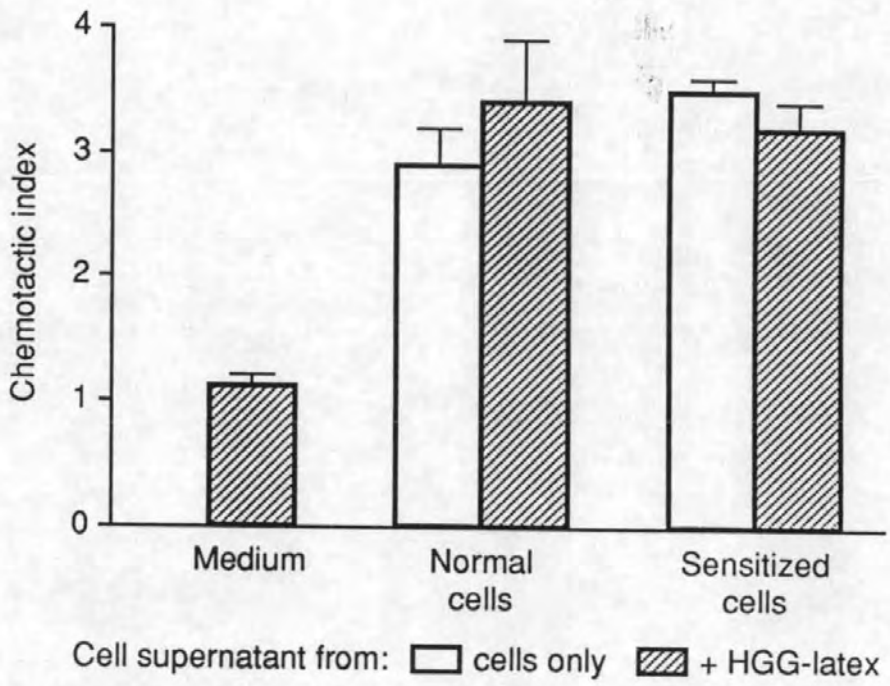


Figure 26

Production of chemotactic factor by pronephric cells from normal fish and from fish which had undergone immersions in HGG-coated latex particles, when incubated with or without HGG-coated latex particles.
Mean ± SE, n=5.

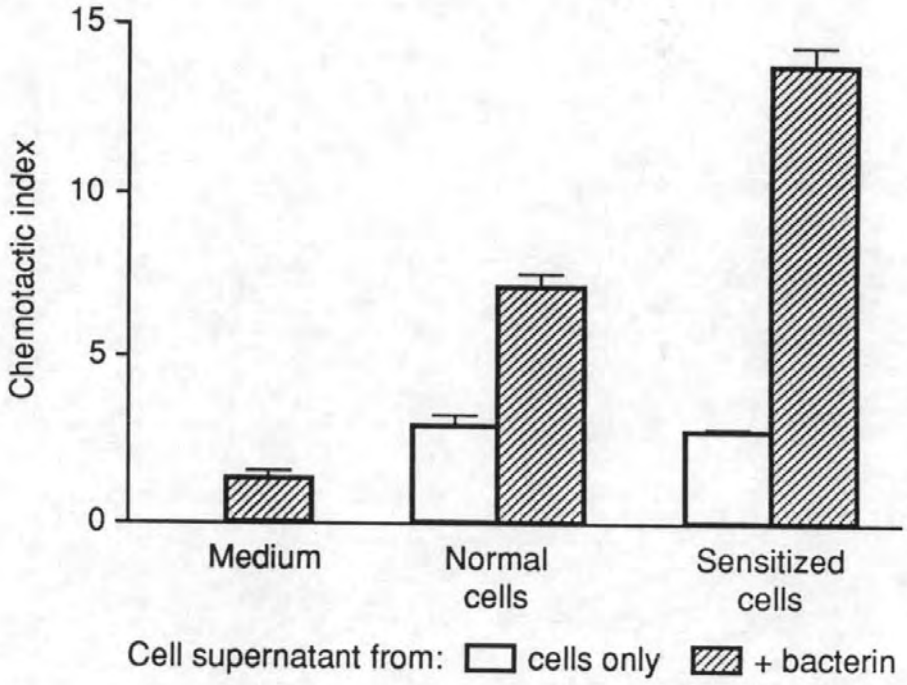


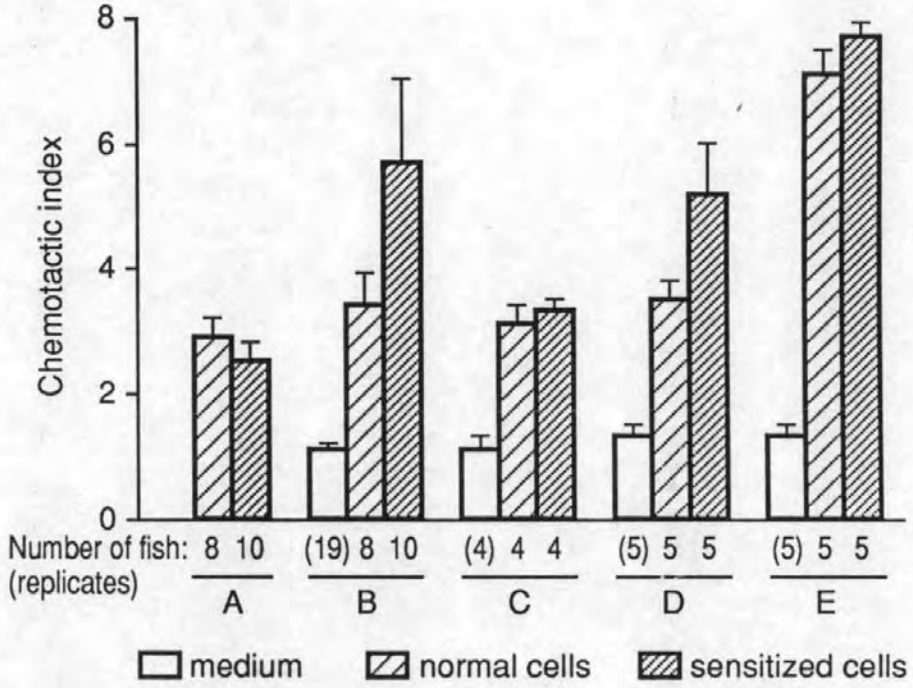
Figure 27

Production of chemotactic factor by pronephric cells from normal fish and from fish injected with *A. salmonicida* bacterin, when incubated with or without *A. salmonicida* bacterin.
Mean ± SE, n=5.

5) Specificity of the cell-mediated immune response following an intraperitoneal injection of HGG in FCA

Supernatants from incubations of normal pronephric cells with either HGG-Latex, keyhole limpet haemocyanin-coated latex particles (KLH-Latex) or chicken gamma globulin-coated latex particles (CGG-Latex), showed no greater chemotactic activity than supernatants from incubations of the cells with medium only. When incubated with *A.salmonicida* bacterin, however, the normal pronephric cells showed significant ($p<0.001$) chemotactic factor production, whereas they did not when incubated without the bacterin (see Fig.28). Supernatants from incubations of KLH-Latex with pronephric cells from the fish which had received an i.p. injection of HGG in FCA, 2 days previously, showed no greater chemotactic activity than supernatants from incubations of the cells with medium only. These HGG-sensitized cells did, however, show chemotactic factor production when incubated with either *A.salmonicida* bacterin ($p<0.001$), HGG-Latex ($p<0.05$) or CGG-Latex ($p<0.01$), whereas they did not when incubated without any antigen. The chemotactic factor production by the sensitized cells on incubation with *A.salmonicida* bacterin, however, was no greater than that of the normal cells when incubated with the bacterin.

The HGG-sensitized cells, therefore, unlike the normal cells, produced chemotactic factor on incubation with HGG-Latex or CGG-Latex, but did not when incubated without antigen. When incubated with either *A.salmonicida* bacterin or KLH-Latex, the response of the sensitized cells showed no significant difference to that of the normal cells.



Supernatant from incubation of:

A = cells only
B = cells + HGG-latex
C = cells + KLH-latex
D = cells + CGG-latex
E = cells + *A.salmonicida* bacterin

Figure 28

Production of chemotactic factor by pronephric cells from normal fish and fish injected with HGG in FCA, when incubated with or without HGG or other antigens. Mean ± SE.

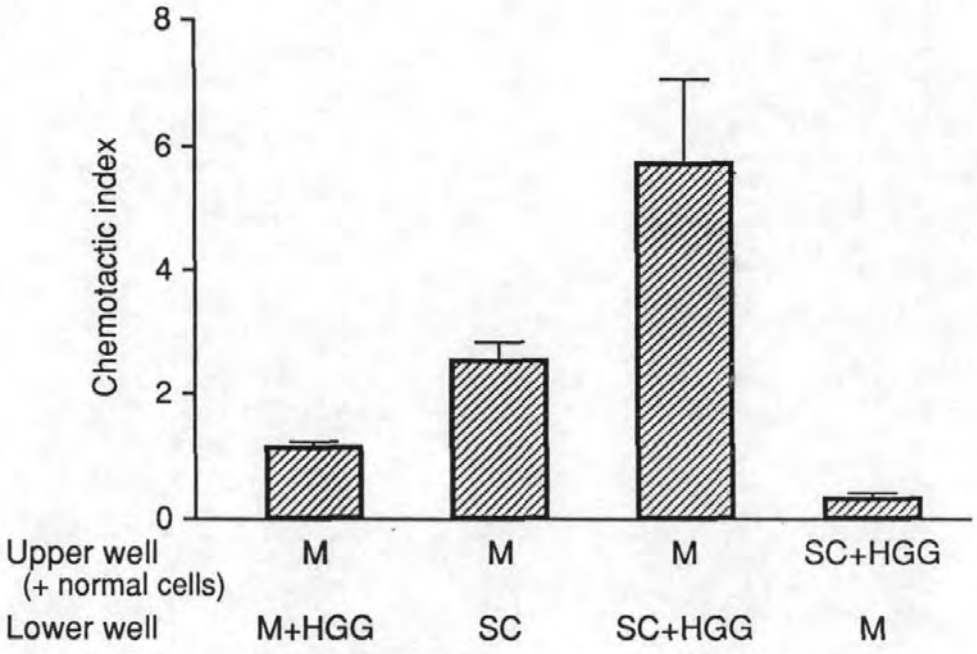
6) Migration inhibiting activity of supernatants from incubations of HGG-sensitized cells with HGG-Latex

Normal pronephric cells showed active migration from normal cell culture medium to supernatants from incubations of HGG-sensitized cells with HGG-Latex ($p<0.05$)(see Fig.29). When normal pronephric cells were actually suspended in supernatants from incubations of HGG-sensitized cells with HGG-Latex, however, they showed greatly reduced migration towards normal cell culture medium compared to if they had been suspended in normal cell culture medium ($p<0.001$).

7) Effects of particle size and opsonization on the cell-mediated immune response to direct immersion immunization with HGG

Prior immersions in soluble HGG, 0.8um HGG-Latex and 0.8μm HGG-Latex opsonized with normal carp serum did not stimulate a significant increase in the cell-mediated immune response to a subsequent i.p. challenge of HGG in FCA, compared to prior immersions in a control bath (see Fig.30).

Prior immersions in 0.8μm HGG-Latex opsonized with immune carp serum , however, did stimulate a significant increase in the cell-mediated immune response to a subsequent i.p. challenge of HGG in FCA, compared to prior immersions in either a control bath ($p<0.01$), soluble HGG ($p<0.05$), 0.8μm HGG-Latex ($p<0.05$) or 0.8μm HGG-Latex opsonized with normal carp serum ($p<0.02$).



M = medium
SC = supernatant from incubation of sensitized cells
SC+HGG = supernatant from incubation of sensitized cells + HGG-latex
M+HGG = supernatant from incubation of medium + HGG-latex

Figure 29

Migration of normal pronephric cells between normal cell culture medium and an HGG-stimulated cell supernatant. Mean ± SE, n=4.

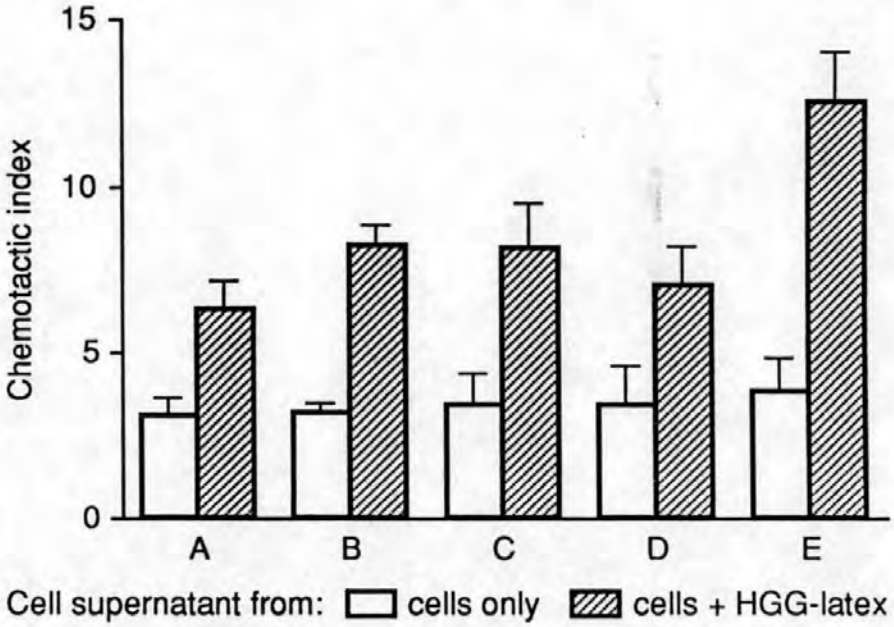


Figure 30

Production of chemotactic factor by HGG-sensitized pronephric cells incubated with or without HGG-coated latex particles. The cells were from fish which had undergone multiple immersions in either HGG-coated latex particles, opsonized or non-opsonized, soluble HGG or a control bath, with a subsequent intraperitoneal HGG in FCA challenge. Mean ± SE, n=5.

DISCUSSION

Results presented in this chapter show that supernatants from incubations of pronephric cells, from carp previously injected with an antigen, with that antigen show greater levels of chemotactic factor-like activity than supernatants from incubations of the cells only. This agrees with the preliminary study of Howell (1987) who also found some chemotactic factor-like activity in the supernatants from incubations of pronephric cells, from carp previously injected with human gamma globulin (HGG), with HGG. It is not clear whether the substance produced by these pronephric cells is directly analagous to mammalian chemotactic factor, but these results suggest that a type of chemotactic factor, or factors, seem to be produced by antigen-specific stimulation, *in vitro*, of antigen-sensitized pronephric cells.

These investigations show that this stimulation of chemotactic factor production seems to occur with both T-dependent (HGG) and T-independent (*Aeromonas salmonicida*) sensitizing antigens. Interestingly, the presence of *A.salmonicida* vaccine in incubations of normal pronephric cells also stimulated chemotactic factor production, compared to incubations of cells alone, although in antigen-sensitized cells the production was greater. The presence of HGG, however, in incubations of normal cells did not stimulate any increased production of chemotactic factor.

The cell-mediated immune system of teleost fish, therefore, seems to be similar to that of mammals in both the production of migration inhibition factor (MIF) (Howell, 1984; Song *et al.*, 1989) and chemotactic factor by antigen-sensitized leukocytes on *in vitro* incubation with the antigen.

In these experiments, this sensitization of the pronephric cells was shown to be due to the antigen and not to due to the adjuvant, injected with the antigen, as cells from fish injected with saline in adjuvant showed no increased production of chemotactic factor on *in vitro* stimulation with antigen.

These investigations found that the cell-mediated immune response in fish occurs much earlier than the corresponding humoral immune response to an intraperitoneal challenge of antigen in adjuvant (see chapter 3). The response was detectable at 24 hrs. post-injection, peaked at 48 hrs. post-injection and was undetectable by 1 week post-injection. This agrees with similar results of Blazer *et al.* (1984) who found the peak cell-mediated immune response of trout to sheep red blood cells (SRBC), as measured by MIF production by antigen-stimulated leukocytes, to occur at 48 hrs. post-injection. Jayaraman *et al.* (1979), looking at the cell-mediated immune response of tilapia to SRBC, again as measured by MIF production, found a response at 5 days post-injection, which corresponds to the experiments presented here, where a measurable response was still found at 4 days post-injection. Howell (1987) found measurable chemotactic factor production by the cells of fish injected 1 and 2 weeks earlier, when incubated with antigen. Unfortunately, however, in the study of Howell (1987), as the cells of control fish were not assayed as well, it cannot be assumed that this chemotactic factor production was due to the earlier antigen injections. Song *et al.* (1989), when challenging fish with a bacterial vaccine, not by injection, but by immersion, found a measurable cell-mediated immune response, as measured by MIF production by antigen-stimulated cells, from 3-106 days post-immersion. Obviously, however, as so little work has been done in this field in fish, it is

impossible to accurately account for any differences between the results of different workers. The results of Song *et al.* (1989) do, however, suggest that immersion challenge with a bacterial vaccine may give a much longer lasting cell-mediated immune response than when the vaccine is administered by injection, though the response to the injected vaccine seems to occur a little earlier.

When incubating antigen-sensitized pronephric cells with antigen, in vitro, the production of chemotactic factor seems to peak within 24 hrs. These experiments detected chemotactic factor production after 18 hrs. incubation. When looking for chemotactic factor production by mitogen-stimulated pronephric cells, Howell (1987) found as high levels in 24 hour incubations as in 36 and 48 hour incubations. In mammals, Altman *et al.* (1973) detected chemotactic factor production in antigen-stimulated lymphocytes in 6 hour cultures and found peak production by 24 hrs. incubation. Zigmond & Hirsch (1973) even found measurable chemotactic factor production by various mammalian antigen-sensitized leukocytes after only a 1 hour incubation with antigen.

The experiments presented here did not find any measurable antigen-sensitization of the pronephric cells when the fish were given immersions only in a bath of the antigen, even when multiple immersions were given. This differs from the results of Song *et al.* (1989) who found that the lymphocytes of glass eels could be antigen-sensitized by 2 immersions of the eels in a bacterial vaccine. The results in this chapter, concerning the cell-mediated immune response to immersions only, however, used HGG, a T-dependent antigen, instead of the bacterin used by Song *et al.*, which was probably a T-independent antigen in fish. This may account for the difference in results. The experiments of Song *et al.* also differed from

those presented here in that Song *et al.* were looking at MIF production by the leukocytes as a measure of the cell-mediated immune response, whereas these experiments were concerned with chemotactic factor production.

The experiments presented here found the specificity of the cell-mediated immune response to injected antigens to be fairly high, although there was cross-reactivity between two similar antigens (HGG and chicken gamma globulin). Pronephric cells from fish injected with HGG in adjuvant showed no sensitization to either keyhole limpet haemocyanin or *A.salmonicida* bacterin. Both Song *et al.* (1989) and Jayaraman *et al.* (1979) found good specificity of the cell-mediated immune response in fish, as measured by MIF production by leukocytes stimulated, *in vitro*, with antigen, even when closely related antigens were used. Ward *et al.* (1969) found good specificity in the chemotactic factor production by antigen-sensitized guinea pig lymphocytes, when stimulated, *in vitro*, with antigen. If the *in vitro* stimulating antigen was not the same as that used to sensitize the cells, then no production of chemotactic factor was stimulated. Jayaraman & Muthukkaruppan (1978b) also found that lizard lymphocytes sensitized to SRBC would only produce MIF on in vitro stimulation with SRBC and would not respond to rat RBC.

As, in these experiments, no cell-mediated immune response could be detected after immersions only in antigen, it was decided to assess the response to immersions in antigen by immersing the fish and then measuring the cell-mediated immune response to a subsequent intraperitoneal (i.p.) challenge of antigen, to see if the prior immersions stimulated a secondary response to the i.p. challenge. Prior immersions in either soluble HGG, latex particle-bound HGG or latex particle-bound

HGG opsonized with normal carp serum did not significantly increase the cell-mediated immune reponse to the i.p. challenge of HGG in adjuvant. However, when the fish were given immersions in latex particle-bound HGG opsonized with immune carp serum, a secondary response to the i.p. challenge was seen. Therefore, as with the humoral immune response (see chapter 3), priming by immersion with a vaccine opsonized with immune serum increases the stimulation of the cell-mediated immune system, compared to priming immersions in the corresponding non-opsonized vaccine.

These experiments also looked at the migration of normal carp pronephric cells when suspended either :- a) in normal cell culture medium with an antigen-stimulated cell supernatant on the opposite side of the filter membrane, or b) when actually suspended in an antigen-stimulated cell supernatant with normal cell culture medium on the opposite side of the filter membrane. When suspended on the opposite side of the filter to the supernatant, more cells migrated through the membrane towards the supernatant compared to when there was cell culture medium on both sides of the membrane. However, when the cells were actually suspended in the supernatant, with cell culture medium on the opposite side of the membrane, far fewer cells migrated through the membrane compared to when normal cell culture medium was on both sides. This migration inhibition effect when the cells are actually suspended in the supernatant may possibly be a dose-dependent effect, with the supernatant showing migration inhibition activity at higher concentrations (the target cells being exposed to a higher concentration of supernatant when actually suspended in it than when being exposed to supernatant diffusing through the membrane). David & David (1972)

found that their mammalian MIF culture supernatants were also chemotactic for mononuclear cells, thus demonstrating the ability of certain supernatants to affect target cells differently under different conditions. The migration inhibition found in the experiments described here may also, possibly, be due to the fact that when the cells are actually suspended in the supernatant, there is no concentration gradient to migrate up. Whatever the actual cause of this migration inhibition effect, the supernatants used here certainly showed no chemokinetic activity when pronephric cells were suspended in the undiluted supernatant. The results presented here, therefore, suggest that a cytokine,or cytokines, produced by the antigen-stimulated sensitized pronephric cells, may actively stimulate migration towards themselves. The existence of two different biological activities (i.e. migration inhibition and chemotaxis or chemokinesis) at different concentrations is still a possibility.

Further work is obviously needed in order to identify the exact cell type, or types, involved in cytokine production and to isolate and purify the factor, or factors, produced by antigen-sensitized fish leukocytes, when stimulated, *in vitro*, with the sensitizing antigen. Ward *et al.* (1970) found MIF, a chemotactic factor for mononuclear cells and a chemotactic factor for neutrophils, all molecularly distinct from each other, being produced by antigen-stimulated sensitized guinea pig lymphocytes on incubation with the antigen. It is, therefore, definately possible that several different factors with different biological activities are produced by antigen-stimulated sensitized fish leukocytes. Jayaraman *et al.* (1979), Blazer *et al.* (1984), Howell (1984) and Song *et al.* (1989) all found MIF activity in the supernatants of antigen-stimulated leukocytes, and these experiments find chemotactic factor activity in the same type of supernatants

Present work, therefore, confirms the preliminary results of Howell (1987), showing chemotactic factor production by antigen-sensitized leukocytes on stimulation *in vitro* with the sensitizing antigen.

CHAPTER 5

Title

In vivo and *in vitro* uptake
and accumulation of antigen.

INTRODUCTION

Researchers have been studying the tissue localization of injected material in fish for over 70 years, and even though there still are some gaps in our knowledge in this field, the fate of injected antigens is fairly well established. Hoskins and Hoskins (1918) looked at the localization of injected materials in the dogfish and found that the spleen and liver were important in removing foreign particles from the circulation, with the gills also seeming to trap foreign particles to a certain extent. Other researchers from the early part of this century (Wislocki, 1917; Mackmull & Michels, 1932) looking at the fate of injected particles in fish all found the spleen and kidney to be major organs of accumulation of foreign matter. Wislocki (1917) found, in the carp, that, similar to Hoskins and Hoskins (1918) findings in the dogfish, the liver was a major organ of accumulation of foreign materials from the blood. Mackmull and Michels (1932) found that, in the cunner, large amounts of injected material accumulated in the heart. This finding was also mirrored by Ellis *et al.* (1976) who looked at the fate of intraperitoneally injected carbon particles in the plaice, which apparently can pass unchanged from the peritoneum into the bloodstream and found large carbon accumulations within cardiac phagocytes. Ellis *et al.* (1976) also found accumulations in the spleen and pronephros, but none in the liver. In contrast, in the hagfish, Tomonaga *et al.* (1986) found a reticuloendothelial cell system in the liver of hagfish, analagous to Kupffer cells in mammals, which phagocytosed many different types of injected particles, from sheep erythrocytes to carbon and latex particles. Therefore, in general, the majority of workers have found the spleen and kidneys to be the major sites of accumulation of injected material, with some workers also finding the liver and heart, and occasionally the gills, to be major organs of accumulation.

The clearance of injected materials from the bloodstream seems to occur relatively quickly. MacArthur *et al.* (1983) looked at the kinetics of the clearance of intravenously injected carbon particles and turbot erythrocytes in plaice. They found the spleen and kidneys to be the major organs of accumulation and found that up to 90% of the particles had been removed from the circulation by 30 mins. post-injection. After that

time the rate of removal slowed down considerably, but by 2-3 hrs. post-injection, only very small amounts of the injected matter was still circulating in the bloodstream.

The uptake and accumulation of foreign matter from direct immersion baths has been a much more recent topic for investigation. Amend and Fender (1976) presented some of the first results, looking at the uptake of soluble bovine serum albumin from a bath, after a 2 minute immersion in a hyperosmotic solution. They thought the skin and lateral line to be the main route of entry into the fish, with the gills only being secondary. However, later work (Hockney, 1984) has shown that uptake over the skin is extremely unlikely, and many think the gills to be a major route of antigen uptake (Alexander *et al.*, 1982). Researchers have also demonstrated the uptake of particles into the gills. Zapata *et al.* (1987) , in electron microscopical studies, found the uptake of a killed bacterial vaccine into the gill epithelial cells, and thence into mononuclear phagocytes lying directly beneath the gill epithelium. Goldes *et al.* (1986) found phagocytosis of kaolin particles by the gills of trout and postulated that branchial phagocytosis by stationary branchial macrophages could explain how antigens from direct immersion baths gain entry to the internal compartment of the fish. Nelson *et al.* (1985) studied the uptake of a killed bacterial vaccine in trout from an immersion bath. Bacterin was observed on the surface of the gills and also in the gut, where antigen persisted for 3 days. post-immersion, after which which it was not detectable. Watson (1984) and Robohm (1986) have noted that antigens applied by direct immersion reach the lumen of the gut and many workers consider the gut to be a major organ of antigen uptake (Davina *et al.*, 1980, 1982; Hart *et al.*, 1988). Lamers (1985), Rombout *et al.* (1986) and Rombout & van den Berg (1989) have shown the uptake of bacterial vaccines, following oral administration, into the epithelial cells of the gut and subsequent transport to intraepithelial macrophages. The uptake of macromolecules by the gut has also been studied by various groups of researchers. McLean & Ash (1986, 1987) Georgopoulou *et al.* (1988) and Doggett (1989) have not only found the uptake of orally and anally intubated macromolecules by enterocytes and intraepithelial macrophages, but have found that these molecules are actually absorbed

and transported into the systemic circulation.

Other studies using radiolabelled killed bacterial cells (Tatner & Horne, 1983; Tatner *et al.*, 1984; Tatner, 1987), have looked at factors affecting the uptake following direct immersion in antigen. Immersion times of greater than 10 seconds did not seem to increase uptake. It was also found that vaccine uptake decreased at lower temperatures and that larger fish took up greater amounts of vaccine. The head end of the fish was found to be more important than the trunk end, during uptake and a hyperosmotic dip prior to immersion in the vaccine did not significantly increase vaccine uptake.

This chapter presents results from studies looking at the uptake and internal accumulation of a radiolabelled *Aeromonas salmonicida* bacterin following immersion in a bath of the bacterin. The uptake and accumulation was studied in both naive fish and fish previously immunized by direct immersion. The uptake and accumulation of an opsonized form of the bacterin is also studied in naive fish.

It is already known that immune complexes, when injected, are trapped more quickly in the pronephros than antigen alone (Secombes *et al.*, 1982a) but no studies have, as yet, been published looking at the fate of immune complexed antigens when administered by direct immersion.

In addition to the *in vivo* studies on antigen uptake noted above, results presented in this chapter also investigate the phagocytosis of particles by carp peritoneal exudate cells. Different particle sizes are used to see what size particles are easily ingested. Shirahama *et al.* (1971) looked at the phagocytosis of different sizes of amyloid bundles in mouse macrophages. Bundles less than 2μm in diameter were ingested by either the extension of cytoplasmic processes around the bundle or by the formation of a cytoplasmic invagination around the amyloid mass. Bundles of 10μm in diameter or larger were treated in a totally different manner, with many cytoplasmic extensions being projected into the amyloid mass. With bundles between 2μm and 10μm in diameter, a mixture of the two forms described above was seen. Mammalian phagocytes can, obviously, ingest a large number of different types and sizes of particle, including sheep erythrocytes (around 8μm in diameter), yeast blastospores (5μm in diameter) (Janusz *et al.*, 1988) and small

lymphocytes (6-10μm in diameter) (Griffin *et al.*, 1976). The external surface characteristics of a particle must also be important as Abo *et al.* (1986) found that human monocytes, which can ingest 8μm sheep erythrocytes could not phagocytose 6μm latex particles. Shirahama *et al.* (1971) also found that amyloid bundles stained with Congo red were much prefered to be ingested by mouse macrophages than unstained bundles of the same size.

Much work has also been done on phagocytosis by fish cells, which seem to be able to phagocytose as large a range of sizes and types of particles as mammalian phagocytes. These include carbon particles (Braun-Nesje *et al.*, 1981; MacArthur & Fletcher, 1985), 5-10μm agarose beads (Johnson & Smith, 1984), yeast blastospores (Braun-Nesje *et al.*, 1981), sheep erythrocytes (Braun-Nesje *et al.*, 1982; Graham, 1989) and 0.81μm latex particles (Tomonaga *et al.*, 1986). The studies presented here look at the phagocytosis of latex particles of various sizes and in various forms. The phagocytosis of uncoated particles, protein-coated particles, and protein-coated then opsonized particles being studied. In mammals it is known that the opsonization of particles with antibody and complement enhances the uptake by phagocytes (Mantovani, 1975). It seems that both antibody and complement are important in the binding of particles by phagocytes, but that fixed complement on the surface of a particle is important in the actual ingestion step of phagocytosis. Fries *et al.* (1987) found that the ingestion of sheep erythrocytes by human phagocytes was enhanced by the opsonization of the erythrocytes with antibody alone but was enhanced even further if opsonization was with both antibody and complement. In fish it has also been found that opsonization enhances the phagocytosis of particles (Fujii, 1981; Griffin, 1983; O'Neill, 1985). It is also known that, as in mammals, complement and antibody opsonizes more effectively than antibody alone (Sakai, 1984; Honda *et al.*, 1986). Complement receptors have also been demonstrated on tilapia macrophages (Saggers & Gould, 1989) and it also seems that certain teleost cells have receptors for human C3 (Ueki *et al.*, 1978). Comparative experiments have also been done looking at the opsonizing effect of mammalian antibody and complement on the phagocytosis of particles by fish cells. There is, however, some

disagreement as some studies have found that opsonization with mammalian antibody and complement does not enhance the phagocytosis of particles by fish phagocytes (Braun-Nesje *et al.*, 1981), whereas others have found that opsonization with human complement does enhance the phagocytosis of particles by fish phagocytes (Johnson & Smith, 1984).

MATERIALS AND METHODS

Peritoneal exudate cell preparation

Carp were injected intraperitoneally with Freunds incomplete adjuvant (FIA)(Difco, Detroit,U.S.A.), at a rate of 0.01cm^3 per gram body weight. It was found that the numbers of peritoneal exudate cells (PECs) induced by this injection peaked at 24 hours post-injection. The fish were, therefore, anaesthetised and injected intraperitoneally with L-15 (Leibovitz) medium (Gibco Ltd.,Scotland) containing 10 units/cm^3 of heparin (Sigma,U.S.A.) with 2% foetal calf serum (FCS)(Flow Laboratories, Rickmansworth, England) at a rate of 0.04cm^3 per gram body weight, at 24 hours post-FIA injection. The peritoneum of the fish was then gently massaged and a small slit was made in the side of the fish at the site of the injection hole and the fluid within the peritoneum (containing PECs) was withdrawn using a pasteur pipette.

After washing twice with L-15 medium containing 0.1% FCS and 2% penicillin and streptomycin (P/S) (Flow Laboratories, Rickmansworth, England), the PECs were then suspended in the same medium and incubated on cover slips for two hours at 15^{o}C to allow the cells to adhere to the glass. The low concentration of FCS was used as Graham (1989) showed that the adherance of trout macrophages to glass was reduced with concentrations of FCS above 0.1% (v/v). The PECs were then ready for use and the medium was changed to L-15 medium containing 5% FCS and 2% P/S until use. These PECs were a mixed population with the vast

majority having macrophage-like, or neutrophil-like morphology. The cells were stained using the α-naphthyl acetate method of staining for non-specific esterase. 60% of the cells stained positively for non-specific esterase (Drury and Wallington, 1980), which is regarded as a reliable cytochemical marker for mammalian macrophages (Kaplow, 1981), although the staining characteristics of teleost macrophages are a little less certain. The cells were kept at 20±2°C throughout their preparation and subsequent experimental use.

Particle uptake

The PECs on the cover slips were incubated with suspensions of latex particles, either plain, HGG-coated or HGG-coated then opsonized (see Experimental Design), in L-15 medium containing 5% FCS and 2% P/S, 0.4cm^3 per cover slip. The incubation concentrations of latex particles used were :-

0.8μm particles :- 10^7/mm^3

3.0μm particles :- $5x10^5$/mm^3

15.0μm particles :- $5.5x10^3$/mm^3

These concentrations for the different sized particles gave the same concentration (w/v) of latex/medium.

After incubation with the particles for 30 minutes at room temperature, each cover slip was dipped 40 times in 0.85% saline and then fixed in absolute methanol for 5 minutes. The cover slips were then immersed , for 5 minutes, in a citric acid/disodium hydrogen phosphate buffer (see Formulae). They were then transfered to May Grunwald stain (BDH Chemicals Ltd., Poole, U.K.), freshly diluted 1:2 in the same buffer, for 5

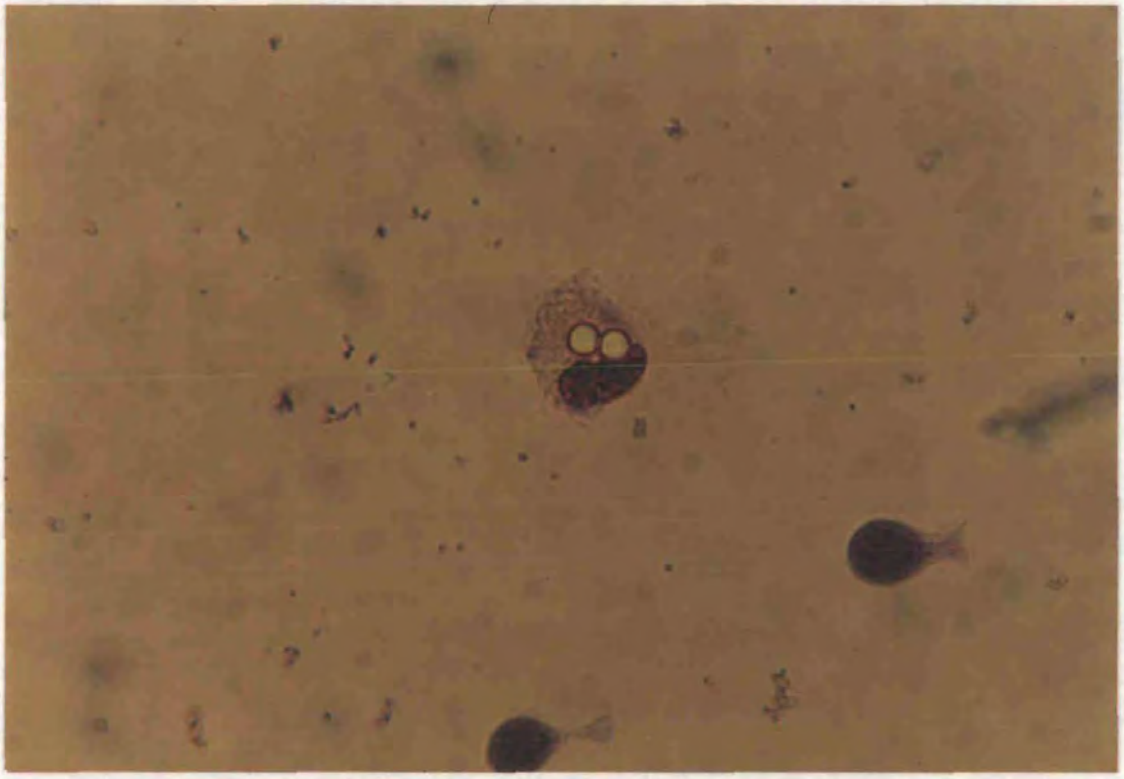
minutes, rinsed once more in the buffer and then immersed in Giemsa stain (BDH Chemicals Ltd., Poole, U.K.), diluted 1:6 in the same buffer, for 15 minutes. The cover slips were then rinsed one final time in the buffer and were then air-dried and mounted on glass slides using DPX. The number of latex particles phagocytosed per PEC, and the percentage of cells phagocytosing was then counted, at 400x magnification on a light microscope (see Fig.31). 100 PECs from each group were counted. For each experiment, 3 replicates were done, using PECs from 3 different fish. This was done to lessen the effect of any differences between PEC populations from different fish.

Preparation of a radiolabelled *Aeromonas salmonicida* bacterin

The method for the preparation of the ^{14}C lysine labelled *A.salmonicida* bacterin is that of Parker (1985). The method is the same as that for the preparation of the normal bacterin (see General Methods) except that the 48 hour incubation in tryptone soy broth at 25^{o}C is replaced by a 72 hour incubation in Shieh & Reddy medium (Shieh & Reddy,1972)(see Formulae) containing 81umol/ml of L-U-^{14}C lysine monohydrochloride (Amersham International plc.,U.K.) with a specific activity of 324 mCi/mmol. This gives a radioactive concentration of ^{14}C in the medium of 0.25μCi/cm^3. Parker (1985) , using this method to radiolabel two different strains of A.salmonicida, found an average specific activity of 6.15 x 10^{-4} cpm/colony forming unit.

Fig.31. Peritoneal exudate cells after incubation with 3.0μm HGG-coated latex particles. (x1333)

The cell in the centre of the picture having phagocytosed two latex particles.



Assessment of the uptake and accumulation of a radiolabelled *Aeromonas salmonicida* bacterin

The uptake and accumulation of a radiolabelled *Aeromonas salmonicida* bacterin was measured by firstly directly immersing fish for 15 minutes in a vaccine bath containing $5x10^9$ radiolabelled cells/dm^3. The fish were then sacrificed at 3 hrs., 30 hrs., 4 days and 11 days post-immersion and gill, pronephros, liver, spleen and hindgut samples were taken from each fish, and were homogenised (see Fig.32). These homogenates were then harvested onto filter discs in a Titertek cell harvester (Skatron, Norway) and the filter discs were then dried at 28^oC for 24 hours. The discs were then weighed and the mass of tissue on the disc was calculated (the mass of a disc with no tissue on it was found to be very constant). These discs were then placed in scintillation vials and 1ml of a scintillation fluid, Cocktail T (BDH Chemicals Ltd., England), was added to each vial. After being capped, the vials were run through a Phillips PW 4700 scintillation counter and the radioactivity in each sample was measured. To take account of any quenching by the tissues or background radioactivity within the tissues, the quenching by each of the tissues in question was calculated by mixing increasing amounts of each tissue with a standard number of radiolabelled bacterin cells. These samples were then run through the scintillation counter (see Table 2) and the quenching of the radioactivity of the bacterin cells by each separate tissue was calculated. It was found that none of the tissues being used, except gill tissue, significantly affected the detection of radiolabelled bacterin within a sample, at the concentrations of tissue being used in this experiment. Gill tissue, however, appeared to contain a measurable

TABLE 2

Quenching properties of different tissues from carp. results are expressed as counts per minute, corrected for the background reading and for a control vial containing scintillation fluid and a clean filter disc + $3x10^5$ radiolabelled bacterin cells.(Mean±SE, n=3)

	mg. of tissue on filter disc (+ 3x10^5 radiolabelled bacterin cells)				
Tissue	0	0.1	0.2	0.4	0.6
Spleen	0.0±1.9	-0.5±1.0	-1.0±1.4	-4.0±2.4	2.0±2.0
Liver	0.0±1.9	-3.0±4.2	2.2±1.3	-0.3±1.7	2.5±2.6
Hindgut	0.0±1.9	4.0±2.7	2.5±1.9	-1.5±2.5	1.0±3.1
Gill	0.0±1.9	2.7±1.5	5.7±2.1	10.0±3.0	16.5±2.4
Pronephros	0.0±1.9	1.3±0.9	-2.1±3.2	-1.5±2.4	2.2±2.0

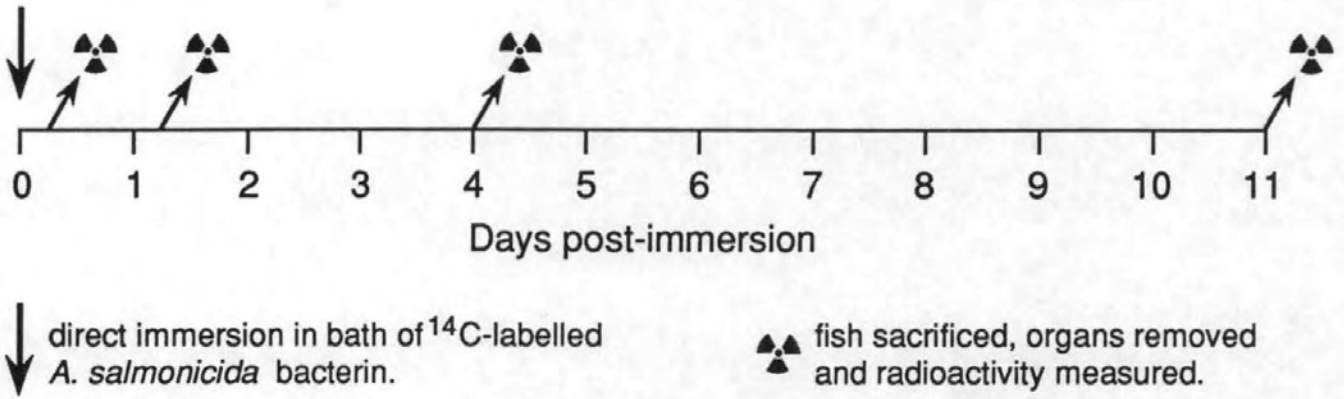


Figure 32

Protocol for studying the uptake and accumulation of a radiolabelled bacterin.

amount of activity within it. Therefore, the concentration of gill tissue in a sample had to be corrected for, when calculating the radioactivity due to radiolabelled bacterin in a sample.

The results for all the samples were also corrected for the background activity present in a control vial containing scintillation fluid and a clean filter disc.

EXPERIMENTAL DESIGN

Uptake of varying sizes of latex particles by peritoneal exudate cells (PECs)

The PECs from 3 different fish were each incubated for 30 minutes with both uncoated and HGG-coated latex particles of 0.8µm, 3.0µm and 15.0µm in diameter. The number of latex particles phagocytosed per PEC, and the percentage of PECs phagocytosing was then calculated for each of the different latex particle preparations. The results were then analysed using ANOVA and the t-test corrected for small samples (McGhee, 1985).

Effects of opsonization on the uptake of latex particles by peritoneal exudate cells (PECs)

The PECs from 3 different fish were incubated for 30 minutes with :-

a) Normal 3.0µm latex particles

b) HGG-coated 3.0µm latex particles

c) HGG-coated 3.0μm latex particles opsonized with normal carp serum

d) HGG-coated 3.0μm latex particles opsonized with serum

e) HGG-coated 3.0μm latex particles opsonized with fresh immune carp serum

For opsonization procedures see General Methods.

The number of latex particles phagocytosed per PEC, and the percentage of PECs phagocytosing was then calculated for each of these different latex particle preparations. The results were then analysed using ANOVA and the t-test corrected for small samples (McGhee, 1985).

Effects of opsonization of a radiolabelled *Aeromonas salmonicida* bacterin and the immune status of the recipient fish on uptake and accumulation of the bacterin following an immersion in a bath of the bacterin

There were 2 main investigations incorporated in this study :-

i) To see if the opsonization of an *A.salmonicida* bacterin would affect the uptake and accumulation of the bacterin in the tissues of normal fish following a single immersion in a bath of the bacterin.

ii) To see if fish which had been pre-immunised, by multiple direct immersions in *A.salmonicida* bacterin, would show a different pattern of uptake and accumulation of the bacterin in their tissues following a single immersion in the bacterin, compared to normal fish.

To study these possibilities, 3 experimental groups were set up :-

1) Normal fish were immersed in a non-opsonized bacterin.
2) Normal fish were immersed in an bacterin opsonized with immune carp serum.
3) Immune fish (immunized by direct immersion once a month for the previous 4 months) were immersed in a non-opsonized bacterin.

There were twenty fish per group, 5 being sacrificed at each separate time interval post-immersion (3 hrs, 30 hrs, 4 days and 11 days)(see Fig.32). Five control fish (which had never been exposed to the radiolabelled bacterin) were also sacrificed. Samples of spleen, liver, pronephros, gill and hindgut from these fish were homogenised, harvested and run through the scintillation counter to assess the radioactivity within the tissues. These results were then analysed using the t-test corrected for small samples (McGhee, 1985).

RESULTS

Uptake of varying sizes of latex particles by peritoneal exudate cells (PECs)

The PECs readily phagocytosed both the 0.8μm and 3.0μm in diameter latex particles (see Fig.33 and Table 3). However, no uptake of the 15.0μm latex particles was observed. There was no difference between the uptake of the normal, uncoated latex particles and the HGG-coated particles, either in numbers of particles ingested or percentage of PECs which phagocytosed particles.

Effects of opsonization on the uptake of latex particles in peritoneal exudate cells (PECs)

i) Particles phagocytosed per PEC

There was no difference between the uptake of the normal, uncoated latex particles, the HGG-coated particles and the HGG-coated particles opsonized with normal carp serum (see Fig.34). However a significantly greater number ($p<0.05$) of the HGG-coated particles opsonized with decomplemented immune carp serum were phagocytized compared to both the uncoated and HGG-coated particles. The uptake of HGG-coated particles opsonized with decomplemented immune serum was not, however, significantly greater than that of the HGG-coated particles opsonized with normal serum.

The greatest uptake of particles was found with the HGG-coated particles opsonized with fresh immune carp serum, which showed a significantly

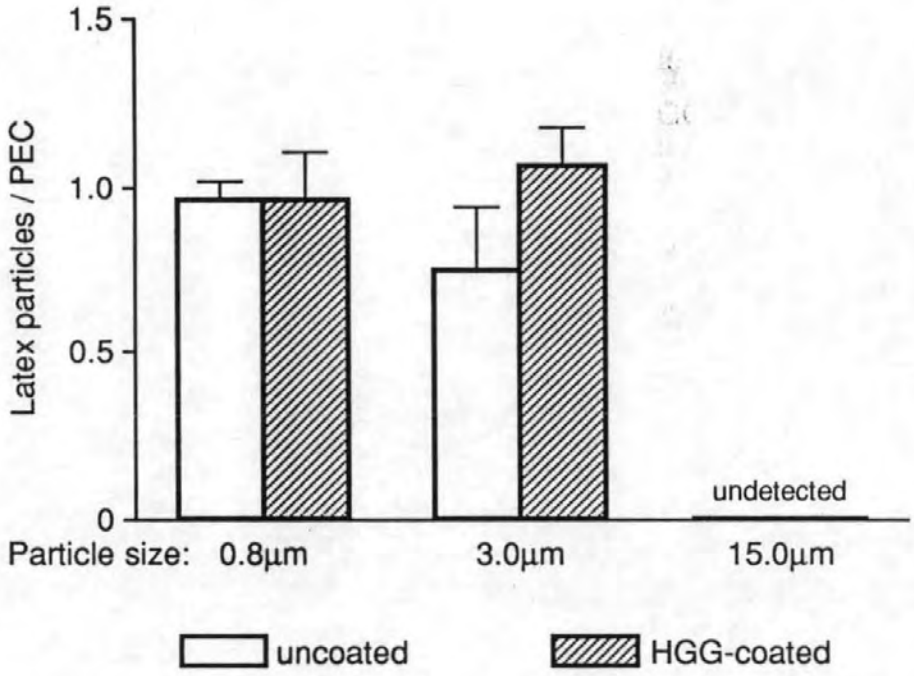


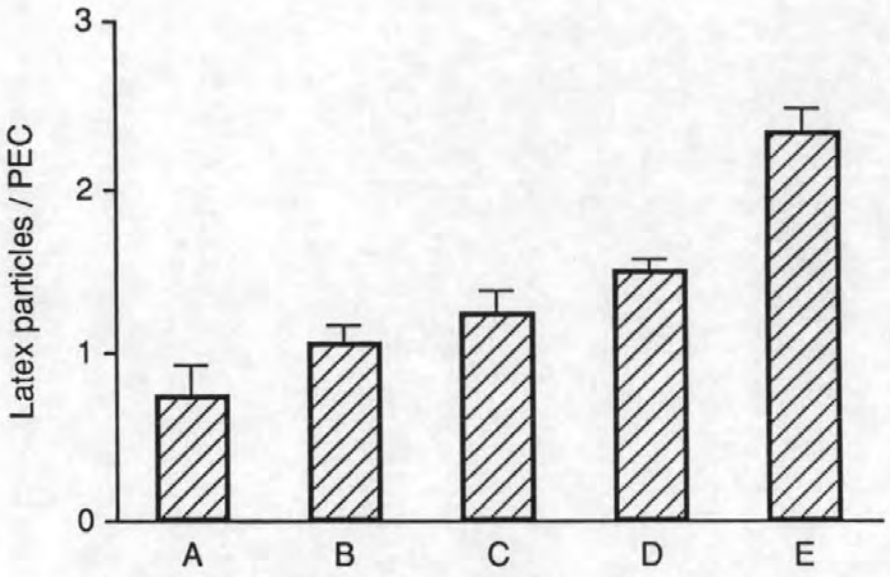
Figure 33

Uptake by peritoneal exudate cells (PECs) of latex particles of varying sizes, both uncoated and HGG-coated.
Mean ± SE, n=3.

TABLE 3

Uptake by peritoneal exudate cells (PECs) of different diameter latex particles, either uncoated, HGG-coated or HGG-coated and opsonized : Percentage of cells phagocytosing. (Mean±SE, n=3)

Latex particles	Percentage of cells Phagocytosing
0.8μm diameter, uncoated	61±6
0.8μm diameter, HGG-coated	74±3
3.0μm diameter, uncoated	67±4
3.0μm diameter, HGG-coated	72±5
3.0μm diameter, HGG-coated and opsonized with normal serum	83±5
3.0μm diameter, HGG-coated and opsonized with decomplemented immune serum	82±5
3.0μm diameter, HGG-coated and opsonized with immune serum	90±3
15.0μm diameter, uncoated	0
15.0μm diameter, HGG-coated	0



A = uncoated
B = HGG-coated
C = HGG-coated and opsonized with normal serum
D = HGG-coated and opsonized with decomplemented immune serum
E = HGG-coated and opsonized with immune serum

Figure 34

Uptake by peritoneal exudate cells (PECs) of 3.0μm diameter latex particles, either uncoated, HGG-coated or HGG-coated and opsonized.
Mean ± SE, n=3.

greater uptake ($p<0.01$) than any other of the particles.

ii) Percentage of PECs phagocytosing

There was no significant difference in the percentage of PECs phagocytosing when incubated with latex particles which were either uncoated, HGG-coated, HGG-coated and opsonized with normal serum or HGG-coated and opsonized with decomplemented immune serum (see Table 3). The only significant increase in the percentage of PECs phagocytosing was with the HGG-coated particles opsonized with fresh immune serum. The percentage of PECs phagocytosing these particles was significantly greater than the percentage of PECs phagocytosing either the uncoated particles ($p<0.02$) or the HGG-coated particles ($p<0.05$), but was not significantly greater than the percentage of PECs phagocytosing the HGG-coated particles opsonized with either normal serum or decomplemented immune serum.

~

Effects of opsonization of a radiolabelled *Aeromonas salmonicida* bacterin and the immune status of the recipient fish on uptake and accumulation of the bacterin following an immersion in a bath of the bacterin

i) Uptake and accumulation in the gills

There was no measurable uptake or accumulation of the non-opsonized bacterin in the gills of the normal fish at any of the timings used, post-

immersion (see Fig.35). However, there was a significant uptake ($p<0.05$) of the opsonized bacterin in the gills of the normal fish at 3 hrs. post-immersion, but there was no measurable accumulation in the gills at any of the later timings. The immune fish did not show any significant uptake or accumulation of the non-opsonized bacterin at any of the timings used, post-immersion.

ii) Uptake and accumulation in the hindgut

There was a significant uptake ($p<0.01$) of the opsonized bacterin in the hindgut of the normal fish at 3 hrs. post-immersion (Fig.36).The uptake of the non-opsonized bacterin in the hindgut of the normal fish at 3 hrs. post-immersion was not significantly greater than the activity in the control but was also not significantly different from that of the opsonized bacterin in the normal fish at the same timing, which was statisticaly significant. The uptake of the non-opsonized bacterin in the hindgut of the immune fish at 3 hrs. post-immersion was not significantly greater than the activity of the control, but, again, was not significantly different from that of the opsonized bacterin in normal fish at the same timing, which was statistically significant.

There was no measurable accumulation of bacterin in the hindgut of any of the fish at any timing later than 3 hrs. post-immersion.

iii) Accumulation in the spleen

The normal fish showed no measurable accumulation of the non-opsonized bacterin in the spleen at any of the timings used, post-

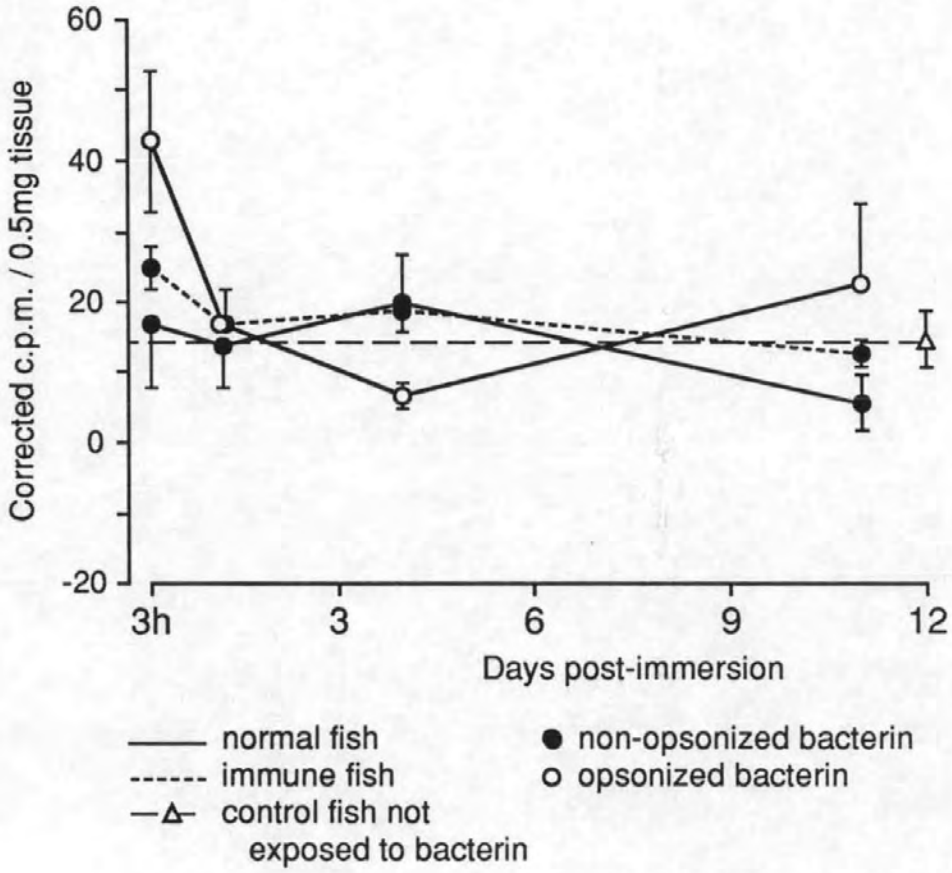


Figure 35

Uptake of radiolabelled *A. salmonicida* bacterin in the gills after a single immersion in a bath of the bacterin.
Mean ± SE, n=5.

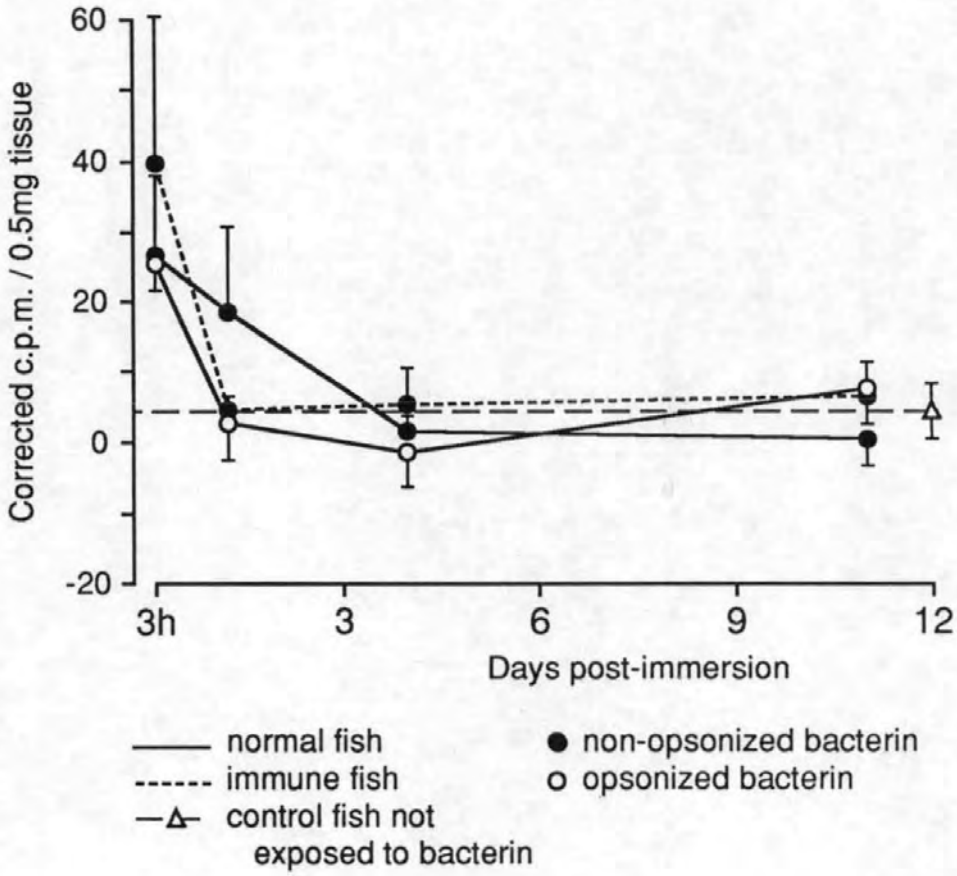


Figure 36

Uptake of radiolabelled *A. salmonicida* bacterin in the hindgut after a single immersion in a bath of the bacterin.
Mean ± SE, n=5.

immersion (see Fig.37). However, the normal fish showed a large accumulation ($p<0.02$) of the opsonized bacterin in the spleen at 3 hours post-immersion. The reading at 30 hrs. post-immersion was elevated but was not significantly greater than the activity in the control. There was no measurable accumulation at any of the later timings. The immune fish showed a significant ($p<0.001$) accumulation of the non-opsonized bacterin in the spleen at 3 hours post-immersion but, again, there was no measurable accumulation at any of the later timings.

iv) Accumulation in the liver

The normal fish showed no measurable accumulation of the non-opsonized bacterin in the liver at any of the timings used, post-immersion (see Fig.38). The accumulation of the opsonized bacterin in the liver of the normal fish at 3 hrs. post-immersion was not significant compared to the activity of the control. However, it was also not significantly different from the accumulation of the non-opsonized bacterin in the liver of the immune fish at 3 hrs. post-immersion, which was statistically significant.These immune fish showed a significant accumulation ($p<0.02$) of the non-opsonized bacterin in the liver at 3 hours post-immersion. There was no detectable accumulation of bacterin in the liver of any of the fish at any of the timings later than 3 hrs. post-immersion.

v) Accumulation in the pronephros

The normal fish showed no accumulation of the non-opsonized bacterin in the pronephros at any of the timings used, post-immersion (see Fig.39).

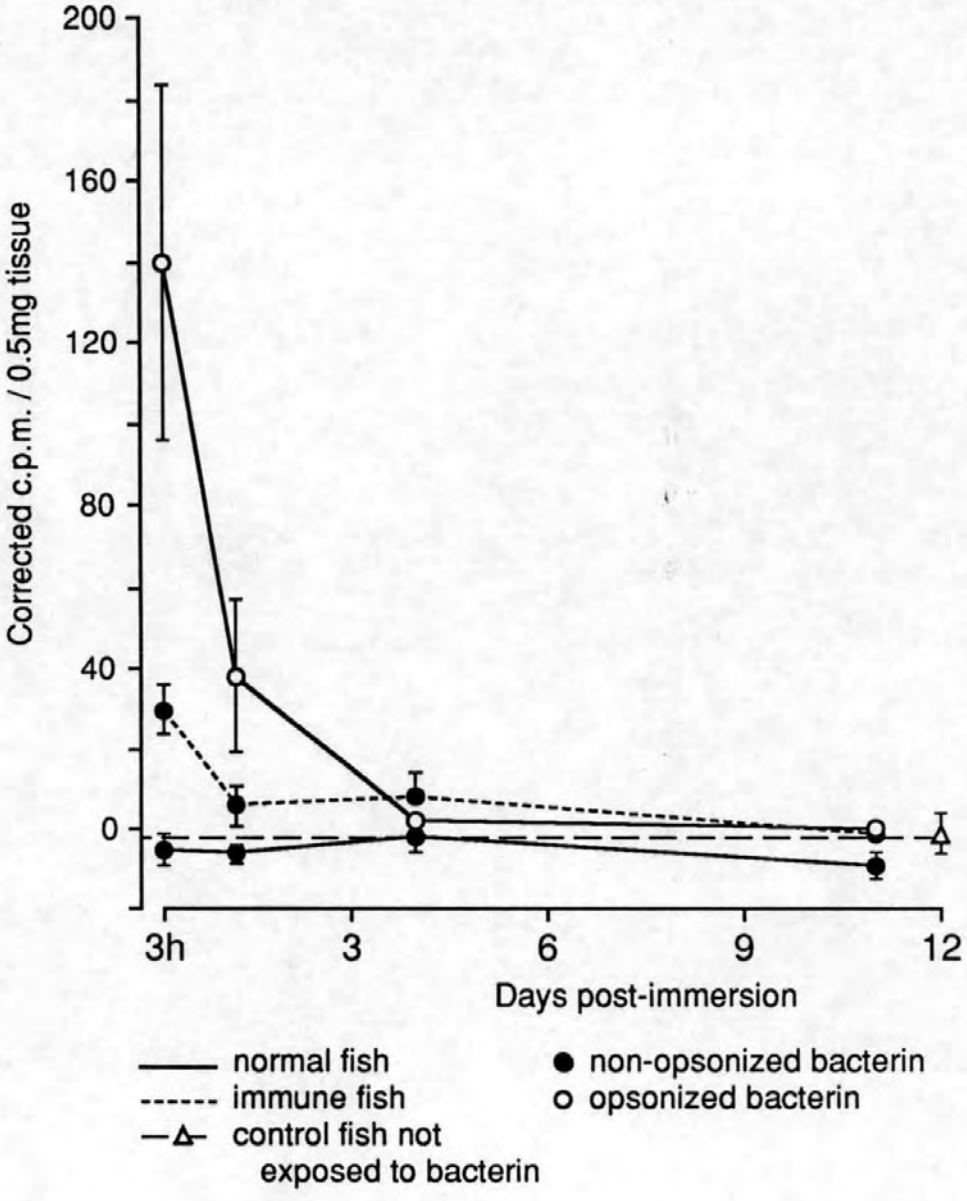


Figure 37

Uptake of radiolabelled *A. salmonicida* bacterin in the spleen after a single immersion in a bath of the bacterin.
Mean ± SE, n=5.

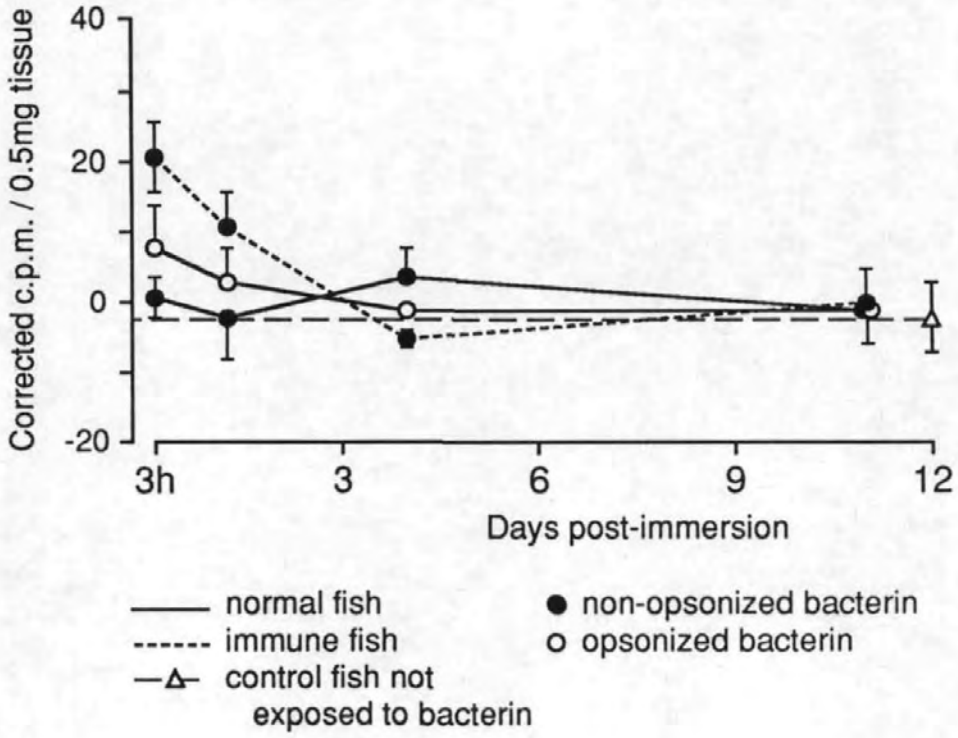


Figure 38

Uptake of radiolabelled *A. salmonicida* bacterin in the liver after a single immersion in a bath of the bacterin.
Mean ± SE, n=5.

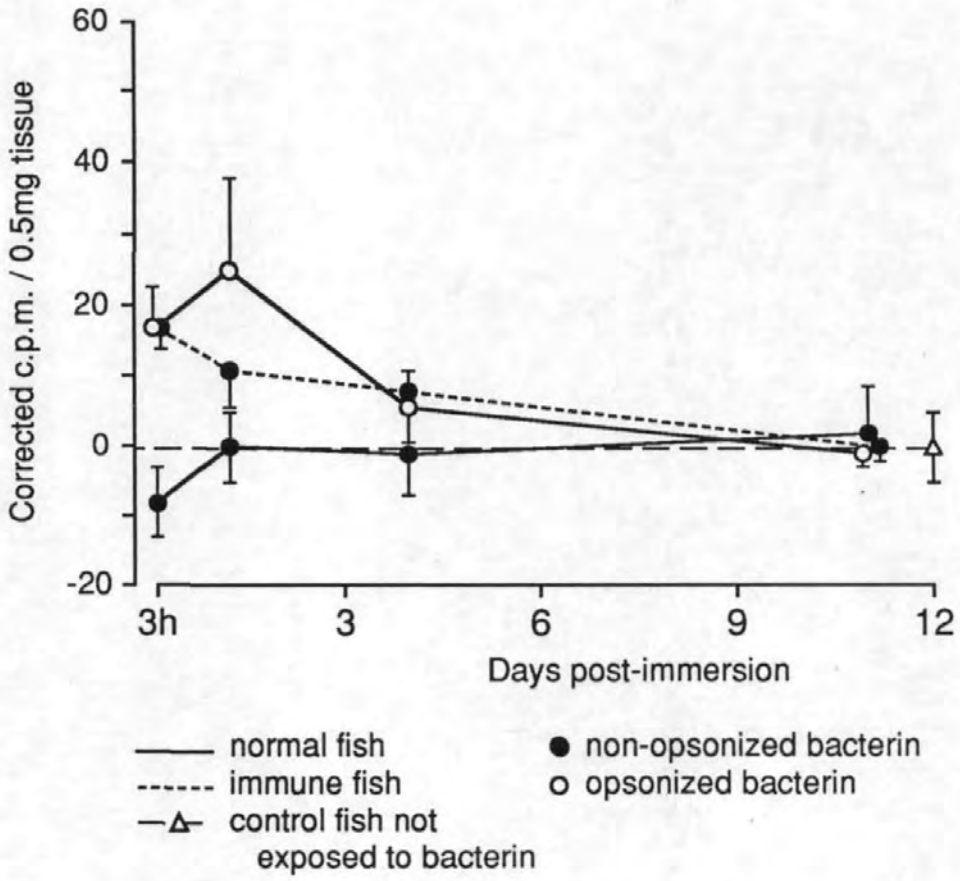


Figure 39

Uptake of radiolabelled *A. salmonicida* bacterin in the pronephros after a single immersion in a bath of the bacterin. Mean ± SE, n=5.

The normal fish did, however, show a significant ($p<0.05$) accumulation of the opsonized bacterin in the pronephros at 3 hours post-immersion. The reading at 30 hrs. post-immersion was also elevated but was not statistically significant compared to the control. There was no significant uptake of the opsonized bacterin in the pronephros of the normal fish at any of the later timings. The immune fish showed a significant ($p<0.02$) accumulation of the non-opsonized bacterin in the pronephros at 3 hours post-immersion, but again, not at any of the later timings.

DISCUSSION

These results show the uptake and accumulation of a radiolabelled *Aeromonas salmonicida* bacterin in fish following a single immersion in a bath of the bacterin. The normal fish showed no statistically significant uptake or accumulation of the non-opsonized bacterin in any of the tissues tested at any of the timings used, post-immersion. This agrees with the findings of Lamers (1985) who found no uptake of an *Aeromonas hydrophila* bacterin into the tissues of carp following an immersion in the bacterin. Tatner *et al.* (1984), however, using a radiolabelled *A.salmonicida* bacterin in trout, detected increased radioactivity in many different tissues of the fish, especially in the thymus, kidney and skin, after a single immersion in the bacterin. Suprisingly, though, this radioactivity was only detectable 5 days post-immersion with no increased radioactivity being detectable at any of the earlier times tested (1,2 and 3 days post-immersion) either in any of the separate tissues or in macerated whole fish samples. Tatner (1987) did, however, find uptake of a radiolabelled *A.salmonicida* bacterin in trout, shortly after having received a single immersion in a bath of the bacterin. However, as macerated whole fish samples only were used in this study, it cannot be known whether the bacterin had actually been internalised.

In contrast to this, Nelson *et al.* (1985) detected a *Vibrio anguillarum* bacterin in the kidney of trout 3 hours after a single immersion in a bath of the bacterin. The presence of bacterin in the kidney was noted until 24 hours post-immersion, after which it was undetectable. Nelson *et al.* also observed bacterin antigens in the gut for 3 days post-immersion, although these did not appear to be intracellular. The fact that these three studies

detected the uptake of a non-opsonized bacterin in normal, non-immune, fish and that the study presented in this chapter and Lamers (1985) did not, could have many possible explanations. Firstly, in the experiments presented here, particularly for uptake in the hindgut, the variability in readings from individual fish may possibly have masked a small amount of uptake. Also, both of the studies detecting uptake were performed on trout whereas both the study of Lamers (1985) and that presented here were performed on carp. There may be differences between the quantitave uptake of bacterins from immersion baths between salmonid and cyprinid fish. There is also the possibility that pre-existing antibody may have been present in the trout used in the studies showing uptake, due to previous exposure to the bacteria. Lastly, both of these other studies used a much higher concentration of the bacterin in the immersion bath (10^{11} cells/dm^3 and $2x10^{11}$ cells/dm^3) than the concentration used in these experiments ($5x10^9$ cells/dm^3). Although other studies (Tatner & Horne, 1983) have found no increase in uptake of a bacterin from an immersion bath at higher bacterin concentrations, there is still a possibility that the relatively low bacterin concentration used in these experiments was a contributing factor to the lack of detection of any uptake of the non-opsonized bacterin in normal fish. It is, of course , also possible that the technique for detecting the presence of radioactivity in a tissue sample, used in these experiments, was not as sensitive as the techniques used for detecting the presence of bacterin in the previously mentioned studies.

The studies presented here, however, did find that if normal fish were immersed in an opsonized bacterin bath, then uptake and accumulation of the bacterin was detectable post-immersion. The opsonized bacterin was detected in the gills, spleen, hindgut and pronephros at 3 hours post-

immersion, the greatest accumulation being in the spleen. No bacterin was detected in any of the tissues tested at 30 hours post-immersion (the next sampling time after 3 hrs.). This large accumulation in the spleen agrees with the findings of Wason (1973), who found that in mice, injected with mouse anti-SRBC before an injection of SRBC, the accumulation of SRBC in the spleen was far greater than if SRBC alone had been injected. White *et al.* (1975) also found that, in chickens, injected human serum albumin localised in the spleen within seconds when complexed with antibody. If the uncomplexed protein was injected, however, it took 25-30 hours to localise in the spleen. The time-course of bacterin uptake found in the study presented here correlates with the findings of Nelson *et al.* (1985) who detected *V.anguillarum* bacterin in the kidney of trout between 3 and 24 hours after a single immersion in a bath of the bacterin, but at no later timing, post-immersion. Opsonization of a bacterin, therefore, seems to increase its uptake in fish when administered by direct immersion, although any hypotheses on the mechanism of this can only be speculative.

These investigations also found that the non-opsonized bacterin could be detected in the tissues of previously immunized fish after a single immersion in a bath of the bacterin, whereas in normal, naive, fish it could not be detected. In these previously immunized fish, the bacterin was detected in the spleen, pronephros and liver at 3 hours post-immersion, but, again, not at any of the later timings. Suprisingly, however, no significant bacterin levels were detected in the gills or hindgut. These experiments, therefore, indicate that prior direct immersion immunization of fish to an antigen increases antigen uptake during a subsequent immersion in a bath of the same antigen. The mechanism of this effect is,

of course, also a matter for speculation. It is known that antigen-specific antibodies can be found in the mucus of fish which have been previously immunized with that antigen by direct immersion (Lobb, 1987) or by oral administration (Rombout, van den Berg, van den Berg, Witte & Egberts, 1989). On subsequent external exposure to the antigen, it is likely that the antigen would be complexed with these mucosal antibodies and, as these studies have shown that opsonization of a direct immersion vaccine increases its uptake, it is possible that the uptake of these mucosal immune complexes would be greater than that of the antigen alone.

The time-course of the uptake and internalisation of a vaccine or soluble antigen from an immersion bath is a factor which does seem to be fairly variable. Tatner *et al.*, (1984) did not find any detectable concentrations of their bacterin in the tissues of trout until 5 days after an immersion in the bacterin, which suggests that, in their experiments, it took 5 days for the fish to accumulate the bacterin in sufficient quantities in certain tissues for detection to be possible.

Anderson *et al.* (1989) also found that if the spleen cells of trout were removed at varying times after the fish had undergone an immersion in antigen, and the antibody production of these cells measured, subsequent antibody production could only be detected if the cells had been removed 4 days or more post-immersion. This was thought to represent a lag period of 4 days for enough antigen to reach the spleen for a measurable immune response to occur. Contrasting with this are the findings of Nelson *et al.* (1985) who found *V.anguillarum* bacterin in the kidneys of trout from 3 to 24 hours after a single immersion in the bacterin and Smith (1982) who also found the uptake of soluble and latex particle-bound bovine serum albumin from an immersion bath to be relatively quick, reaching the

kidneys and spleen between 8 and 24 hours post-immersion. Tatner (1987) also found bacterin uptake in whole macerated trout samples shortly after an immersion in a bath of the bacterin. The investigations presented in this chapter also found the uptake and accumulation of the bacterin from the immersion bath to be fairly swift, with bacterin being detectable in the tissues at 3 hrs. post-immersion. Strangely, with both the opsonized and non-opsonized bacterins in both the immune and normal fish, no bacterin could be detected in any of the tissues by 30 hrs. post-immersion or at any other of the later timings. One might imagine that the bacterin would accumulate in certain lymphoid tissues and be retained there, for optimal antigen presentation to occur, as in mammals it is known that antigens can be retained in secondary lymphoid organs for very long periods of time (Roitt *et al.*, 1985). Lamers (1985) also demonstrated in carp that injected bacterial antigens can be retained in the melano-macrophage centres of the lymphoid organs for up to 12 months post-injection. Of course, it has to be remembered that, in these experiments, it was the presence of radioactivity in the tissues which was being measured, and the presence of bacterin being linked indirectly to this. From these results it can only be assumed that the radioactivity was lost, and not necessarily the antigenic portions of the bacterin. It is possible that the radioactivity incorporated within the bacterin cells is dissociated from the antigenic portions of the bacterin, once the processes of antigen presentation begin.

The studies on phagocytosis in carp peritoneal exudate cells (PECs), found in this chapter, agree with the findings of Mantovani (1975) and Fries *et al.* (1987) in mammals and those of Sakai (1984) and Honda *et al.* (1986) in fish. These studies all found that opsonization of particles

with antibody alone does enhance the phagocytosis of those particles, but the effect is enhanced even further by additional opsonization with complement. The results presented here agreed with these findings and also found that opsonization with whole fresh non-immune serum did not significantly enhance phagocytosis of the particles. O'Neill (1985), however, found that opsonizing with normal trout plasma did enhance the phagocytosis of yeast cells. Yeast cells are, however, far more antigenically diverse than the human gamma globulin (HGG), used as the antigen in these experiments. It is quite possible that there would be naturally occuring antibodies in normal blood which would bind to a yeast cell and which would not bind to a protein like HGG.

These studies also investigated the phagocytosis, by carp PECs, of different sizes of latex particles, both in uncoated and HGG-coated form. Both the 0.8μm and 3.0μm particles were readily phagocytosed, however no ingestion of the 15μm diameter particles was observed. In general, it seems that the largest size of particle that normal fish macrophages and granulocytes are reported to be able to ingest is approximately 8-10μm in diameter. It is known that sheep erythrocytes (approximately 8μm in diameter) and agarose beads of 5-10μm in diameter can be ingested by fish phagocytes (Johnson & Smith, 1984; Graham, 1989), however, evidence of the total ingestion of any larger particles, including the 15μm latex particles used in these experiments, has not been published. In mouse macrophages, Shirahama *et al.* (1971) also thought a particle diameter of 10μm to be a critical size with amyloid bundles over this size not being ingested.

CHAPTER 6

Title

General Discussion

The work presented in this thesis has been largely concerned with the immune responses of carp, both humoral and cell-mediated, following direct immersion in a bath of an antigen, looking in particular at the effects of opsonization of the antigen and the particle size of the antigen on the subsequent response. The phagocytic uptake by carp peritoneal exudate cells (PECs) of antigen-coated latex particles of different sizes and of opsonized antigen-coated particles was also studied in order to discover any links between the phagocytic uptake of these particles by carp PECs and the immune response following direct immersion immunization with these same particles. The uptake and accumulation of a radiolabelled bacterial vaccine from an immersion bath was also investigated, with the effects of opsonization of the vaccine and the effects of the immune status of the recipient fish being studied.

These studies found that the opsonization of immersion vaccines with immune carp serum significantly increased the immunological memory for both the humoral and the cell-mediated immune responses following immersion. This effect was only seen when the vaccines were opsonized with immune serum, opsonization with normal serum having no detectable effect. It was also found that opsonization of a bacterial immersion vaccine with immune carp serum increased the uptake and accumulation of the vaccine in carp immersed in the vaccine.

The exact mechanisms by which these effects are mediated and their site, or sites, of action are, as yet, unknown. Anderson *et al.* (1984) proposed two theories concerning the initial processes of the immune response to externally applied antigens. A selective uptake theory suggesting that the discrimination of the antigen is through specific

receptors at the external surface, and a non-selective theory which suggests that the determination of an active immune response against a specific antigen occurs after its internalisation. The fact that opsonization seems to increase the uptake of a direct immersion vaccine suggests that Fc or complement receptors may be involved. If this is the case then whether this effect is mediated by increased uptake at external epithelial surfaces or by increased trapping in the lymphoid organs is still a matter for speculation. It has already been shown that, if administered by intraperitoneal injection, an antigen is trapped better in the lymphoid organs if in the form of an immune complex (Secombes *et al.*, 1982a). However, the effect of opsonization on uptake from immersion baths, shown here, may well be a combination of both these factors (i.e. increased uptake at the epithelial surfaces and increased accumulation in the lymphoid organs).

The phagocytic uptake of particles by carp PECs was also found to be enhanced by the opsonization of the particles with fresh immune serum, but yet again no increase in uptake was found when the particles were opsonized with normal carp serum.

These studies therefore conclude that opsonization with fresh immune carp serum increases both the uptake of and the immune response to an antigen, when delivered by direct immersion, and also the phagocytic uptake of particulate antigens by carp peritoneal leukocytes.

On the question of the effect of the particle size of an immersion vaccine on its efficacy of vaccination, these investigations found that, when administered by direct immersion, a protein antigen stimulated the immune system less when administered in soluble form than when coated onto latex particles. All the vaccine particle sizes used in these

experiments (0.05μm to 15μm) were found to stimulate a greater immune response than the soluble antigen. When the phagocytic uptake of these particles was assessed in carp PECs, however, it was found that the PECs were unable to ingest the largest size of particle used in the direct immersion experiments (15μm). As it is assumed that a systemic immune response to an antigen administered by direct immersion must involve some uptake of the antigen into the fish, this suggests that either the antigen is detached from the 15μm latex particles before absorption or that the mechanism by which these 15μm particles are taken up by the carp during direct immersion may not actually involve any cellular ingestion of the particles. The findings in this thesis only show, of course, that it is carp peritoneal exudate cells which cannot ingest the 15μm particles. It is possible that the epithelial phagocytic cells may have different phagocytic qualities and be able to ingest these large particles. There is evidence of particle uptake in both intestinal (Davina *et al.*, 1982; Rombout *et al.*, 1986; Rombout & van den Berg, 1989) and gill (Goldes *et al.*, 1986; Zapata *et al.*, 1987) absorptive cells, but the largest of the particles used in any of these studies were only 5μm in diameter.

Volkheimer (1977) found that large particles could pass through the gut wall of both mammals and birds into the bloodstream. This mechanism, called persorption, did not involve any phagocytosis of the particles, and seemed to occur by the passage of the particles through intercellular gaps between the intestinal epithelial cells. Using starch granules and PVC particles, amongst others, it was demonstrated that particles of up to 150μm in diameter could pass through the gut into the bloodstream, although the optimum size seemed to be from 7-70μm. This phenomenon seems to occur relatively quickly and, in humans, the peak detection of

starch granules in the blood occurs only 90 mins. after ingestion. It is known that, during direct immersion immunization of fish, a certain proportion of the vaccine is to be found in the gut lumen due to swallowing (Watson, 1984; Robohm, 1986). Whether some mechanism similar to that of persorption has any effect on the uptake and subsequent immune response of fish to swallowed antigens is a matter for conjecture, although it might account for the successful priming by direct immersion using HGG coated onto 15μm diameter latex particles, observed in this study. It is even possible that some mechanism similar to persorption is responsible for a proportion of the uptake of all the particle sizes used in these direct immersion experiments. Obviously further work is needed to establish the exact mechanism of uptake by fish of antigens from the external aqueous environment and the respective roles of the gut and gills as portals of antigen entry.

In these investigations, to achieve a more complete understanding of the immune reactions of fish following antigenic challenge, and of special interest in these studies, to antigenic challenge by direct immersion, it was necessary to develop a technique to assess the cell-mediated immune response of fish. The technique used here, involving the assay of chemotactic activity in the supernatants from incubations of pronephric cells with antigen confirmed that fish leukocytes do, in fact, produce a chemotactic cytokine (or cytokines). Many cytokines, produced in mammals, have now been demonstrated in fish. Macrophage activating factor (Smith & Braun-Nesje, 1982; Secombes, 1987; Graham, 1989), migration inhibition factor (Jayaraman *et al.*, 1979; Blazer *et al.*, 1984; Howell, 1984), an interferon-like factor (Graham & Secombes, 1990), T-cell growth factor (Caspi & Avtalion, 1984; Grondel & Harmsen, 1984) and

now chemotactic factor have been shown to be active components of cell communication in fish. Surely it is only a matter of time before other cytokines, already known in mammals, are sought in fish, thus leading us further towards a better understanding of this complex subject of cell-mediated immunity.

It is well known in the mammalian field that certain antigens stimulate the humoral and cell-mediated immune systems to very different degrees (Dennert & Tucker, 1972). For example it is known that formalinised sheep red blood cells (SRBC), on a primary injected challenge with the antigen, can stimulate a good cell-mediated immune response but with virtually no humoral response in both fish (Mohan, 1977) and lizards (Jayaraman & Muthukkaruppan, 1978a). The dose of an injected antigen also seems to be important in determining the comparative levels of response of the cell-mediated and the humoral immune systems. It has been shown in mammals (Mackaness *et al.*, 1974), reptiles (Jayaraman & Muthukkaruppan, 1978a,b) and fish (Jayaraman *et al.*, 1979; Rijkers *et al.*, 1980) that very low doses of an injected antigen can stimulate a good cell-mediated response but with virtually no humoral response. If the injected dose is slightly greater, the cell-mediated response seems to be less with the humoral response being detectable until at fairly high doses, almost the entire response is humoral with virtually no detectable cell-mediated response. Jayaraman & Muthukkaruppan (1978a) also looked at the kinetics of both the cell-mediated and the humoral responses following injection of SRBC in the lizard. If a low dose injection was given, initially the cell-mediated response was good but with very little humoral response (as before) but as the cell-mediated reponse waned, the humoral response increased. In comparison to this, if a high dose of

antigen was given, initially the humoral response was good, with very little cell-mediated response, but as the humoral response decreased, the cell-mediated response increased, seemingly to compensate. Jayaraman & Muthukkaruppan (1978) concluded from these experiments that the induction of antibody synthesis needs a higher threshold level of antigen for its full expression, in contrast to the minimal antigenic dose required for the cell-mediated response. This evidence, as well as confirming the need for the study of both the humoral and the cell-mediated immune systems in any immune response under investigation, suggests that very different results might be obtained when investigating the immune reactions of an animal to an antigen, if several different antigenic doses were used.

The experiments presented in this thesis found that although immunological memory, for both the humoral and cell-mediated immune systems, could be stimulated by direct immersion immunization with an antigen, no actual detectable immune response could be found following immersions only. This still leaves the question of how direct immersion immunization against pathogens can confer protective immunity to a virulent challenge with the pathogenic organism, which it clearly seems to achieve (Gould *et al.*, 1979; Johnson *et al.*, 1982a, 1982b; Newman & Majnarich, 1982). Perhaps immunological memory alone, induced by direct immersion immunization, is enough to sufficiently speed up the protective response, during virulent challenge. It is also possible that the secretory immune system, which was not studied in these investigations, plays a far greater role in the protection of fish against water-borne pathogens than is generally realised. Obviously the secretory immune system is the first immune system to encounter any external antigens, and therefore is the first line of immune defence. The studies of Rombout *et al.*,

(1986), Lobb (1987) and Rombout, van den Berg, van den Berg, Witte & Taverne-Thiele (1989) all found either skin or gut mucus antigen-specific antibody titres increased after either immersion or oral vaccination with antigen. The interactions between systemic immunological memory and response and the secretory immune system in fish are, as yet, virtually a mystery. Further investigations into the secretory immune system and how it relates to the systemic immune system must surely increase our understanding of direct immersion immunization and the mechanisms of response to it.

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