IMMUNOLOGIE VAN DE KARPER

GER RIJKERS

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INHOUD

VOORWOORD

Proefschriften worden in het algemeen nauwelijks gelezen. In de meeste gevallen beperkt men zich tot de laatste stelling, het dankwoord en de levensloop. Hiervoor zijn 3 (goede) redenen aan te wijzen:

1) de tekst is meestal in het Engels gesteld,
2) het onderwerp is erg gespecialiseerd en zeker voor een leek nauwelijks te begrijpen,
3) wetenschappelijke teksten zijn saai.

In dit gedeelte van het proefschrift wilde ik vertellen wat ik gedurende 3 jaar met "die karpers" gedaan heb. De tekst is in het nederlands, verder heb ik geprobeerd ook het 2e en 3e argument te ondervangen.
Ik hoop dat U na het lezen van dit gedeelte met mij de volgende stelling kunt onderschrijven:

Immunologie is niet moeilijk.

ALGEMEEN

Om zinvol te kunnen praten over immunologie is het noodzakelijk om eerst iets te vertellen over cellen, DNA, RNA, aminozuren, eiwitten etc. Iedereen heeft deze termen vast wel eens ergens gehoord, maar de betekenis en onderlinge samenhang zal niet iedereen duidelijk zijn.

CELLEN

Een cel kunnen we omschrijven als de kleinste eenheid binnen een organisme die nog tot alle basisfuncties in staat is. Als basisfunctie beschouwen we het in leven blijven en het in staat zijn om zich te delen. Omdat definities nooit zo erg duidelijk zijn (definitie van een stoel?) volgen hier enkele voorbeelden.

Zoals een huis is opgebouwd uit stenen, een rekenmachientje uit chips, zo zijn dieren en planten opgebouwd uit cellen. Hogere dieren en planten zijn opgebouwd uit zeer veel cellen (de mens b.v. uit 2 à 300.000.000.000.000 cellen). Uit de definitie van een cel volgt dat er ook organismen kunnen voorkomen bestaande uit één enkele cel. Voorbeelden hiervan zijn bacteriën en het pantoffeldiertje. Het pantoffeldiertje heeft het vermogen tot bewegen, voedsel- en zuurstofopname verenigd in die ene cel. De cellen van hogere organismen zijn gespecialiseerd, dat wil zeggen elke cel heeft z'n eigen functie.

Specialisatie van cellen bij dieren

cel	functie
rode bloedcel	transport van zuurstof
darmcel	opname van voedsel
spiercel	beweging

Een cel moet aan een aantal vereisten voldoen om in leven te kunnen blijven en om zijn speciale functie uit te kunnen oefenen. Deze vereisten zullen we bespreken aan de hand van een voorbeeld: de plasma cel. De functie van een plasmacel is het produceren van antilichamen of antistoffen (daar komen we later nog uitvoerig op terug). Binnen een cel kunnen we verschillende onderafdelingen onderscheiden: een informatiecentrum (de kern), energiecentrales (mitochondriën), fabrieken (ribosomen), afvalverwerkingsbedrijven (lysosomen), inpakafdelingen (Golgi-apparaat) en verder nog een infrastructuur (endoplasmaties reticulum). Zie fig. 1.

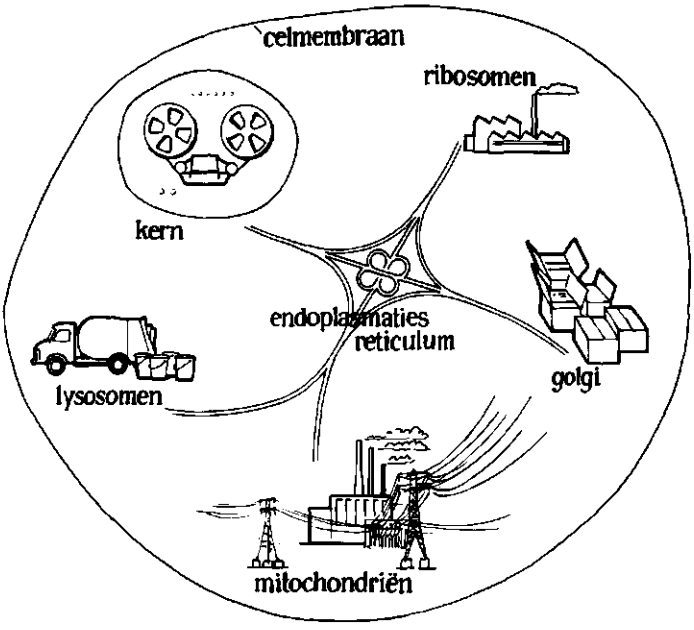


Fig. 1: De onderdelen van een cel.

Om te voorkomen dat deze processen in de soep lopen (de afvalverwerking gaat het informatiecentrum opruimen) zijn al deze onderafdelingen van elkaar gescheiden door een tussenwandje (membraan). De cel als geheel is ook omgeven door een wand, de celmembraan. Deze celmembraan zorgt er niet alleen voor dat het hele zaakje bij elkaar gehouden wordt, maar ook dat grondstoffen (voor de fabrieken en de energiecentrale) naar binnen of naar buiten kunnen.

Een organisme ontstaat door versmelting van een eicel met een zaadcel. Door deling van deze ene cel ontstaan dan 2 cellen, die zich weer delen, enz. Dat dit snel kan aantikken is bekend uit de parabel van de graankorrel en het schaakbord (fig. 2). Wanneer we links bovenaan met 1 graankorrel beginnen, op het 2e vakje 2 korrels, op het 3e vakje 4, enz., bereiken we vrij snel de wereld graan productie. In fig. 2 zien we ook waar we uitkomen voor een volwassen mens als we links boven op het schaakbord beginnen met een eicel en een zaadcel.

Bij deling wordt de erfelijke informatie (opgeslagen in de kern) keurig verdeeld over de twee cellen. Hierbij gaat geen informatie verloren, want voordat een cel zich deelt, wordt eerst alle informatie gecopieerd zodat de twee nieuwe cellen weer de oorspronkelijke hoeveelheid bezitten. Het gevolg hiervan is dat elke cel dus alle erfelijke informatie bezit.

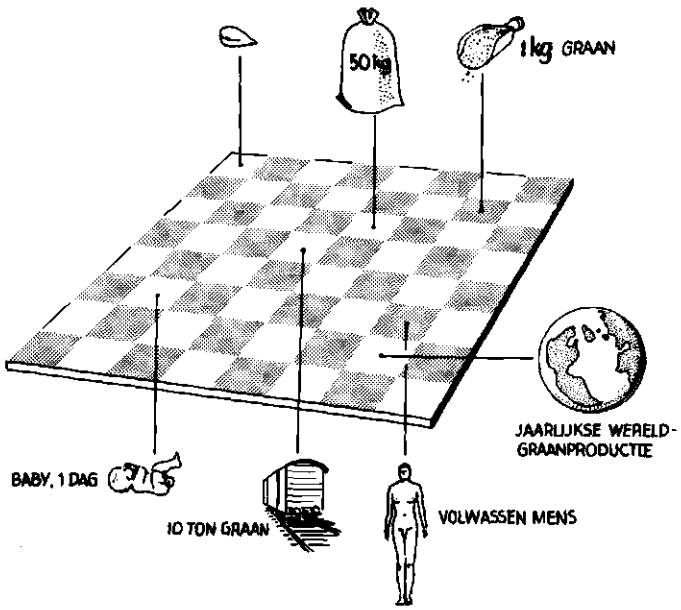


Fig. 2: De graankorrel op het schaakbord.

De erfelijke informatie in de kern is gecodeerd. We kunnen dit vergelijken met een computer, waar de informatie op een magneetband (DNA) gecodeerd ligt. Als er een bepaalde opdracht moet worden uitgevoerd, b.v. de produktie van antilichamen, dan wordt in de kern het benodigde stukje informatie overgeschreven op "boodschapper RNA". Deze boodschapper brengt de opdracht (in code) over naar de ribosomen, die zich buiten de kern verspreid in de cel bevinden. Er zijn verschillende

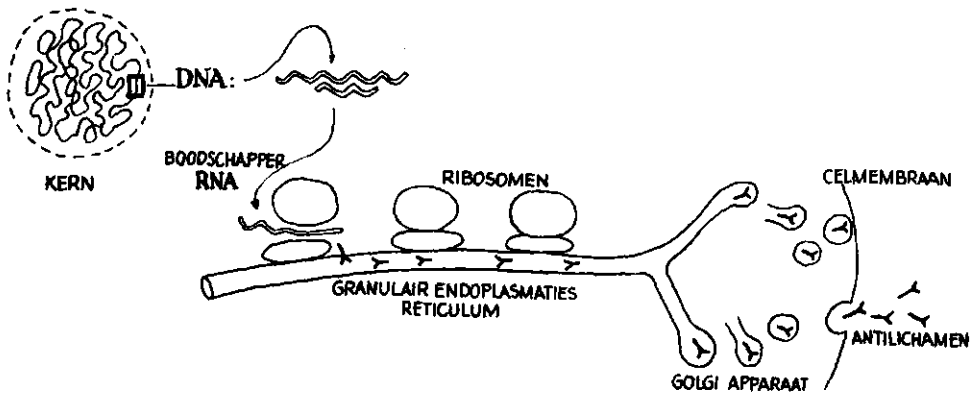


Fig. 3: Aanmaak van eiwitten.

soorten ribosomen. Zo zijn er losse ribosomen die eiwitten maken voor eigen gebruik, zoals b.v. eiwitten die nodig zijn voor de vervanging van versleten onderdelen. Ribosomen die eiwitten maken die bestemd zijn voor de export (zoals b.v. antilichamen) liggen op het endoplasmaties reticulum zodat de eiwitten meteen naar de inpakafdelingen getransporteerd kunnen worden. Endoplasmaties reticulum met ribosomen erop noemt men granulair endoplasmatisch reticulum (GER); kant-en-klare eiwitten worden door het Golgi-apparaat ingepakt in blaasjes. Door middel van deze blaasjes worden de eiwitten uitgescheiden (fig. 3).
Eiwitten zijn opgebouwd uit aminozuren. Er zijn 20 verschillende aminozuren. De eigenschappen van een eiwit worden bepaald door het aantal, de samenstelling en de volgorde van de aminozuren. We kunnen eiwitten vergelijken met woorden. Woorden zijn opgebouwd uit letters. Er zijn 26 verschillende letters. De betekenis van een woord wordt bepaald door het aantal, de samenstelling en de volgorde van de letters.

IMMUNOLOGIE

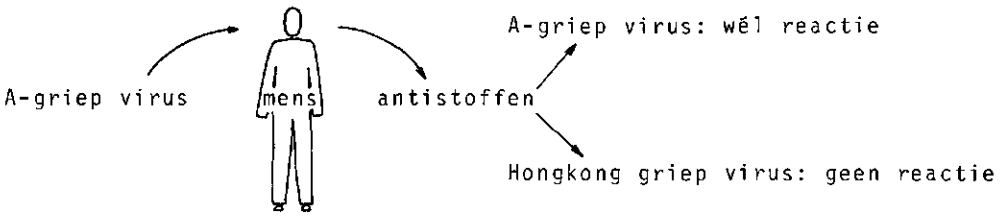
Alle gewervelde dieren zijn uitgerust met een bewakingssysteem (immuunsysteem), dat er voor zorgt dat binnengedrongen vreemde stoffen of cellen onschadelijk gemaakt worden. Deze vreemde zaken kunnen bijvoorbeeld bacteriën zijn of virussen of eiwitten. Een immuunsysteem moet om die ongewenste bezoekers (die we antigenen noemen) te herkennen, onderscheid kunnen maken tussen "vreemd" en "eigen". Onder eigen verstaan we de cellen, eiwitten, hormonen enz. waaruit het lichaam is opgebouwd. Dit vermogen om onderscheid te kunnen maken is erg belangrijk omdat het natuurlijk niet de bedoeling van het immuunsysteem is om het eigen lichaam af te breken.

Een voorbeeld uit de praktijk kan veel van de immunologie duidelijk maken. Als je een griepje oploopt kun je een paar dagen goed ziek zijn. Het griepvirus is dan je lichaam binnengedrongen en is zich daar naar hartelust aan het vermenigvuldigen. Na enige tijd ben je echter weer genezen. Wat er zich in de tussentijd heeft afgespeeld is het volgende: het immuunsysteem heeft het virus als vreemd herkend en het daarna onschadelijk gemaakt.

Antigenen en antistoffen

Vreemde stoffen roepen een bepaalde reactie op wanneer ze bij mens of dier worden ingespoten. De vreemde stoffen noemen we met een verzamelnaam antigenen. Men heeft gevonden dat voor het oproepen van een immunologische reactie niet een compleet eiwit of een hele cel nodig is. Een groepje van 6-7 aminozuren is al voldoende. Zo'n eenheid noemen we antigene determinant.

Als reactie op het inspuiten van een antigeen worden antistoffen geproduceerd. Deze antistoffen binden specifiek met het antigeen: dat wil zeggen ze reageren alléén met het antigeen waarmee het dier is ingespoten en nergens anders mee. Specificiteit is één van de belangrijkste kenmerken van een immunologische reactie.



Antistoffen, die ook wel immunoglobulines worden genoemd, komen voor in het bloed, maar ook in de rest van het lichaam. Bij mensen komen verschillende soorten immunoglobulines voor. De belangrijkste zijn

IgG en IgM (Ig = immunoglobuline, G en M geeft de klasse aan).
In figuur 4 staan IgG en IgM schematisch getekend. Het gearceerde gedeelte van het immunoglobuline bindt (specifiek) aan het antigeen. We zien dat IgG 2 bindingsplaatsen heeft, IgM zelfs 10.

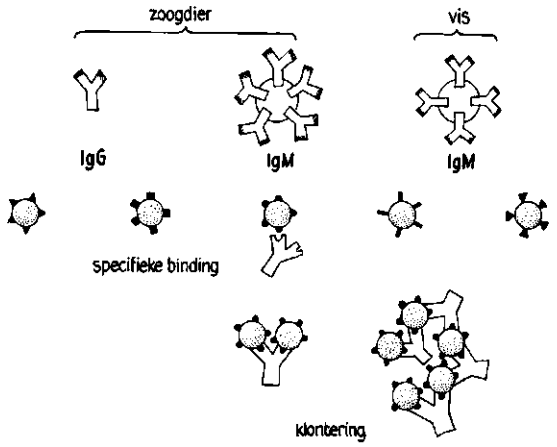


Fig. 4: Antistoffen

We zien dat immunoglobulines zich kunnen binden aan lichaamsvreemde stoffen. Dit alleen is echter niet voldoende om b.v. een ziekteverwekkende bacterie onschadelijk te maken. Dit onschadelijk maken kan op twee manieren gebeuren: de eerste manier is door macrofagen (fig.5). Deze holle bolle Gijzen zijn grote cellen die graag rommel opruimen. Ze doen dat niet specifiek; alles wat vreemd is wordt opgepeuzeld (macrofaag betekent letterlijk veelvraat).

Fig. 5: Macrofaag

Omdat immunoglobulines meerdere bindingsplaatsen hebben, zijn ze in staat om antigenen aan elkaar te doen klonteren. Deze samengeklonterde antigenen zijn dan extra lekker voor macrofagen. Een tweede manier waarop antigenen onschadelijk gemaakt worden nadat antilichamen gebonden zijn is de volgende.
In het bloed bevindt zich een hele reeks van stoffen die we met een verzamelnaam het complementsysteem noemen. Wanneer een antilichaam gebonden is aan een antigeen (fig. 6) verandert er iets aan het antilichaam waardoor het eerste deel van het complementsysteem (C1) gebonden wordt. C1 activeert dan C2, C3 activeert C4 enz. totdat het laatste deel C9 geactiveerd wordt. C9 is een stof die de membraan van de vreemde cel doet oplossen, waardoor de vreemde cel gedood wordt.

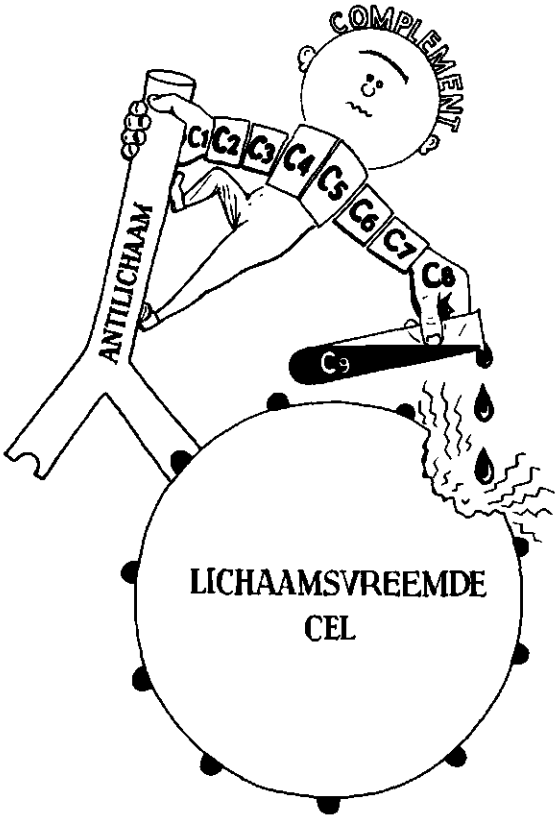


Fig. 6: Werking van complement.

De belangrijkste kenmerken van het immuunsysteem zijn de specificiteit en de vorming van (immunologisch) geheugen.
Tegen griep kun je je in laten enten. Je krijgt dan een injectie met gedood griepvirus. Omdat het virus dood is kan het zich niet meer ver-

menigvuldigen en kun je van zo'n injectie niet ziek worden. Het immuunsysteem herkent het griepvirus echter wél als vreemd en reageert daarop. Er worden antistoffen gevormd, maar er wordt ook immunologisch geheugen opgewekt. Na een griepspuit ben je niet meer vatbaar. Dit betekent echter niet dat je dan niet meer met het virus besmet kunt raken. Het virus dringt nog wel het lichaam binnen, maar het immuunsysteem reageert zo snel en zo doeltreffend dat het virus al onschadelijk gemaakt is voordat het je ziek heeft kunnen maken. Je bent dan immuun voor de griep. Het immunologisch geheugen, dat daarbij gevormd wordt, houdt lang aan. De bekende DTKP prikken maken je gedurende de rest van je leven immuun voor difterie, tetanus, kinkhoest en polio. Toch biedt een griepspuit over het algemeen maar bescherming voor één jaar; het volgend jaar kun je toch weer de griep krijgen. Dit wordt niet veroorzaakt door een kortdurend immunologisch geheugen maar heeft alles te maken met de specificiteit van het immuunsysteem. Een griepepidemie krijgt - net als orkanen - een naam: de A-griep, de Hongkonggriep enz. Men gebruikt die verschillende namen omdat het telkens weer een ander virus betreft. Alhoewel die verschillende virussen wel allemaal griep veroorzaken, verschillen ze onderling zó sterk dat het immuunsysteem zich niet meer herinnert daarmee ooit in aanraking te zijn geweest. Een A-griep spuit biedt dan ook geen bescherming tegen een infectie met het Hongkong-griep virus.

Humorale en cellulaire immuniteit

In de immunologische reacties die we tot nu toe besproken hebben wordt de vreemde stof onschadelijk gemaakt met behulp van antilichamen. Men noemt deze vorm van verdediging humoral immuniteit. Humoraal betekent vloeibaar. Een dergelijke naam is gekozen omdat deze vorm van immuniteit overgebracht kan worden door een dier met serum van een immuun dier in te spuiten. De cellen die betrokken blijken te zijn bij humorale reacties heten B-lymfocyten.

Een andere vorm van immuniteit is de zogenaamde cellulaire immuniteit. Voorbeelden uit de praktijk zijn de afstoting van een getransplanteerd orgaan en de Mantoux-reactie (krasjes bij TBC-controle). Bij een cellulaire immunologische reactie worden geen antilichamen gemaakt, de vreemde cel wordt onschadelijk gemaakt door direct contact met een lymfocyt (fig. 7). De lymfocyten betrokken bij cellulaire immuniteit noemt men T-lymfocyten. De twee kenmerken van een immunologische reactie specificiteit en geheugen gelden voor zowél humorale als cellulaire reacties.

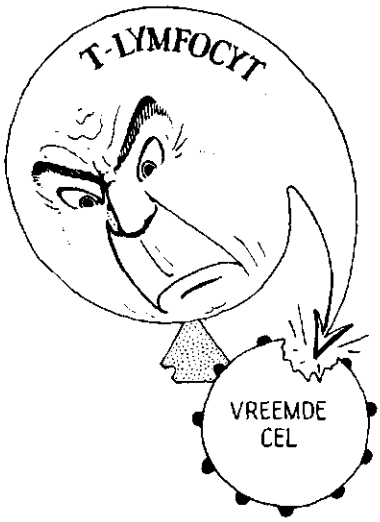


Fig. 7.

Organen

Belangrijke immuunorganen bij de mens zijn milt, thymus (zwezerik), beenmerg, lymfeklieren en in mindere mate tonsillen (amandelen) en appendix (blinde darm). T-lymfocyten komen uit de thymus en B-lymfocyten worden aangemaakt in het beenmerg. Vissen bezitten geen beenmerg of lymfeklieren. De belangrijkste organen bij een vis zijn thymus, milt, kopnier en nier (fig. 8).

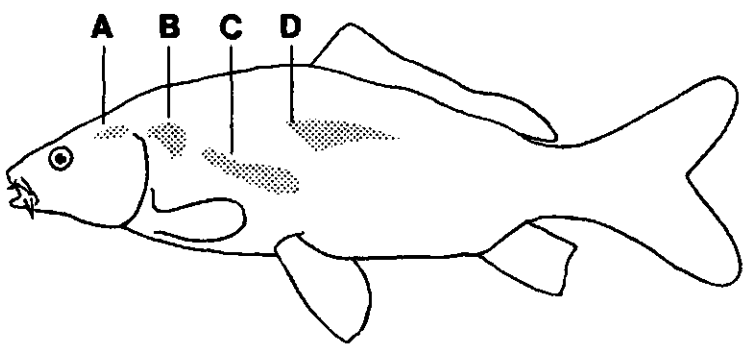


Fig. 8: Immuunorganen bij de karper

De thymus bestaat vrijwel uitsluitend uit lymfocyten, in milt, kopnier en nier komen naast lymfocyten ook veel rode bloedcellen en granulo-

cyten voor. Behalve in de immuunorganen komen lymfocyten ook voor in het bloed waar ze behoren tot de witte bloedlichaampjes.

Immunologische reactie

Wat gebeurt er nu precies als een vreemde stof het lichaam is binnengedrongen. We hebben al besproken dat antilichamen specifiek een vreemde stof kunnen binden. Lymfocyten hebben antilichamen aan hun celoppervlak. Door middel van deze oppervlakte-antilichamen zijn ze in staat om vreemde stoffen te herkennen. Deze herkenning is specifiek, d.w.z. een lymfocyt herkent maar één bepaalde vreemde stof en een vreemde stof wordt maar door één lymfocyt (of een groepje identieke lymfocyten) als vreemd herkend. In figuur 9 is deze specifieke herkenning vergeleken met een aangerande maagd (lymfocyt) die met haar hand (oppervlakte-antilichaam) uit een hele reeks van verdachten haar aanvaller (vreemde cel) herkent aan de vorm van zijn oren (antigene determinant).

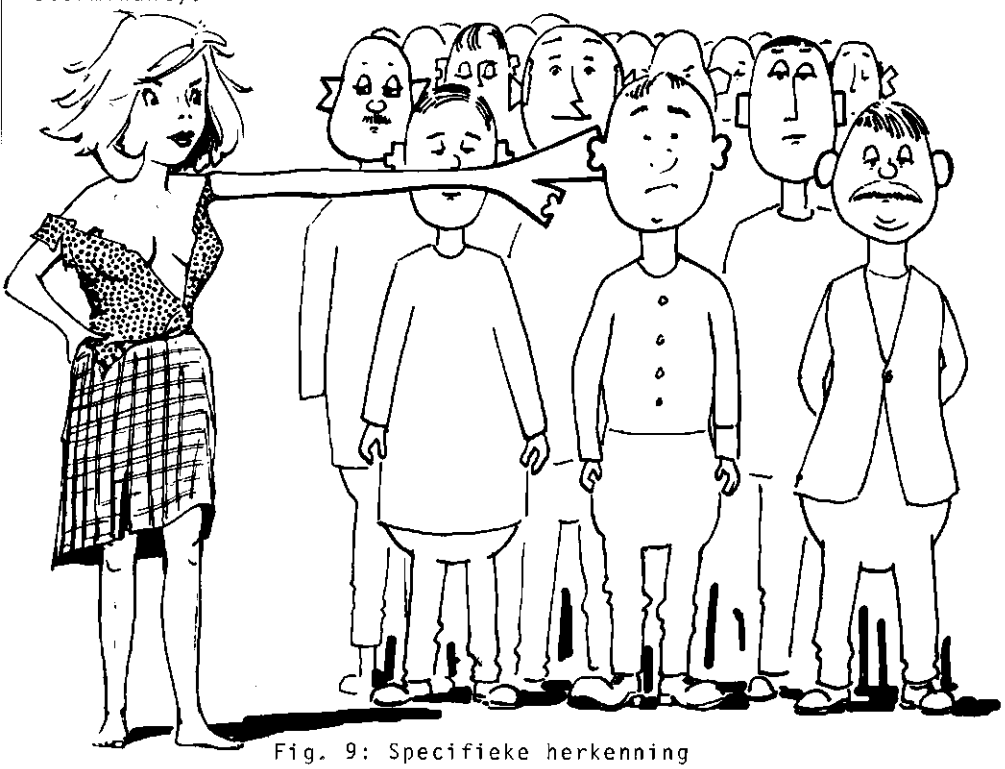


Fig. 9: Specifieke herkenning

Nadat de herkenning heeft plaats gevonden (tussen vreemde stof A en lymfocyt a) gaat de desbetreffende lymfocyt zich delen. Op die manier ontstaan er veel lymfocyten die allemaal specifiek zijn voor de vreemde stof A. Daarna veranderen (differentiëren met een mooi woord) de

lymfocyten in plasmacellen. Plasmacellen produceren veel antilichamen (specifiek gericht tegen vreemde stof A) en deze antilichamen komen onder andere in het bloed terecht. De manier waarop antilichamen een vreemde stof herkennen en onschadelijk maken hebben we reeds besproken.

Nadat herkenning heeft plaatsgevonden veranderen gelukkig niet alle lymfocyten in een plasmacel. Een gedeelte wordt namelijk geheugencel. Deze geheugencellen zorgen ervoor dat als er weer een infectie met vreemde stof A optreedt het hele proces van herkenning tot en met antilichaamproductie veel sneller en heftiger verloopt.
Als voorbeeld dient een grafiek van het aantal plasmacellen in de kopnier van een karper tijdens een eerste en een herhaalde reactie (fig. 10).

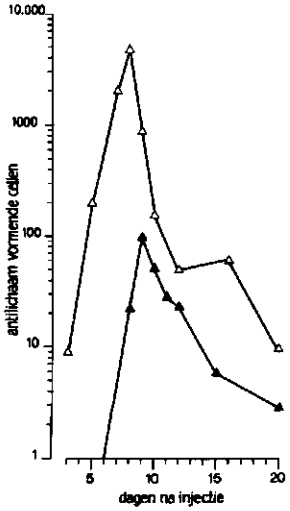


Fig. 10: Een eerste (▲) en herhaalde (△) immunologische reactie in de kopnier van een karper ingespoten met schape rode bloedcellen.

TECHNIEKEN

In dit hoofdstukje worden enkele technieken besproken die gebruikt zijn bij het onderzoek van het immuunsysteem van de karper.

Rozet test

Met behulp van de rozet test is het mogelijk om het aantal lymfocyten gericht tegen een bepaalde vreemde stof te bepalen. We maken daarbij gebruik van het feit dat de oppervlakte-antilichamen van een lymfocyt een vreemde stof kunnen binden.

Van een karper die ingespoten is met konijne rode bloedcellen (KRBC) verwijderen we de milt. De milt wordt fijngeknipt en door een fijnmazig gaasje gewreven zodat we een oplossing krijgen bestaande uit losse miltcellen. Aan deze celsuspensie voegen we KRBC toe en vervolgens zetten we dit mengseltje een tijdje in de koelkast. Als er in de milt lymfocyten aanwezig waren specifiek gericht tegen KRBC, dan binden de oppervlakte-antilichamen van de lymfocyt aan de KRBC. Op die manier ontstaat er een krans van KRBC rondom zo'n lymfocyt: een rozet (fig. 11). Door op verschillende dagen na inspuiten het aantal rozetten te tellen kun je een idee krijgen hoe de lymfocyten reageren op deze lichaamsvreemde stof.

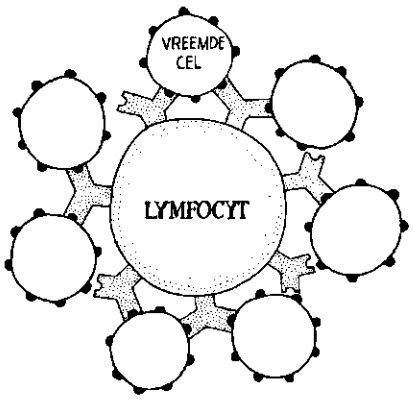


Fig. 11: Rozet vormende cel.

Plaque test

Bij de plaque test bepalen we het aantal plasmacellen dat antilichamen uitscheidt tegen een bepaalde vreemde stof of cel. Als vreemde cel gebruiken we nu niet KRBC maar schape rode bloedcellen (SRBC). Ook hier maken we weer een celsuspensie en mengen deze met SRBC. Bovendien wordt complement aan het mengsel toegevoegd. Dit mengseltje laten we tussen twee microscoop glaasjes lopen. De SRBC en de karpercellen zijn zodanig verdund dat 1) tussen de glaasjes een monolayer

ontstaat, d.w.z. de cellen liggen tegen elkaar aan in een enkele laag; 2) er veel meer SRBC zijn dan karpercellen. Als er in het mengsel plasmacellen aanwezig waren dan gaan deze cellen tussen de glaasjes rustig door met de taak waar ze mee bezig waren, namelijk het maken en uitscheiden van antilichamen. De antilichamen binden zich aan de SRBC, die immers in overmaat aanwezig zijn, vervolgens wordt het complement geactiveerd en de SRBC in de buurt van de plasmacel worden gedood. Op die manier ontstaat er rondom de plasmacel een gat dat na verloop van tijd met het blote oog kan worden waargenomen. Door het aantal gaten (plaques) te tellen komen we te weten hoeveel plasmacellen er aanwezig waren in een bepaald orgaan. Foto's van plaques staan in appendix I en II.

Schubtransplantatie

Een methode om de cellulaire immuniteit te testen is door schubben te transplanteren en vervolgens te bekijken hoe lang het duurt voordat zo'n schub afgestoten is.

Bij de karper liggen de schubben als dakpannen over elkaar heen. Het is mogelijk om bij een vis een schub te verwijderen en daarvoor een schub van een andere vis in de plaats te zetten. In figuur 12 staat aangegeven hoe we dat ongeveer gedaan hebben.

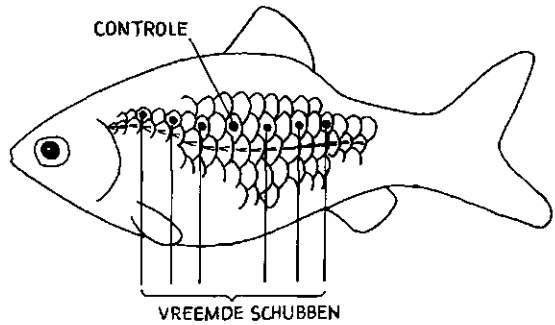


Fig. 12: Schema voor schubtransplantatie.

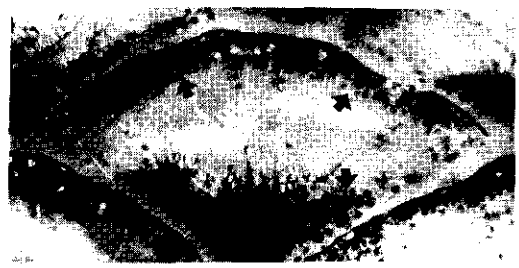


Fig. 13: Schub van een vis. Pigmentcellen zijn aangegeven met een pijl.

Een getransplanteerde schub wordt herkend als lichaamsvreemd en het immuunsysteen neemt daarom maatregelen: de pigmentcellen, die in een normale schub rond zijn (fig. 13), vertakken zich. Wat later krijgt de getransplanteerde schub een melkwit-achtige schijn over zich. Dit wordt veroorzaakt door de lymfocyten die op de vreemde schub afkomen en deze afbreken. Na een aantal dagen verdwijnen de lymfocyten weer en blijft er alleen nog maar een doorschijnend stukje bot over. Op dit moment beschouwen we de schub als afgesoten.

IMMUNOLOGIE VAN DE KARPER

In het voorafgaande is de karper al af en toe ter sprake gekomen in verband met het immuunsysteem. In appendix I van dit proefschrift staan de resultaten vermeld van onderzoek verricht aan een tropisch visje *Barbus conchonius* (prachtbarbeel in het nederlands). Aandacht werd besteed aan de ontwikkeling van het humorale en cellulaire immuunsysteem. Humorale immuniteit werd onderzocht door bij dieren van verschillende leeftijden te kijken naar het aantal plaque vormende cellen dat in de milt ontstaat na inspuiten met SRBC. Cellulaire immuniteit werd onderzocht met schubtransplàntaties. De resultaten laten zien dat dieren van 3 maanden al kunnen reageren op SRBC, maar dat het daarna nog wel 6 maanden duurt voordat dit vermogen maximaal is. De cellulaire immuniteit ontwikkelt zich sneller: dieren van 6 maanden oud stoten vreemde schubben even snel af als 9 maanden oude dieren.

In appendix II staat hoe we de plaque test bij de karper uitgevoerd hebben. Het grootste probleem was het vinden van een geschikte complement bron. Het bleek dat karper-complement niet het meest geschikt was. Veel beter was kopvoorn, sneep, barbeel en brasem. Omdat de kopvoorn, sneep en barbeel in Nederland erg zeldzaam of zelfs beschermd zijn, hebben we besloten de optimale omstandigheden uit te werken voor brasem-complement. Brasems zijn er gelukkig genoeg, er is zelfs een meer naar genoemd.

In appendix III is gekeken naar de reactie van het immuunsysteem van de karper op SRBC bij verschillende temperaturen. Eerst hebben we gekeken in welke organen antilichaam producerende cellen voorkomen. In de kopnier en nier zit ongeveer 90% van het totaal aantal "plaque vormende cellen" (PFC). Slechts 5% zit in de milt en verder komen er nog geringe aantallen PFC voor in het bloed, het hart en de thymus. In figuur 10 staat weergegeven hoe het aantal PFC in de kopnier verloopt bij 24^{o}C. Het hoogste aantal PFC wordt bereikt op dag 9 na inspuiten. Als we de karpers bij een lagere temperatuur houden, dan duurt het langer voordat de piek van de PFC reactie bereikt wordt.
Vissen zijn koudbloedige dieren, ze hebben geen vaste lichaamstemperatuur zoals zoogdieren maar de temperatuur van hun lichaam wordt bepaald door de omgevingstemperatuur. Bij lagere temperaturen verlopen alle levensprocessen van een vis trager. Het is daarom niet verwonderlijk dat ook de immuunreactie trager verloopt. Wat echter opviel was dat de hoogte van de reactie (het maximale aantal antilichamen vormende cellen) gelijk was bij alle temperaturen. Van uitstel komt in dit geval dus geen afstel. Een tweede interressant aspect van dit experiment blijkt als je in een grafiek de temperatuur uitzet tegen de dag waarop

de piekreactie bereikt wordt (fig. 14). We zien dat de grafiek geen rechte lijn vormt maar dat er een "knik" in zit.

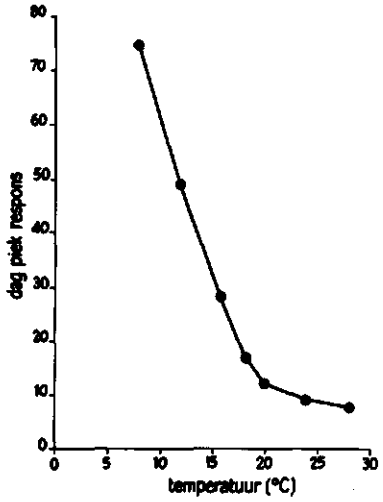


Fig. 14: Verband tussen de temperatuur en de immunologische reactie.

Dat betekent dat er in de immunologische reactie (het hele proces van herkenning van de vreemde stof tot aan de productie van antilichamen) stappen zijn die verschillen in temperatuurgevoeligheid.

In appendix IV staan experimenten beschreven die gedaan zijn om iets te weten te komen over vorming van immunologisch geheugen. We hebben al besproken dat als je een dier met een vreemde stof inspuit er niet uitsluitend specifieke antilichamen gevormd worden maar ook geheugencellen. De geheugencellen zorgen ervoor dat na een tweede injectie met dezelfde vreemde stof de reactie sneller en feller is. De mate van geheugenvorming kun je dus aflezen aan de hoogte van de herhaalde reactie.

Karpers zijn ingespoten met 3 verschillende hoeveelheden SRBC (1 miljard, 10 miljoen en 100.000 cellen/dier) en langs 2 verschillende routes: in de spieren of in de bloedbaan. De hoogste dosis SRBC geeft de hoogste eerste reactie terwijl bij de laagste dosis nauwelijks een reactie te meten valt. Als je echter na 1 maand de geheugenvorming test door een tweede injectie toe te dienen dan blijkt dat de beste reactie wordt gevonden in dieren die met de middelste dosis geimmuniseerd worden. Na 6 maanden en 1 jaar is de situatie nog extremer: de hoogste reactie wordt bereikt in dieren geimmuniseerd met de laagste dosis. Dat betekent dat de dosis die in een eerste reactie nauwelijks iets doet, het beste is voor de geheugenvorming. Je kunt dit fenomeen ook vertalen in termen van vaccineren (inenten) en bescherming tegen een ziekte (immuniteit). Het idee heerst nog steeds dat een vaccin

een zo hoog mogelijke antilichaamproductie op moet wekken wil het bescherming bieden. Op grond van deze experimenten zou je het tegenovergestelde kunnen beweren: een vaccin biedt pas dan optimale bescherming als het bij een eerste injectie geen antilichamen opwekt.

In de laatste twee hoofdstukken, appendix V en VI, wordt beschreven wat voor effecten antibiotica op het immuunsysteem van de karper kunnen hebben.

Antibiotica zijn stoffen die de groei van bacteriën remmen. De bekendste zijn penicilline, streptomycine, chlooramphenicol, tetracycline. Antibiotica worden bij de mens gebruikt voor de bestrijding van infectieziektes. In de veehouderij worden ze behalve voor de bestrijding van ziektes ook preventief (ter voorkoming van ziektes) gebruikt. Een bijkomende reden voor het gebruik is de groeibevorderende werking op de dieren. Het werkingsmechanisme is niet voor alle antibiotica gelijk. Penicilline b.v. verstoort de vorming van een goede celwand, waardoor de bacterie, als hij gaat groeien, uit elkaar klapt. Dit kunnen we vergelijken met de gevolgen van een kapotte buitenband van een fiets; de binnenband klapt pas als je hem oppompt. Tetracycline en chlooramphenicol remmen de eiwitfabriek van bacteriën.

Antibiotica remmen de groei van bacteriën. Alleen remming van de groei is natuurlijk niet voldoende om een bacterie uit te schakelen, immers zodra je stopt met de behandeling gaan de bacteriën weer rustig verder met groei en deling. Daarom zal altijd het immuunsysteem nodig zijn om de, door antibiotica in bedwang gehouden bacteriën, definitief onschadelijk te maken.
Het antibioticum dat we gebruikt hebben in onze experimenten is oxytetracycline (oxyTC). De scheikundige formule staat in figuur 15.

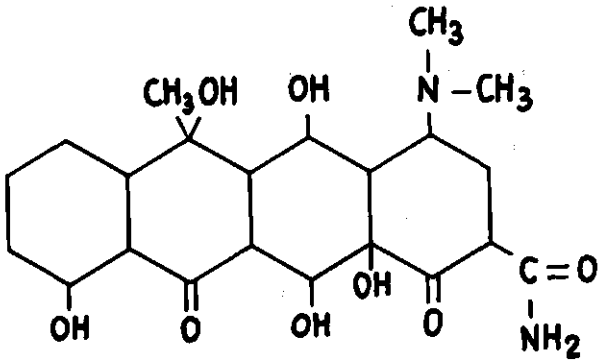


Fig. 15: Oxytetracycline

Het antibioticum is op twee verschillende manieren toegediend aan de karper.

1. door karpers te voeren met korrels waarin oxyTC meegemengd is. Dit is ook de manier waarop antibiotica in de visteelt worden toegediend.
2. door karpers om de 3 dagen met een oxyTC-oplossing in te spuiten.

Bij de behandelde karpers hebben we gekeken of het immuunsysteem nog goed werkte. Het bleek dat het bij karpers, die met oxyTC waren ingespoten, veel langer duurde voordat getransplanteerde schubben werden afgestoten dan bij controle-dieren. Er waren zelfs schubben die helemaal niet meer werden afgestoten. Injecties met oyxTC remmen dus in sterke mate de cellulaire immuunreacties. De humorale immuniteit wordt sterk geremd door zowel injecties als voeren van oxyTC:

test	controle	oxyTC voer	oxyTC injectie
aantal rozet vormende cellen in de milt	16	4	2,5
aantal antilichaam producerende cellen:			
- in de milt	34	2	2
- in de kopnier	86	15	5
- in de middennier	55	15	4

Zowel het aantal rozet vormende cellen als het aantal antilichaam producerende cellen is sterk verminderd tijdens een humorale respons in met antibiotica behandelde dieren.

In latere experimenten hebben we dit effect op de humorale immuniteit verder onderzocht. Daarbij bleek dat vooral de eerste reactie gevoelig voor antibiotica was, herhaalde reacties werden niet beïnvloed.

Uit deze experimenten kun je concluderen dat er erg voorzichtig met antibiotica moet worden omgesprongen. Immers, wanneer je een ziek dier, dat een bepaalde infectie heeft, met antibiotica gaat behandelen dan gebeuren er twee dingen:

a. de bacterie wordt door het antibioticum in zijn groei geremd
b. het immuunsysteem van het dier wordt onderdrukt.

Als het dier tijdens de antibioticumkuur besmet wordt met een virus, een schimmel of een bacterie die niet gevoelig is voor het antibioticum dan kan het immuunsysteem deze micro-organismen niet meer onschadelijk maken. Tenslotte is het de vraag wie zich na beëindiging van de antibioticum behandeling het snelst herstelt: de bacterie of het immuunsysteem.

THE IMMUNE SYSTEM OF CYPRINID FISH

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AAN MIJN OUDERS

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GER T. RIJKERS

THE IMMUNE SYSTEM OF CYPRINID FISH

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H.C. van der Plas,
hoogleraar in de organische scheikunde,
in het openbaar te verdedigen
op woensdag 1 oktober 1980
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen.

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CONTENTS

STELLINGEN

I

De nier bij lagere vertebraten is vergelijkbaar met het beenmerg van zoogdieren.

Turpen, J.B. (1980) in: Development and Differentiation of Vertebrate Lymphocytes (J.D. Horton, Ed.). Elsevier/North Holland Biomedical Press, pp. 15-24.
dit proefschrift.

II

Antibiotica zijn immuunsuppressief.

dit proefschrift

III

Een positieve gemengde haemagglutinatie reactie vormt geen bewijs voor de multispecificiteit van natuurlijke antilichamen.

Sigel, M.M., Lee, J.C., McKinney, E.C. & Lopez, D.M. (1978). Mar. Fish. Rev., 40, 6-11.

IV

Het idee van Gorczynski en Steele dat gemuteerde somatische genen in het genoom van geslachtscellen kunnen worden ingebouwd is attractief en verdient nader onderzoek.

Gorczynski, R.M. & Steele, E.J. (1980) P.N.A.S., 77, 2871-2875.

V

Het bestuderen van wetenschappelijke literatuur buiten het eigen vakgebied met als enig doel om tot een stelling te komen is in strijd met de geest van het promotiereglement.

VI

De beslissing over het al dan niet toekennen van spreektijd op een internationaal congres kan worden vereenvoudigd door bij de aanmelding niet een geschreven maar mondelinge, door de auteur op een casettebandje ingesproken, samenvatting te verlangen.

VII

Indien fietsers werkelijk als volwaardige weggebruikers worden beschouwd, dan behoren vrijliggende fietspaden te worden voorzien van verlichting en wegmarkering.

VIII

De aandacht die een "laatste stelling" in de media krijgt bewijst de overschatting van de maatschappelijke betrokkenheid van academici.

IX

De exportsymbolen Frau Antje en Hansje Brinker zijn te prefereren boven het duo van Agt/van der Klauw als zijnde representatief voor de Nederlandse bevolking.

Proefschrift van G.T. Rijkers
The Immune System of Cyprinid Fish
Wageningen, 1 oktober 1980.

The immune system of cyprinid fish. Oxytetracycline and the regulation of humoral immunity in carp (*Cyprinus carpio*)
G.T. Rijkers, R. van Oosterom and W.B. van Muiswinkel
(submitted for publication).

ABBREVIATIONS

ABC	antigen binding cell(s)
AcBSA	acetylated bovine serum albumin
BALT	bronchus associated lymphoid tissue
B cell	bursa (equivalent)derived lymphocyte
BGG	bovine gamma globulin
BSA	bovine serum albumin
C1-C9	components of the complement system
CD	circular dichroism
CG	chicken globulin
CH_{50}	complement concentration bringing about 50% lysis of a standard dose indicator erythrocytes
conA	concanavalin A
CRP	C-reactive protein
CVF	cobra venom factor
DNP	dinitrophenol
DTH	delayed type hypersensitivity
EDTA	ethylenediaminetetraacetic acid
ERM	enteric redmouth disease
Fab	antigen binding fragment of immunoglobulin
Fc	crystallizable fragment of immunoglobulin
FHM	fat head minnow
FSP	fructosan specific protein
GALT	gut associated lymphoid tissue
HA	haemagglutinating
H chain	heavy chain
HGG	human gamma globulin
HL	haemolysing
HMW	high molecular weight
HRBC	horse red blood cells
HSA	human serum albumin
Ig	immunoglobulin
IHN	infectious haemopoietic necrosis
IPN	infectious pancreatic necrosis
J chain	joining chain
K_f	functional association constant
KLH	keyhole limpet hemocyanin
K_o	intrinsic association constant
L chain	light chain
LMW	low molecular weight

LPS	lipopolysaccharide
MI	migration inhibition
MLR	mixed leukocyte reaction
MST	median survival time
MW	molecular weight
NIP	3-iodo-4-hydroxy-5-nitrophenyl acetic acid
NNP	3,5-dinitro-4-hydroxy-phenyl acetic acid
OSA	O-antigen of *Salmonella abortus*
oxyTC	oxytetracycline
Pen	penicillin
PFC	antibody forming cells/plaque forming cells
PHA	phytohaemagglutinin
PPD	purified protein derivate of tuberculin
PVP	polyvinylpyrrolidone
PWM	pokeweed mitogen
RBC	red blood cells
RE cells	reticulo-endothelial cells
RFC	rosette forming cells
S_{20}	sedimentation coefficient (in Svedberg units)
SΔQ	sum of squared differences
sIg	surface immunoglobulin
SRBC	sheep red blood cells
T cell	thymus derived lymphocyte
TNP	trinitrophenol
UV	ultra violet
VHS	virus haemorrhagic septicemia
WC	white cells

ALPHABETICAL LIST OF FISH SPECIES

common name	scientific name
arrowana	*Osteoglossum bicirrhosum*
Atlantic hagfish	*Myxine glutinosa*
barbel	*Barbus barbus*
bigmouth buffalo	*Ictiobus ciprinellus*
black-spot barb	*Barbus filamentosus*
bluegill	*Lepomis macrochirus*
blue gourami	*Trichogaster trichopterus*
blue striped grunt	*Haemulon sciurus*
bowfin	*Amia calva*
bream	*Abramis brama*
brook lamprey	*Lampetra reissneri*
brown trout	*Salmo trutta*
carp	*Cyprinus carpio*
channel catfish	*Ictalurus punctatus*
chinook salmon	*Oncorhynchus tshawytsha*
chub	*leuciscus cephalus*
cod	*Gadus gadus*
coho salmon	*Oncorhynchus kisitch*
Crusian carp	*Carassius carassius*
cunner	*Tautogolabrus adspersus*
dab	*Limanda limanda*
dace	*Leuciscus leuciscus*
dogfish	*Scyliorhinus caniculus*
eel	*Anguilla anguilla* (Litman, Kreutzmann)
eel	*Anguilla chrysypa* (Nardi)
eel	*Anguilla vulgaris* (von Hagen)
fat head minnow	*Pimephales promelas*
flounder	*Platichthys flesus*
gar	*Lepisosteus platyrhincus* (McKinney, Clem)
gar	*Lepisosteus osseus* (Acton)
goldfish	*Carassius auratus*
grouper	*Epinephelus itaria*
guitarfish	*Rhinobatus productus*
guppy	*Lebistes reticulatus*
horned shark	*Heterodontus francisci*
ice fish	*Notothenia rossii*
Japanese eel	*Anguilla japonica*

common name	scientific name
killifish	*Fundulus heteroclitus*
lane snapper	*latjanus synagris*
lemon shark	*Negaprion brevirostris*
leopard shark	*Triakis semifasciata*
margate	*Haemulon album*
Mozambique mouthbrooder	*Tilapia mossambica*
nase	*Chondrostoma nasus*
nurse shark	*Ginglymostoma cirratum*
Pacific hagfish	*Eptatretus stouttii*
paddlefish	*Polyodon spathula*
perch	*Perca fluviatilis*
pike	*Esox lucius*
plaice	*Pleuronectes platessa*
pumpkin seed	*Lepomis gibbosus*
purple-headed barb	*Barbus nigrofasciatus*
rainbow trout	*Salmo gairdneri*
rock bass	*Ambloplites rupestris*
rosy barb	*Barbus conchonius*
sablefish	*Anoplopoma fimbria*
salmon	*Salmo salar*
sea lamprey	*Petromyzon marinus*
skate	*Raja naevus*
smooth dogfish	*Mustelus canis*
snapper	*Lutjanus griseus*
sockeye salmon	*Oncorhynchus nerka*
southern ray	*Dasiatis americana*
spanner barb	*Barbus lateristriga*
sting ray	*Dasiatis centroura*
sunfish	*Centrarchidae sp.*
surfperch	*Cymatogaster aggregata*
white perch	*Morone americana*
winter flounder	*Pseudopleuronectes americanus*

GLOSSARY

allograft ..... graft derived from one animal and transplanted to a genetically different animal of the same species.

anamnestic reaction ..... the manifestation of immunological memory whereby a second or subsequent exposure to an antigen leads to a greater or more rapid reaction than the first.

anaphylaxis ..... a major type of immediate hypersensitivity dependent on the formation of antigen-antibody complexes. The reaction is accompanied by pathological symptoms in tissues and organs due to the release of pharmacologically active agents.

autograft ..... a graft in which the donor and recipient are the same individual.

delayed type hypersensitivity ..... a state of increased reactivity to an antigen, depending on previous sensitization, giving rise to a specific inflammatory reaction in the area where the antigen is localized.

migration inhibition ..... the inhibition of the movement of cultured macrophages by a factor released by sensitized lymphocytes.

mixed leukocyte reaction..... the transformation of leukocytes into blast cells in mixed cultures of leukocytes from normal allogeneic individuals

xenograft..... a graft between individuals of different species.

OPENING REMARKS

There are several reasons for studying the immune system of fish:

1) From a phylogenetic point of view fish are interesting because they are the first group of animals in which an immune system characterized by the presence of immunoglobulins occurs.
2) The immune system of poikilotherms - including fish - is dependent upon the environmental temperature offering the unique possibility to manipulate the immune response by a mere variation in temperature.
3) When studying the ontogeny of the immune response oviparous fish are particular interesting because the early stages and free swimming larvae are readily accessible to experimental work.
4) A better understanding of the immune system of fish may aid in prophylaxis and therapy of fish diseases in fish culture.

In the General Introduction relevant data about fish immunology will be discussed. Since it is not the intention of this thesis to review the whole field of comparative immunology, data are restricted to fishes although a comparison with invertebrate defence mechanisms and mammalian immune systems will be made when needed.

During the last 5-10 years the interest in fish immunology has accumulated in a vast number of publications. However the data are difficult to unify: only in the introduction of this thesis 69 species are mentioned. During evolution the different classes of fish have diverged long before mammals were present. For this reason it is difficult, if not impossible, to draw definite conclusions when comparing data of species belonging to different classes. In this respect it is worthwhile to mention the observation of Heuzeroth, Resch, Richter & Ambrosius (1973) who studied the degree of similarity of immunoglobulins. The differences between classes of fishes were as large as the differences between other vertebrate orders (e.g. amphibians and mamals). Even within one class of fish data are difficult to compare due to a variety of immunization schedules and antigens used. Moreover, species may differ in their preferential temperature. In the Epilogue an attempt will be made to give a general and more personal idea about the immune system of bony fish.

In most experiments described in this thesis carp (*Cyprinus carpio* L. 1758) were used. This animal was chosen because it is an excellent experimental animal for biological studies. In addition it is worthwhile to mention that this species, *Tilapia* and salmonids form the base for large scale fish culture in the world.

GENERAL INTRODUCTION

CHAPTER I
CELLS AND ORGANS

Cells

At the light microscopical as well as electron-microscopical level lymphocytes, plasma cells, mononuclear phagocytes and granulocytes of fish closely resemble their mammalian counterparts.

Lymphocytes are small round cells with a large nucleus and a small rim of basophilic cytoplasm (Weinreb, 1963; Ferguson, 1976b; Mattison & Fänge, 1977; Kreuzmann, 1977; Davina, Rijkers, Rombout, Timmermans & van Muiswinkel, 1980).

Plasma cells possessing an eccentric nucleus with a prominent nucleolus and a cytoplasm packed with rough endoplasmic reticulum have been observed in rainbow trout (Chiller, Hodgins, Chambers & Weiser, 1969; Etlinger, Hodgins & Chiller, 1978).

Monocytes and macrophages have been described in fish but a confusing nomenclature is used by many authors (see review of Ellis, 1977a). The same holds true for granulocyte identification. A description of the morphology and a discussion on the nomenclature of these cells is published by Barber & Westermann (1975, 1978), Ellis (1977a) and Davina et al. (1980).

- Lymphoid cells as well as mononuclear phagocytes and granulocytes of mammals and fish are quite comparable when morphological criteria are used.

Lymphoid organs

The most primitive vertebrates, the hagfish lack on morphological grounds a definite thymus and spleen (Harboe, 1963). Haematopoietic foci are present in the lamina propria of the entire gut lenght (Good, Finstad, Pollara & Gabrielsen, 1966). The pronephros, which contains nephrostomes, is transformed partly into lymphoid tissue (Gérard, 1954; Fänge, 1966). In peripheral blood of sea lamprey, cells were observed which resembled mammalian lymphocytes. A primitive spleen is located in an invagination of anterior gut tissue. Erythro-, thrombo-, granulo- and lymphopoiesis occurs in this organ. A primitive bone marrow, located in the fibrocartilaginous provertebral arch, has been observed. The bone marrow contains haematopoietic tissue; proliferation of lymphoid cells was observed after stimulation with bovine gamma globulin (BGG). A definite thymus is absent in lamprey. However, small groups of lymphoid cells in the epithelium of the pharyngeal gutter are considered as a primitive thymus (Good et al., 1966).

From the evolutionary point of view, the guitarfish (a primitive Elasmobranch) is the first representative where a thymus is found. It is a fully developed, encapsulated lymphoid organ with a well organized cortex and medulla. In the spleen a red and white pulp area is found. Furthermore lymphoid tissue was found in the gut and in the gonads. In the more advanced Elasmobranchs, the leopard shark and nurse shark, a well developed thymus and spleen were present. Abundant lymphoid tissue was also found along the gastrointestinal tract and in the gonads. The kidney did not contain lymphoid cells. Cells, identical to mammalian plasma cells, were found among the lymphoid cells in spleen and gonads (Good et al., 1966).

The Chondrostean paddlefish has a well-developed thymus organized into lobules. Peripheral blood contains large, medium and small lymphocytes. The spleen is well developed and clearly divided into red and white pulp. Plasma cells were observed in spleen and in haematopoietic tissue overlying the heart. The holostean bowfin has a thymus with some degree of organization into cortex and medulla. The spleen is a discrete organ with tissue components distributed into red and white pulp (Good et al., 1966). In holostean fish a unique organ is found, the meningeal myeloid tissue, which is actively involved in haemopoiesis and thought to be primitive bone marrow (Scharrer, 1944; McLeod, Sigel & Yunis, 1978).

In teleost fish, no uniformity exists in the histological appearance of lymphoid organs. For instance the thymus of eel (von Hagen, 1936) and Mozambique mouthbrooder (Sailendri & Muthukkaruppan, 1975) is clearly divided into a cortex and a medulla whereas this distinction can hardly be made in the salmon and rainbow trout (Ellis, 1977b; Grace & Manning, 1980). A spleen with red and white pulp is found in Mozambique mouthbrooder (Sailendri & Muthukkaruppan, 1975), perch (Pontius & Ambrosius, 1972) and pike (M.G. Vos, pers. comm.). In plaice this division is less clear, the area occupied by the white pulp being relatively small (Ellis & de Sousa, 1974), whereas in carp (Secombes & Manning, 1980) and rainbow trout, spleen is mainly red pulp (Anderson, 1974; Grace & Manning, 1980). The kidney is an important lymphoid organ in teleosts. The excretion function of pronephros is completely lost in adult fish and the organ shows mainly haemopoietic and lymphoid cells (Ellis & de Sousa 1974). Some sinuses in pronephros are surrounded by endocrine cells (Secombes & Manning, 1980). Lymphoid tissue of mesonephros is situated in between nephric tubules. In Mozambique mouthbrooder no organized lymphoid tissue was observed in mesonephros (Sailendri & Muthukkaruppan, 1975). Furthermore individual or small groups of lymphoid cells are situated in between intestinal epithelial cells as well as in the lamina propria (Bullock, 1963; Pontius & Ambrosius, 1972; Krementz & Chapman, 1975; Reiffel & Travill, 1977; Zapata, 1979a; Davina et al., 1980). Teleost fish lack bone marrow, bursa of Fabricius and lymph nodes.

- In cartilaginous and bony fish a discrete thymus and spleen are found. In bony fish the kidney contains lymphoid cells. With the exception of sea lamprey and Holosteans, fish lack bone marrow. The cellular organization of thymus and spleen is strongly species dependent.

Function of lymphoid organs

After injection of carp with a soluble antigen (human gamma globulin (HGG)), antigen trapping occurred in spleen, pronephros and to a lesser degree in mesonephros. Using a cellular antigen (*Aeromonas salmonicida*), mesonephros played a major part in antigen trapping. Thymus and liver were not involved in this proces (Secombes & Manning, 1980). Antigen binding cells (or rosette forming cells) were detected in spleen and pronephros of rainbow trout after immunization with sheep red blood cells (SRBC) (Chiller, Hodgins, Chambers & Weiser, 1969). Unfortunately mesonephros was not included in this study. Electron microscopy revealed that lymphocytes, blast-like cells, macrophages and cells resembling eosinophils bound the antigen. Antigen binding cells have also been observed in pronephros and spleen of goldfish, but only low numbers were found in thymus (Ruben, Warr, Decker & Marchalonis, 1977; Warr, DeLuca, Decker, Marchalonis & Ruben, 1977). Antibody producing cells have been demonstrated in spleen and pronephros of bluegill (Smith, Potter & Merchant, 1967), rainbow trout (Chiller, Hodgins & Weiser, 1969; Anderson, 1978), perch, (Pontius & Ambrosius, 1972) and Mozambique mouthbrooder (Sailendri & Muthukkaruppan, 1975). Furthermore antibody forming cells were detected in the mesonephros of rainbow trout and goldfish (Anderson, 1978; Neale & Chavin, 1971). Organ cultures of snapper and grouper revealed that antibody synthesis occurred not only in spleen and pronephros but also in the thymus (Ortiz-Muniz & Sigel, 1968, 1971). In Mozambique mouthbrooder antibody forming cells were present in the thymus (Sailendri, 1973). In other teleost species studied, thymus contained no or very low numbers of antibody forming cells. In liver of rainbow trout and several shark species antibody forming cells were detected (Chiller, Hodgins & Weiser, 1969; Gitlin, Perricelli & Gitlin, 1973).

Ontogenetic studies in salmon (Ellis, 1977b) and carp (Grace & Manning, 1980), suggested that the spleen is not vital for immunological maturity since lymphocytes of thymus and kidney carry surface immunoglobulin and display mixed leucocyte reactions at a time when spleen in only present in a rudimentary form. Splenectomy in killifish did not affect allograft rejection (Goss, 1961). In snapper, splenectomy had no effect on the antibody response against bovine serum albumin (BSA) (Ferren, 1967). On the other hand, the spleen of blue gourami is supposed to be a major lymphoid organ (Yu, Sarot, Filazzola & Perlmutter, 1970).

Adult thymectomy in Mozambique moutbrooder and salmon had no effect upon allograft rejection (Sailendri, 1973; Botham, Grace & Manning, 1980). Thymectomy in 4 months old Mozambique mouthbrooder prolonged survival of allografts and thymectomy in 2 months old animals totally suppressed the anti-SRBC response (Sailendri, 1973). The pronephros has been regarded as a phylogenetic analogue of bone marrow (Zapata, 1979b) and/or lymph node (Ellis, 1977a).

- The thymus of bony fish may be looked upon as a primary lymphoid organ important for the continuous production of lymphoid cells. Kidney (pronephros and mesonephros) can be considered as a stem cell compartment, a primary lymphoid organ

and a peripheral lymphoid organ. It seems that the spleen does not play an important role in the immune response of most bony fish.

CHAPTER 2

NON-LYMPHOID DEFENCE MECHANISMS

Non-lymphoid cellular defence

Phagocytosis of foreign material both serves as a defence mechanism in itself and as an initial step in the onset of the specific immune response. In mammals, mononuclear phagocytes as well as cells belonging to the granulocyte series posses phagocytic capacity.

In the holostean gar studies *in vivo* and *in vitro* showed that monocytes and macrophages were capable to phagocytize bacteria, yeast and SRBC. These cells destroyed the engulfed micro-organisms, as revealed by electron microscopy. Granulocytes appeared to be inert *in vivo* and *in vitro* uptake of particles (McKinney, Smith, Haines & Sigel, 1977). In plaice, monocytes and thrombocytes were the only phagocytic cell types in peripheral blood (Ellis, 1976; Ferguson, 1976b). Macrophages found in pronephros, mesonephros, spleen, thymus, heart, mesentary and peritoneal fluid, consisted of three types: 1) free rounded cells resembling monocytes 2) reticuloendothelial (RE) cells lining blood sinuses and 3) melano-macrophages. After intraperitoneal injection of carbon particles, phagocytosis was performed predominantly by the ellipsoids of the spleen and the network of RE cells throughout haemapoietic tissue of pronephros and mesonephros (Ellis, Munroe & Roberts, 1976). Macrophages lining the atrial endocardium were also involved in phagocytosis (Ferguson, 1975). Following phagocytosis some macrophages in pro- and mesonephros and spleen formed aggregates with melano-macrophages (Ellis et al., 1976, M.G. Vos, pers. comm.). Amazingly, granulocytes were not phagocytic in plaice (Ellis, 1976).

In contrast to the situation in gar and plaice, neutrophilic granulocytes in rainbow trout and goldfish have phagocytic properties (Finn & Nielsen, 1971a, 1971b; Watson, Shechmeister & Jackson, 1963; Weinreb & Weinreb, 1969). Eosinophilic granulocytes of goldfish phagocytize bacteria (Watson et al., 1963; Weinreb & Weinreb, 1969). Similar observations have been made in the cunner (Mackmull & Michels, 1932), carp (Pliszka, 1939) and guppy (Jakowska & Nigrelli, 1953).

Thrombocytes have been described to be phagocytic (Yokoyama, 1960; Fänge, 1968; Ferguson, 1976b). It remains to be demonstrated for these cells if the uptake of foreign material is followed by intracellular digestion.

There are a number of publications dealing with natural or experimental infections in fish. An extensive discussion of these reports falls beyond the scope of this thesis. In this context it is only worthwhile to mention that phagocytic cells are involved in inflammatory reactions evoked by viruses (Finn, 1970), bacteria Post, 1963) and parasitic infections (Joy & Jones, 1973).

In some studies the clearance of carbon or antigenic material from blood was used as an indication for the phagocytizing capacity of the animal. Following intravenous or intramuscular injection of T_2 bacteriophage into lemon shark, phage particles were completely eliminated from the circulation by day 4-5 (Sigel, Acton,

Evans, Russell, Wells, Painter & Lucas, 1968). In brown trout and carp, kept at optimal temperatures MS2 bacteriophage is cleared from the bloodstream within 7 days. In the icefish, kept at 2°C this process takes 42-56 days (O'Neill, 1980). The effect of temperature on non lymphoid defence will be discussed in Chapter 7.

- Phagocytosis in fish is accomplished by mononuclear phagocytes. In some species granulocytes are also involved in this process.

Non-lymphoid humoral defence

In higher vertebrates a number of components have been described with a potent anti-bacterial and/or anti-viral activity which function in a non-specific way. Although some components act totally independent of the immune system others are activated by antibody (e.g. complement).

The following components present in higher vertebrates have been described for fish. Their biological significance and their relation to the specific defence system, especially under circumstances were the immune system functions poorly (low temperatures), are largely unknown at present.

a) complement

Complement is a group of serum components involved in both specific and non-specific defence. In mammals the complement system consists of a series of at least 18 proteins (including C1-C9) which can be activated in two ways. In the classical pathway complement is activated by contact with the altered Fc part of an antibody molecule after binding with an antigen. In the alternative pathway activation is accomplished by contact with bacterial cell wall polysaccharides. Complement activity has been demonstrated in sera from all vertebrate classes (Gewurz, Bugster, Muschel, Finstad & Good, 1965; Gewurz, Finstad, Muschel & Good, 1966; Legler, Evans & Dupree, 1966; Legler, Evans & Dupree, 1967; Gigli & Austin, 1971; Ross & Jensen, 1973). Fish sera in which complement activity has been demonstrated are shown in TABLE 1.

Basic properties of mammalian complement (thermolability, requirement of Ca^{++}, Mg^{++}) are shared by fish complement. However, the temperature range over which complement remains active is far greater for fishes: at 4°C perch and carp complement retains its haemolytic activity (Pontius & Ambrosius, 1972; own observations). For heat-inactivation of fish complement lower temperatures are required than to inactivate mammalian complement (TABLE 1).

As in other poikilotherms complement is in most cases not exchangable between unrelated fish species. SRBC, sensitized by rainbow trout antibodies, were haemolyzed only in combination with isologous serum (rainbouw trout) or with serum from closely related species as coho salmon, sockeye salmon and chinook salmon (Chiller, Hodgins & Weiser, 1966). For SRBC, sensitized with blue gill antibodies, blue gill, pumpkin seed and white perch serum are effective complement sources (Smith, Potter & Merchant, 1967). Rosy barb and rainbow trout serum are suitable complement sources for SRBC sensitized with rosy barb antibodies (Rijkers &

TABLE 1 Characteristics of fish complement

Class	Species		CH_{50}	Heat inactivation (°C)	CVF activation	EDTA inactivation	Ca/Mg restoration	reference
AGNATHA	lamprey	*Petromyzon marinus*	< 2, 0		< 5	-	?	1,2,10
	hagfish	*Eptatetrus stoutii*	< 2		20-40			1
CHONDRICHTHYES	lemon shark	*Negaprion brevirostis*				+	-	10
	nurse shark	*Ginglimostoma cirratum*	300-400		< 5	+	-	1,10
CHONDROSTEANS	paddlefish	*Polyodon spathula*	50-75, 7.5	50	< 5	+	+	1,2,10
HOLOSTEANS	gar	*Lepisosteus osseus*		54		+	+	2,10
	bowfin	*Amia calva*	60	52		+	+	2,10
TELEOSTS	bigmouth buffalo	*Ictiobus cyprinellus*	50	50		+	-	2,10
	blue gill	*Lepomis macrochirus*		56				3
	carp	*Cyprinus carpio*	100-150	53, 42	< 5	+	+	1,4,5,10
	catfish	*Ictalurus punctata*	27	50		+	+	2,10
	eel	*Anguilla chrysypa*		40-42				6
	goldfish	*Carassius auratus*	11	50		+	+	2,10
	perch	*Perca fluviatilis*		50				7
	rainbow trout	*Salmo gairdneri*		45				8,9

CH_{50} = complement titre; CVF activation = complement depletion by cobra venom factor; Ca/Mg restoration of complement activity after inactivation by EDTA.
1) Day et al. (1970); 2) Legler et al. (1967); 3) Smith et al (1967); 4) Cushing (1945); 5) Rijkers et al. (1980a); 6) Nardi (1938); 7) Pontius & Ambrosius (1972); 8) Chiller et al. (1969); 9) Anderson (1978). 10) Gigli & Austen (1971).

Van Muiswinkel, 1977). In the haemolytic plaque assay using SRBC and immune carp cells, sera of carp, bream, barbel, chub and nase were capable of bringing about haemolysis (Rijkers, Frederix-Wolters & Van Muiswinkel, 1980a). It appears that isologous serum or serum from closely related species is effective as complement source in the haemolytic plaque assay. However, in the plaque assay of rosy barb, serum from purple-headed barb, black-spot barb and spanner barb do not display complement activity (Rijkers & Van Muiswinkel, 1977). In the plaque assay of carp, serum of goldfish is ineffective (Rijkers et al., 1980a). It is therefore unlikely that functional interchangability of complement can be used as a taxonomical criterium.

Fish complement is present not only in serum. In rainbow trout a heatlabile anti-*Vibrio* activity was observed in mucus, presumably complement + antibody (Harrell, Etlinger & Hodgins, 1976).

Only few data exist on isolated components of fish complement. Ross & Jensen (1973b) have isolated and purified the first component (C1n) of the complement system of nurse shark. C1n was imcompatible with rabbit immunoglobulin but formed an intermediate complex with sheep erythrocytes sensitized with nurse shark antibody. These intermediates could be specifically lysed with guinea pig serum devoid of C1. On the other hand, whole guinea pig complement did not react with SRBC sensitized with shark antibody. It was concluded that the first component of shark complement can initiate the cascade reaction of the mammalian complement system provided that it is allowed to react with an immune complex containing a compatible antibody (Ross & Jensen, 1973a). In addition Gigli & Austen (1971) have demonstrated the presence of C9 in nurse shark serum which reacted with the cellular intermediate EAC 14235678 prepared with guinea pig serum.

Cobra venom factor (CVF) depletes the terminal complement components by activating the complement sequence at C3. The complement activating effect of CVF requires the cooperation of the alternative pathway system. This alternative complement activity was present in hagfish serum and in hemolymph of horseshoe crab (*Limulus polyphemus*) and in sipunculid worm (*Golfingea sp.*) but absent in lamprey, nurse shark, paddlefish and carp sera (Day, Gewurz, Johannsen, Finstad & Good, 1970). Classical complement activity was not observed in hagfish, lamprey, horseshoe, crab and sipunculid worm. These findings suggest that the terminal components of the complement system, which can be activated without antibody, appeared early during evolution. A complement system, which requires antibody-mediated activation is only found in the vertebrates were immunoglobulin has been demonstrated.

b) Lysozyme

Lysozyme, an enzym with bacteriolytic properties, is present in serum, mucus and phagocytic cells of many fish species (Luk'yanenko, 1965; Fletcher & Grant, 1968; Fletcher & White, 1973b; Ourth, 1980). The molecular weight (15×10^3) is similar to mammalian lysozyme. The different electroporetic mobility reflects differences in amino acid composition (Fletcher & White, 1973b).

Variations in lysozyme activity between individual members within one species are considerable. In general, serum lysozyme activity in carnivorous fishes like pike and perch is higher than in omnivorous species like carp (Luk'yanenko, 1965). After immunization of carp with *Aeromonas punctata* the highest lysozome activity coincides with the serum antibody peak (Vladimirov, 1972).

Using immunohistochemical techniques, lysozyme activity could be demonstrated in monocytes and neutrophils (Murray & Fletcher, 1976). These cell types probably contribute to the serum lysozyme activity since the number of monocytes and neutrophils increases concommitantly with serum lysozome levels after intravenous injection of latex beads (Fletcher & White, 1973b).

c) Interferon

Interferon is a substance produced during viral infections in order to increase cell resistance to different types of viruses. The antiviral action of interferon is species specific, i.e. mouse interferon does not prevent virus replication in human cell lines. In fish, interferon production has been demonstrated both *in vivo* and *in vitro*.

Virus haemorrhagic septicemia (VHS) is a disease caused by a rhabdovirus (Egtved virus). In rainbow trout the disease occurs mainly at water temperatures of 6-12°C and disappears spontaneously at temperatures over 14-15°C. Following injection of rainbow trout kept at 15°C with Egtved virus, serum was collected after 3, 9 and 14 days. The antiviral activity of these sera tested *in vitro* was maximal on day 3. This was shown by protection of rainbow trout gonad cells against challenge with Egtved virus, but also to infectious haemopoietic necrosis (IHN) virus or infectious pancreatic necrosis (IPN) virus. This broad antiviral activity is species specific since fat head minnow (FHM) cells are not protected after treatment with this serum against the virus challenge. The arguments for the interferon nature of the serum factor were enforced since it appeared to be non-sedimentable, non-dialysable, heat and low pH stable, trypsin labile and RNase resistant (de Kinkelin & Dorson, 1973). Rainbow trout and brown trout injected with rhabdovirus strain 23.75 synthesize interferon with maximum titres reached within 2 days. The interferon synthesis preceeds the neutralizing activity due to specific antibody (de Kinkelin, Baudouy & Le Berre, 1977).

Attempts to infect FHM cells with reovirus type 2 were not succesful. FHM cells exposed to this virus developed resistance and produced an antiviral substance which appeared to be interferon (Oie & Loh, 1969). GFA, a virus-like component, is capable of inducing interferon synthesis in a cell line of the blue striped grunt (Beasly, Sigel & Clem, 1966).

d) C-reactive protein

C-reactive protein (CRP) is a protein which appears in mammalian serum during the acute phase of infection with micro-organisms. CRP binds to phosphoryl choline residues which are present in cell wall glycopeptides of various bacteria, fungi and

parasites. CRP can cause agglutination, precipitation and complement activation (Siegel, Rent & Gewurz, 1974; Pepys, Balz, Mussalam & Doenhoff, 1980).

A serum component with CRP properties has been described for plaice, dab, cod, flounder and dogfish (Baldo & Fletcher, 1973; Pepys, Dash, Fletcher, Richardson, Munn & Feinstein, 1978). CRP precipitated aqueous extracts from bacteria, fungi and nematodes. The precipitation reaction could be inhibited by phosphoryl choline. Immunoelectrophoresis revealed a precipitation line in the α_2-region, excluding the possibility of an immunoglobulin nature because antibody activity was restricted to the β-region in their experiments (Baldo & Fletcher, 1973). According to Pepys et al. (1978) CRP of plaice, flounder and dogfish closely resemble human CRP in molecular weight (135-160 x 10^3) and E.M. morphology (two pentameric discs).

In contrast to mammals, CRP in plaice is not an acute phase protein but a normal serum constituent which may provide the animal with a permanent defence line against invading micro-organisms.

e) Natural hemagglutinins

In representatives from all classes of fish, natural agglutinins have been detected. Their activity is usually directed against xenogeneic erythrocytes, more precisely towards carbohydrate moieties on surface membranes.

This natural agglutinins share the following properties which distinguish them from immunoglobulins:

1) they are composed of identical subunits, so no distinction between heavy and light chains can be made.
2) no interchain disulfide bridges occur, the molecule is linked by noncovalent bonds.
3) upon electrophoresis no heterogeneity in amino acid composition of the subunits is displayed.

A protein with agglutinating activity to human erythrocyte "O" antigens has been demonstrated in lamprey serum (Litman, Frommel, Finstad, Howell, Pollara & Good, 1970b). The molecule which consisted of 4 non-covalent linked subunits (M.W. 90.000) dissociated spontaneously. In addition, the circular dichroic (CD) spectra and electrophoretic mobility differed markedly from Ig.

In non-immune lamprey serum a 48 S protein was observed with agglutinating activity to horse erythrocytes (Marchalonis & Edelman, 1968b). The molecule is composed of low molecular weight subunits linked by noncovalent bonds. Ca^{++} requirement and amino acid composition of the protein resembled natural invertebrate hemagglutinin illustrating the evolutionary position of Agnatha between invertebrates and vertebrates.

In normal eel serum an agglutinin is found which reacts specifically with human O-erythrocytes (Springer & Desai, 1970). The agglutinin appeared to be a protein with a molecular weight of 123.000 (7.2 S), but without significant amounts of carbohydrate characteristic for immunoglobulin (Desai & Springer, 1972). The molecule consists of

TABLE 2 Natural occurring hemagglutinins

Species	Name substance	M.W.	S_{20}	subunits & conformation	carbo-hydrate %	predominant amino acids	Ca^{++} dependence	reference
PLANTS								
meadow mushroom *Agaricus campestris*		64.000	4.8	4 x 16.000	4	asp,gln,asp,glu		1
jack bean	concanavalin A	68.000	4		0.4	asp,ser		2,3
EVERTEBRATES								
snail *Helix pomotia*		79.000	5.3	6 x 13.000	6-7	asp,glu		4
oyster *Crassotrea virginica*			33.4	n x 20.000	8.8		+	5
horseshoe crab *Limulus polyphemus*		400.000	13.5	6 x 3 x 22.500	0	asp,glu	+	6,7,8
crayfish *Parachaeraps bicarinatus*		81.000		6 x 13.500			+	9
VERTEBRATES								
nurse shark	FSP	280.500	10.6	4 x 71.000	0	asp,glu		10
eel		123.000	7	3 x 4 x 10.000		asp		11

MW = molecular weight; S_{20} = sedimentation coefficient; asn = asparagine; gln = glutamine; asp = aspartic acid; glu = glutamic acid; ser = serine; FSP = fructosan specific protein.
1) Sage & Connett, 1969; 2)Agrawal & Goldstein, 1968; 3) Olson & Lienen, 1967; 4) Hammerström, 1974; 5) Acton & Weinheimer, 1974; 6) Clem & Leslie, 1970; 7) Marchalonis & Edelman, 1968a; 8) Finstad, 1972; 9) Jenkin, 1977; 10) Harisdangkul, Kabat, McDonough & Sigel, 1972b; 11) Springer & Desai, 1970.

3 subunits, each subunit composed of 4 identical polypeptide chains, joined by disulfide bridges. CD spectra of this molecule differ significantly from those of human 7S immunoglobulin (Jirgensons, Springer & Desai, 1970).

In rainbow trout natural hemagglutinins against rabbit, mouse and human erythrocytes were present. Whereas induced antibodies were always eluted with the first high molecular weight peak from a Sephadex G-200 column, natural hemagglutinins were eluted in the first and third protein peak. Induced antibodies were precipitable by 22% Na_2SO_4 and were β-globulins in immunoelectrophoresis, natural hemagglutinins were nonprecipitable by 22% Na_2SO_4 and were faster than β-globulins (Hodgins, Weiser & Ridgway, 1967). Moreover, such a low molecular weight agglutinin similar to the natural agglutinin was not induced after exposure to various antigens (Hodgins, Wendling, Braaten & Weiser, 1973).

In nurse shark serum a protein was found which specifically precipitates fructosans. This protein (FSP) was antigenically unrelated to shark immunoglobulin (Harisdangkul, Kabat, McDonough & Sigel, 1972a).
The molecular weight was 280,500 (10.6 S) and it consisted of 4 noncovalent linked subunits. No carbohydrate was detected (Harisdangkul, Kabat, McDonough & Sigel, 1972b). CD spectra of purified FSP from nurse shark resembled more closely to phytohemagglutinin or concanavalin A than to vertebrate immunoglobulin (Pflumm, Wang & Edelmann, 1971).

In TABLE 2 some characteristics of agglutinins from plant, invertebrates and vertebrate origin are given. Natural occuring hemagglutinins in invertebrates are large molecular weight proteins with an electrophoretic mobility similar to that of immunoglobulins (Marchalonis, 1977). In the majority of invertebrate species, hemagglutinins possess an opsonic activity, indicating a role in the defence system (Acton & Weinheimer, 1974). The biological significance of agglutinins of plant origin is not clear.

- A number of humoral defence factors have been observed in fish: complement, lysozyme, interferon and C-reactive protein. In general they resemble their mammalian counterparts in functional and physiochemical characteristics. Furthermore, non-antibody hemagglutinins are present in fish serum which resemble invertebrate agglutinins.

CHAPTER 3

IMMUNOGLOBULINS

According to the World Health Organization immunoglobulins (Ig's) are those proteins of animal origin with known antibody activity and certain proteins related to antibody by chemical structure. In man five classes of serum Ig's are found, as illustrated in TABLE 3. All these immunoglobulin classes are serologically related because they share the same light chains. Two antigenically different types of light chains, termed κ or λ, are found in all classes. The heavy chains define the immunoglobulin classes. Heavy chains, which differ in a variety of properties, including molecular weight (M.W.) and amino acid sequence, have been given the designations: μ, γ, α, δ and ε. The molecular formula of IgM $(L_2\mu_2)5$ indicates that 5 sub-units, each composed of 2 light and 2 heavy chains, are linked together.

TABLE 3 Characteristics of immunoglobulin classes in man

Class	S_{20}	Molecular weight ($\times 10^3$)			Carbohydrate content (%)	Formula
		Native	H-chain	L-chain		
IgM	18-20	950	70	22.5	9-12	$(L_2\mu_2)5^*$
IgG	6.7	150	53	22.5	2	$L_2\gamma_2$
IgA	7-11	150-400	64	22.5	6-10	$(L_2\alpha_2)n$
IgD	6.2-6.8	180	60	22.5	12.7	$L_2\delta_2$
IgE	8.2	190	72.5	22.5	11.7	$L_2\varepsilon_2$

S_{20} = sedimentation coefficient; H-chain = heavy chain; L-chain = light chain; $^*L$ can be λ or κ; n can be 1, 2 or higher.
Data from Marchalonis (1977).

Two major properties by which proteins can be resolved are electric charge and molecular weight. Upon zone electrophoresis in starch blocks at pH 8.6 mammalian immunoglobulins migrate to the cathode and were originally termed γ-globulins. From early studies it was concluded that fish lacked immunoglobulins because γ-globulins were not detected in serum (Engle, Woods, Paulsen & Pert, 1958; Good & Papermaster, 1961; Clem & Sigel, 1963; Post, 1966). This conclusion turned out to be incorrect since carp and plaice immunoglobulin for instance migrate as β-globulins (Ambrosius, 1966; Baldo & Fletcher, 1973). On the other hand, proteins which migrate as γ-globulins do not necessary represent immunoglobulins: transferrin and C-reactive protein migrate also as γ-globulins (see also Chapter 2). Therefore reports in which the presence or absence of immunoglobulin is based upon electrophoresis alone should be interpreted with great care.

Immunoglobulin of Agnatha

On base of the failure of detecting circulating antibody after immunization with BSA and the absence of serum globulin upon immunoelectrophoresis, Good & Papermaster (1961) concluded that Pacific hagfish lack an immune response. When using other antigens (heat-killed *Brucella abortus*, typhoid-paratyphoid vaccine, hemocyanin, bacteriophage T_2) with or without complete Freund's adjuvant, no agglutinating, complement-fixing, neutralizing or antigen binding antibodies could be detected (Papermaster, Condie & Good, 1962; Good & Papermaster, 1964). The cause for this failure in eliciting antibody responses probably lies in poor animal husbandry (low water temperatures, starvation) especially since later studies have shown that hagfish are capable of mounting a humoral immune response. After immunization of hagfish with keyhole limpet hemocyanin (KLH) the production of specific antibodies could be demonstrated by passive haemagglutination and immunodiffusion in agar gels (Thoenes & Hildemann, 1969). Fractionation of immune serum showed the antibodies to be present in the macroglobulin fraction (28S). A smaller component (7S) which lacked antibody activity was antigenically identical with the 28S component. The 28S component was thought to be IgM (possibly a dimer of 19S molecules) while the 7S component represented monomeric IgM (Thoenes & Hildemann, 1969).

Natural occurring agglutinins for SRBC were heatlabile since hagfish serum heated at 56°C for 30 min lost all activity. Antibodies induced after repeated immunization with SRBC were heat stable (Linthicium & Hildemann, 1970).

More recently Raison, Hull & Hildemann (1978a) succeeded in raising specific antibodies against group A streptococcal carbohydrate in hagfish. Gel filtration and sodium dodecyl sulfate/polyacrylamide gel electrophoresis under non-reducing conditions indicated that the intact immunoglobulin had a molecular weight of approximately 160,000. The electrophoretic mobility of heavy chains was identical with that of murine μ chains. The tertiary structure of hagfish immunoglobulin was very labile (complete dissociation in 0.005 M 2-mercaptoethanol) suggesting the absence of disulfide bonding of the polypeptide chains (Raison, Hull & Hildemann, 1978b). The hexose content of hagfish immunoglobulin is 3.4% (W/W) (Hildemann, cited in Litman, 1977).

Upon repeated immunization the sea lamprey is capable of producing neutralizing antibodies to bacteriophage f2. The antibody activity is localized in 6.6S and 14S fractions of immune lamprey serum. The 6.6S and 14S fractions were antigenically identical. The yield of 14S material was insufficient for further characterization. The 6.6S molecule contained heavy (H) and light (L) chains with a molecular weight of 70,000 and 22,000 respectively. Lamprey H chains resembled murine μ chains in molecular weight and electrophoretic mobility. H and L chains were not covalently linked and dissociated spontaneously (Marchalonis & Edelman, 1968b). Therefore the molecular weight of the intact immunoglobulin (180,000) could only be estimated (Marchalonis & Cone, 1973).

Stimulation of sea lamprey with hemocyanin or BSA did not evoke production of circulating antibody, while high doses *Brucella* induced an antibody response (Pollara, Finstad & Good, 1966). The antibodies (9S) had a molecular weight of 150,000-200,000 and showed upon immunoelectrophoresis an α globulin arc. The protein with γ mobility lacked antibody activity (Finstad & Good, 1964; Pollara, Finstad, Good & Bridges, 1966). When lamprey was immunized with human "O" erythrocytes, antibody production was demonstrated (Pollara, Litman, Finstad, Howell & Good, 1970). These antibodies were associated with the 9S serum fraction. Upon electrophoresis antibody activity resided in the anodal migrating fraction with a charge density characteristic for an α globulin rather than a γ globulin (Good, Finstad & Litman, 1972). A molecular weight of 320,000 was obtained for the purified immunoglobulin (Litman, Howell, Finstad, Good & Pollara, 1969). The native molecule spontaneously dissociates into antigenically identical subunits of approximately 150,000 and 75,000 M.W. After reduction and alkylation no counterparts of heavy and light chains were obtained but subunits with a M.W. of 90,000. It was suggested that the natural form of lamprey immunoglobulin is represented by a tetramer comprised of 4 equivalent subunits held together by noncovalent bonds (Litman, Frommel, Finstad, Howell, Pollara & Good, 1970b).

- Studies on immunoglobulins in Agnatha result in conflicting conclusions regarding structural conformations. According to Marchalonis lamprey immunoglobulin is comprised of heavy and light chains (Marchalonis & Edelman, 1968b) while Litman suggests that the molecule contains identical subunits (Litman et al., 1970b). This disagreement can be explained by considering the nature of the antigens used for immunization. The immunoglobulins of Marchalonis & Edelman (1968b) were induced by viral protein antigens while Litman et al. (1970b) may have characterized an inducible class of antibody to cell surface carbohydrate.

Natural agglutinins to xenogeneic erythrocytes have been described for lower vertebrates (see also Chapter 2). The natural occurring agglutinin for SRBC in hagfish was already mentioned (Linthicium & Hildemann, 1970). Sera from the arctic lamprey contained natural agglutinins which reacted with various vertebrate erythrocytes. Antibodies induced after repeated immunization with SRBC were heat stable and displayed a high degree of specificity for SRBC, whereas natural agglutinins lost their activity after heating serum at 46°C for 30 min. (Fujii, Nakagawa & Murakawa, 1979b). Normal sea lamprey serum has been shown to contain natural agglutinating activity for SRBC (Gewurz, Finstad, Muschel & Good, 1966). This activity was blocked by EDTA and was not stimulated by immunization. Marchalonis & Edelman (1968b) described a natural agglutinating activity towards horse erythrocytes in sea lamprey which resided in the 48S serum fraction.

- Natural agglutinins can be functional distinguished from inducible antibodies by their heat stability. A physiochemical comparison reveals that natural agglutinins resemble more invertebrate agglutinins than vertebrate immunoglobulins.

(TABLE 2). The lamprey antibody described by Litman et al. (1970b) may represent an inducible form of natural agglutinins. As far as their immune capacity is concerned Agnatha might represent an intermediate between invertebrates and vertebrates, possessing defence system characteristics of both groups.

Immunoglobulin of Chondrichthyes

After immunization of leopard shark with bacteriophage T_2 antibody activity is present in the 17S macroglobulin serum fraction. Late antisera contained antibody activity in the 7S fraction also (Suran, Tarail & Papermaster, 1967). The immunoglobulin fractions could be purified from shark serum by gel filtration and ion exchange chromatography (Clem & Small, 1967). The 7S and 17S immunoglobulins appeared to be identical in immunodiffusion using rabbit anti shark serum (Suran et al., 1967). After extensive reduction and alkylation followed by gel filtration in 5 M guanidine-HCl both immunoglobulins could be separated in H and L-chains. H chains of 7S and 19S were indistinguishable by fingerprints of tryptic digests, disc electrophoretic patterns, antigenic properties and molecular weight (77,000). The MW of shark H chains is comparable to mammalian μ chain. By similar criteria L chains were identical to each other but different from H chains. H and L chains account for about 75 and 25% respectively of the mass of the immunoglobulin molecule. Based upon the M.W. of the native immunoglobulin molecule, the polypeptide chains and the mass ratios of these chains shark 7S immunoglobulin is composed of 2H and 2L chains. The 17S molecule consists of a pentamer of disulfide-linked subunits, each subunit being composed of 2H and 2L chains (Clem & Leslie, 1966). Properties of other Chondrichthyes are listed in TABLE 4. All these species possess pentameric as well as monomeric immunoglobulin. The pentameric nature of the 17S immunoglobulin has been confirmed by electron microscopy. Dogfish 17S immunoglobulin showed a five branched cyclic symetry and dimensions similar to mammalian IgM (Feinstein & Munn, 1969).

Interrelationship between 7S and 17S molecules

- Newborn nurse shark have essential no serum immunoglobulin. During further development the synthesis of 17S Ig preceeds 7S Ig synthesis (Fidler, Clem & small, 1969).
- The 18S antibody to *Salmonella typhimurium* O antigen is 150-200 times more efficient in agglutination reactions than 7S antibody (Schulkind, Robins & Clem, 1969).
- Antibody activity during the first 10-12 months after immunization with *Salmonella typhimurium* was restricted to 17S immunoglobulin. Not until 1 year after immunization antibody activity was observed in 7S immunoglobulin (Clem & Leslie, 1966).
- Administration of radiolabeled 7S and 17S immunoglobulin has demonstrated that 7S IgM is neither a precurser nor a degradation product of 17S IgM.
  The half life of lemon shark immunoglobulin was 4-5 days. Furthermore it was demonstrated that 17S IgM is predominantly intravascular whereas 7S IgM is distributed both extravascularly and intravascularly (Clem, Klapper & Small, 1969; Small, Klapper & Clem, 1970).

On base of these data Clem & Leslie (1969) concluded that the two molecular forms of shark IgM functionally mimic mammalian IgM and IgG molecules.

An interesting exception to the pentameric IgM found in most Chondrichthyes is the immunoglobulin molecule of stingray. H and L chains are similar to mammalian μ and light chains in molecular weight and amino acid compositions, but the molecular

TABLE 4 Physicochemical properties of cartilaginous and bony fish immunoglobulin

Class and species		S_{20}	Molecular weight ($\times 10^{-3}$)			Carbohydrate content %	Formula	Reference
			Native	H-chain	L-chain			
CHONDRICHTHYES								
horned shark	(*Heterodontus francisci*)	19	900	69	23	9.3	$(L_2\mu_2)_5$	1
		7	180	69	23	11.3	$L_2\mu_2$	1
smooth dogfish	(*Mustelus canis*)	17	982	72	20	8.7	$(L_2\mu_2)_5$	2,3
		7	198	73	21	7.6	$L_2\mu_2$	2,3
lemon shark	(*Negaprion brevirostris*)	19	850	71	23	3.7	$(L_2\mu_2)_5$	4
		7	160	71	23	3.5	$L_2\mu_2$	4
leopard shark	(*Triakis semifasciata*)	17		77				5,6
		7		77				5,6
nurse shark	(*Ginglimostoma cirratum*)	19		70	22	3.5	$(L_2\mu_2)_5$	7
		7		70	22	3.6	$L_2\mu_2$	7
sting ray	(*Dasiatis centroura*)	11	360	72	22		$(L_2\mu_2)_2$	8
southern ray	(*Dasiatis americana*)	17		70	23	9.3	$(L_2\mu_2)_5$	9
CHONDROSTEANS								
paddlefish	(*Polyodon spathula*)	14	662	58	21	6.8	$(L_2\mu_2')_4$	10
	(*Polyodon spathula*)	19	870	75	24	9.0	$(L_2\mu_2)_4$	11
			180	75	24	8.1	$L_2\mu_2$	11
HOLOSTEANS								
bowfin	(*Amia calva*)	14	600-900	70	24	10.7	$(L_2\mu_2)_4$	12,13
		6		52	24	9.1	$L_2\mu_2$	12,13
gar	(*Lepisosteus platyrhineus*)	14	650	70	22		$(L_2\mu_2)_4$	14,15
	(*Lepisosteus osseus*)	14	610	58	23	4.9	$(L_2\mu_2')_2$	16,17,18
TELEOSTS								
carp	(*Cyprinus carpio*)	15	720	71	24		$(L_2\mu_2)_4$	19,20
	(*Cyprinus carpio*)	14	608	77	24	6.8	$(L_2\mu_2)_4$	21,22,23
goldfish	(*Carassius auratus*)	16		76	23			19
catfish	(*Ictalurus punctatus*)	14	610	70	23	5.5	$(L_2\mu_2)_4$	16,24
grouper	(*Epinephelus itaria*)	16	900	70	22	3.5	$(L_2\mu_2)_4$	25,26
		6	120	40	22	1.1	$(F_{ab}')_2$	25,26
rainbow trout	(*Salmo gairdneri*)	16		70	22		$(L_2\mu_2)_4$	27
brown trout	(*Salmo trutta*)	17	670					28
coho salmon	(*Oncorhynchus kisutch*)	17	756	75	26		$(L_2\mu_2)_4$	29
margate	(*Haemulon album*)	15	900	70	22			30
		7	180	70	22			30
plaice	(*Pleuronectes platessa*)	12		70	22	8.8		31
pike	(*Esox lucius*)	15	638-651	60	23	9.2	$(L_2\mu_2)_4$	32,33

S_{20} = sedimentation coefficient, H-chain = heavy chain; L-chain = light chain. 1) Frommel, Litman, Finstad & Good (1971); 2) Marchalonis & Edelman (1965); 3) Marchalonis & Edelman (1966); 4) Clem & Small (1967); 5) Suran, Tarail & Papermaster (1967); 6) Litman, Frommel, Chartrand, Finstad & Good (1971e); 7) Clem, DeBoutsud & Sigel (1967); 8) Marchalonis & Schonfeld (1970); 9) Johnston, Acton, Weinheimer, Niedermeier, Evans, Shelton & Bennett (1971); 10) Acton, Weinheimer, Dupree, Russell, Wolcott, Evans, Schrohenloher & Bennett (1971d); 11) Pollara, Suran, Finstad & Good (1968); 12) Litman, Frommel, Finstad & Good (1971a); 13) Litman, Frommel, Finstad & Good (1971b); 14) Bradshaw, Clem & Sigel (1969); 15) Bradshaw, Clem & Sigel (1971); 16)Acton, Weinheimer, Hall, Niedermeier, Shelton & Bennett (1971b); 17) Acton, Evans, Weinheimer, Dupree & Bennett (1970); 18) Acton, Weinheimer, Dupree, Evans & Bennett (1971c); 19) Marchalonis (1971); 20) Shelton & Smith (1970); 21) Ambrosius, Richter & König (1967); 22) Richter, Frenzel, Hädge, Kopperschläger & Ambrosius (1973); 23) Andreas, Richter, Hädge & Ambrosius (1975); 24) Hall, Evans, Dupree, Acton, Weinheimer & Bennett (1973); 25) Clem & Small (1970); 26) Clem (1971); 27) Dorson (1972); 28) Ingram & Alexander (1979); 29) Cisar & Fryer (1974); 30) Clem & Leslie (1969); 31) Fletcher & Grant (1969); 32) Clerx (1978); 33) Clerx, Castel, Bol & Gerwig (1980).

TABLE 5 Physicochemical properties of J chains.

Species	Associated with HMW Ig	Associated with LMW Ig	Molecular weight	Carbohydrate content %	Electrophoretic mobility [+]	Co-elution with L chains [++]	Reference
Nurse shark	+	-	~12.000	?	?	?	1
Leopard shark	+	-	~20.000	?	fast	+	2
Catfish	+		~15.000	7	fast	+	3,4
Man	+	-	~15.000	7	fast	+	5

[+] electrophoretic mobility in polyacrylamide disc electrophoresis (10 M urea, pH 9) after reduction and alkylation of immunoglobulin.

[++] gel filtration of sulfonated immunoglobulin on Sephadex G-200 in 5 M guanidine-HCl.

1) Klapper & Clem, 1972; 2) Klaus, Halpern, Koshland & Goodman, 1971; 3) Weinheimer, Mestecky & Acton, 1971; 4) Mestecky, Kulhavy, Schrohenloher, Tomana & Wright, 1975; 5) Koshland, 1975.

high negative charge. Using this isolation technique, J chain analogues have been demonstrated in nurse shark (Klapper & Clem, 1972; McCumber & Clem, 1976), leopard shark (Klaus, Halpern, Koshland & Goodman, 1971; Weinheimer, Mestecky & Acton, 1971) and channel catfish (Weinheimer et al., 1971; Mestecky, Kulhavy, Schrohenloher, Tomana & Wright, 1975a, b). The physiochemical characteristics of fish J chain are compared with human J chain in TABLE 5. J chain is an evolutionary conservative protein since rabbit antisera to human J chain also react with dogfish and leopard shark J chain (Koshland, 1975).

J chains probably play an important role in the assembly of IgM and IgA monomers into polymers (Kownatzki, 1973). J chains however, are not always essential for polymer assembly as illustrated by the apparent absence of J chain in polymeric IgM of gar (Mestecky et al., 1975), paddlefish (Weinheimer et al., 1971), pike (Clerx, 1978) and carp (Zikan, 1974). Moreover, mildly reduced high molecular weight immunoglobulin of carp, which lacks J chain, can reassociate *in vitro* into native molecules (Richter & Ambrosius, 1978).

- In bony fish pentameric or tetrameric immunoglobulin is found. Physicochemical characteristics of fish immunoglobulin closely resemble mammalian IgM.

Specificity and affinity

Goldfish anti-BSA antibodies showed the same degree of specificity towards the antigen as rabbit anti-BSA antibodies (Everhardt & Shefner, 1966).

Studies on antigen-combining sites require well defined antigenic determinants. Dinitrophenol (DNP) is a suitable molecule for this purpose. In mammals the affinity of antibodies increases during the immune response. This phenomenon is called maturation and holds true for IgG but for IgM this is questionable (discussed in Fiebig, Hörnig, Scherbaum & Ambrosius, 1979).

Nurse sharks immunized with DNP substituted KLH produce both HWM and LMW anti-DNP antibodies (Voss, Russell & Sigel, 1969). Giant grouper respond to DNP by the production of HWM and LMW antibodies (Clem & Small, 1968, 1970). In both experiments antibodies with a high affinity ($K_o = 10^5 - 5 \times 10^6\ M^{-1}$) and low affinity ($K_o = 10^4\ M^{-1}$) were produced. An increase in affinity was never observed.

Antibodies produced by carp immunized with DNP conjugated to human serum albumin (DNP-HSA), possessed 4 measurable combining sites per molecule. The intrinsic affinity (K_o) to the monovalent hapten DNP-L-lysine varied from $10^4 M^{-1} - 2.5 \times 10^6 M^{-1}$. During the initial phase of the immune response the affinity doubled but over the following 25 months period it remained constant (Fiebig & Ambrosius, 1975, 1977). The functional affinity (K_f) of the anti-DNP antibodies for the multivalent hapten DNP-T_4 bacteriphage was 10^3-10^7 fold greater than the K_o (Gruhn, Fiebig & Ambrosius, 1977). K_f values increased considerably after secondary immunization with DNP-HSA, up to $10^{12}\ M^{-1}$ (Fiebig, Gruhn & Ambrosius, 1977). Carp immunized with thymus independent DNP conjugates (DNP-Ficoll) synthesize antibodies with K_o similar to antibodies elicited by the thymus dependent DNP-HSA (Fiebig, Gruhn, Scherbaum & Ambrosius, 1979).

K_f however is only 4×10^1 - 4×10^2 higher than their corresponding K_o values. A possible explanation for this observation is the involvement of T-like cells in the differentiation of different B-like cell clones (Fiebig, Scherbaum, Nuhn & Ambrosius, 1979).

- The intrinsic affinity of fish immunoglobulin is relatively low and does not increase during the immune response. The functional affinity however is high.

Immunoglobulin levels

Data shown in TABLE 6 demonstrate that immunoglobulin levels in serum of fish are comparable to immunoglobulin levels in man. Calculations based upon data obtained from passively transferred Ig in sharks indicate that the synthetic rate in these species is comparable to that in man (100-150 and 80 mg Ig/kg body weight/day respectively) (Small, Klapper & Clem, 1970).

At this point the statement of Clem (1970) that the only "primitive" aspect of fish immunoglobulins is the limited number of immunoglobulin classes seems to be correct.

TABLE 6 Serum immunoglobulin levels in fish

	absolute amount (mg/ml)	relative amount (Ig/total protein)	reference
smooth dogfish	9		1
lemon shark	10-12		2
nurse shark	5- 6	50%	3
sting ray		35%	4
paddlefish	17	40%	5
carp	1.7	6%	6
brown trout	6- 7	10%	7
man (IgM)	0.5- 1	0.5 - 1%	8
man (total)	10-20	9 -20%	8

1) Marchalonis & Edelman (1968b); 2) Clem & Small (1967); 3) Fidler, Clem & Small (1969); 4) Marchalonis & Schonfeld (1970); 5) Legler, Weinheimer, Acton, Dupree & Russell (1971); 6) Richter, Frenzel, Hädge, Kopperschläger & Ambrosius (1973); 7) Ingram & Alexander (1979); 8) Marchalonis (1977).

Location of immunoglobulin

In addition to serum, antibodies have been detected in intestinal and surface mucus of plaice (Fletcher & Grant, 1969). Mucus antibodies were similar to serum antibodies in electrophoretic mobility and carbohydrate and amino acid composition. Natural hemagglutinins found in the water soluble fraction of Pacific hagfish mucus were immunologically related to serum hemagglutinins and showed a similar heat-sensitivity (Spitzer, Downing, Koch & Kaplan, 1976). Bradshaw, Richard & Sigel (1971) reported the presence of antibody to a variety of antigens in surface mucus of gar, snapper

and bowfin. Antibody activity in serum and mucus was totally removed by treatment with 2-mercaptoethanol or rabbit anti-gar IgM serum. In rainbow trout anti-*Vibrio anguillarum* agglutinins could be detected in surface mucus 3-6 weeks after maximum serum titers were attained. Serum and mucus agglutinins were identical in Ouchterlony double diffusion and immunoelectrophoresis. Agglutinating antibody to *Salmonella paratyphi* was found in skin mucus of channel catfish after intraperitoneal injection of bacteria. By immunodiffusion skin mucus gave a precipitation line identical with serum immunoglobulin (Ourth, 1980).

It is not known by which mechanism antibodies gain access to mucus. It is possible that antibodies produced in lymphoid organs are secreted in mucus or that they are produced locally by mucosal plasma cells. When plaice were fed heat-killed *Vibrio anguillarum*, high antibody titres were found in intestinal mucus in almost complete absence of circulating antibody (Fletcher & White, 1973a). This observation favors the idea that local antibody synthesis exists in fish.

● Immunoglobulin is present in serum and mucus of skin and intestine.

CHAPTER 4
HUMORAL IMMUNITY

Following antigen injection it takes some time before the first antibodies appear in serum. Antibody gradually increases and reaches a plateau followed by a decline. After a second injection with the same antigen the latent period is shorter, the response is accelerated and higher antibody levels are reached. A secondary response is only observed when the animal has formed immunological memory after the first injection. Most antibodies belong to the IgM class during the first days of a primary response. IgG is the predominant antibody class during the later phase of the primary response and especially during the secondary response in mammals.

The first stage of the humoral immune response involves the elimination of antigen from the circulation. In mammals and fish this elimination proces is accomplished by mononuclear phagocytes. Phagocytosis is discussed in Chapter 2 (non-lymphoid defence). However, it is obvious that a distinction between specific and non-specific defence is only arbitrary in this respect. Antigen trapping and presentation are essential steps in the induction of an immune response.

Antibody response

The Atlantic hagfish failed to produce antibodies to the soluble antigens BSA and KLH and to the corpuscular antigen *Brucella abortus* (Finstad & Good, 1966). Attempts to induce antibody formation in Pacific hagfish were unsuccessful when using a wide variety of antigens: *Brucella abortus*, typhoid, paratyphoid A and B vaccine, BSA, KLH, bacteriophage T_2 and actinophage MSP-8 (Papermaster, Condie & Good, 1962; Papermaster, Condie, Finstad & Good, 1964). More recently it was shown that Pacific hagfish were able to produce specific antibody to KLH and SRBC, but repeated immunization was needed to obtain moderate antibody titres (Thoenes & Hildemann, 1969; Linthicium & Hildemann, 1970). A high antibody activity to group A streptococcal carbohydrate was detected in hagfish immunized intravenously with streptococci (Raison, Hull & Hildemann, 1978a). Hagfish synthesize bactericidal antibodies after injection with the gram negative bacterium *Salmonella typhosa* (Acton, Weinheimer, Hildemann & Evans, 1969). These bactericidins showed a lesser degree of specificity (since they cross-reacted with heterologous gram negative bacteria) and a shorter induction period than other hagfish antibodies (Acton, Weinheimer, Hildemann & Evans, 1971).

In the sea lamprey no antibodies were detected after immunization with diphteria toxoid, BSA, BGG, SRBC, rabbit RBC, KLH, typhoid H and typhoid O antigens. A weak primary but a clear-cut secondary response was obtained when using *Brucella abortus* and bacteriophage T_2 as immunizing agent (Papermaster et al., 1964; Finstad & Good, 1966). Later studies of Marchalonis & Edelman (1968) and Litman et al. (1970), which were designed to characterize immunoglobulins, showed that lamprey did produce antibodies to bacteriophage F_2, *Brucella* and human RBC. In brook lamprey primary and secondary responses to SRBC were demonstrated in which both haemagglutinating and haemolysing

antibodies were involved (Fujii, Nakagawa & Murakawa, 1979a).

- It is concluded that in spite of initial experimental failures, antibody responses are inducible in the most primitive vertebrates; the Agnatha. However, the humoral immune system of hagfish is poorly developed. Raison, Hull & Hildemann (1978b) proposed that hagfish lack a humoral immune system equivalent to the usual B lymphocyte-plasma cell system. Immunoglobulins would exist primarily in a membrane bound state, functioning as lymphocyte surface receptors. The presence of serum immunoglobulin could be the result of shedding of receptors rather than active secretion of immunoglobulin.

Chondrichthyes have been shown to produce specific antibody after immunization with a variety of antigens. Among them were viral antigens such as influenza virus PR8 (Clem & Sigel, 1963; Sigel & Clem, 1965; Clem, DeBoutand & Sigel, 1965), bacteriophage T_2 (Papermaster et al., 1964; Suran, Tarail & Papermaster, 1967) and poliovirus (Clem & Sigel, 1963), furthermore bacterial antigens such as *Salmonella paratyphi* (Clem & Sigel, 1963) and *Brucella abortus* (Finstad & Good, 1966; Frommel, Litman, Finstad & Good, 1971). Also xenogeneic erythrocytes like SRBC (Finstad & Good, 1966) and chicken RBC (Sigel & Clem, 1966), proteins like BSA, BGG and KLH (Clem & Sigel, 1963; Papermaster et al., 1964; Finstad & Good, 1966; Sigel & Clem, 1966; Suran et al., 1967), carbohydrates like streptococcal group A carbohydrate (Clem & Leslie, 1971; Clem, McLean & Shankey, 1975; Sledge, Clem & Hood, 1974) and *Pneumococcus* type III and VIII polysaccharides (Clem et al., 1975) and haptens (DNP) conjugated to protein carriers (Leslie & Clem, 1969; Voss, Russell & Sigel, 1969; Voss, Rudikoff & Sigel, 1971; Sigel, Voss & Rudikoff, 1972) were used.

In lemon shark and nurse shark kept at 27-30°C, maximum antibody titres were obtained 30-40 days after immunization with PR8 or Sendai virus. After restimulation with PR8 virus, renewed antibody synthesis could be detected, but not in the order of a real secondary response (Sigel & Clem, 1965). Other studies of Sigel & Clem (1966) with nurse shark indicated that although the animals showed a primary response to BSA and chicken RBC, clear-cut secondary responses were lacking. Lemon shark, kept at 26-28°C produce antibodies to BSA after a latent period of 10-12 days. Maximum titres were obtained after 25-40 days with antibody levels remaining at a plateau up to 90 days after injection. No anamnestic response was observed after a second injection (Clem & Small, 1967). Only after a weak primary response a heightened secondary response could be obtained. According to Sigel, Lee, McKinney & Lopez (1978) the humoral immune response of sharks resembles a mammalian response to thymus-independent antigens. In mammals thymus-independent antigens do not evoke true secondary responses (Baker, Stashak, Amsbaugh, Prescott & Barth, 1970). Sigel et al. (1978) suggest that

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The chondrostean paddlefish produced antibodies after immunization with *Brucella abortus* or *Salmonella paratyphi* (Fish, Pollara & Good, 1966). Antibodies remained detectable for long periods after secondary stimulation (Pollara, Finstad & Good, 1966). Definite primary and secondary antibody responses were obtained with protein antigens like BSA, BGG and KLH (Finstad & Good, 1966).

Initial studies on the humoral immune response of the holostean gar showed that the animals did produce antibodies to BSA but that a secondary response was absent (Sigel & Clem, 1965; Clem & Sigel, 1965, 1966). In a later study, Bradshaw, Clem & Sigel (1969) demonstrated that the gar produced antibody to diphteria toxoid and BSA after a latent period of 11-30 days. With both antigens a clear secondary response was obtained (80-fold enhanced antibody titres for diphteria toxoid). Antibodies produced during a secondary response persisted for over 275 days. The bowfin showed a barely detectable primary antibody response to bacteriophage T_2 and KLH. The secondary response was vigorous for T_2 but not impressive for KLH (Papermaster, Condie, Finstad, Good & Gabrielsen, 1963; Papermaster et al., 1964).

The capability of teleost fish to respond upon antigenic stimulation with specific antibody production has been demonstrated since the beginning of this century. Most of the early studies were performed in order to obtain protective immunity against epizootic diseases in cultured fish. This aspect, in combination with vaccin development, will be discussed in a separate paragraph in this chapter.

Primary and secondary antibody responses have been observed in teleost fish using protein, cellular and carbohydrate antigens (see review of Cushing, 1970; Carton, 1973; Corbel, 1975). Three typical examples will be given.

1) Goldfish produce antibody after a latent period of 7-10 days when immunized with BSA at 25°C. Peak titres were reached by day 20. In a secondary response anti-BSA antibodies were detected 3 days after immunization. Peak antibody titres, reached at day 15, exceeded those of a primary response (Trump & Hildemann, 1970).

2) In Mozambique mouthbrooder kept at 30°C, maximum antibody titres were obtained on day 11 after immunization with SRBC, the latent period was only 2 days. Following a second injection with SRBC, antibodies were detected within 2 days, the 8-fold enhanced peak titre was reached on day 8 (Sailendri & Muthukkaruppan, 1975).

3) Brown trout, kept at 20°C were immunized with *Salmonella typhimurium* lipopolysaccharide (a carbohydrate antigen). In a primary response antibodies were detected after 14 days, maximum titres between day 56 and 63. In a secondary response the latent period was 14 days, the enhanced peak was reached 34-40 days after injection (Ingram & Alexander, 1980).

The length of the latent period and the kinetics of the antibody response are influenced by the following factors:

a) the ambient temperature. The effect of temperature on the immune response is discussed in Chapter 7. Even when keeping 2 different species at the same temperature

similar kinetic profiles are not to be expected because these species may have different preferential temperatures. For example peak antibody titres in a primary response at 15°C after immunization with MS_2 bacteriophage are reached in brown trout on day 35 and in carp on day 42 (O'Neill, 1980).

b) the type of antigen. In brown trout, kept at 20°C, maximum titres to *Salmonella typhi* were reached after 49 days, to KLH after 43 days (Ingram & Alexander, 1976a).

c) the nature of the antigen. Busch (1978) injected rainbow trout with preparations of enteric red mouth disease (ERM) bacterium. The latent period was shorter in animals injected with water soluble preparations than in those receiving organic solvent soluble preparations (13 and 28 days respectively). Moreover, a shorter latent period and higher antibody titres were obtained after injecting formalin killed bacteria than after the same procedure with phenol killed bacteria.

d) the route of administration. In dace, kept at 18°C antibody was detected 10 days after an intraperitoneal injection of horse serum whereas the latent period took 12 days after intramuscular injection. Moreover a higher percentage of animals responded to intramuscular than to intraperitoneal injected antigen (Harris, 1973a).

e) the antigen dose. A longer lag period was observed when immunizing carp with low doses pig serum. However, the peak day of the response was not dependent of antigen dose (Ambrosius & Schäker, 1964).

● From the data mentioned above it may be clear that it is almost impossible to draw definite conclusions when concerning the kinetics of the antibody response. Only a general conclusion can be drawn: the capacity to mount a humoral antibody response and the formation of immunological memory is well developed in teleost fish. The capacity of Chondrichthyes to develop immunological memory is questionable.

Cellular aspects

After antigen injection it takes a certain time before the first circulating antibodies appear (latent or lag period). During that period antigen is eliminated from the circulation (clearance) due to trapping by macrophages and dendritic cells in lymphoid organs (Ellis et al., 1976; Secombes & Manning, 1980). Subsequently B-like lymphocytes start to proliferate and differentiate into plasma cells. Plasma cells synthesize and secrete specific antibody. In fish, two techniques have been used to study these cellular aspects of the humoral immune response: the rosette assay and the plaque assay. With the rosette assay, originally developed by Zaalberg (1964) and Biozzi, Stiffel, Mouton, Liacopoulos-Briot, Decreusfond & Bouthillier (1966) the number of antigen binding cells (ABC) can be determined. In this assay cells carrying surface immunoglobulin or secreting antibody specific for the antigen used react *in vitro* with this antigen. When xenogeneic erythrocytes are used as antigen, cells surrounded by erythrocytes (rosettes) are formed. Cells with a single layer of erythrocytes are ABC but cells with a multiple layer of erythrocytes repre-

sent antibody secreting cells. In the plaque assay, originally developed by Jerne & Nordin (1963) only antibody forming cells are visualized.

In brook lamprey, immunized with a high SRBC dose ABC could be detected after 4 days in spleen and blood. Maximum numbers were obtained on day 12. After a low dose immunization maximum ABC numbers were found on day 8. This observation is in contrast to data mentioned above where it was shown that low antigen doses extend the latent period. Multilayered ABC were observed in spleen and blood. Thus, although lamprey lack plasma cells on morphological grounds (Finstead & Good, 1966) cells which actively secrete antibody are present (Fujii, Nakagawa & Murakawa, 1979b). In rainbow trout ABC were observed in spleen and pronephros after immunization with SRBC. Cells with the ultrastructural morphology of plasma cells were surrounded by multiple layers of SRBC. Unfortunately the immunization schedule used in this study does not allow us to draw conclusions about the kinetics of the ABC response (Chiller, Hodgins, Chambers & Weiser, 1969). ABC were found in goldfish thymus, spleen and pronephros from day 2 onwards, maximum numbers being reached at day 8. Multilayered ABC were present in spleen and pronephros (Warr, Deluca, Decker, Marchalonis & Ruben, 1977). Also in studies on the carrier-hapten effect the rosette assay has been used to monitor the response. Relevant experiments are discussed in Chapter 6.

Studies of the humoral immune response using the plaque assay have been performed only in Teleosts. The species studied are bluegill (Smith, Potter & Merchant, 1967), rainbow trout (Chiller, Hodgins & Weiser, 1969), goldfish (Neale & Chavin, 1971), perch (Pontius & Ambrosius, 1972) and Mozambique mouthbrooder (Sailendri & Muthukkaruppan, 1975).

- The cellular aspects of the humoral immune response have been studied in a limited number of teleosts species.

  First plaque forming cells (PFC) were observed 2 days after immunization. Peak of the PFC response preceeds in all cases the peak of serum antibody and is reached at the same day in different lymphoid organs. An accelerated as well as enhanced PFC response was demonstrated after secondary immunization.

Vaccination and protective immunity

In early studies it has been shown that teleost fish are capable of producing antibodies against bacterial antigens (reviewed in Ridgway, Hodgins & Klontz, 1966 and Avtalion, Wojdani, Malik, Shahrabani & Duczyminer, 1973). These studies were aimed to develop vaccination schedules which provide protective immunity against epizootic diseases in cultured fish. Most attention has been devoted to the main bacterial diseases: vibriosis (caused by *Vibrio anguillarum*), enteric redmouth disease (*Yersinia ruckeri*), furunculosis (*Aeromonas salmonicida*), columnaris disease (*Flexibacter columnaris*) and bacterial kidney disease (*Corynebacterium* spp.).

Injections of *Vibrio anguillarum* bacterin evoked antibody titres in chinook salmon and coho salmon. Immunized animals were protected against a natural challenge

up to 6 months post-injection (Antipa, 1976). Several reports describe antibody responses against *Aeromonas salmonicida* after intramuscular or intraperitoneal injections (Krantz, Reddecliff & Heist, 1963, 1964; Post, 1966; Maisse & Dorson, 1976). No mortality occurred in immunized animals upon challenge with live bacteria. High agglutination titres against *Flexibacter columnaris* were obtained in rainbow trout after parenteral vaccination. Susceptibility of vaccinated animals to challenge with virulent columnaris bacteria was greatly reduced (Becker & Fujihara, 1978). It can be concluded that vaccination by injecting killed bacteria evokes a humoral antibody response and provides the animals with protective immunity upon challenge with live bacteria.

In large scale fish culture immunization of individual animals is hardly applicable for economical reasons. Therefore mass vaccination methods have been developed:
a) Oral immunization. This method has been used for about 40 years and consists of mixing vaccin with food. A humoral antibody response has been observed both in blood and mucus of intestine and skin after feeding plaice with *Vibrio anguillarum* antigens (Fletcher & White, 1973a). Oral immunization has yielded positive (e.g. Braaten & Hodgins, 1976; Fryer, Rohovec & Garrison, 1978) but also negative results (Schachte, 1678; Udey & Fryer, 1978). Efficacy of oral immunization is reviewed in Snieszko (1970), Anderson (1974) and Evelyn (1977). In most cases oral vaccination has not been satisfactory because of the variable results and the discrepancy between laboratory and field studies.
b) Spray vaccination. In this method the animals are removed from water and under high pressure vaccin is sprayed on fish (Evelyn, 1977; Gould, O'Leary, Garrison, Rohovec & Fryer, 1978). Results obtained thusfar suggest that this technique is more effective than oral immunization.
c) Bath immunization. In a first version of this technique the fish were placed in a vacuum chamber in a hyperosmotic vaccin solution. During a short period the atmospheric pressure was reduced (Amend, 1976). In a second version antigen uptake was accomplished by merely placing the animals in a hyperosmotic vaccin solution (Amend & Fender, 1976). Recently it has been shown that direct immersion is as effective as hyperosmotic infiltration (Egidius & Anderson, 1979; Antipa, Gould & Amend, 1980). In coho salmon, high agglutination titres were observed against *V. anguillarum* and *A. salmonicida* after hyperosmotic infiltration with a bivalent bacterin (Antipa & Amend, 1977).

- Based upon the results obtained thusfar, bath immunization seems to be superior to oral or spray vaccination. It is a promising method in the prevention of infectious diseases in cultured fish.

Relative little attention has been paid to the cellular aspects of the antibody response in developing vaccination schedules. Anderson (1978) adapted the plaque assay to detect antibody forming cells in rainbow trout injected with the O-antigen of *Yersinia ruckeri*. In animals kept at 17°C first PFC were detected after 7 days, the

peak occurred on day 11 (Anderson, Roberson & Dixon, 1979b). PFC were also detected after immersion of the animals in the antigen solution (Anderson et al., 1979a). The minimal antigen concentration required to evoke a response was 5 µg/ml (Anderson et al., 1979c). After flush exposure of rainbow trout at 11°C first PFC were observed on day 9 while highest PFC numbers were found on day 14-16 (Anderson et al., 1979d). Another method to study the immune status of rainbow trout after bath immunization has been developed by Chilmonczyk (1977, 1978b). Lymphocytes of trout after survival of a natural VHS infection were induced to blast transformation *in vitro* with VHS virus, whereas control animals did not. Specific lymphocyte stimulation was also obtained in vaccinated and subsequently challenged animals.

Anaphylaxis

In mammals anaphylactic reactions are caused by interaction of antigen with a special antibody class (IgE) bound to mast cells. This interaction leads to degranulation of mast cells (release of histamine).

Anaphylactic reactions were demonstrated in perch, sunfish, rock bass, and goldfish (Dreyer & King, 1948). Sensitization after intraperitoneal injections of either horse serum or ovalbumin elicited signs of anaphylaxis after re-exposure to homologous antigen following a latent period of at least 10 days. The anaphylactic reaction was specific and characterized by folding and curling of fins, equilibrium disturbance, sinking to the bottom and diminished movement. These signs persisted for 6 hours. Unsensitized fish demonstrated no anaphylatic symptoms.

Later reports failed to repeat these observations. Clem and Leslie (1969) attempted to demonstrate anaphylaxis in the margate by two different approaches. In the first approach animals were intravenously injected with margate antisera to BSA. Animals were injected intravenously with BSA 4 to 24 hours later but no reaction followed. In the cutaneous approach, animals were injected intravenously with BSA and Evans blue 4 to 24 hours after subcutaneous injection of margate antiserum to BSA. No obvious blueing was noted at the site of injection. Harris (1973b) was unable to demonstrate an anaphylactic reaction in dace and chub using horse serum or egg albumin. However, in plaice and flounder intradermal injections with fungal extracts cause immediate erythema (Fletcher & Baldo, 1974). Immediate hypersensitivity reactions occurred in channel catfish and goldfish following immunization and challenge with different antigens (solubilized protozoa, BSA, versatol). The reactions (disorientation, vertical swimming, increased opercular movement and gasping) were specific for the sensitizing antigen and could be passively transferred to nonsensitized recipients with serum from sensitized animals (Goven, Dawe & Gratzek, 1980).

Mast cells have been described in fish (Ellis, 1977a) and it has been shown that these cells contain histamine (Roberts, Young & Milne, 1972). An IgE-like molecule has not been found in fish. If IgM can mimic the homocytotropic IgE function or that mast cells of fish are activated by another mechanism is not known.

Anaphylatic reactions have been demonstrated in a number of teleost species. If

immunoglobulin is involved in these reactions is not known.

CHAPTER 5
CELLULAR IMMUNITY

Cellular immunity involves all specific immune responses mediated by a combination of living lymphoid and mononuclear phagocytes and their non-antibody effector molecules. Cell mediated immunity includes the mixed leukocyte reaction, migration inhibition, delayed type hypersensitivity, transplantation immunity and tumor immunity. A related but non antigen specific reaction used for demonstration of T cell activity is the *in vitro* response to certain mitogens. The capacity of fish lymphocytes to respond to "T cell mitogens" is discussed in chapter 6.

Mixed leukocyte reaction (MLR)

McKinney et al. (1976, 1980) failed to demonstrate positive MLR using cells from sharks, snappers and gars. In xenogeneic combinations, catfish leukocytes as target cells evoked positive MLR by human peripheral blood cells but the reciprocal combination gave negative results. In rainbow trout it was observed that homologous mixtures of peripheral blood leukocytes or pronephros cells obtained from 2 individual fish revealed marked proliferative responses. Maximum responses were observed on day 7 with pronephros cells and on day 9 with peripheral blood lymphocytes (Etlinger, Hodgins & Chiller, 1976b; 1977). In blue gill a positive mixed lymphocyte reaction was obtained with pronephros cells at 32°C but not at 22°C. Positive MLR has been observed in 12 out of 15 random two-way cultures. Negative reactions were not correlated with a low responsiveness to phytohaemagglutinin (PHA) by either cell preparation (Cuchens & Clem, 1977).

- In a number of Teleosts positive MLR has been observed while in otheres negative results were obtained.

Migration inhibition (MI)

Jayaraman, Mohan & Muthukkaruppan (1979) assessed the cell mediated immune response of Mozambique mouthbrooder to SRBC using the migration inhibition (MI) technique. Formalinized SRBC, which have been shown to activate T-helper cells specifically without the generation of plaque forming cells in mice (Dennert & Tucker, 1972) evoked an enhanced MI response without a detectable PFC response. Optimal MI responses were recorded when using relatively low SRBC doses. In the gar MI reactions were induced in kidney and peripheral blood cells after a secondary allograft rejection (McKinney, McLeod & Sigel, 1980). The lectins PHA, concanavalin A (con A) and pokeweed mitogen (PWM) also evoked MI reactions (McKinney, Ortiz, Lee, Sigel, Lopez, Epstein & McLeod, 1976). The migrating cells were identified as macrophages but it was presumed that lymphocytes were involved in producing inhibitory substances. In the elasmobranch dogfish an MI response to an antigen extract of the nematode *Proleptus obtusus* could be demonstrated (Morrow & Harris, 1978).

- MI reactions have been obtained in all fish species tested thus far.

Delayed type hypersensitivity (DTH)

Adult sea lampreys were injected intramuscular with complete Freund's adjuvant followed 21 days later by a challenge with old tuberculin. Within 48 hours all sensitized animals showed typical delayed type skin reactions (Finstad & Good, 1966; Good, Finstad & Litman, 1972). In hagfish, no evidence for DTH to BGG or tuberculin was found (Papermaster, Condie & Good, 1962). In bowfin a DTH reaction was observed 3 days after challenge with *Ascaris* antigen. The challenge was given 30 days after initial sensitization. The reaction consisted of a well defined indurated area in the axial musculature (Good & Papermaster, 1964). In the horned shark, guitarfish and paddle fish, moderate to severe inflammatory reactions occurred after repeated injection with BGG in complete Freund's adjuvant. According to Finstad & Good (1966) the reactions were due to DTH. In rainbow trout a positive delayed corneal reaction to purified protein derivate of tuberculin (PPD) has been demonstrated 1 month after immunization with complete Freund's adjuvant. The reaction reached a peak in 3-5 days and lasted 12 days. Unimmunized controls injected intracorneally with PPD only showed a transient and faint clouding reaction (Ridgway, Hodgins & Klontz, 1966).

● Delayed type hypersensitivity reactions have been demonstrated in all classes of fish, except hagfish.

Transplantation immunity

Pacific hagfish - one of the most primitive vertebrates - was supposed to be incapable of rejecting allografts (Good & Papermaster, 1961; Papermaster, Condie, Finstad & Good, 1964). However, later studies revealed that the capacity to recognize and reject skin allografts was well developed in this species (Hildemann & Thoenes, 1969). Allgraft destruction involved inflammatory reactions, lymphocyte infiltration, capillary hemorrhage and pigment cell destruction. In animals kept at 18-19°C first-set allografts showed a chronic type of rejection illustrated by a median survival time (MST) of the grafts of 72 days. Second-set grafts placed one month later were rejected within 28 days. When second-set grafts were placed immediately after the first rejection process was completed, an acute rejection occurred within 14 days. Rejection of first-set skin allografts in sea lamprey, kept at 18-21°C starts during the 3th week after transplantation. Between 42 and 47 allografts are completely rejected (Finstad & Good, 1966; Perey, Finstad, Pollara & Good, 1968). The rejection of second-set grafts, placed 39 days after first-set grafting starts in the second week. By 18 days most grafts were completely destroyed (Perey et al., 1968).

Sting rays, belonging to the class of Chondrichthyes, kept at 18-28°C showed by day 21 the onset of first-set graft rejection. Survival end points ranged from 30-53 days. Second-set grafts evoked a more severe inflammatory reaction, most grafts showed survival times of less than 12 days (Perey et al., 1968). In the horned shark, kept at 22°C four successive sets of skin allografts yielded MST's of 41, 17, 9 and

7 days respectively (Borysenko & Hildemann, 1970). Lymphocytes and mononuclear cells were present in the inflammatory exudate but no plasma cells (Finstad & Good, 1966).

In the holostean gar, first-set allografts were rejected in an acute manner (McKinney, McLeod & Sigel, 1980). During the first 8 days after transplantation both autologous and allogeneic grafts were invaded by inflammatory cells. Subsequently, allografts proceeded to focal and extensive necrosis of the connective tissue by day 10-18 while chromatophores cleared. Cells infiltrating the allografts were identified as lymphocytes, monocytes and granulocytes. Second-set allografts were all rejected by day 12. Another interesting fish is arrowana belonging to the superorder *Osteoglossomorpha* which includes fish near or just above the holostean level of organization. In arrowanas maintained at 25°C first-set skin allografts were rejected in a sub-acute manner (MST 17.9 days) whereas second-set grafts showed an acute rejection characterized by an MST of 5.1 days (Borysenko & Hildemann, 1969). Histopathologic manifestations of allograft rejection include hyperplasia, lymphocyte infiltration, hemostasis, pigment cell granulation and donor cell replacement by host tissue (Hildemann, 1970).

In the Chondostrean paddlefish, kept at 18-25°C, first-set skin allografts were rejected between day 42-68. Second-set grafts were rejected within 12 days. The second-set rejection was characterized by pronounced hemorrhage, followed by necrosis (Perey et al., 1968).

In teleost fish, kept at optimal temperatures, allografts are always rejected in an acute manner, i.e. rejection times shorter than 14 days. Transplantation immunity has been extensively studied in goldfish using the scale graft technique. Following transplantation both autografts and allografts become revascularized, the time required for restoration of circulation varies from 1 day at 32°C to 12-15 days at 10°C (Hildemann, 1957). Allografts become overgrown with hyperplastic host tissue and elicit capillary leakage and vasodilation in the contact zone with recipient tissue. The end point of donor tissue breakdown has been estimated by a biological test, i.e. regrafting scales on the donor. It was found that allografts survived up to the time that clearing of the dense hyperplastic tissue grown over the graft started. The MST of first-set allografts in goldfish kept at 25°C was 7.2 days. Second-set grafts, placed 25 days after first-set grafting, induce early and severe inflammation and were rejected rapidly (MST 4.7 days) (Hildemann & Haas, 1960). With an increasing number of allografts, ranging from 1-9 grafts per animal, no significant antigen dosage effect on the MST of first- or second-set allografts was observed (Hildemann, 1957). A definite secondary immune response to tissue allografts was obtained already at 6-8 days following first grafting.

The following experiments have been performed to demonstrate that allograft rejection is a cell-mediated immune reaction. Lyophilized grafts or grafts heated at 48°C for 20 minutes did not provoke transplantation immunity as manifested by the finding that second-set grafts were not rejected faster. Probably no isohaemagglutinating antibodies are involved in the normal process of graft rejection since no

alteration in serum titre of recipient animals towards red blood cells of donor animals was observed after scale rejection. In another set of experiments animals were immunized with whole blood from donor animals. Four intraperitoneal injections yielded a haemagglutination titre of 1:526 whereas 2 intramuscular injections gave rise to a serum titre of 1:42. When these animals received scale allografts from their respective blood donors no secondary response was evident in animals injected by the intraperitoneal route (MST 7.2 days) but a strong immunity was expressed in animals injected by the intramuscular route (MST 5.6 days) (Hildemann, 1958).

Sailendri (1973) studied allograft rejection in Mozambique mouthbrooder. In animals kept at 25°C, allografts showed swelling, hyperplasia and severe inflammation on the second day after transplantation. Later expansion of melanophores occurred. Inflammation disappeared at the 6th day and desintegration of melanophores was completed 2 days later marking the survival end-point of the grafts. The grafts were invaded by leukocytes from day 3 onwards, with a maximum on day 6. The rejection process of second-set allografts was accompanied by a more severe inflammation. Second-set allografts, transplanted as long as 4 month after first-set, were rejected in an accelerated way, indicating a long lived immunological memory. Xenografts evoked a more severe inflammation and faster rejection than allografts. Antigen dosage (3-18 allografts per animal) had no effect on the MST of the grafts. Second-set allografts transplanted from a third-party donor were rejected in a primary fashion.

- In Agnatha and cartilaginous fish allografts are rejected in a chronic way (MST > 30 days). Sub-acute rejection (MST approximately 20 days) occurs in Chondostreans and Holosteans, while Teleosts show an acute rejection of allografts (MST < 14 days). Second-set grafts are always rejected faster than first-set. It has been demonstrated that allograft rejection is a manifestation of cellular immunity.

Ontogeny of transplantation immunity

Adult rainbow trout, kept at 16-18°C, reject skin grafts in 19-21 days. First-set grafts are invaded by large numbers of lymphocytes on day 5 post grafting followed by maximal infiltration on day 9. In 26 day old trout lymphocytes are not present in the graft before day 7 reaching a maximum at day 12. However, the total range of rejection times falls within that of adults (Botham, Grace & Manning, 1980).

In adult carp, kept at 22-23°C, lymphocyte infiltration in first-set skin grafts starts 2 days after grafting, reaching a maximum by day 4-6. Allograft breakdown commences by day 8 while total rejection occurs by day 14 post grafting. In 2-4 weeks old animals invading lymphocytes are seen first between day 4-8 after grafting and graft breakdown starts at day 16. In 2 months old carp lymphocyte infiltration also starts by day 4-8, but graft breakdown commences by day 11. It is concluded that carp as young as 16 days post hatching are capable of mounting an allograft response but complete maturation of the cellular immune system takes over 2 months (Botham et al.,

1980).

The MST of first-set allografts in 4.5 month old Mozambique mouthbrooder is the same as in adults (5.4 and 5.2 days respectively). In younger animals (1.5 months) scale allografts survived for 10.9 days (Sailendri, 1973).

- In oviparous Teleosts the capacity of larvae to reject allografts starts 16-45 days after fertilization.

CHAPTER 6

LYMPHOCYTE HETEROGENEITY

The lymphocytes of higher vertebrates can be divided in 2 major subsets according to their origin, function and characteristics. Cells derived from the thymus (T cells) are involved in cellular immune reactions such as: allograft rejection, graft versus host reaction, mixed lymphocyte reaction, delayed type hypersensitivity and helper function in antibody formation. B lymphocytes, which are derived from the bursa of Fabricius in birds and from a bursa-equivalent in mammals, mediate humoral immunity by producing antibodies. T cells function predominantly by direct contact with antigen while plasma cells (B cell derived) produce antibodies which either directly or indirectly act upon antigens. In TABLE 7 some characteristics of mammalian T and B cells are listed.

TABLE 7 Characteristics of T and B lymphocytes in adult mammals

Feature	T lymphocyte	B lymphocyte
origin	thymus	bone marrow and/or bursa-equivalent
Thy-1 antigen	present	absent
membrane bound Ig	absent	present
Fc receptor	absent	present
C_3 receptor	absent	present on some B cells
mitogen response	PHA, ConA	LPS
"carrier-reactive"	+	-
"hapten-reactive"	-	+
function	helper cell, suppressor cell, killer cell	plasma cell precursor

In this Chapter data will be presented which are required to answer the question whether fish lymphocytes can be classified in T and B subsets or not.

Thy-1 antigen

Thy-1 was shown by Reif & Allen (1963) to be a surface antigen present on mouse thymocytes and nervous tissue. Later studies revealed that Thy-1 antigen could be used as a marker for mouse T cells (Raff, 1971). Most anti-Thy 1 sera are iso-antisera but T cell specific antisera can also be obtained by immunizing rabbits with mouse brain cells and absorbing the antiserum with mouse erythrocytes and liver cells (Golub, 1971; Thiele, Stark & Keeser, 1972). Strictly speaking these antisera cannot be denominated anti-Thy-1 sera but they are useful as a T cell marker.

Cuchens & Clem (1977) prepared a rabbit antiserum against bluegill brain tissue. In combination with complement this antiserum killed about 70% of pronephros lymphocytes. Cells surviving the antiserum treatment responsed to LPS (a mammalian B cell

mitogen, see below) but not to PHA (a mammalian T cell mitogen) whereas untreated pronephros cells can be stimulated by both LPS and PHA. These results suggest that bluegill lymphocytes responsive to PHA carry surface antigens related to murine Thy-1 antigen.

Surface immunoglobulin (sIg)

In mammals T cells lack sIg in the sense that they do not react with antisera directed against serum Ig (Raff, 1970). It has been established that the vast majority of fish lymphocytes, including thymocytes bear surface immunoglobulin (TABLE 8). This conclusion is based upon the observation that a rabbit antiserum raised against fish serum immunoglobulin reacts with surface material of lymphocytes. The positive reaction of the anti-immunoglobulin sera with thymocytes is probably not due to cross-reactivity with cell surface carbohydrates because (1) components with an electrophoretic mobility of immunoglobulin polypeptide chains can be extracted from thymocytes (see below) and (2) a positive reaction can be obtained using antisera directed against immunoglobulin light chains which lack a carbohydrate moiety (Fiebig & Ambrosius, 1976; Clem, McLean, Shankey & Cuchens, 1977). Interestingly, quantitative and qualitative differences in sIg on lymphocytes from different lymphoid organs exist in fish. Carp peripheral blood and pronephros lymphocytes carry more sIg molecules per cell than thymocytes (Fiebig, Scherbaum & Ambrosius, 1977). Lymphocytes of goldfish and Crusian carp are heterogeneous in the quantitative distribution of sIg: fluorescence intensity of spleen >> pronephros > thymus (Warr, DeLuca, Decker, Marchalonis & Ruben, 1977; DeLuca, Warr & Marchalonis, 1978). A lower quantity of sIg on skate thymocytes compared with splenocytes was suggested by Ellis & Parkhouse (1975). In rainbow trout thymocytes possessed at least 10-fold lower amounts of sIg compared with splenocytes (Yamaga, Etlinger & Kubo, 1977). In blue gill, however, quantification of sIg revealed that there was little difference between lymphocytes from peripheral blood, spleen, pronephros and thymus in this respect (Clem, McLean, Shankey & Cuchens, 1977).

In order to further characterize sIg on carp lymphocytes, Fiebig & Ambrosius (1976) radioiodinated peripheral blood, pronephros and thymus lymphocytes and extracted surface proteins with non-ionic detergents. Protein was analysed on sodium-dodecylsulfate-polyacrylamide-electrophoresis. Surface Ig was never present in a tetrameric form, as in serum IgM but as monomers (H_2L_2). This monomeric Ig on pronephros lymphocytes had a M.W. of 220,000 while sIg of thymocytes had a M.W. of 260,000. Peripheral blood lymphocytes carried both forms. Furthermore sIg was present on all lymphocytes as HL subunits (M.W. 110,000). Thymocytes, apart from H and L chains, showed 2 additional components upon electrophoresis, with M.W.'s of 100,000 and 35-40,000. The last component was not observed on pronephros lymphocytes. In goldfish sIg of splenocytes and thymocytes differ in solubilization characteristics with non-ionic detergents and in H chain electrophoretic mobility. The H chains of splenocytes were comparable to mammalian μ chains whereas thymocyte H chains express-

TABLE 8 Surface immunoglobulin on fish lymphocytes

Species	Percentage surface immunoglobulin-positive lymphocytes					Reference
	Thymus	Pronephros	Mesonephros	Spleen	Peripheral blood	
CHONDRICHTYES						
skate (*Raja naevus*)	60-80			60-80	60-80	{1}
TELEOSTS						
rainbow trout (*Salmo gairdneri*)	99	96		99	90	{2}
carp (*Cyprinus carpio*)	65-68	30-58		25-45	30-58	{3}
Crusian carp (*Carassius carassius*)	100	100		100		{4}
goldfish (*Carassius auratus*)	97	76		80		{5}
blue gill (*Lepomis macrochirus*)	>90	>90	<5	>90		{6}

1) Ellis & Parkhouse, 1975; 2) Etlinger et al., 1976b; 3) Emrich et al., 1975; 4) DeLuca et al., 1978; 5) Warr et al., 1976; 6) Clem et al., 1977.

ed a faster mobility (Warr, DeLuca & Marchalonis, 1976). Furthermore, sIg of splenocytes differs from serum Ig in a deficiency of 10,000 d in the H chain of splenocyte sIg (Warr & Marchalonis, 1977). The H chain peak of pronephros sIg was very broad compared with the sharp H chain peak obtained from thymocyte sIg (Ruben, Warr, Decker & Marchalonis, 1977). Characterization of sIg of rainbow trout splenocytes showed the presence of 3 major components with M.W.'s of approximately 100,000, 65,000 and 25,000. In contrast thymocytes contained several high M.W. components (70-150,000) but lacked peaks in the L chain region (Yamaga, Etlinger & Kubo, 1977). Bluegill sIg is quite similar to serum Ig, and M.W.'s of H chains isolated from thymocytes is similar to that isolated from other lymphoid tissue (Clem, McLean, Shankey & Cuchens, 1977).

- It can be concluded that in fish nearly all lymphocytes carry sIg which is present in a monomeric form or as HL subunits. However, there is some heterogeneity (both quantitative and qualitative) in sIg among lymphocytes from different lymphoid organs.

Hapten-carrier effect

Cellular co-operation during an immune response between different lymphocyte populations can be studied using the hapten carrier effect. A hapten is a small, in itself non-antigenic molecule (such as DNP) which is covalently coupled to a large "carrier" molecule, generally a protein. The hapten-carrier effect basically involves preimmunization with a carrier, followed by a booster with the hapten-carrier complex. This schedule results in an enhanced primary response to the hapten. In mammals, T cells react with the carrier moiety while B cells respond to the hapten.

Hapten-carrier immunization was used in brook lamprey to explore lymphocyte heterogeneity at the lowest phylogenetic level of the vertebrates. Carrier priming did not enhance the following anti-hapten response (Fujii, Nakagawa & Murakawa, 1979b).

For a number of teleosts the hapten-carrier effect now has been demonstrated using two basically different techniques. A first technique in which the number of hapten binding cells is determined has been used in goldfish. Two or eight days after carrier priming (HRBC) animals were immunized with the hapten tri-nitrophenol (TNP) conjugated to HRBC. The TNP-binding pronephros lymphocytes were shown to be hapten specific since preincubation of sensitized cells with TNP-glycine completely eliminates the response. In goldfish kept at 24°C, maximum TNP binding activity appears at day 8 after challenge. High dose priming is uneffective in generating helper activity. Helper memory evoked by low dose carrier priming is short lived: TNP-HRBC challenge 8 days after HRBC priming does not result in an anti-TNP response (Ruben, Warr, Decker & Marchalonis, 1977).

The other approach to demonstrate the hapten-carrier effect (titration of hapten specific serum antibodies) was followed in winter flounder. Carrier preimmunization with chicken globulin (CG) or KLH was followed 3 weeks later by immunization with the

haptens NIP (3-iodo-4-hydroxy-5-nitrophenyl acetic acid) and NNP (3,5-dinitro-4-hydroxy-phenyl acetic acid) covalently coupled to CG or KLH. Antibody titres were determined by the ability of serum to inactivate haptenated bacteriophages. Pre-immunization with the carrier enhanced the antibody response to the hapten in a specific way: a response to NIP-CG could only be detected after preimmunization with CG, not after preimmunization with KLH (Stolen & Mäkelä, 1975, 1976). Weiss & Avtalion (1977) preimmunized carp with either native BSA, methylated BSA or acetylated BSA as carriers. Three weeks later animals were immunized with the hapten penicillin conjugated to the carrier in various epitope densities (Pen_3-BSA and Pen_5-BSA). It was shown that 2 factors were important in obtaining a maximal enhancement of the primary anti-hapten response: a) preimmunization with a modified carrier molecule rather than its native form and b) a low hapten density on the hapten-carrier conjugate.

Ruben et al. (1977) and Warr et al. (1977) have attempted to separate "carrier-reactive" and "hapten-reactive" lymphocyte populations using the nylon wool column adherence method (Julius, Simpson & Herzenberg, 1973). Pronephros and thymus cells of goldfish, which were primed with HRBC and challenged with TNP-HRBC, were used. Hapten-reactive cells were only found in pronephros, not in thymus. These hapten-reactive cells were retained in the nylon-wool column. Thus, hapten-reactive and carrier-reactive cells could be physically separated by their adherence to nylon-wool columns.

- In conclusion it is evident that cell-cell cooperation in humoral immune responses is demonstrated for a number of teleost fish indicating that there are at least 2 different lymphoid cell populations. On the other hand this phenomenon probably does not yet occur at the level of Agnatha.

Cell surface receptors

The only report on C_3 receptors on cell surfaces in fish deals with a fibroblast cell line of goldfish. It appears that the goldfish fibroblasts have a receptor for mouse (AKR) C_3 but the function of this receptor is unknown (Ueki, Fukushima, Hyodoh & Kimoto, 1978).

- As far as we know nothing is published so far about C_3 and Fc receptors on fish lymphocytes.

Mitogen response

In higher vertebrates, T and B lymphocytes can be distinguished by their *in vitro* response to different mitogens. In contrast to a particular antigen, which stimulates only a small fraction of lymphocytes, mitogens stimulate substantial parts of lymphocyte populations non-specifically. After stimulation large lymphoblasts are formed, which will undergo mitosis and finally develop into effector cells. In mammals the mitogens phytohaemagglutinin (PHA) and concanavalian A (Con A) stimulate predominantly T cells; lipopolysaccharide (LPS) and polyvinylpyrrolidone (PVP) only B cells.

Blood cells of Pacific hagfish , synthesize DNA and proliferate in response to PHA. Relative long incubation periods and high PHA concentration are required compared with mammalian lymphocyte cultures (Tam, Reddy, Karp & Hildemann, 1977).

Peripheral blood lymphocytes from nurse shark respond to Con A, but not to PHA (Lopez, Sigel & Lee, 1974). Separation on Ficoll-Isopaque gradients yielded 2 populations of lymphocytes of which one responded to PHA and Con A and the other population only to Con A. It was concluded that sharks possess lymphocytes capable of responding to mitogens which are stimulators for mammalian T cells.
Since it was possible to inhibit PHA responsive cells by the addition of Con A responsive cells the authors suggest that nurse shark possessed suppressor cells (Sigel, Lee, McKinney & Lopez, 1978).

Within the teleost family, most work on mitogen induced lymphocyte transformation has been carried out with rainbow trout. Bogner & Ellis (1977) demonstrated that *in vivo* peripheral blood lymphocytes respond to PHA, Con A and LPS with the formation of lymphoblasts. *In vitro*, peripheral blood lymphocytes could be stimulated by PHA, Con A, LPS and PPD (Etlinger, Hodgins & Chiller, 1975, 1976a; Chilmonczyk, 1978a). Simultaneous exposure to both LPS and PHA led to significant higher stimulations than by each mitogen alone, suggesting that there are 2 different subpopulations of peripheral blood lymphocytes (Chilmonczyk, 1978a). Rainbow trout thymocytes respond to the T cell mitogen Con A but not to the B cell mitogen LPS. Leukocytes from pronephros respond to LPS but not to Con A and PPD, while splenocytes could be stimulated by LPS, PPD and Con A (Etlinger et al., 1976a).
Peripheral blood lymphocytes of snapper could be stimulated by PHA (McKinney et al., 1976), In contrast to the clear-cut organ distribution of mitogen responsiveness in rainbow trout, bluegill lymphocytes from pronephros, thymus and spleen can be stimulated by PHA, Con A and LPS (Cuchens, McLean & Clem, 1976).

Morphological consequences of lymphocyte transformation have been studied in rainbow trout splenocytes (Etlinger, Hodgins & Chiller, 1978). Con A induced blast cells are larger and possess a lighter (May-Grünwald/Giemsa) staining nucleus than lymphocytes, have a higher cytoplasm-nucleus ratio and an increased amount of mitochondria and rough endoplasmic reticulum (RER). Plasma cells occur infrequently in Con A stimulated cell cultures. Blast cell morphology of LPS and PPD cultures is similar to those with Con A. LPS and PPD induce mitogenesis revealed by the presence of about 7% plasma cells after 7 days of culture (Etlinger, Hodgins & Chiller, 1976b).

- Lymphocytes of fish respond *in vitro* to mitogens by blast transformation. A different organ distribution for cells responding to T- and B-cell mitogens is found in some species.

Do fish possess T and B lymphocytes?

In the literature 3 different answers have been given to the question above mentioned a) yes, they do, b) no, only B cells, and c) no, only T cells.

a) Taking the above mentioned data into account, Marchalonis concluded that fish lymphocytes can be divided into 2 subsets, one with T-like properties and the other with B-like properties (Marchalonis, Warr & Ruben, 1978; Warr & Marchalonis, 1978) The major argument against this conclusion is that in fish (and in lower vertebrates in general) both "T" and "B" cells carry sIg whereas in mammals T cells do not. This argument is countered by stating that mammalian T cells dò carry sIg. In fish, thymocyte sIg differs from serum Ig and pronephros sIg. Thymocyte sIg cross-react with rabbit anti-Ig sera because of the phylogenetic distance between mammals and fish. Indeed, when using chicken antiserum against Fab fragments of serum Ig, thymocytes of man and mouse carry cross-reacting sIg (Jones, Graves & Orlans, 1976; Marchalonis, Warr, Bucana, Hoyer, Szenberg & Warner, 1977; Szenberg, Marchalonis & Warner, 1977). The reverse experiment, goldfish anti-carp serum Ig incubated with carp thymocytes, should consequently yield negative results.

b) On the other hand McKinney et al. (1976) suggest that no heterogeneity exists in fish lymphocytes. In fact they state that fish do not possess T lymphocytes. Their arguments are the following.

1) Organ cultures of thymus synthesize antibody. 2) During a humoral immune response in fish no shift from IgM to IgG occurs. This shift is stimulated by T cells in mammals (Katz & Benacerraf, 1972). 3) Although the hapten-carrier effect has been demonstrated in fish, it remains to be proven that carrier reactive cells are T cells or lymphocytes at all. 4) The assumption that every fish lymphocyte stimulated by a mammalian T cell mitogen (PHA) is a real T cell is premature. For instance, blood cells from the tunicate, *Ciona intestinalis*, which lacks a thymus respond to PHA (Tam et al., 1977). Furthermore, under certain circumstances, PHA can stimulate mammalian B cells (Greaves, Owen & Raff, 1974).

Therefore McKinney et al. (1976) provisionally concluded that fish lymphocytes act principally as B cells and that the observed T cell functions reflect multipotentiality.

c) Reciprocal transplantation of thymic primordia between diploid and triploid amphibian embryos (*Rana pipiens*) suggested that a) thymus lymphocytes arise within the thymus; b) virtually all lymphocytes are thymus derived (Turpen, Volpe & Cohen, 1973; 1975). If this is true for lower vertebrates in general, fish lymphocytes should be classified as T cells.

The difficulty in assessing T and B equivalents in lower vertebrates can be illustrated by summarizing relevant data concerning the most primitive class of fish: the Agnatha. On morphological ground these animals lack a definite thymus (Good, et al., 1966; Riviere, Cooper, Reddy & Hildemann, 1975);
Allografts are rejected (Perey, Finstad, Pollara & Good, 1968; Hildemann & Thoenes, 1969); Peripheral blood lymphocytes respond to PHA (Cooper, 1971; Tam et al., 1977); Hapten-carrier effect is absent (Fujii et al., 1979b). The animals respond to (a limited variety of) antigens by the production of specific antibody (Linthicium & Hildemann, 1970; Fujii et al., 1979a); no mammalian type plasma cells were observed

(Good et al., 1966).

- At this moment it is impossible to classify fish lymphocytes in definite T and B cell subsets, although data for more advanced species are more consistent. More data are required on ontogeny of lymphocytes, effects of thymectomy and behaviour of separated "T" and "B" cells in reconstitution experiments. Until then it is preferred to speak in terms of functional T- and B-like fish lymphocytes

CHAPTER 7

FACTORS AFFECTING THE IMMUNE RESPONSE

Temperature

Since fish are poikilothermic vertebrates, the ambient temperature will influence all metabolic processes including the immune response. From a theoretical viewpoint this phenomenon offers interesting possibilities, e.g. dissociation of the immune response into discrete steps by a mere variation in water temperature. The temperature dependence of the immune system also has some practical consequences.

Non lymphoid defence

It has been shown in carp that phagocytosis occurred at a relative slow rate at temperatures which prevented antibody synthesis (see below) (Avtalion et al., 1973; Wojdani, Katz, Shahrabani & Avtalion, 1979). The rate of MS2 bacteriophage clearance from the blood of carp and rainbow trout decreases with lowering temperatures (O'Neill, 1980). The same effect was observed in nurse shark and lane snapper (Russell, Taylor & Sigel, 1976). The complement system in fish functions very well at low temperatures (Chapter 2). Natural agglutinins to SRBC and *Salmonella typhosa* in the gar display a higher activity at 4°C than at 22°C. In paddlefish also "cold" agglutinins were found (Legler, Weinheimer, Acton, Dupree & Russell, 1971). It is possible that "cold" agglutinins represent a compensatory mechanism for the immunosuppressive effect of low temperatures in fish (Bradshaw & Sigel, 1969).

● Non lymphoid defence systems are relative temperature independent.

Cellular immunity

In Chapter 5 data have been presented which indicate that allograft rejection is a manifestation of cellular immunity. In a number of publications it has been shown that allograft rejection is delayed at low temperatures (Goss, 1961; Stutzman, 1967; Botham, Grace & Manning, 1980). Hildemann (1957) studied the effect of water temperature on the median survival time of allografts in goldfish. At all temperatures studied a clear-cut secondary response were observed (Table 9). Both first set and second set allograft rejection was strongly temperature dependent. In Mozambique mouthbrooder the temperature effect on cellular immunity was much less pronounced (Table 9) (Sailendri, 1973). It was found in goldfish that the relationship between MST and temperature was not linear but that a break occurred between 20 and 25°C (Hildemann & Cooper, 1963). Similar observations have been made in amphibians (Cohen, 1966) and reptiles (Borysenko, 1970). Grafted frogs (Macela & Romanovsky, 1969) and turtles (Borysenko, 1970), maintained at low temperatures, reject allografts in an accelerated fashion when transferred to higher temperatures during allograft rejection. Therefore antigen processing and recognition must take place at low temperatures. Borysenko (1979) postulated that at low temperatures an early but post recognition event (cell proliferation?) is primarily affected while at higher

temperatures effector functions are the limiting factor. If this holds true for fish remains to be investigated.

TABLE 9 Influence of temperature on allograft rejection in teleost fish

Temperature (°C)	Median Survival Time (days)			
	Goldfish(1		Mozambique mouthbrooder(2	
	First-set	Second set	First-set	Second-set
10	40.5	19.5		
16	20.5	13.9		
17			9.4	N.D.
19	12.6	8.0		
21	8.3	5.4		
25	7.2	4.7	7.8	5.8
27			7.0	5.0
28	6.3	4.4		
30			5.2	4.2
32	4.3	3.2	5.1	N.D.

Data taken from Hildemann, 1957 (1) and Sailendri, 1973 (2).
N.D. = not done.

Humoral immunity

Early studies on the effect of temperature on the humoral immune response suggested that fish were not able to produce circulating antibody below 10°C (reviewed in Avtalion et al., 1973). More recent reports demonstrated also that certain species lack a humoral response below 9-12°C: In the Japanese eel anti-*Vibrio anguillarum* titres were obtained more rapidly at higher temperatures within the 15-23°C range. No antibody production was detected at 11°C (Muroga & Egusa, 1969). Carp kept at 12°C did not produce antibodies against BSA (Avtalion, 1969a).

Very interesting studies have been performed on the immune responsiveness of cold-water fish. Sablefish, a habituant of the North Pacific, produced agglutinating antibodies at temperatures of 5-8°C within 1 month after injection of foreign erythrocytes (Ridgway, Hodgins & Klontz, 1966). In the Antarctic teleost, the icefish, kept at 2°C neutralizing antibody could be detected 42-56 days after immunization with MS2 bacteriophage (O'Neill, 1979, 1980). It therefore is more appropriate to state that the temperature range over which antibody production can take place is related to the normal environmental temperature range of the species considered.

The most extensive studies on the effect of temperature on humoral immunity have been performed by Avtalion and co-workers. Carp kept at 25°C (high temperature) produce agglutinating antibodies against BSA after a lag period of 10 days. When kept at 12-14°C (low temperature) no antibody could be detected up to 77 days after immunization. When carp, immunized and subsequently kept at 14°C for 35 days, were

transferred to 25°C, antibodies were detected 7 days later. Animals immunized and kept at 25°C for 8 days before transfer to 14°C produce antibodies at this low temperature (Avtalion, 1969a). Similar results have been obtained when using *Aeromonas punctata* as immunizing agent (Avtalion, Wojdani, Malik & Shahrabani, 1973). Later studies showed that a temperature sensitive event during the response, was situated between the 3rd and 4th day after primary stimulation. This was illustrated by the fact that only carp kept at high temperatures for more than 3 days after immunization produce antibodies in the cold (Avtalion, Weiss & Moalem, 1976). Carp were able to mount a secondary response against BSA at low temperatures provided that priming took place at 25°C (Avtalion, 1969b, 1969c; Avatalion, Malik, Lefler & Katz, 1970). Animals were primed with the O-antigen of *Salmonella abortus* (OSA) at high temperatures and transferred to 12°C before the appearance of circulating antibody. After a simultaneous immunization with OSA and BSA, only antibodies against the bacterial antigen were produced. Thus, memory formation at low temperatures was immunologically specific (Avtalion et al., 1973).

Three different approaches have been used to demonstrate that the function of helper cells was primarily affected by low temperatures.

a) Carp, which were injected with rabbit gamma globulin as a carrier, before their transfer to low temperatures, were able to produce anti-hapten (penicillin) antibodies following immunization with the hapten-carrier conjugate in the cold (Avtalion et al., 1976).

b) Acetylated BSA (AcBSA) does not evoke antibody production in carp. An injection with native BSA, given 40 days after AcBSA priming, gives rise to a secondary type anti-BSA response. AcBSA has lost its ability to stimulate antibody synthesis but retained its potential memory formation (Weiss & Avtalion, 1977). The anti-BSA response in *Tilapia* (hybrid of *T. aureus* x *T. nilotica*) kept at low temperatures was of a secondary type when priming with AcBSA occurred at high temperatures (Avtalion, Wishkovsky & Katz, 1980).

c) Penicillin conjugated to BSA at low epitope density (Pen_5BSA) stimulate carp for both anti-hapten and anti-carrier antibodies (Avtalion & Milgrom, 1976). Preimmunization with BSA enhanced the primary anti-hapten response at low temperatures (Weiss & Avtalion, 1977).

The optimal temperature for LPS stimulation of blue gill pronephros lymphocytes was 22°C, while responses to PHA and Con A were optimal at 32°C (Cuchens & Clem, 1977). These results are consistent with Avtalion's hypothesis that T-like cells require a higher temperature than B-like cells (Avtalion, Weiss, Moalem & Milgrom, 1975). However, studies in rainbow trout revealed that the optimal temperature for stimulation by PHA, Con A and LPS was always 28°C (Chilmonczyk, 1978a).

In carp kept at 25°C both low dose (0.01-0.1 mg/kg) and high dose (10-50 mg/kg) tolerance to BSA could be induced (Serero & Avtalion, 1978). At low temperatures, only high dose tolerance could be induced (Avtalion et al., 1980).

On base of the data discussed above Avatalion et al. (1973) proposed a model for temperature sensitive stages during the humoral immune response. Following primary antigenic stimulation, the initial steps of the response such as phagocytosis, priming and tolerance induction are relative temperature insensitive. The first temperature sensitive event might be cellular interaction and/or differentiation. Cellular multiplication of memory cells and antibody forming cells are temperature insensitive in this model. However, the effector phase of the humoral response (antibody synthesis and release) is again a relative temperature sensitive event.

- Both cellular and humoral immune responses of fish are temperature dependent. Immune responses can take place within the normal temperature range of the species. There are steps in the immune response differing in temperature-sensitivity.

From the practical point of view the temperature dependence of the immune response is important because it has been shown that seasonal variations in the incidence of infectious diseases in fish are correlated with changes in water temperature (Besse, Levaditi, Guillon & de Kinkelin, 1965; Schäperclaus, 1965; Roberts, 1975). The optimal temperatures for growth and replication of infectious micro-organisms varies (Reichenbach-Klinke, 1976). For instance the growth rate of *Aeromonas salmonicida* and *A. hydrophila* (causative agent of furunculosis) was optimal at water temperatures of about 20°C (Groberg, McCoy, Pilcher & Fryer, 1978). Maximum mortality in salmonids infected with the causative agent of bacterial disease, *Corynebacterium* spp. occurs in the range of 7-12°C (Sanders, Pilcher & Fryer, 1978). The optimal temperature for the *in vitro* replication of pathogenic rhabdoviruses specific for salmonids is 14°C, for related viruses specific for warmwater fishes it is 20-22°C (Ahne, 1978).

- From the immmunological point of view, animals in large scale fish culture should be kept at their optimal temperature. Lowering the temperature will diminish or inhibit the immune capacity of fish while the circumstances might become favourable for cold adapted pathogenic micro-organisms at the same time.

Stress

The importance of social factors in fish populations has been underestimated in the first reports on fish immunology. The negative results of Papermaster et al. (1962) and Good & Papermaster (1964) in obtaining immune responses in Agnatha were probably caused by poor animal husbandry since later investigators (Thoenes & Hildemann, 1969; Linthicium & Hildemann, 1970) succeeded in demonstrating antibody production.

Fish under crowded conditions produce pheromone-like crowding factors (Pfeiffer, 1974). These crowding factors inhibit growth and reproduction and depress the heart contraction rate (Pfuderer, Williams & Francis, 1974). Moreover, the immune response of crowded blue gourami to infectious pancreatic necrosis virus was depressed. It

was demonstrated that pheromone-like factors, released in crowded fish populations, were reponsible for the observed immunosuppression (Perlmutter, Sarot, Yu, Filazzola & Seeley, 1973). Carp kept under crowded conditions showed a relatively high susceptibility to experimental induced *Aeromonas* infections (Avtalion et al., 1973). Miller & Tripp (1979) studied the effect of captivity on the immune response of the killifish. Fish maintained at the laboratory for 1-2 months showed a lower anti-SRBC response than freshly captured specimens, while no effect on allograft rejection was observed. The suppressive factor -specific for humoral immune responses- was present in serum since it could be transferred from laboratory animals to freshly captured fish. At present it is clear that physiological and morphological effects of stress in fish are to a large extent homologous with those in mammals (Mazeaud, Mazeaud & Donaldson, 1977; Peters, 1979).

- Stress can cause immunosuppression. This should be kept in mind, not only when studying immune responses under laboratory conditions, but also when devising rearing systems for large scale fish culture.

Antibiotics and antimetabolites

Antibiotics are frequently used in animal husbandry for the prevention and control of infectious diseases. Data on the immunosuppressive effect of antibiotics in mammals are given in appendices V and VI. Reports on the effects of antibiotics in fish deal with cellular immunity. In killifish the antibiotics chloramphenicol, tetracycline, puromycin and cycloheximide prolonged the MST of allografts (Goss, 1961; Cooper, 1964). Furthermore, corticosteroids, nucleic acid and amino acid analogues and folic acid analogues also prolonged allograft survival (Goss, 1961; Hildemann & Cooper, 1963). Similar observations have been made in goldfish (Levy, 1963; Stutzman, 1967).

- The limited experimental data available today suggest that antibiotics can exert immunosuppressive effects in fish.

Environmental pollution and irradiation

Although immunosuppressive effects of environmental pollutants in mammals and birds are well documented (Vos, 1977; Vos, Faith & Luster, 1980) little is known about effects of these compounds in fish. It has been observed that DDT can suppress both humoral and cellular immune responses in goldfish (Zeeman & Brindley, 1975, 1976, 1979) when used in doses which are immunosuppressive in mammals and birds (Vos, 1977). A marked reduction in the number of lymphocytes in the spleen of guppy and brown trout was observed after exposure to DDT (Walsh & Ribelin, 1975). Zinc appeared to suppress the immune response of zebrafish against *Proteus vulgaris* but not against infectious pancreatic necrosis virus (Sarot & Perlmutter, 1976).

Few reports exist dealing with effects of radiation on the immune system of fish. Severe atrophy in kidney, spleen and thymus was found in channel catfish

A careful use of this drug is recommended since oxyTC can exert immunosuppressive effects during a primary response.

SAMENVATTING

Dit proefschrift behandelt enkele aspecten van het cellulaire en humoral immuunsysteem van karperachtige vissen.

In appendix I wordt de ontwikkeling van het cellulaire en humorale immuunsysteem bij de prachtbarbeel (*Barbus conchonius*) beschreven. In 3-4 maanden oude dieren kon een humorale anti-schape rode bloedcellen (SRBC) reactie worden aangetoond, maar de hoogte van de reactie was lager dan in 9 maanden oude volwassen dieren (90 en 700 plaque vormende cellen(PVC)/10^6 witte cellen, respectievelijk). Cellulaire immuniteit werd onderzocht met de schubtransplantatie techniek. Tussen 6 en 9 maanden oude dieren bestond geen signifikant verschil in de gemiddelde afstotingstijd van allogeen getransplanteerde schubben (respectievelijk 8 en 8.3 dagen).

Uit deze experimenten kan de conclusie worden getrokken dat bij 3 tot 4 maanden oude dieren het vermogen om op SRBC te reageren aanwezig is, maar dat het humorale immuunsysteem pas 5-6 maanden later volgroeid is. Cellulaire immuniteit bereikt het volwassen niveau na 6 maanden of eerder.

In het vervolg van het onderzoek werd om een aantal redenen de karper (*Cyprinus carpio*) gekozen als proefdier. Allereerst moest de plaque test van Jerne, waarmee het aantal antilichaam-producerende cellen kan worden bepaald, worden aangepast voor het werk met de karper (appendix II). Het bleek dat serum van de brasem (*Abramis brama*) een betrouwbaarder complement bron vormde dan allogeen karper serum. Gebruik makend van de plaque test werd de kinetiek van de anti-SRBC respons onderzocht (appendix III). Het bleek dat bij de karper de nier (pro- en mesonephros) een belangrijk antilichaam-producerend orgaan is. De milt droeg slechts voor 5% aan het totale aantal PVC bij. Wanneer karpers bij hoge temperaturen gehouden werden (20-24°C) vertoonden zij een karakteristieke primaire en secundaire respons. Bij lagere temperaturen (10-18°C) wordt de piek van de primaire PVC respons later bereikt, maar de hoogte van de respons blijft gelijk. De relatie tussen de temperatuur en het tijdstip waarop de piek-respons bereikt wordt, suggereert dat er bij de karper tenminste 2 stappen in de immuunrespons zijn met een verschillende temperatuurgevoeligheid. De karakteristieke eigenschappen van een secundaire respons verdwijnen geleidelijk bij lagere temperaturen. De vorming van immunologisch geheugen zou daarom ook temperatuur afhankelijk kunnen zijn. De invloed van antigeen dosering en de route van antigeen toediening op het ontstaan van immunologisch geheugen werd onderzocht in appendix IV. Immunisatie met een lage antigeen dosering toegediend langs de intramusculaire weg was optimaal voor de vorming van immunologisch geheugen. Dit vermogen om een secundaire respons op te wekken was specifiek voor het antigeen dat gebruikt werd bij de primaire immunisatie, en bleef gedurende ten minste 10 maanden op een hoog niveau.

In appendix V en VI werd het effect van het antibioticum oxytetracycline (oxyTC)

op het immuunsysteem van de karper onderzocht. Het voeren van oxyTC had geen effect op de afstoting van allogeen getransplanteerde schubben, maar in dieren die met oxyTC werden ingespoten was de afstotingstijd signifikant verlengd van 8.5 naar 11-20 dagen. Het remmend effect van oxyTC op de humorale immuun respons kon worden aangetoond op het niveau van antigeen bindende én antilichaamproducerende cellen. Gedurende een primaire respons werden de PVC aantallen teruggebracht tot 5% van normaal. Secundaire responsen werden niet signifikant geremd door oxyTC. Een zeer selectief gebruik van dit antibioticum wordt aanbevolen omdat het onder bepaalde omstandigheden immunosuppressief kan werken.

CURRICULUM VITAE

Ger Rijkers werd op 13 oktober 1952 als Gerrit Tjalling Rijkers te Zeelst (N.Br.) geboren. Het voorbereidend wetenschappelijk onderwijs werd gevolgd aan het Peelland College (voorheen Pius XII College) te Deurne alwaar in 1971 het diploma Atheneum B werd behaald. In dat zelfde jaar werd de studie biologie aangevangen, de plaatsingscommissie bepaalde dat dit aan de Landbouwhogeschool te Wageningen zou zijn. Het ingenieursexamen in de (cel)biologie werd in april 1977 afgelegd, met moleculaire biologie en celbiologie als hoofdvakken en plantkunde als bijvak.

Op 11 juli 1977 werd een begin gemaakt met een promotieonderzoek aan de afdeling Experimentele Diermorfologie & Celbiologie onder (bege)leiding van Prof. Dr. J.F. Jongkind en Dr. W.B. van Muiswinkel. Dit onderzoek werd mogelijk gemaakt door een 3-jarig promotie-assistentschap van de Landbouwhogeschool. Het betreffende project (000.772) had als titel: "De bouw en functie van het immuunsysteem bij de karper (*Cyprinus carpio*)". In de week van 11 juli 1980 werd het manuscript van dit proefschrift bij de drukker ingeleverd.

Vanaf half augustus 1980 is hij werkzaam in de klinische immunologie aan het Wilhelmina Kinderziekenhuis bij Dr. B.J.M. Zegers te Utrecht.

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APPENDIX I

Developmental Immunobiology, J.B. Solomon and J.D. Horton eds.

THE IMMUNE SYSTEM OF CYPRINID FISH
THE DEVELOPMENT OF CELLULAR AND HUMORAL RESPONSIVENESS IN THE ROSY BARB (BARBUS CONCHONIUS)

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INTRODUCTION

The basic properties of mammalian lymphocytes - the recognition of nonself and the subsequent differentiation and proliferation - appear to be common to lymphocytes from all vertebrates. Although a number of investigations concerning phylogenetic aspects of the immune response are known[1, 2] comparatively little information is available about structure of lymphoid organs and function of lymphocyte populations in poikilothermic vertebrates. Most observations in this respect have been done on Amphibia[3, 4]. Some data about the kinetics of the immune response in fish have been published[5, 6]. There are indications that co-operation between 2 populations of lymphoid cells is needed during the humoral immune response in fish[7, 8]. Rejection of allografts is a common feature in ectothermic vertebrates[9]. In teleost fish an acute rejection is observed within 2 weeks after grafting. The immunological nature of the rejection is demonstrated by a second-set rejection time of 4-6 days[10].

Information about the development of the immune system in fish is lacking or very scarce[11, 12]. The present study was performed to add some information about the functional development of the cellular and humoral immune system in Cyprinid fish.

MATERIALS AND METHODS

Animals: Rosy barbs (Barbus conchonius) of both sexes were used at an age between 3 and 30 months (table I). The animals were bred at the laboratory and kept at 24°C in full glass aquaria. Daily feeding was performed with Trouvit pellets (Trouw & Co, The Netherlands) or tubifex worms. They were acclimatized to the experimental aquaria for at least 1 week before injection or grafting.

Antigen: Sheep red blood cells (SRBC) were obtained from the Department of Cell Biology and Genetics, Erasmus University, Rotterdam. For immunization they were washed 3 times with phosphate buffered saline (PBS) and adjusted to a concentration of 2×10^{10}/ml.

Immunization: All animals were immunized with 10^8 SRBC in 5 µl PBS using a 50 µl Hamilton syringe. In preliminary experiments the most effective immunization turned out to be dorsal, intramuscular injection.

TABLE I

Some characteristics of Barbus conchonius

Age in months	Number of animals	Standard length* (cm ± s.e.)	Weight (g ± s.e.)	Number of white spleen cells (x 10^5 ± s.e.)
3	14	1.45 ± 0.06	0.085 ± 0.01	1.0 ± 0.2**
4	28	3.27 ± 0.05	1.13 ± 0.07	1.4 ± 0.1
9	24	4.45 ± 0.08	2.41 ± 0.14	2.8 ± 0.3
30	3	6.46 ± 0.03	11.1 ± 0.3	11.2 ± 0.2

* Standard length is total length without tail.

** Determined in 3 specimen.

Cell suspensions and bleeding: Anaesthesia was performed by means of MS-222 (tricaine methanesulfonate, Sandoz Inc, 150 mg/l) in aquarium water. Fish were bled by cutting the tail. The amount of blood obtained from one animal ranged from 1 µl (4 months old) to 20 µl (9 months old). Subsequently the spleen was removed and brought into cold Hank's balanced salt solution (HBSS, Difco, Detroit) supplemented with 5% newborn calf serum. The spleen was minced with scissors and squeezed through a nylon-gauze filter to give a single cell suspension. Sera were stored at -90°C.

Complement: A preliminary experiment was carried out to select a suitable complement donor. We prepared fresh complement from several Barbus species (B. conchonius, B. lateristriga, B. filamentosus and B. nigrofasciatus), grass carp and rainbow trout. The isologous complement of B. conchonius turned out to contain a high level of haemolytic factors to lyse SRBC in the presence of B. conchonius antibodies. The complement was absorbed for 15 minutes at 0°C with packed SRBC. After centrifugation the supernatant was absorbed for another 10 minutes at 20°C. After centrifugation the supernatant was collected and stored at -90°C.

Serology: Haemolysing (HL) and haemagglutinating (HA) antibodies were detected in sera of individual animals using standard techniques[13]. The sera were diluted with PBS to yield a starting volume of 30 µl. Microtitreplates were incubated for 2-3 hours at 24°C.

Plaque forming cell assay: To detect the presence of antibody forming cells the plaque-technique of Jerne in an agar-free medium was used[14]. The monolayer plaque assay slides were produced according to the technique of Majoor et al.[15]. A mixture of 0,1 ml cell suspension, 0,1 ml SRBC (5 x 10^8/ml) and 10 µl complement was prepared and incubated as a monolayer between two glass slides. After an incubation of 2 hours at 24°C the slides were scored for the number of haemolytic plaques

using a dissection microscope. Part of the spleen cell suspension was used for white cell counting. To lyse the erythrocytes the suspension was incubated for 2 x 15 minutes in 0,75% NH_4Cl in 0,17 M tris-HCl pH 7,2[16].

Scale transplantation: For scale transplantation a modification of the Hildemann technique[17] was used. Repeated anaesthesia with MS-222 (150 mg/l) caused a considerable loss of animals. For grafting and subsequent daily inspection we placed the fish in a petri dish (Fig. 1). The bottom plate of the dish is filled with cotton wool, drained in aquarium water. The fish is placed on the cotton wool and the lid is closed immediately. In addition to one autograft, as a control, three to six allografts per recipient are made. Grafting is done in the row of scales just above the lateral line. Daily inspection of the scales was carried out using a low-power microscope.

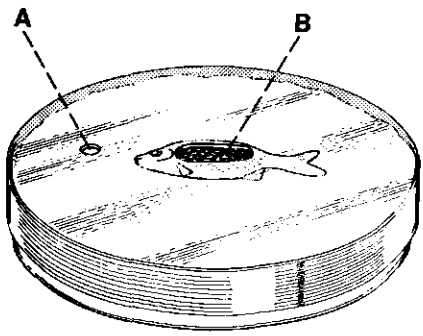

Fig. 1. A simple device for scale transplantation in fish.
A) Hole to supply aquarium water.
B) Oval hole enabling scale transplantation.

RESULTS

PFC-response to sheep erythrocytes: A group of 64 male, 9 months old animals were immunized with SRBC. On day 2, 4, 6, 7, 8, 9, 11, 14 and 17 after immunization 4 animals were killed for detection of PFC in the spleen. A plaque with an antibody producing cell in the center is shown in fig. 2. The first plaques appeared on day 4 after immunization (Fig. 3). Thereafter the number of plaques increased and reached a maximum on day 7 (700 PFC/10^6 white spleen cells (WSC)). In the subsequent period the number of plaques decreased to less than 10 PFC/10^6 WSC. Twenty eight days after the primary injection 18 animals received a secondary injection with SRBC. Groups of 3 animals were killed on day 3, 5, 8 and 16 after the antigen injection to determine the number of PFC's in the spleen. The spleens of 6 animals were pooled and tested on day 6. The first plaques appeared after 3 days, the peak response occured on the fifth day (4200 PFC/10^6 WSC). On day 16, the last day tested, there were 11 PFC/10^6 WSC. A group of 28, 4 months old animals were immunized with SRBC. On day 2, 4, 6, 8, 9, 10 and 13 after the injection of the antigen 4

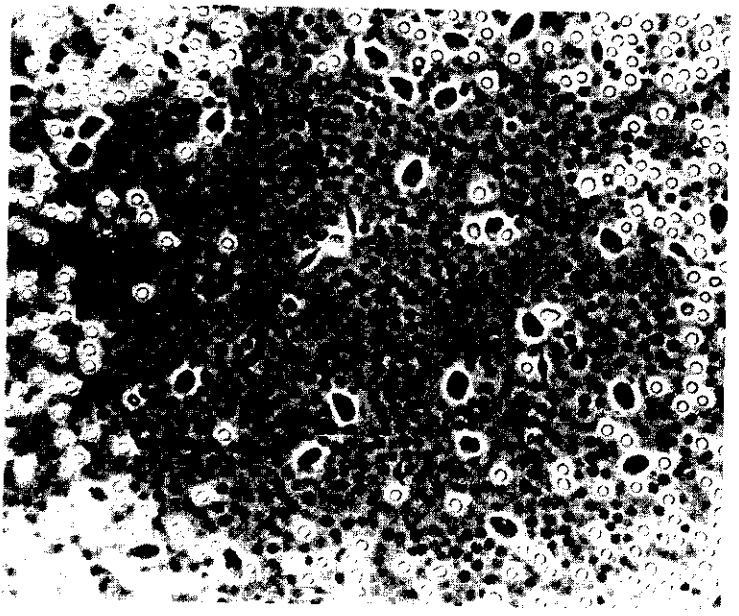

Fig. 2. A phase contrast photomicrograph of a plaque, produced by a plaque forming spleen cell (PFC). Around this antibody producing cell an area of lysed sheep erythrocytes is visible. Fish erythrocytes (FE) and thrombocytes (T) are also present.

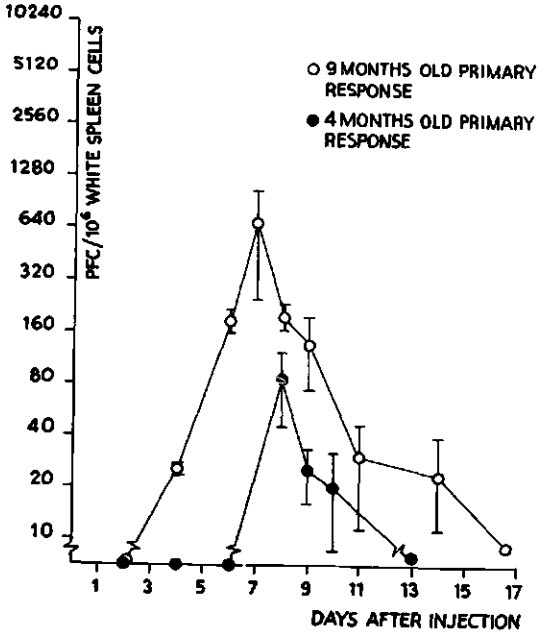

Fig. 3. Primary immune response of Barbus conchonius to SRBC. Each point represents the mean value from 4 animals ± s.e..

TABLE II

Immune response of Barbus conchonius to SRBC

Age in months	PFC/10^6 WSC	HA-titre
3	30 ± 15	-
4	91 ± 43	80 ± 53
9	699 ± 434	987 ± 683

Plaque forming cells (PFC) were detected 7-8 days after injection of the antigen, the haemagglutination (HA) titre was determined on day 13-14. The data represent the mean value from 3-4 animals ± standard error.

animals were tested for the presence of PFC in the spleen. The first plaques appeared on day 8 (91 PFC/10^6 WSC, see fig. 3). Day 8 also turned out to be the peak day of the PFC-response. During the subsequent period the number of plaques decreased and dropped under 10 PFC/10^6 WSC on day 13. Additionally a group of 3 animals (3 months old) were tested for the presence of PFC in the spleen 7 days after immunization with SRBC. At that time 30 PFC/10^6 WSC were detected (table II). The animals were homogenized in HBSS and centrifuged after removal of the spleen. The HA-titre in the supernatant of the homogenate turned out to be 1:58 ± 21. Comparing animals with the age of 4 months (young) and 9 months (old) we see that the PFC-peak response is reached about the same time after immunization (fig. 3). However, the number of PFC/10^6 WSC or PFC/spleen is higher in old animals. Furthermore, the time interval in which PFC's can be detected is shorter for young animals. It is worthwhile to mention that some young animals did not respond at all, even at the peak day. As visualized in table I, 3 months old animals have a relatively high number of white spleen cells. Their peak PFC response was lower than in 4 months old animals.

Antibody response to SRBC: Sera of immunized animals were analysed for the presence of agglutinating and lytic antibodies. HA and HL-titres in 9 months old animals reached a maximum (1:987 and 1:40 respectively) on day 14 of the primary response (fig. 4b). The HA-titre surpassed the detection limit (dilution 1:20) for the first time on day 11. The HL-titre was always lower than the HA-titre. Following a secondary injection of SRBC, 28 days after primary immunization, HA antibodies were detectable for the first time on day 3 and reached a maximum on day 5 (1:2229). Eleven days after the peak the HA-titre still was 1:80. HL antibodies appeared on day 5 and a maximum was reached on day 8 (1:213). HA and HL antibody responses in 4 months old fish were very low (fig. 4a). The highest value for HA antibodies was observed on day 13 (1:80), the last test day. HL antibodies first appeared on day 9, a maximum was reached on day 10 (1:35).

Scale transplantation: Seven 9 months old animals and six 6 months old animals

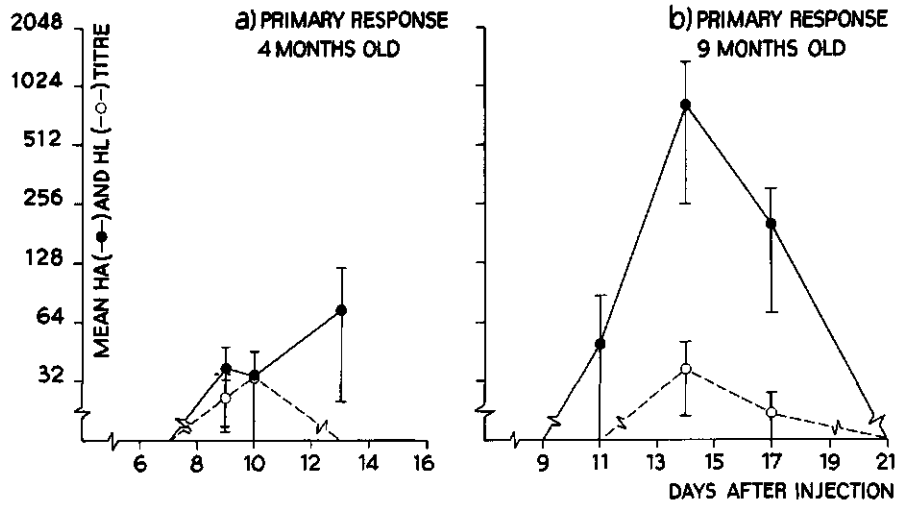

Fig. 4. Primary antibody response of Barbus conchonius to SRBC. Each point represents the mean value of 4 animals ± s.e..

were used for scale transplantation. After transplantation the following stages of rejection of the allografts were distinguished:

a) scales become overgrown by hyperplastic host-tissue. This results in a white appearance.
b) orange pigment cells turn into red.
c) black pigment cells are branching.
d) clearance of the hyperplastic host-tissue.

The beginning of the clearing phenomenon is considered to be the survival end point (S.E.P.) of the graft[17]. The median survival time (M.S.T.) was calculated from the S.E.P.'s. Autografts survived well in all cases. The MST of Barbus conchonius (8,0 days) falls within the range of MST's reported for other cyprinid fish. Barbus filamentosus has an MST of 8,6 days, Carrasius auratus of 7,2 days, both at 25°C[10]. The MST's of 6 and 9 months old animals showed no significant difference (table III).

TABLE III

Allogeneic scale graft rejection in Barbus conchonius

Age in months	Number of grafts per experiment	Temperature (°C)	Median Survival Time (days ± s.e.)
6	17	24	8,3 ± 0,2
9	6	24	8,0 ± 0,5

DISCUSSION

The present study describes the functional maturation of the immune system in Barbus conchonius. The humoral immune system was studied by injecting the animals with SRBC. The peak response was determined by counting the number of PFC/10^6 WSC and measuring HA and HL-titres in serum. It is known that the immunocompetent cells in teleost fish are localized not only in the spleen, but also in the thymus and head-kidney[18]. Therefore the HA and HL titration data provide a better estimation of the immune capacity of the animal than the number of PFC/10^6 WSC.

Smith et al.[19] and Chiller[20] reported that in fish isologous complement or complement from closely related species is a prerequisite for haemolysing sensitized SRBC. Our results with isologous complement of Barbus conchonius confirm their observations, however, closely related species as B. filamentosus, B. nigrofasciatus and B. lateristriga failed as a suitable complement donor. In our experiments serum of Salmo gairdneri was as active as B. conchonius complement. This is a striking observation because these 2 species are not related.

The primary response showed a maximum of 700 PFC/10^6 WSC on day 7 after injection of SRBC. This figure is comparable with other teleost fish as Tilapia mossambica (287 PFC)[6], Salmo gairdneri (165 PFC)[20] and Perca fluviatilis (848 PFC)[5]. A second injection with the same antigen elicited a higher PFC response (6-fold increase) at day 5. This illustrates the immune nature of the PFC response. Young animals (4 months old) showed a lower PFC response (90 PFC/10^6 WSC) and HA and HL-titres. It is concluded that 4 months old animals have the competence to respond to SRBC but their humoral immune system is still not full-grown. Plaques were detected in the spleen of 3 months old animals 7 days after injection of the antigen. However, the number of plaques was very low (0-6 plaques/spleen).

For studying cellular immunity we modified the scale transplantation technique of Hildemann[17]. No significant difference between the M.S.T.'s of adult (9 months) and young-adult (6 months) animals was detected (table III). Taking allograft rejection as a probe for cellular immunity it is concluded that the cellular immune system in B. conchonius reached the adult stage at 6 months or earlier. In viviparous Cymatogaster, newborn fish - with an age of 5 months - have an MST of 13 days, whereas the MST for adult animals is 7 days[11]. The difference in the maturation of cellular responsiveness between Cymatogaster and Barbus might be explained by a difference in ambient temperature (17°C and 24°C respectively).

Studies in mice indicate that the maturation of humoral responsiveness takes at least 2 months (ref. 21, 22 and own observations). Therefore it is tempting to say that the development of the immune responsiveness in cyprinid fish is comparable with the same process in mammals taking into account the temperature difference between cold and warmblooded vertebrates.

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APPENDIX II

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THE HAEMOLYTIC PLAQUE ASSAY IN CARP *(CYPRINUS CARPIO)*

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A haemolytic plaque assay for the enumeration of antibody forming cells in the carp (*Cyprinus carpio*) is described. Serum of bream (*Abramis brama*) turned out to be a more reliable complement source than allogeneic carp serum. The addition of 3—5% bream serum to a mixture of immune lymphoid carp cells and xenogeneic erythrocytes gave optimal results. Higher amounts of bream complement inhibited plaque formation. Plaque formation was also suppressed when inactive or heat-inactivated bream or carp serum was added to a mixture containing normal bream complement.

INTRODUCTION

For many years the plaque assay of Jerne and Nordin has been a useful tool for in vitro quantitation of antibody secreting cells or plaque forming cells (PFC). Although it was originally developed to detect cells which secrete antibodies against cellular surface antigens of erythrocytes, it has also been used to detect PFC against soluble antigens by coating indicator erythrocytes with proteins, polysaccharides or haptens (Jerne et al., 1974).

Recently the technique has been adapted for use with cells from vertebrate classes other than mammals such as amphibians (Horton et al., 1976), reptiles (Rothe and Ambrosius, 1968; Kanakimba and Muthukkaruppan, 1972) and birds (Janković et al., 1972).

The haemolytic plaque assay has been used in the following fish species: blue gill, *Lepomis macrochirus* (Smith et al., 1967), rainbow trout, *Salmo gairdneri* (Chiller et al., 1969), perch, *Perca fluviatilis* (Pontius and Ambrosius, 1972), Mozambique mouthbrooder, *Tilapia mossambica* (Sailendri and Muthukkaruppan, 1975), goldfish, *Carassius auratus* (Warr et al., 1977) and the rosy barb, *Barbus conchonius* (Rijkers and Van Muiswinkel, 1977).

Carp, (*Cyprinus carpio*) together with *Tilapia* and a few salmonids constitute the basis for large scale fish culture (Weatherly and Cogger, 1977).

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For economical reasons the study of the immune system of carp under mass culturing conditions is of great importance (Avtalion et al., 1976; Fiebig and Ambrosius, 1977; Rijkers et al., 1980). Research has been hampered by lack of a sensitive method for monitoring the humoral immune response in carp. In the present study a haemolytic plaque assay in liquid medium is described.

MATERIALS AND METHODS

Animals

Carp (*Cyprinus carpio*), ranging from 6 to 18 months of age and 75 to 300 g in weight, were either bred at the laboratory or obtained from the Organisation for Improvement of Inland Fisheries (Nieuwegein, The Netherlands). They were kept in aquaria with running tap water at temperatures of 18 or 24°C and fed daily with pelleted food (Trouvit K30, Trouw and Co., Putten, The Netherlands).

Swiss (Cpb : SE(S)) random-bred female mice, 20 weeks old were used. They were purchased from the Centre for Laboratory Animals (Wageningen, The Netherlands).

Sheep red blood cells (SRBC)

Blood was collected from the jugular vein of 6 sheep into heparinised vacutainers® (B-D). The blood samples were pooled and an equal volume of Alsever's solution added. The SRBC were stored at 4°C for a maximum period of 2 weeks. For the plaque assay, SRBC were washed 3 times with phosphate buffered saline (PBS) and finally diluted in Hank's balanced salt solution (HBSS, Difco, Detroit, U.S.A.) to a concentration of 5×10^8 SRBC/ml. HBSS was supplemented with 5% newborn calf serum (NCS) and antibiotics (40 I.U. mycostatine, 200 I.U. penicillin and 0.2 mg streptomycin per 100 ml). Carp were injected intramuscularly (i.m.) in the dorsal region with 10^9 SRBC (0.05 ml of 2×10^{10} SRBC/ml PBS). Mice were injected intraperitoneally (i.p.) with 5×10^8 SRBC.

Complement sources

Pooled sera of pig, goat, cow, sheep, horse and dog were obtained from the Foundation for Blood Group Studies, Wageningen. The chicken serum was a gift from the Department of Animal Husbandry, Agricultural University, Wageningen. The amphibian and fish sera and sera or coelomic fluid of invertebrates (indicated in Table 1) were collected from animals kept in our laboratory. Bream serum was collected at the Tjeukemeer Field Station of the Limnological Institute (Oosterzee, The Netherlands). Blood was collected by caudal puncture and allowed to clot for 2 h at 0°C. Sera with a high natural haemolytic activity against SRBC were absorbed for 20 min at 0°C with an equal volume of packed SRBC. All sera were stored at −20°C. Inactivation of bream or carp complement was accomplished by incubating serum for 10—30 min at 42°C.

TABLE 1

INFLUENCE OF COMPLEMENT SOURCE ON THE DEVELOPMENT OF PLAQUES BY IMMUNE CARP CELLS

Complement activity was determined in the haemolytic plaque assay with a head-kidney suspension of carp immunised with 10^9 SRBC.

Complement source		Plaques	Positive/total
Mammals			
including rabbit, guinea pig, man, cow, goat, sheep, horse, dog		—	pooled sera
Birds			
chicken		—	pooled sera
Amphibia			
axolotl	*Ambystoma mexicanum*	—	0/3
Fish			
carp	*Cyprinus carpio*	+	31/56
bream	*Abramis brama*	+	55/58
chub	*Leuciscus cephalus*	+	5/8
nase	*Chondrostoma nasus*	+	2/2
barbel	*Barbus barbus*	+	2/4
rosy barb	*Barbus conchonius*	—	pooled sera
goldfish	*Carassius auratus*	—	0/7
grass carp	*Ctenopharyngodon idella*	—	0/4
roach	*Rutilus rutilus*	—	0/4
bleak	*Alburnus alburnus*	—	0/3
tench	*Tinca tinca*	—	0/3
rainbow trout	*Salmo gairdneri*	—	pooled sera
labyrinthic catfish	*Clarias lazera*	—	0/5
perch	*Perca fluviatilis*	—	0/3
Invertebrates			
earthworm [a]	*Lumbricus terrestris*	—	pooled coelomic fluid
locust [a]	*Locusta migratoria*	—	pooled coelomic fluid
pond snail [a]	*Lymnaea stagnalis*	—	pooled haemolymph

[a] Haemolymph or coelomic fluid tested.

Plaque assay

Cell suspensions were prepared as described previously (Rijkers and Van Muiswinkel, 1977) and suspended in HBSS supplemented with NCS and antibiotics. The plaque technique in agar-free medium as described by Zaalberg et al. (1968) was used. Monolayer plaque assay slides were produced according to the technique of Majoor et al. (1975). Briefly, 0.1 ml cell suspension, 0.1 ml SRBC (5×10^8/ml) and complement were mixed and incubated between two glass slides at 25°C for 2 h. The number of haemolytic plaques was scored using a low-power dissecting microscope with dark-

field illumination. Doubtful plaques were examined under a high-power phase-contrast microscope.

RESULTS

Complement sources

Sera of 28 animal species were tested for their ability to lyse SRBC in the plaque assay with immune carp cells. Only five complement sources were capable of lysing sensitised SRBC (Table 1). These were sera from carp, barbel (*Barbus barbus*), bream (*Abramis brama*), chub (*Leuciscus cephalus*) and nase (*Chondrostoma nasus*), all fish belonging to the teleost family of the Cyprinidae.

Among the sera with complement activity, bream serum was superior even to allogeneic carp serum. Fifty-five out of 58 individually tested bream sera were able to lyse sensitised SRBC. A plaque developed with bream serum is shown in Fig. 1. The highest number of plaques was observed with cells from the main lymphoid organs of carp (head-kidney, kidney, spleen) when bream serum was used.

Complement concentration

The optimal complement concentration in the plaque assay of carp was

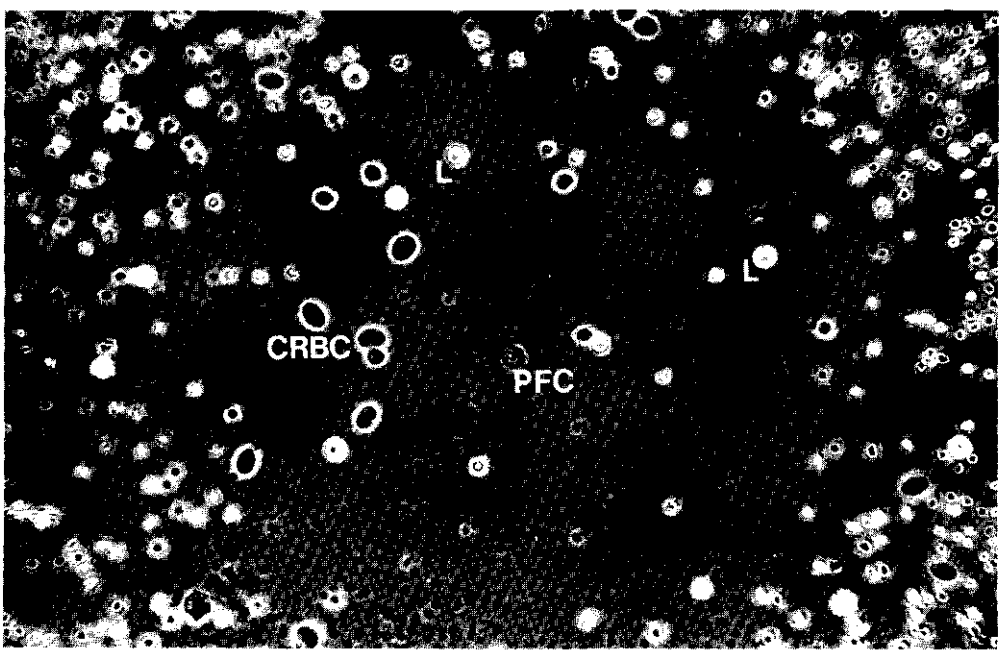

Fig. 1. A phase-contrast photomicrograph of a plaque forming cell (PFC) from the head-kidney of carp. Several carp red blood cells (CRBC) and head-kidney leucocytes (L) are found within the area of lysed sheep red blood cells.

determined by adding increasing amounts of bream serum to a mixture of SRBC and head-kidney cells of an immunised carp (Fig. 2). Low numbers of haemolytic plaques were developed when 1% bream serum was added. Three to 5% serum appeared to be optimal, while higher concentrations inhibited plaque formation.

In a comparative experiment the complement dependence of SRBC lysis after sensitisation with mouse antibody was determined using rabbit serum as complement source. The minimal amount of rabbit complement needed to develop any plaques was 3% (Fig. 2). Higher plaque numbers were observed with increasing complement concentration (optimum 10%). Inhibition with super-optimal complement levels as observed in carp was not found in mice.

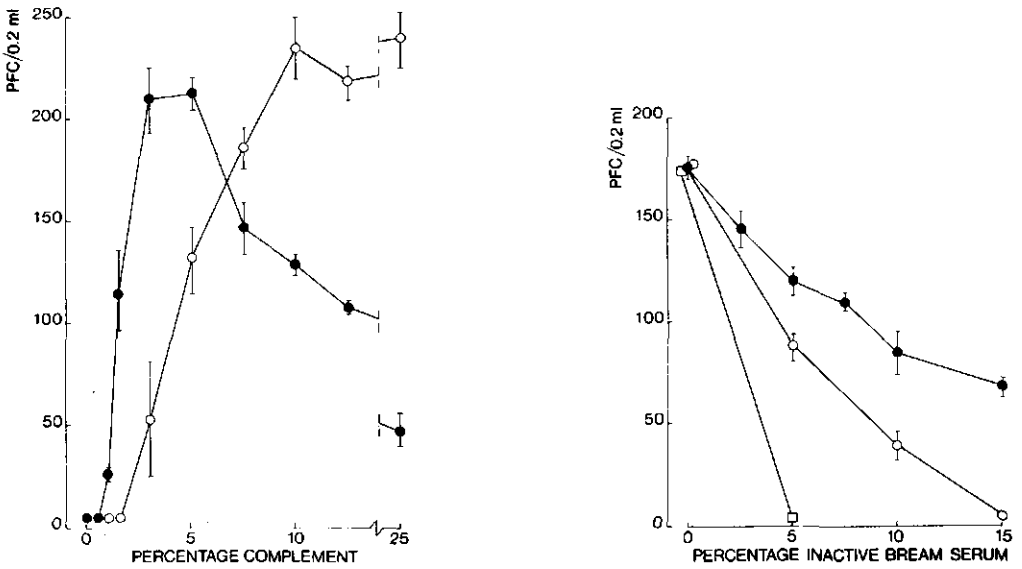

Fig. 2. Effect of complement concentration on number of plaques. ● = Plaques developed by a head-kidney cell suspension of carp, injected twice with 10^9 sheep red blood cells (SRBC) i.m. Secondary response, 10 days after injection. Pooled bream serum was used as complement source. ○ = Direct plaques developed by a spleen cell suspension of mice, immunised with 5×10^8 SRBC i.p. Primary response, 4 days after injection. Pooled rabbit serum was used as complement source. Each point represents the arithmetic mean (±1 standard error, n = 4) of the number of plaques in 0.2 ml cell suspension.

Fig. 3. Inhibition of plaque formation in carp by inactive bream serum. Plaques developed by a head-kidney cell suspension of carp, injected twice with 10^9 sheep red blood cells (SRBC) i.m. Secondary response, 10 days after injection. Increasing amounts pooled heat-inactivated (20 min, 42°C) bream serum (●) or 2 individual inactive normal bream sera (○, □) were added to a mixture containing lymphoid cells, SRBC and 5% bream serum. Each point represents the arithmetic mean (±1 standard error, n = 4) of the number of plaques in 0.2 ml cell suspension.

PFC inhibition

In carp, the inhibition of plaque formation was also observed when normal inactive or heat-inactivated bream serum was added to a mixture containing optimal bream complement concentration (Fig. 3). Inhibition was more pronounced when natural inactive bream serum was used. PFC inhibition was also observed when carp serum was used as complement source; 20% inactive or inactivated carp serum added to a mixture containing 5% active carp serum completely inhibits plaque formation.

In mice, the addition of inactivated rabbit complement did not alter the number of plaques developed by an optimal complement concentration.

DISCUSSION

In all plaque assays for lower vertebrates, allogeneic complement is used, with the exception of the clawed toad, *Xenopus laevis*, where guinea pig complement is satisfactory (Horton et al., 1976). Conversely, amphibian sera are able to serve as complement source for SRBC sensitised with rabbit-anti-SRBC antibodies (Ruben et al., 1977).

Other claims for the cooperation of lower vertebrate antibodies with mammalian complement have not been tested in the haemolytic plaque assay (Inebi and Horne, 1979). In the present study in carp, xenogeneic serum from a related species, the bream, was used as complement source. Ability to haemolyse sensitised SRBC in vitro was present in 95% of the bream sera tested, while for carp the figure was only 50%.

Whereas mammalian complement requires a temperature of 56°C for heat-inactivation, complement of lower vertebrates is more heat labile. For teleost fishes inactivation temperatures from 42 to 53°C are reported (Feldman and Miller, 1960; Hodgins et al., 1967; Chiller et al., 1969; Gigli and Austin, 1971). In our experiments sera of bream and carp lost the ability to develop plaques after incubation at 42°C for 10 and 30 min respectively. Surprisingly, incubation for 60 min at 37°C had no effect upon the resulting number of plaques.

Reduced plaque numbers may be due to failure of antibody secretion by plasma cells or inhibition of complement-mediated haemolysis. In a tube haemolysis experiment, with SRBC sensitised with carp-anti-SRBC antibodies, 3—5% bream serum was optimal, while higher concentrations inhibited haemolysis as measured by 541 nm absorbance. This suggests that reduced plaque numbers may be caused by inhibition of complement activity.

In mice, the addition of inactivated serum to a mixture of SRBC, immune spleen cells and complement had no effect upon the number of plaques. In frogs, serum complement levels are reduced during hibernation (Green and Cohen, 1977). However, addition of serum from hibernating frogs had no inhibitory effect on the high complement titres observed in sera from non-hibernating animals. Thus, inhibition of plaque formation in carp by

inactive serum seems to be a unique finding. With super-optimal complement concentrations reduced plaque numbers were also observed. Since complement cannot be the limiting factor an inhibiting or "anti-complementary" factor must be assumed. This assumption is strengthened by the finding that when the concentration of "anti-complementary" factor is raised artificially by addition of inactive or inactivated serum to an optimal amount of active complement, the same suppression phenomenon is observed. The nature of this 'anti-complementary' factor is not clear. It may be that specific inhibitors resembling the C_1-esterase inhibitor or C_{3b}-inactivator of mammals, are present in higher concentrations.

While individual carp sera may be a reasonable complement source in the haemolytic plaque assay of carp, 4 vol. of inactive carp serum added to 1 vol. active carp serum completely inhibit plaque formation. The development of haemolytic plaques was completely inhibited when equal volumes of pooled bream complement and naturally inactive bream serum were mixed. Therefore it is not advisable to pool fish sera as a complement source. Although complement is critical, the haemolytic plaque assay in carp presented here offers the possibility of monitoring the immune response in this species in a sensitive and quantitative way.

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APPENDIX III

The immune system of cyprinid fish. Kinetics and temperature dependence of antibody-producing cells in carp (*Cyprinus carpio*)

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SUMMARY

After immunization of carp with sheep red blood cells spleen accounts for only 5% of the total number of plaque forming cells (PFC). In addition, thymus, peripheral blood and heart contained low numbers of PFC (< 0.5, 1 and 0.5% respectively). Pronephros and mesonephros were the major antibody producing organs (53 and 40% of total PFC respectively). The temperature dependence of the antibody forming cell response in spleen, pronephros and mesonephros was studied in animals kept at 12-24°C. Lowering temperatures induced a delay in the peak of the primary response but had no effect on the magnitude of the response. The temperature-peak day relationship indicated that there are steps in the primary immune response of carp differing in temperature sensitivity. The anamnestic character of the secondary response was clearly demonstrated at 24 and 20°C but lost at 18°C.

INTRODUCTION

Both cellular and humoral immune responses in poikilothermic vertebrates are temperature dependent. At low temperatures allograft rejection proceeds at a relative slow rate (Hildemann, 1957; Cohen, 1966; Borysenko, 1970) and antibody production is diminished (Pliszka, 1939; Ambrosius & Schäker, 1964; Harris, 1973; Paterson & Fryer, 1974) or completely absent (Nybelin, 1935; Barrow, 1955; Avtalion, 1969a, b; Avtalion, Malik, Lefler & Katz, 1970). Upper and lower temperature limits of the immune response are closely related to the ecological temperature range of the species considered (Ridgway, Hodgins & Klontz, 1966; Tait, 1969; O'Neill, 1980). The relationship between temperature and allograft survival time in goldfish indicated that there were at least 2 temperature sensitive stages in the process of allograft rejection (Hildemann, 1957). According to studies in different animals, the first phase of the immune response including antigen processing and recognition is relative temperature independent (Cone & Marchalonis, 1972; Avtalion, Wojdani, Malik, Shahrabani & Duczyminer, 1973). The first temperature sensitive event might be the subsequent interaction between T- and B-like cells (Avtalion, Weiss & Moalem, 1976; Marchalonis, 1977; Cuchens & Clem, 1977). A possible explanation is a block in "T-helper" activity (Cuchens, Mclean & Clem, 1976) or an increase in "T-suppressor" activity at low temperatures (Avtalion, Wishkovsky & Katz, 1980). The following cellular multiplication and differentiation of B-like cells are considered to be temperature independent (Avtalion et al., 1973). According to the same authors the second temperature sensitive event is the synthesis and release of antibodies by

plasma cells (Avtalion et al., 1973).

Most studies mentioned above are centered around the influence of temperature on the effector phase of the immune response, e.g. antibody levels in serum or allograft survival time. In this paper the kinetics of the plaque forming cell response at different temperatures was studied in order to obtain more insight in the temperature dependence of the inductive and proliferative phase of the humoral immune response in poikilotherms.

MATERIALS AND METHODS

Animals

Outbred carp (*Cyprinus carpio*) were obtained from the Organization for Improvement of Inland Fisheries (O.V.B., Nieuwegein, The Netherlands) or bred in our laboratory. Animals from 6 and 18 months old, weighing 75-300 g, were used. They were kept in aquaria with aerated water (pH 7.4-7.8, NO_2 < 0.3 ppm, 4°DH) and fed daily on pelleted dry food (K30, Trouw & Co, Putten, The Netherlands). Animals were kept at 12, 16, 18, 20 or 24°C. They were acclimatized for at least 2 weeks at each temperature before antigen injection.

Antigen and immunization

Sheep red blood cells (SRBC) were obtained from 6 animals. Pooled blood was mixed with an equal volume of Alsever's solution. The cells were washed 3 times with phosphate buffered saline (PBS, pH 7.2) before use. Carp were immunized by an intramuscular (i.m.) injection of 10^9 SRBC in 0.05 ml PBS in the dorsal region.

Plaque forming cell assay

Plaque forming cells (PFC) were detected using the method described previously (Rijkers, Frederix-Wolters & van Muiswinkel, 1980a). Bream (*Abramis brama*) serum was used as complement source. Plaques were developed at 25°C for 2 h unless stated otherwise. Viability of lymphoid cells was determined with the dye exclusion assay (0.2% trypan blue in PBS).

RESULTS

Organ distribution of PFC

In order to obtain an idea about the relative importance of different lymphoid organs of carp, the number of PFC in spleen, pronephros, mesonephros, heart, peripheral blood, thymus, liver and intestine was determined. Pronephros and mesonephros contribute for about 90% of the total PFC number after i.m. injection with SRBC (Table I). Spleen accounts only for about 5% while thymus, heart and peripheral blood contain very low percentages of PFC (≤ 1%). PFC were not detected in liver and intestine. Similar results were obtained when carp were injected intravenously

TABLE I. Organ distribution of plaque forming cells (PFC) in carp at 20°C.

Organ	PFC/10^6 white cells	PFC/organ	% of total PFC
Thymus	0.3 ± 0.04 *	120	< 0.5
Spleen	25 ± 1	17,100	5
Pronephros	548 ± 30	184,000	53
Mesonephros	87 ± 4	138,500	40
Heart	71 ± 7	1,950	0.5
Peripheral blood	15 ± 1	3,000	1
Liver	0	0	-
Intestine	0	0	-

Animals were injected twice with 10^9 SRBC (i.m.) with an interval of 1 month. The number of PFC was determined 12 days after the last injection (peak of the response).
* Arithmetic mean ± 1 S.E. (n=4).

with the same antigen. After oral administration of SRBC considerable numbers of PFC were detected in pronephros and mesonephros and to a lesser degree in spleen but not in intestine.

PFC kinetics

The number of antibody forming cells in suspensions of spleen, pronephros and mesonephros was determined at succesive days after i.m. injection with 10^9 SRBC in carp which were kept at 24°C (Fig. 1). The first PFC could be detected at day 6. Peak response was reached on day 9 (93 ± 43 PFC/10^6 WC in pronephros). Following a secondary injection 1 month after the first, PFC were already observed on day 3. The peak of the response on day 8 was a 50-fold higher than in the primary response (4800 ± 2360 PFC/10^6 WC in pronephros). PFC numbers diminished in the period after day 8 but up to 20 days after injection PFC were observed.

To assess peak primary and secondary anti-SRBC response in carp which were kept at 20°C, animals were tested on a few selected days (Fig. 2). Peak of both the primary and secondary response was reached around 12 days after injection. The secondary injection gave rise to only 10 times more PFC (95 ± 28 and 1104 ± 582 PFC/10^6 WC in pronephros respectively).

In carp kept at 18°C, the first PFC appeared in spleen, pronephros and mesonephros 11 days after primary injection with 10^9 SRBC i.m. (Fig. 3). Peak response was reached on day 17 (379 ± 185 PFC/10^6 WC in pronephros). Afterwards PFC numbers rapidly declined but PFC remained present in pronephros up to 30 days after injection. When a secondary injection was given 45 days after primary this did not result in a significant higher peak response in mesonephros or pronephros (304 ± 101 PFC/10^6 WC), although first PFC appeared 3-4 days earlier and remained for a longer

period. Peak secondary response was reached in spleen and mesonephros on day 15, in pronephros on day 17.

At 16°C, maximum numbers of PFC could be detected in spleen, pronephros (131 ± 29 PFC/10^6 WC) and mesonephros at around 27 days after injection (Fig. 4).

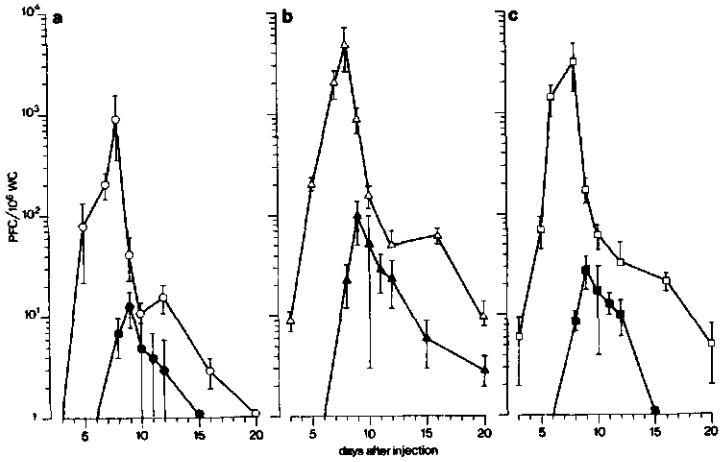

Fig. 1. Kinetics of primary (closed symbols) and secondary (open symbols) anti-SRBC response in spleen (a), pronephros (b) and mesonephros (c) of carp (6 months old) kept at 24°C. Animals were injected twice with 10^9 SRBC. The interval between 2 i.m. injections was 1 month. PFC/10^6 WC = plaque forming cells per 10^6 viable white cells. Each point represents the arithmetic mean ± 1 S.E. (n=4).

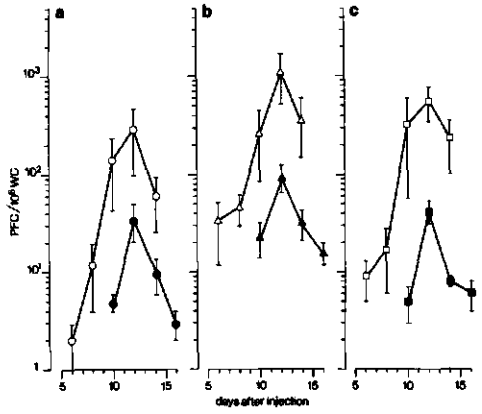

Fig. 2. Kinetics of primary (closed symbols) and secondary (open symbols) anti-SRBC response in spleen (a), pronephros (b) and mesonephros (c) of carp (6 months old) kept at 20°C. Animals were injected twice with 10^9 SRBC. The interval between 2 i.m. injections was 1 month. PFC/10^6 WC = plaque forming cells per 10^6 viable white cells. Each point represents the arithmetic mean ± 1 S.E. (n=4).

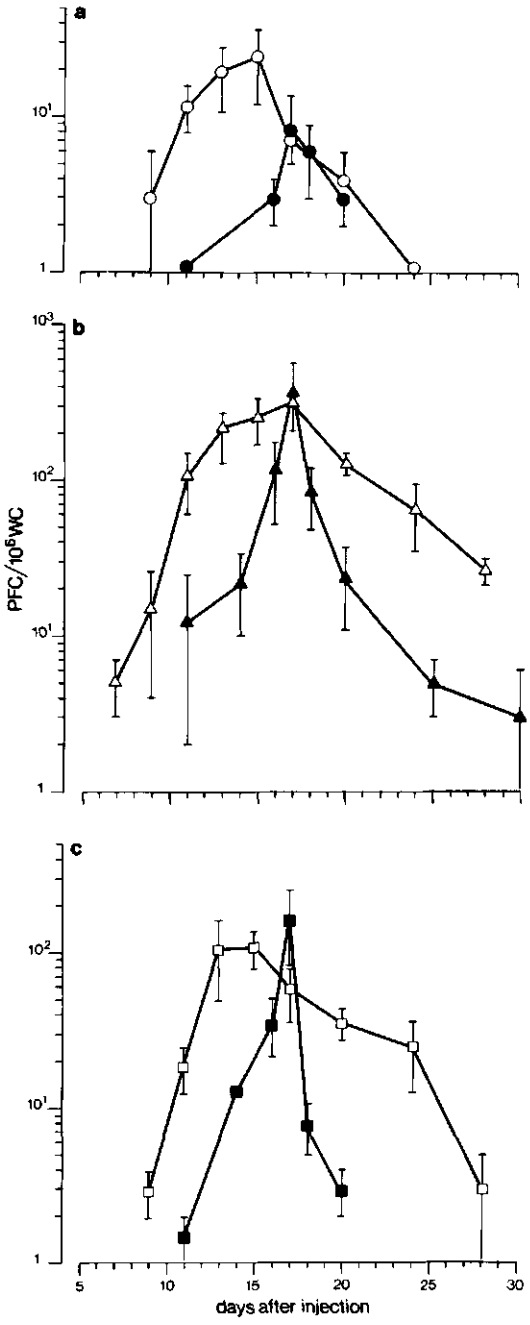

Fig. 3. Kinetics of primary (closed symbols) and secondary (open symbols) anti-SRBC response in spleen (a), pronephros (b) and mesonephros (c) of carp (18 months old) kept at 18°C. Animals were injected twice with 10^9 SRBC. The interval between 2 i.m. injections was 45 days. PFC/10^6 WC = plaque forming cells per 10^6 viable white cells. Each point represents the arithmetic mean ± 1 S.E. (n=4).

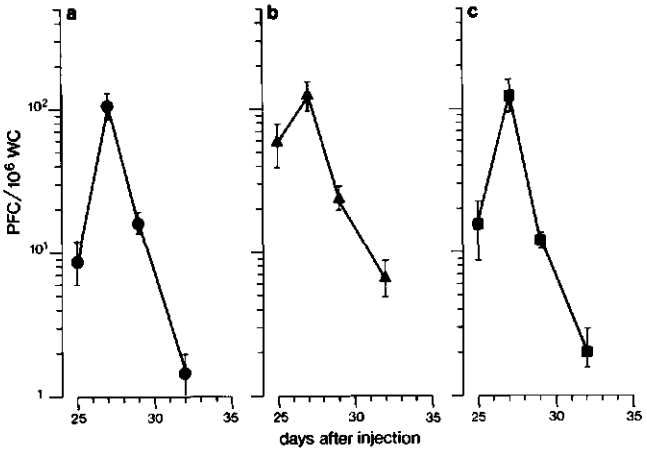

Fig. 4. Kinetics of primary anti-SRBC response in spleen (a), pronephros (b) and mesonephros (c) of carp (6 months old) kept at 16°C. Animals were i.m. injected with 10^9 SRBC. PFC/10^6 WC = plaque forming cells per 10^6 viable white cells. Each point represents the arithmetic mean ± 1 S.E. (n=4).

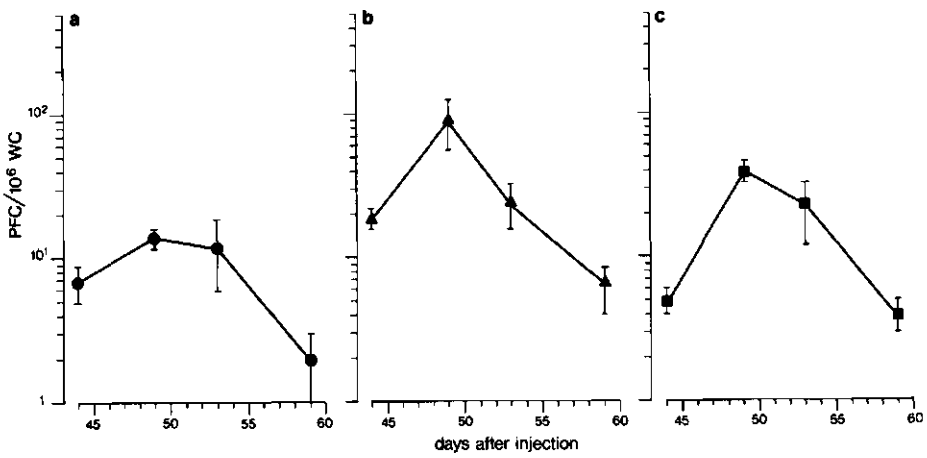

Fig. 5. Kinetics of primary anti-SRBC response in spleen (a), pronephros (b) and mesonephros (c) of carp (6 months old) kept at 12°C. Animals were i.m. injected with 10^9 SRBC. PFC/10^6 WC = plaque forming cells per 10^6 viable white cells. Each point represents the arithmetic mean ± 1 S.E. (n=4).

Peak primary response was reached around day 49 in carp kept at 12°C (91 ± 35 PFC/10^6 WC in pronephros; Fig. 5).

To ascertain the observation that carp are able to develop PFC at low temperatures, PFC slides were incubated at 16 or 12°C - ambient temperature of the animals considered - instead of the ususal 25°C for plaque incubation. It appeared

that both at 16 and 12°C plaques were formed. However, the speed of development was low and attained levels did not meet values obtained at the 25°C incubation (Table II). It is concluded that antibody production and secretion continues at low temperatures, but at a reduced rate.

TABLE II. Effect of incubation temperature on plaque development.

Ambient temperature (°C)	PFC incubation temperature (°C)	PFC incubation time (hours)	PFC/10^6 white cells in pronephros
16	25	2	61 ± 20*
16	16	5	26 ± 20
12	25	2	19 ± 3
12	12	5	4 ± 1
12	12	22	6 ± 1

Animals kept at 16°C were tested for the presence of plaque forming cells (PFC) in pronephros 25 days after an i.m. injection of 10^9 SRBC. Animals kept at 12°C were tested 44 days after injection.
* Arithmetic mean ± 1 S.E. (n=4).

In summarizing the results in can be seen that with lowered temperatures the peak of the primary response in delayed (Fig. 6) but the magnitude of the response is unaltered. The anamnestic character of the secondary response however, is gradually lost when lowering water temperatures.

DISCUSSION

The role of the pronephros in antibody production in teleost fish has been demonstrated in a number of studies (Smith, Potter & Merchant, 1967; Chiller, Hodgins, Chambers & Weiser, 1969; Chiller, Hodgins & Weiser, 1969; Pontius & Ambrosius, 1972; Sailendri & Muthukkaruppan, 1975). In the toad *Bufo marinus*, plasma cells were most abundant in intertubular lymphoid tissue of the kidney after injection with bovine serum albumin (BSA) (Cowden, Dyer, Gebhardt & Volpe, 1968). It was suggested that also in other lower vertebrates the kidney might be the major site for antibody production. However, in the tortoise *Agrionemys horsfieldii*, the kidney played no role in antibody production after immunization with SRBC (Rothe & Ambrosius, 1968). In rainbow trout (*Salmo Gairdneri*) PFC were found in mesonephros after immunization with O-antigen of the bacterium *Yersinia ruckeri*, but numbers were not as high as in spleen (Anderson, 1978). In perch (*Perca fluviatilis*) mesonephros contained lower PFC numbers than spleen and pronephros after injection with SRBC (Pontius & Ambrosius, 1972). In goldfish (*Carassius auratus*)

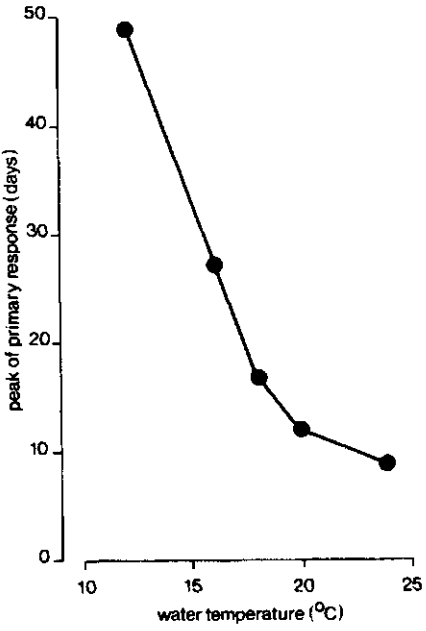

Fig. 6. Temperature dependence of humoral anti-SRBC response in carp.

pronephros and mesonephros are major sites of antibody production against *Salmonella typhi* (Neale & Chavin, 1971). In our experiments with carp, high PFC numbers were found in mesonephros. This organ together with pronephros contributed for 90% of the total PFC illustrating the importance of the kidney as a whole for antibody formation.

In most higher and lower vertebrates the spleen is an important PFC containing organ. In carp only low PFC numbers were found in spleen. However, it cannot be excluded that this organ plays an important role in the differentiation of B-like cells into plasma cells. Since PFC were observed in peripheral blood it is possible that proliferation and differentiation of B-like cells takes place in spleen and that maturating plasma cells migrate through the bloodstream and subsequently home in other organs (e.g. kidney). To verify this assumption splenectomy should be performed. Results of splenectomy experiments in other teleost fish are conflicting. Splenectomy in gray snapper (*Lutjanus griseus*) did not affect the antibody response against BSA (Ferren, 1967). Studies of Yu, Sarot, Filazzola & Perlmutter (1970) on the effect of splenectomy in blue gourami (*Trichogaster trichopterus*) suggest that the spleen is a major lymphoid organ in this species. Unfortunately splenectomy in carp is not possible because the spleen is interspersed with liver and pancreas and in most cases not a distinct organ but fragmented along the intestine.

Harris (1973) showed that dace (*Leuciscus leuciscus*) are capable of producing

antibodies over their complete environmental temperature range (2-18°C). The influence of temperature upon antibody production could be a general physiological effect as described for other metabolic processes (Huisman, 1974; Brett, 1979). A logarithmic relation between temperature and oxygen consumption or standard metabolism was observed by Brett (1976). In our experiments it turned out that a linear relationship exists between peak day and low temperatures (12-18°C) but the slope of the graph changed at 20°C and higher (Fig. 6). The possibility of a general physiological effect of temperature on the immune response as a whole is therefore unlikely. In this respect our results are in agreement with the ideas of Avtalion et al. (1976) and Marchalonis (1977) indicating that there are less and more temperature sensitive steps in the immune response.

In all instances plaque assay slides were incubated at 25°C. For carp kept at low temperatures (12-16°C) this is well beyond their ambient temperature. To assure that carp were capable of performing an immune response at 12 and 16°C PFC slides were also incubated at these temperatures. About 30% of the plaques were visualized at low temperatures compared with the situation at 25°C. It is admitted that a longer incubation period for plaque development was needed. It is possible that at 12°C higher PFC numbers can be reached *in vivo* because our *in vitro* system was not devised for long incubation periods.

In contrast to Avtalion et al. (1970) who did not show antibody formation in carp at 12°C using titration techniques, we were able to demonstrate a clear PFC response at this temperature (maximum day 49). Since no exponential relationship between temperature and peak day was observed and the magnitude of the response remained constant over the whole temperature range with carp of the same age, we expect that carp are able to mount an immune response at temperatures below 12°C. However, in those cases lag periods will become very long and the protective effect of such a response is therefore questionable.

Criteria to assess immunological memory in vertebrates are an increased rate of antibody production and higher levels of antibody (Marchalonis, 1977). At 24°C a true secondary response was found, illustrated by an early appearance of the first PFC and a 50 x higher peak than in the primary response. At 20°C, a 10 x higher secondary response is reached on the same day as during the primary response. During the secondary response at 18°C the first PFC appear earlier but the peak coincides with the primary response. It must be mentioned that the animals were primed with high antigen dosis (10^9 SRBC/animal) and that the interval between primary and secondary injection was about 1 month. Lower priming dose and prolonged interval between primary and secondary injection lead to a higher secondary response (Rijkers, Frederix-Wolters & van Muiswinkel, 1980b). Whereas the magnitude of the primary response is not affected by lower temperatures, the anamnestic character of the secondary response is gradually lost. This might indicate that the formation of memory cells is also a relative temperature sensitive process.

The relationship between temperature and peak day of the humoral immune response in carp (Fig. 6) closely matched the relationship between temperature and cellular immune response (allograft survival time) in goldfish (Hildemann, 1957). It is concluded that in bony fish humoral and cellular immunity are affected by temperature in a similar way.

ACKNOWLEDGEMENTS

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APPENDIX IV

Phylogeny of Immunological Memory
M.J. Manning, editor

THE IMMUNE SYSTEM OF CYPRINID FISH. THE EFFECT OF ANTIGEN DOSE AND ROUTE OF ADMINISTRATION ON THE DEVELOPMENT OF IMMUNOLOGICAL MEMORY IN CARP (Cyprinus carpio)

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INTRODUCTION

One of the most characteristic features of the immune system is the phenomenon of immunological memory. Memory means that a second contact with the same antigen evokes an enhanced response. Immunological memory in mammals applies to both cellular and humoral immune responses. Memory in cell-mediated reactions is manifested by the phenomenon that second-set grafts are rejected faster than first-set grafts. Criteria to assess immunological memory in humoral immune responses are an earlier rise in antibody formation, attainment of higher antibody levels and predominance of IgG[1]. Moreover, memory is specific for a given antigen and not a general enhancement of immune competence.

The capacity to discriminate between "self" and "non-self" is present throughout all vertebrates and invertebrates[2]. A cell-mediated immune system is found in annelids, e.g. earthworms which are capable to reject transplanted xeno- and allografts[3]. Upon repeated grafting transplants are rejected faster but this primitive form of memory is not donor specific and only short-lived[4]. It is not clear whether invertebrates possess a specific humoral immune system since immunoglobulins have not been demonstrated[5]. This does not imply that invertebrates are devoid of humoral defence mechanisms since in snails serum factors have been demonstrated which promote recognition and subsequent phagocytosis of foreign material[6], while in silk moths substances with a complement-like activity were found in hemolymph[7].

In the first vertebrates (jawless and cartilaginous fish) allografts are rejected in a chronic way[8]. However, the most advanced bony fish (teleosts) show an acute rejection of first-set allografts and accelerated rejection of second-set grafts[9,10]. When cyprinid fish are kept at 25°C - a normal ambient temperature for the species - cellular immune reactions proceed even faster than in mammals[11,12]. At the level of cellular immunity, secondary responses seem to be comparable in teleosts and mammals. As far as humoral immunity is concerned there have been difficulties in demonstrating clear-cut secondary responses in

in lower vertebrates[13-15]. In goldfish the magnitude of the secondary response was not enhanced but differences in the kinetics of antibody production were taken as evidence for immunological memory[16]. In other studies secondary responses were obtained which met the usual criteria for immunological memory[17-19]. Since teleost fish possess only one IgM like class of immunoglobulins[20-21], the criterium of predominance of IgG antibodies during the secondary response is not valid for this group.

In mice the apparent paradox exists that priming with a low antigen dose which evokes no or only a low primary response, is optimal for memory development. Priming with a high antigen dose, which results in a clear primary response, gives a poor secondary response[22,23]. It has been shown that antibody produced during the primary immunization, acts through a feedback mechanism that limits the magnitude of the secondary response to subsequent antigenic challenge, both *in vivo* and *in vitro*[24,25].

In this paper duration and specificity of immunological memory in carp, a teleost fish, is compared with the same process in mammals. The effect of different routes of administration and antigen dosages on the development of immunological memory is studied.

MATERIALS AND METHODS

Animals. Common carp (*Cyprinus carpio*) were obtained from the Organization for Improvement of Inland Fisheries (Nieuwegein) or bred in our laboratory. They were kept in aquaria with running tap water at 18°C and fed daily with dry food (K 30, Trouw & Co, Putten) with a "Scharflinger" automatic feeder. Animals, weighing 200-600 g, were 1 year old at start of the experiments.

Antigens. Sheep red blood cells (SRBC) were obtained from the Department of Animal Husbandry, Agricultural University (Wageningen). Horse red blood cells (HRBC) were purchased from the National Institute of Public Health (Bilthoven). Cells were washed 3 times with phosphate buffered saline (PBS) pH 7.2 before use.

Immunization. Animals were injected with SRBC either intramuscular (i.m.) or intravenous (i.v.). A priming dose of 10^5, 10^7 or 10^9 SRBC was i.m. injected in the dorsal region (0.05 ml) or i.v. in the caudal vein (0.5 ml). Control animals were i.m. injected with 0.05 ml PBS. At 1, 3, 6 or 10 months after primary injection, 5 animals out of each group were i.m. injected with 10^9 SRBC. Fourteen days after the challenge the animals were killed and the number of antibody producing cells in different lymphoid organs was determined.

Plaque forming cell assay. The number of antibody producing cells in spleen, pronephros and mesonephros was determined using the haemolytic plaque assay adapted for carp[26]. Bream (*Abramis brama*) serum was used as complement source.

After incubation for 2 hours at 25°C the plaque forming cells (PFC) were scored under a low-power dissection microscope. The number of viable cells in cell suspensions was counted in a haemocytometer using a dye exclusion assay (0.2% Trypan Blue in PBS). Results are expressed as number of plaque forming cells per 10^6 viable white cells (PFC/10^6 WC).

RESULTS

Primary response against SRBC

The results with carp injected with different dosages of SRBC are shown in Fig. 1. The number of PFC in spleen, pronephros and mesonephros was determined 17 days after injection (peak of the response). Similar experiments with non-immune carp showed no background PFC. Animals injected i.v. or i.m. with 10^5 SRBC poorly responded (0.2-2 PFC/10^6 WC). With increasing SRBC dose a marked increase in PFC response was observed. Animals i.v. injected with 10^7 and 10^9 SRBC gave rise to higher PFC levels compared with i.m. injected animals, although differences were only significant for spleen at the highest antigen dose. Thus, after a primary injection the magnitude of the peak response at day 17 is correlated with the antigen dose.

When animals kept at 18°C are i.m. injected with 10^9 SRBC with an interval of 1 month between 2 injections, the same peak value is reached on the same day (day 17, Table 1.). However, in the secondary response the first PFC appear 3 days earlier than in the primary response resulting in a ten-fold difference on day 14. In order to allow an assessment of immunological memory all PFC assays in

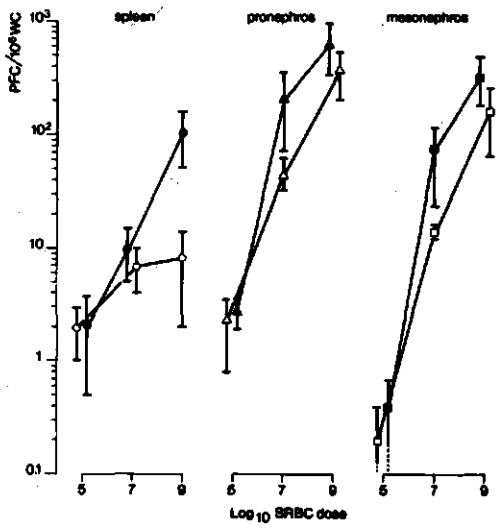

Fig. 1. Dose-dependency of the primary anti-SRBC response in carp. Animals, kept at 18°C, were intramuscular (○,△,□) or intravenous (●,▲,■) injected with 10^5, 10^7 or 10^9 SRBC. On day 17 the number of PFC in spleen, pronephros and mesonephros was determined. Each point represents the aritmetic mean ± 1 S.E.(n=4)

TABLE 1

IMMUNE RESPONSE OF CARP AGAINST SRBC

Type of response	PFC/10^6 white pronephros cells		
	day 14	day 17	day 20
primary	22 ± 12 *	379 ± 185	24 ± 13
secondary	230 ± 75	304 ± 101	128 ± 16

Animals, kept at 18°C were injected 2 times with 10^9 SRBC (i.m.). The second injection was given 1 month after the first. *Arithmetic mean ± 1 S.E. (n=4).

ted with the antigen dose.

When animals kept at 18°C were i.m. injected with 10^9 SRBC with an interval of 1 month, the same peak value is reached on day 17 (Table 1). However, in the secondary response the first PFC appear 3 days earlier than in the primary response resulting in a ten-fold difference on day 14. In order to allow an assessment of immunological memory all PFC assays in the following experiments were performed 14 days after the last injection.

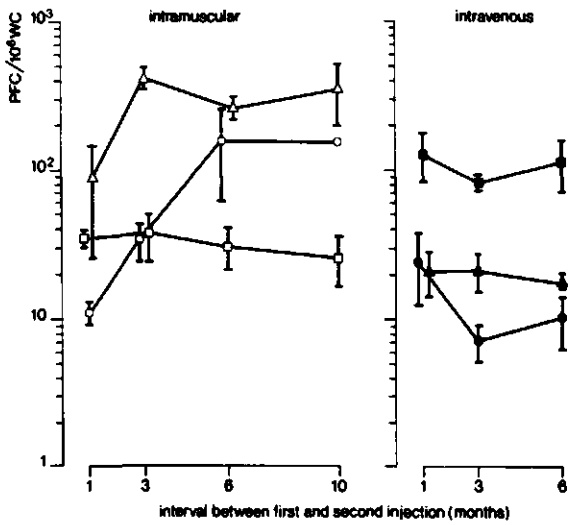

Fig. 2. Development of immunological memory in carp spleen. Animals were primed with 10^5 (○,●), 10^7 (△,▲) or 10^9 SRBC (□,■) either intramuscular or intravenous. At 1, 3, 6 or 10 months after priming 5 animals out of each group were challenged with 10^9 SRBC (i.m.). The number of PFC in spleen was determined 14 days after challenge. Each point represents the arithmetic mean ± 1 S.E..

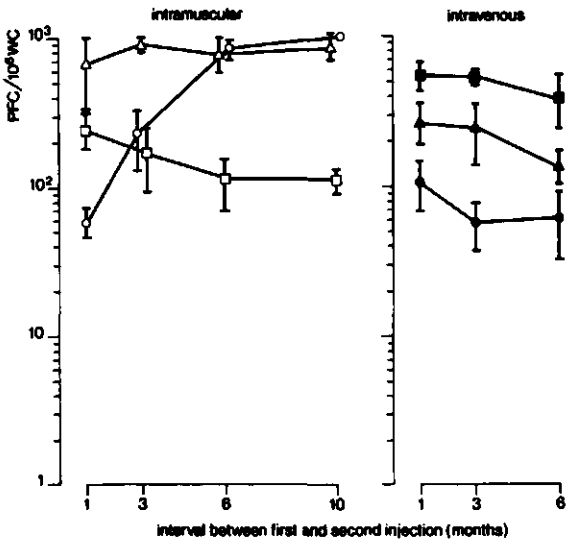

Fig. 3. Development of immunological memory in carp pronephros. For further explanation see Fig. 2.

Development of immunological memory

In order to study the effect of different dosages and routes of antigen administration on the formation of immunological memory, animals were divided into 7 groups. Three experimental groups were injected i.v. and 3 other groups i.m.. Different dosages SRBC (10^5, 10^7 and 10^9) were injected along each route. A group of PBS injected animals served as control.

Five animals out of each group received an i.m. injection with 10^9 SRBC 1 month after primary injection and were tested for PFC 14 days later. Control animals developed only 3 $\pm$ 1, 24 $\pm$ 15 and 6 $\pm$ 3 PFC/10^6 WC in spleen, pronephros and mesonephros respectively. The response of 10^5 SRBC i.m. primed animals did not exceed these values significantly (Figs. 2-4). The 10^9 SRBC i.m. group gave a clear secondary response (e.g. 254 $\pm$ 75 PFC/10^6 WC in pronephros) but the highest PFC levels were obtained with animals primed with only 10^7 SRBC i.m. (664 $\pm$ 350 PFC/10^6 WC in pronephros; Figs. 2-4). In all i.v. primed groups a secondary response was observed. After priming along this route PFC levels were higher with increasing priming dosages.

Challenge at 3 months after priming produced the following picture. Control animals developed 17 $\pm$ 12, 49 $\pm$ 12 and 29 $\pm$ 17 PFC/10^6 WC in spleen, pronephros and mesonephros respectively. Interestingly, the response of 10^5 SRBC i.m. primed animals showed for the first time a clear secondary character and was comparable with the response of the 10^9 SRBC i.m. primed group (227 $\pm$ 99 and 171 $\pm$ 80

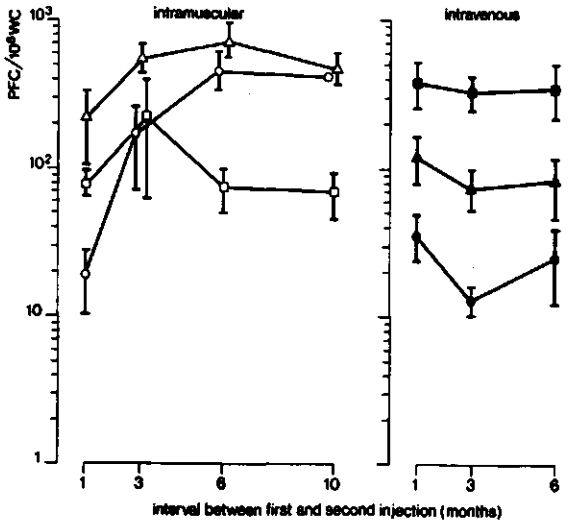

Fig. 4. Development of immunological memory in carp mesonephros. For further explanation see Fig. 2.

PFC/10^6 WC in pronephros respectively). The response of the 10^7 SRBC i.m. group remained at a quite high level (899 $\pm$ 106 PFC/10^6 WC in pronephros). In the i.v. primed animals PFC levels slightly declined compared with the situation 2 months earlier. The response of the 10^5 SRBC i.v. group was comparable with control values.

Another 3 months later the whole procedure was repeated. In control animals 4 $\pm$ 2, 17 $\pm$ 4 and 8 $\pm$ 2 PFC/10^6 WC were observed in spleen, pronephros and mesonephros respectively. Amazingly the response of the 10^5 SRBC i.m. group was still increasing and became equal to the high PFC level of 10^7 SRBC i.m. primed animals (784 $\pm$ 218 and 829 $\pm$ 139 PFC/10^6 WC in pronephros). In the 10^9 SRBC i.m. and all i.v. groups PFC levels remained nearly constant.

In addition, the animals which were primed i.m. received a booster 10 months after priming. PFC levels were similar to the situation at 6 months after priming, indicating a long-lived memory. Highest secondary responses were achieved with 10^5 and 10^7 SRBC i.m. primed animals (1094 and 828 $\pm$ 158 PFC/10^6 WC in pronephros). Animals primed with 10^9 SRBC i.m. remained at a level of about 100 PFC/10^6 WC in pronephros.

In general the development of immunological memory in spleen (Fig. 2) and mesonephros (Fig. 4) runs parallel with the picture described for pronephros (Fig. 3).

Specificity of the humoral immune response

In order to test the specificity of the immune response and memory formation SRBC primed and control animals were injected with HRBC. It appeared that only PFC directed against the booster antigen were present (Table 2.). The immune response against SRBC and HRBC turned out to be highly specific and no cross-reactivity between SRBC and HRBC was observed in this system. The magnitude of the anti-HRBC response in 10^9 SRBC i.v. primed animals was not enhanced compared with the response of control animals which were injected with PBS. It is concluded that the developed memory cells were specific for the priming antigen. No indications for a general enhancement of immune competence were found.

TABLE 2

SPECIFICITY OF THE HUMORAL IMMUNE RESPONSE IN CARP

First injection	Second injection	Test antigen	PFC/10^6 WC spleen	pronephros	mesonephros
PBS i.m.	10^9 HRBC i.m.	HRBC	6.4 ± 2.4*	16.0 ± 8.1	4.5 ± 0.9
		SRBC	0.2 ± 0.2	1.1 ± 0.7	0.3 ± 0.1
10^7 SRBC i.v.	10^9 SRBC i.m.	HRBC	0.1 ± 0.1	0.9 ± 0.3	0
		SRBC	9.8 ± 5.2	32.0 ± 10.5	15.0 ± 5.1
10^9 SRBC i.v.	10^9 HRBC i.m.	HRBC	7.3 ± 2.8	13.7 ± 7.6	5.0 ± 1.5
		SRBC	0.4 ± 0.3	0.8 ± 0.6	0.1 ± 0.1

Carp, which were kept at 18°C, were intravenous (i.v.) injected with 10^9 or 10^7 SRBC or intramuscular (i.m.) with 0.05 ml PBS. Ten months later all animals received an i.m. injection with 10^9 SRBC or HRBC. The number of PFC in spleen, pronephros and mesonephros was determined 14 days after the last injection.
*Arithmetic mean ± 1 S.E. (n=3).

DISCUSSION

Our results with carp show that the priming route is important for the magnitude of the secondary response. Moreover, differences in antigen priming dose-secondary response relationship were observed. When carp were primed i.v. the magnitude of the primary and secondary response increased with increasing antigen doses. After i.m. priming with a high antigen dose (10^9 SRBC) a high primary but a poor secondary response was observed. Priming with 10^7 SRBC was optimal for memory formation. A low priming dose (10^5 SRBC) resulted in a low primary but in

a high secondary response when the booster injection was given at least 6 months later.

It appeared that the priming dose-secondary response relationship in carp after i.m. priming is similar to mammals after i.v. priming[27,28]. It is tempting to speculate that antibody feedback inhibition is responsible for this phenomenon. It has been shown in rodents that IgM is more susceptible to suppression than IgG[29]. Yet, antibody feedback cannot fully explain the observed differences in memory development after priming with different antigen doses. E.g. the secondary response of 10^9 SRBC i.m. primed animals should increase when the interval between primary and secondary injection is prolonged. A second phenomenon which might play a role is antigen persistence. Since the primary response after low dose priming is very meager, antigen is not eliminated by antibody induced lysis or phagocytosis. Antigen(fragments) may persist and stimulate the formation of memory cells. This might be the reason why it took so long for the 10^5 SRBC i.m. primed animals to develop a high secondary response.

In trout, the primary immune response against MS2 bacteriophage increased with higher antigen dose. The same dose dependency was found after a secondary stimulation[19]. In carp, immunized with bovine serum albumin (BSA) ranging from 0.1-5 mg/kg, no significant differences in titres during the primary response were obtained[30]. High BSA doses were found to be tolerogenic in primary and secondary response. At low temperatures (14°C) low BSA doses (0.04-0.2 mg/kg) turned out to be tolerogenic too[30]. In the teleost *Tilapia mossambica* the primary PFC response to SRBC was found to be proportional to the amount of antigen injected[31]. In all studies the antigen was administered intraperitoneal[19,30] or i.v.[31]. This might explain the observed antigen dose-secondary response relationship, especially since i.v. priming in our experiments resulted in the same relationship.

The controversial data on secondary responses in lower vertebrates is centered around at least 2 factors: the antigen used and the period between first and second contact with the antigen. In this respect it is stated that characteristic secondary responses can only be obtained with antigens which induce a true primary response[32]. The second factor is the period between first and second antigen contact. This is visualized by our finding that no anamnestic response was observed in animals primed with 10^5 SRBC i.m. when a booster injection was given 1 month later. However, a second injection 6 months after the low dose priming resulted in a clear secondary response. It is evident that temperature is important too for primary and secondary responses in ectothermic vertebrates [30]. Results of studies in our laboratory concerning the effect of temperature on the immune response in carp at 12-24°C will be presented elsewhere.

The presented results might have practical consequences in the field of prophylaxis of diseases, especially vaccination schedules. However, it must be kept in mind that we used SRBC which is a corpuscular, non-pathogenic antigen. The immune system will probably respond in another way to proliferating pathogens.

The most striking observation was that a high priming dose (10^9 SRBC) was not optimal for memory formation; in other words: high dose vaccination does not always result in optimal protection. Priming with a 100 x lower antigen dose resulted in a 10 x higher secondary response; the developed memory remained at this high level for at least 10 months. A low priming dose (10^5 SRBC) can lead to a high secondary response when the second injection is given at least 6 months after priming. When dealing with expensive vaccins it is worthwhile to consider that low priming doses can be used if not an immediate protection is required.

If similar results can be achieved when a vaccin is administered by routes applicable in mass fish culture (e.g. oral immunization or hyperosmotic infiltration) is still an open question.

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APPENDIX V

Aquaculture, 19 (1980) 177—189

THE IMMUNE SYSTEM OF CYPRINID FISH. THE IMMUNOSUPPRESSIVE EFFECT OF THE ANTIBIOTIC OXYTETRACYCLINE IN CARP (*CYPRINUS CARPIO* L.)

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ABSTRACT

Rijkers, G.T., Teunissen, A.G., Van Oosterom, R. and Van Muiswinkel, W.B., 1980. The immune system of cyprinid fish. The immunosuppressive effect of the antibiotic oxytetracycline in carp (*Cyprinus carpio* L.). *Aquaculture*, 19: 177—189.

The effect of oxytetracycline (oxyTC) upon the immune system of carp was investigated. OxyTC was administered by feeding with oxyTC-containing pellets or by intraperitoneal injection. In order to study cellular immunity, allogeneic scale transplantation was carried out. Oral administration of oxyTC had no influence upon the median survival time (MST) of the scales. However, injections with oxyTC significantly prolonged the MST from 8.5 to 11—20 days. Thus, cellular immunity was not affected by oral administration of oxyTC, but injections did have a dramatic immunosuppressive effect.

To investigate the effect of oxyTC upon humoral immunity, animals were injected with rabbit red blood cells (RaRBC). During the primary and secondary response the number of rosette forming cells (RFC) in the spleen was determined. Control animals (not treated with oxyTC) developed an anti-RaRBC response up to 25000 RFC/10^6 white spleen cells but oxyTC-treated animals always showed reduced RFC numbers. In some cases the RFC number in oxyTC-treated animals was comparable with background levels in non-immunized control animals (4000 RFC/10^6 white spleen cells). Thus, irrespective of the route of administration, the humoral immune response is depressed by oxyTC. It is concluded that both humoral and cellular immune responses of carp are suppressed during treatment with oxyTC.

Preliminary observations showed an increased number of granulocytes in the spleen of oxyTC-treated animals. It is tempting to speculate that in those cases where specific lymphoid defence mechanisms are blocked, the phagocytic defence system becomes more active.

INTRODUCTION

There are several ways for a diseased animal to eliminate pathogenic micro-organisms. Under normal circumstances the pathogen is attacked either by phagocytic cells (granulocytes, mononuclear phagocytes) or in a more specific way by lymphoid cells (humoral and cell-mediated immunity).

In addition, antibiotics and chemotherapeutics can be used for the prevention and control of diseases (Braude et al., 1953; Guest, 1976). At present, penicillins, tetracyclines, macrolides, aminoglycosides, chloramphenicol, methylenblue, nitrofurans and antifungals are employed (Sanford, 1976).

It is known that antibiotics and chemotherapeutics can evoke acute toxic effects in animals (Martin, 1973) and increase the risk of raising resistant pathogens (Swann, 1969; Finland, 1975). Moreover, some antibiotics can interfere with certain steps in eukaryotic protein synthesis (Watson, 1975). Most probably the mitochondrial protein synthesis is inhibited (De Vries and Kroon, 1970).

Numerous studies in mammals and birds have been performed on the interaction between antibiotics and the immune system, with contradictory results. A stimulatory effect of antibiotics upon the immune response is reported by Popović et al. (1973). Their results show that pigs treated with oxytetracycline (oxyTC) produce significantly higher agglutinin titers after injection with a brucella vaccine than non-treated controls. According to other reports antibiotics have no effect upon the immune response. In birds, feeding with oxyTC did not interfere with antibody production after bovine serum albumin (BSA) injection (Glick, 1968). Several studies deal with immune suppression after treatment with antibiotics. In pigs, oxyTC depressed the formation of immunity against experimental *Erysipelothrix* infection (Fortushnyi et al., 1973). In mice injections with oxyTC depressed the anti-sheep erythrocyte response (Nikolaev and Nazarmukhamedova, 1974). Feeding of oxyTC to rats and mice interfered with the antibody production against *Salmonella enteritidis* (Slanetz, 1953).

Antibiotics are in common use for the prevention (Schäperclaus, 1967; Bauer et al., 1969) or control of diseases in cultured fish (Schäperclaus, 1969; Ghittino, 1972; Van Duijn, 1973). In order to prevent the development of drug resistant bacteria the amounts of antibiotics used are generally high (Van Duijn, 1973; Roberts and Shepherd, 1974).

In contrast to the data mentioned above, virtually nothing is known about the effects upon fish. Kreutzmann (1977) demonstrated that erythropoiesis in the eel was disturbed after treatment with chloramphenicol or oxyTC. Reports dealing with the immune system describe effects on cellular immunity. Antibiotics and antimetabolites can delay allograft rejection in fish (Hildemann and Cooper, 1963; Levy, 1963; Cooper, 1976). At present no data concerning the effects of antibiotics upon humoral immunity in fish are available.

This study was performed in order to obtain information about the possible side effects of antibiotics upon the immune system of fish. We have chosen oxyTC because of its frequent use in fish culture (Herman, 1969).

MATERIALS AND METHODS

Animals

Carp (*Cyprinus carpio*, Linnaeus 1758) were obtained from the Organization for Improvement of Inland Fisheries (OVB) at Nieuwegein, The Netherlands. They were kept in aquaria with running tap water at a temperature of 20 ± 1°C. Animals were fed with pelleted dry food (Trouvit K30, Trouw & Co, Putten, The Netherlands). The daily amount of food (2.5% of their body weight) was supplied by means of a "Scharflinger" automatic feeder. At the start of the experiment two size classes were selected from a group of 800 carps, aged 6 months: (1) small animals with a total length between 11 and 13 cm (15–49 g); (2) large animals between 15 and 17 cm (62–105 g).

Antibiotic

Oxytetracycline (oxyTC) was administered either by intraperitoneal (i.p.) injection of a solution of 50 mg/ml Engemycine® (Mycofarm, De Bilt, The Netherlands) or orally by feeding fish with Trouvit-pellets supplemented with 2000 ppm oxyTC.

Scale transplantation

It is known that repeated MS-222 anaesthesia causes a considerable loss of animals in this species (Rijkers and Van Muiswinkel, 1977). Therefore carps were not anaesthetized during scale transplantation and subsequent daily inspection. Five scales in the row above the lateral line were removed with a pair of fine forceps and kept for 5 min at maximum in sterile phosphate buffered saline (PBS). Each fish received four allografts, taken from two donors, and one autograft as a control. After transplantation allogeneic scales became overgrown by hyperplastic host tissue, which resulted in a white appearance. At the same time some scales exhibited vasodilatation and branching melanophores. The beginning of the clearance of the hyperplastic host tissue is considered to be the survival end point (SEP) of the graft (Hildemann, 1957). The median survival time (MST) was calculated from the SEP's.

Immunization

Rabbit red blood cells (RaRBC) were obtained from the Foundation of Blood Group Studies, Wageningen, The Netherlands. The cells were washed three times with PBS before use. Each animal received 10^9 RaRBC, injected intramuscularly (i.m.) in the dorsal region. In some cases a second injection was given 24 days later.

Rosette test

Cell suspensions were prepared as described earlier (Rijkers and Van Muiswinkel, 1977). The number of rosette forming cells (RFC) in the spleen was determined according to a slight modification of the method of Zaalberg (1964) and Biozzi et al. (1966). Briefly, 4×10^6 white spleen cells and 24×10^6 RaRBC were mixed and adjusted to a volume of 1 ml. The medium used was Hank's Balanced Salt Solution (HBSS, Difco, Detroit, U.S.A.) supplemented with 0.4% New Born Calf Serum and 1% antibiotics (20 I.U. mycostatine, 100 I.U. penicillin and 0.1 mg streptomycin per ml). The cell suspension was incubated overnight at 4°C and subsequently rotated for 10 min (10 rpm) at room temperature (Multi Purpose Rotator, Wilten, Etten-Leur, The Netherlands). Rosettes were counted under a phase-contrast microscope (objective 20 ×). Two types of rosettes were distinguished: (1) rosettes with a single layer of RaRBC, probably cells with receptors for RaRBC on their surface; (2) rosettes with a multiple layer of RaRBC representing cells which secrete antibodies against RaRBC.

Blood sampling and testing

Blood samples were collected by caudal puncture (Steucke and Schoettger, 1967). The hematocrit value was determined using a standard technique for fish (Amlacher, 1976). Sera were stored at −20°C. Serum levels of oxyTC were determined by the agar well diffusion method using *Bacillus cereus* (ATCC 11778) as assay organism (Bennett et al., 1966). A calibration curve was prepared using oxyTC dissolved in normal carp serum. The bacteria were a gift from the National Institute of Public Health (Bilthoven, The Netherlands

Experimental set-up

Animals were divided into five groups, which were kept in equal sized aquaria. Control animals received no treatment (group I, $n = 28$). Others received intraperitoneal (i.p.) injections of phosphate buffered saline (PBS) every 3 days (group II). Group III was fed with pellets containing 2000 ppm oxyTC. Groups IV and V received oxyTC by i.p. injection every 3 days (dosag in Table I). Groups II—V consisted of 50 animals each.

In scale transplantation experiments each group consisted of six fish (three large and three small). In rosette tests, six (primary response) or four (secondary response) animals were used for each determination.

Student's *t*-test was used to test the significance of differences between groups.

RESULTS

Growth effects

No difference in growth rate was observed between non-treated and PBS-injected animals (Table I, groups I and II). Feeding with oxyTC-containing pellets had a significant growth promoting effect ($P < 0.005$; group III). However, injections with oxyTC diminished growth ($P < 0.005$; groups IV and V). The growth inhibition was more pronounced with higher oxyTC doses. Differences between the two weight-classes became smaller during the experiment, because small animals grew faster than large ones in all experimental groups, except group I.

TABLE I

Oxytetracycline (oxyTC) treatment and growth in carp

Experimental group	Treatment	Size class	Weight at start of the experiment (g)	Weight after treatment for one month (g)	Percentile increase in weight
I: Control	None	Small	39 ± 2*	50 ± 4	28
	None	Large	75 ± 2	101 ± 4	35
II: PBS-injection	0.15 ml PBS/animal	Small	30 ± 2	41 ± 2	37
	0.40 ml PBS/animal	Large	77 ± 1	96 ± 4	25
III: OxyTC-feeding	Pellets supplemented with 2000 ppm oxyTC	Small	35 ± 1	54 ± 2	55
		Large	77 ± 2	112 ± 3	45
IV: OxyTC-injection low dose	60 mg oxyTC/kg fish	Small	31 ± 1	39 ± 2	25
	60 mg oxyTC/kg fish	Large	77 ± 2	89 ± 3	15
V: OxyTC-injection high dose	180 mg oxyTC/kg fish	Small	35 ± 2	42 ± 2	20
	180 mg oxyTC/kg fish	Large	84 ± 2	94 ± 3	12

All experimental groups (28—50 animals/group) were fed daily with pellets weighing 2.5% of their initial body weight. Control animals received no treatment (group I). Group II received an intraperitoneal (i.p.) injection of phosphate buffered saline (PBS) every 3 days. The other groups received the antibiotic daily by feeding with oxyTC-supplemented pellets (group III) or by i.p. injection every 3 days (Groups IV and V). Weighing of the animals was performed at the start of the experiment and 1 month later.
*Arithmetic mean (± 1 standard error).

After 48 days of feeding with antibiotics, animals in group III showed for the first time demonstrable levels of oxyTC in their serum; the maximum concentration observed was 1.25 μg oxyTC/ml. Injections with oxyTC gave rise to an earlier appearance of higher antibiotic levels in serum. After 16 days of treatment animals of group IV had 5 μg oxyTC/ml and group V 10—15 μg oxyTC/ml serum. In general oxyTC levels were influenced by the time between the last oxyTC injection and blood sampling.

TABLE II

Rosette forming cell response in the spleen of carp treated with oxytetracycline (oxyTC)

Experimental group	Days under treatment at the day of immunization	Number of rosette forming cells ($\times 10^3$) per 10^6 white spleen cells					
		Primary response		Secondary response			
		9 days after ag* injection		7 days after ag injection		13 days after ag injection	
		Single	Multiple	Single	Multiple	Single	Multiple
I: Control	—	16	4	20 ± 0.5	12.5 ± 3	13 ± 2	5 ± 1
II: PBS-injection	15	n.d.**	n.d.	16 ± 2.5	8.0 ± 0.5	14 ± 0.5	3 ± 1
	35	11 ± 1	5 ± 1	5 ± 1	4.5 ± 1	14 ± 2	11 ± 2.5
III: OxyTC-feeding	15	4 ± 0.5	0	1.5 ± 0.5	0	1.5 ± 0.5	3 ± 1
	35	8.5 ± 1	4 ± 1	9 ± 2	5.5 ± 1.5	2 ± 1	1 ± 0.5
IV: OxyTC-injection low dose	15	2.5 ± 0.5	0	n.d.	n.d.	4 ± 1	8 ± 3
	35	1.5 ± 0.5	0.5	14.5 ± 5.5	5 ± 2	5 ± 1	2 ± 0.5
V: OxyTC-injection high dose	15	1 ± 0.5	0	n.d.	n.d.	5 ± 1	4 ± 2.5
	35	n.d.	n.d.	3 ± 0.5	2 ± 0.5	n.d.	n.d.

All animals were injected with 10^9 rabbit red blood cells (RaRBC) intramuscularly (i.m.). For the secondary response a second i.m. injection of 10^9 RaRBC was given 24 days later. Two types of rosettes were counted: (1) rosettes with a single layer of RaRBC; (2) rosettes with a multiple layer of RaRBC. Each point represents the arithmetic mean (± 1 standard error) of six (primary response) or four (secondary response) animals.

* ag = antigen (RaRBC).

** n.d. = not done.

TABLE III

Differential blood cell counts in oxytetracycline (oxyTC)-treated carp

Experimental group	Number of cells ($\times 10^7$) per ml*			
	Erythrocytes	Lymphocytes + monocytes	Granulocytes	Thrombocytes
I: Control	135 ± 2	4.6 ± 0.3	0.58 ± 0.20	0.32 ± 0.02
V: OxyTC-injection** high dose	110 ± 1	3.0 ± 0.3	0.66 ± 0.06	0.30 ± 0.03

* Arithmetic mean (± 1 standard error) of five animals.
** Cell counts were performed 37 days after start of oxyTC treatment.

TABLE IV

Granulocytes in the spleen of carps treated with oxytetracycline (oxyTC)

Experimental group	Percentage granulocytes of splenic white cells		
	9 Days after primary injection	7 Days after secondary injection	13 Days after secondary injection
I: Control	n.d.*	7.0 ± 0.6	4.2 ± 0.6
II: PBS-injection	n.d.	4.5 ± 0.3	6.9 ± 2.5
III: OxyTC-feeding	n.d.	5.9 ± 1.0	5.0 ± 0.7
IV: OxyTC-injection low dose	13.4 ± 1.0	19.0 ± 2.7	15.5 ± 1.7
V: OxyTC-injection high dose	21.7 ± 2.1	32.6 ± 12.2	n.d.

Animals were injected with 10^9 rabbit red blood cells (RaRBC) intramuscularly (i.m.) at 35 days after start of PBS injection or oxyTC treatment. A secondary injection (10^9 RaRBC, i.m.) was given 24 days later. Cells were counted in a hemocytometer under a phase-contrast microscope (objective 20 ×). Each point represents the arithmetic mean (± 1 standard error) of six (primary response) or four (secondary response) animals.
* n.d. = not done.

splenic leucocytes were granulocytes. However, the low dose oxyTC-injection group (IV) had 10—20% and the high dose oxyTC-injection group (V) showed even higher numbers of granulocytes (20—30%).

DISCUSSION

The effect on the immune status of animals is rarely taken into consideration when chosing an antibiotic for therapeutic use. Yet, adverse side effects are

unwanted because the immune system has to co-operate with the antibiotic. The antibiotic generally inhibits growth of the pathogen allowing the immune system to eliminate the invaded micro-organisms.

The data about the effects of antibiotics upon the immune system are rather confusing. Many reports are known describing positive, negative or no effects on the immune system. However, this picture is based on effects of different antibiotics studied in different animal species. Moreover, the parameters used (e.g. susceptibility to diseases) reveal overall effects on the well-being of an animal, which is not exclusively dependant on the immune status. No studies are available concerning the effects of antibiotics upon basic immunological processes such as antigen recognition and antibody production.

Besides being used for prevention and control of diseases, antibiotics are used in animal husbandry because of their growth promoting effect. Growth of freshwater fish such as *Labeo* and carp is promoted by supplementing food with oxyTC (Mitra and Ghosh, 1967; Sukhoverkhov, 1967). In our experiments with carp the same growth promoting effect was found after feeding with oxyTC-containing pellets. This growth promotion might be caused by changes in the intestinal flora or gut epithelium allowing a more efficient food uptake.

In order to study humoral immunity, animals were injected with RaRBC instead of the usual sheep red blood cells (SRBC). SRBC and carp lymphocytes look quite alike under the phase-contrast microscope. Therefore RaRBC which are smaller than SRBC, were used, allowing easy detection of the central lymphoid cell in a rosette. No data are available about the kinetics of antigen recognizing and antibody producing cells after injection of this antigen in cyprinid fish. The time schedule for the rosette test was based upon the work of Warr et al. (1977), who showed that maximum numbers of splenic RFC appeared at day 8 after injection of goldfish with horse erythrocytes.

In the scale transplantation experiments we observed no effect of orally administered oxyTC, but injections of oxyTC delayed allograft rejection. This observation is in agreement with that of Hildemann and Cooper (1963) who found that in the teleost *Fundulus heteroclitus*, injections with tetracycline prolonged survival times of allografts.

Antibiotics have an influence not only on the peak height of the humoral immune response but also on the kinetics of the cells involved. For instance, the peak day of antibody producing cells after immunization of mice with SRBC is influenced by streptomycin (Toshkov and Slavcheva, 1968); in chickens chlortetracycline affects the kinetics of the antibody response to *Salmonella cholerasuis* (Procházka et al., 1968). These facts must be taken into account when comparing our data on RFC in different experimental groups during the primary and secondary response against RaRBC.

In control groups, injected with RaRBC, maximum percentages of RFC scored were 1.5—3. This is in the same order of magnitude as reported by Chiller et al. (1969) for rainbow trout and Warr et al. (1977) for goldfish. OxyTC suppressed the number of RFC independent of the route of administration.

Taking oxyTC serum levels into account when regarding the effects of oxyTC on the humoral and cellular immune response, the humoral immune system might be more sensitive to low levels of antibiotics. This is suggested by the observation that serum levels of oxyTC were low and humoral immunity was suppressed while cellular immunity was not affected in animals which were fed with oxyTC-containing pellets.

Animals injected with oxyTC showed raised levels of granulocytes in their spleen. The function of granulocytes in fish is not yet clear. A mast cell function is suggested by Ellis (1977) and Barber and Mills Westermann (1975, 1978). It is known that mammalian granulocytes fulfill an important role in non-specific defence. It is tempting to speculate that in those cases where specific lymphoid defence mechanisms are blocked, the phagocytic system becomes more active.

The presented results have shown that oxyTC dramatically suppresses the immune system of carp. Cellular immunity is suppressed when injecting the antibiotic, but not by oxyTC feeding. Humoral immunity however is suppressed in all cases. Thus, bacterial growth and the specific defence mechanism of fish are suppressed by oxyTC at the same time. Consequently any non-bacterial infection or an invasion by resistant bacteria causes serious problems.

Since the concentration of oxyTC used in commercial fish farming is the same as that used in our feeding experiments we recommend a selective and very cautious use of this antibiotic. It may be useful to develop proper vaccination methods for the prevention of the major diseases in large-scale fish culture. In this respect more basic research on issues like raising long-term immunological memory in fish is needed.

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APPENDIX VI

or by a decomposition product (Sanford, 1976). An adverse effect of antibiotics which is rarely taken into account is the effect on the immune system. It has been demonstrated that antibiotics can induce immunosuppression in man (Daikos and Weinstein, 1951; Daniel et al., 1964), laboratory animals (Stevens, 1953; Weisberger et al., 1964), domestic animals (Fortushnyi et al., 1973; Lyashenko, 1966), birds (Lakhotia and Stephens, 1972; Panigrahy et al., 1979) and fish (Hildemann and Cooper, 1963; Levy, 1963).

Reports on the effects of antibiotics in fish mainly deal with cellular immunity. It appeared that antibiotics can delay allograft rejection (Hildemann and Cooper, 1963; Levy, 1963). In a previous paper (Rijkers et al, 1980a) we reported the immunosuppressive effect of oxytetracycline (oxyTC) in carp. It was shown that both cellular and humoral immunity were depressed after feeding or injecting the antibiotic. In this paper the effect of oxyTC upon the regulation of humoral immunity in fish is studied in more detail. On base of the differential effects of oxyTC on primary and secondary immune responses we propose a model for the cellular interaction during the humoral immune response in fish.

MATERIALS AND METHODS

Animals

Carp (*Cyprinus carpio*) were bred in our laboratory. They were kept in aquaria with running tap water at 20°C. Animals were fed daily on pelleted dry food (K30, Trouw & Co, Putten, The Netherlands), amounting 2.5% of their body weight, by means of a "Scharflinger" automatic feeder. Eight to twelve months old animals, weighing 100-300 g were used.

Antibiotic

Oxytetracycline (oxyTC) was administered either by intraperitoneal (i.p.) injection of a 50 mg/ml Engemycine® solution (Mycofarm, De Bilt, The Netherlands) or orally by feeding pellets supplemented with 2000 ppm oxyTC.

Antisera

Goat antiserum to rabbit IgG conjugated to peroxidase (GAR/IgG/PO) was obtained from Nordic (Tilburg, The Netherlands). Rabbit antiserum to pike immunoglobulin heavy chains (RAP/Ig/H) was prepared and described by Clerx (1978). Rabbit antiserum to carp immunoglobulin (RAC/Ig) was prepared as described previously (Davina et al., 1980).

Quantitation of immunoglobulin and protein levels

The enzyme-linked immunosorbent assay (ELISA) and rocket electrophoresis were used to quantitate immunoglobulin levels using normal carp serum as a standard. In the ELISA, essentially the procedure of Voller et al. (1976) was followed. Carp serum was coated to polystyrene microhaemagglutination plates in two-fold serial dilutions starting with 1:12,000. RAP/Ig/H (1:100) and GAR/IgG/PO (1:100) were used as antiserum and enzyme labeled antiglobulin respectively. After incubation with substrate (hydrogen peroxide and 5-aminosalicylic acid) 450 nm absorbance was measured with a Titertek® Multiskan (Flow, McLean, USA).

In the rocket electrophoresis according to Laurell (1972) gels made up of 1% RAC/Ig and 1% agarose (No. 4, Nordic) in high resolution buffer, pH 8.8 (Gelman, Ann Harbor, USA) were used. Prior to the electrophoretic run (110 V, 21 h, room temperature) samples were carbamylated according to Weeke (1968).

Total serum protein was determined according to Lowry et al. (1951) using bovine serum albumin (BSA, Organon Teknika, Oss, The Netherlands) as a standard.

Antigen and immunization

Sheep red blood cells (SRBC) were obtained from the Department of Animal Husbandry, Agricultural University, Wageningen. Cells were washed 3 times with phosphate buffered saline (PBS, pH 7.2) before use. 10^9 SRBC were injected intramuscularly (i.m.) in the dorsal region. If needed a second injection was given 1 month later.

PFC assay

Antibody forming cells (PFC) were determined with the haemolytic plaque assay adapted for carp as described previously (Rijkers et al., 1980b). Bream (*Abramis brama*) serum was used as complement source. PFC slides were incubated at 25°C for 2 h.

Experimental set-up

Animals were divided into 3 groups. Control animals received no oxyTC. OxyTC-feeded animals received pellets containing 2000 ppm oxyTC (50 µg oxyTC/g fish/day). OxyTC-injected animals received an i.p. injection of Engemycine® every 3 days (20 µg oxyTC/g fish/day). Primary and secondary anti-SRBC responses and serum analysis were carried out after treatment of the animals for 6 weeks. During the anti-SRBC response oxyTC treatment was continued.

RESULTS

Serum analysis

Feeding oxyTC had no effect on total serum protein concentration, while oxyTC-injection reduced protein levels only by 20%. In contrast to total protein, serum immunoglobulin levels were markedly reduced after oxyTC feeding (decrease 40-75%) and oxyTC injection (55%) (TABLE I).

TABLE I

The effect of oxytetracycline on serum protein and immunoglobulin levels in carp

Treatment	Protein	Immunoglobulin (U/ml)	
	(mg/ml)	Rocket	ELISA
Control	4.63 ± 0.42 *	150 ± 36	116 ± 11
OxyTC-feeding	4.72 ± 0.30	40 ± 10	102 ± 19
OxyTC-injection	3.71 ± 0.10	67 ± 37	n.d. **

Protein and immunoglobulin (Ig) determinations were carried out in sera of normal carp (control) and animals treated with oxytetracycline (oxyTC) for 6 weeks. Ig concentration was determined with the rocket and ELISA technique and expressed as arbitrary units (U/ml). *Arithmetic mean ± 1 S.E. (n=4), **n.d.= not done.

Primary and secondary anti-SRBC response

Animals were injected with 10^9 SRBC (i.m.). At day 12 after injection (peak of the response) spleen, pronephros and mesonephros were removed for determination of the number of antibody forming cells. Control animals showed PFC levels comparable with results obtained earlier (Rijkers et al., 1980c). PFC numbers in spleen, pronephros and mesonephros were reduced by approximately 85% after feeding with oxyTC. OxyTC injection caused an even stronger inhibition (95%, Fig. 1).

To investigate the effect of oxyTC on the formation of immunological memory and the secondary response, animals which were treated for 2 weeks were injected with a priming dose of 10^9 SRBC (i.m.). A second injection was given 4 weeks later. Control animals tested on day 12 (peak of the response) showed a 10-fold enhancement of PFC numbers compared with the primary response. Surprisingly, the secondary response in oxyTC-feeded and oxyTC-injected animals was not significantly different from the response in control animals (Fig. 2).

In conclusion oxyTC caused a strong inhibition of the primary anti-SRBC response

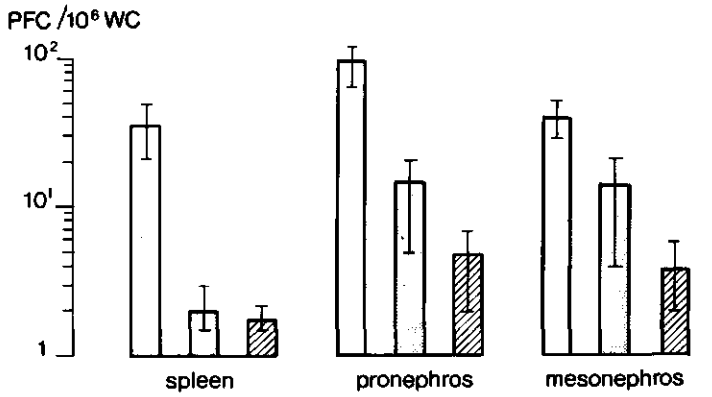

Fig. 1. Primary anti-SRBC response in carp. All animals were given an intramuscular injection with 10^9 SRBC after treatment for 6 weeks. The number of plaque forming cells (PFC) per 10^6 white cells (WC) in spleen, pronephros and mesonephros of control (open bars), oxyTC-feeded (shaded bars) or oxyTC-injected animals (hatched bars) was determined 12 days after injection. Each bar represents the arithmetic mean ± 1.S.E. (n=4).

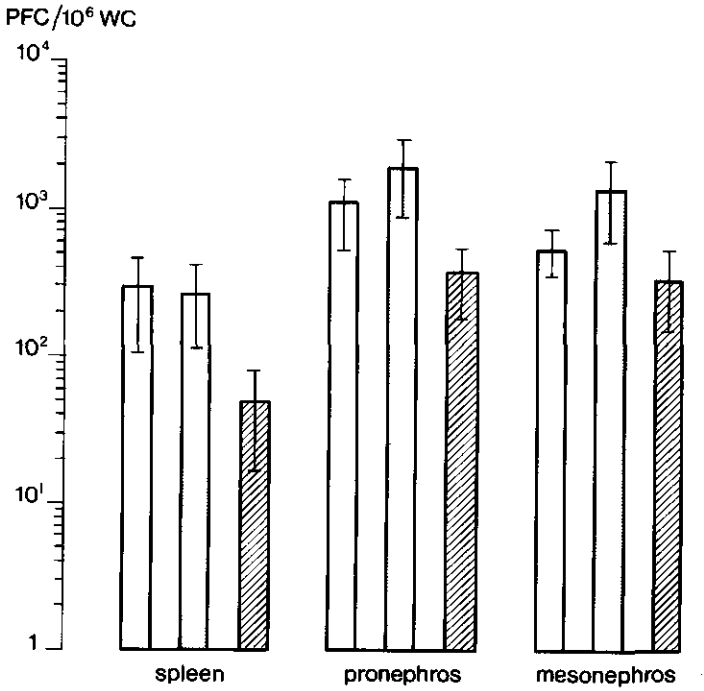

Fig. 2. Secondary anti-SRBC response in carp. All animals were given a primary intramuscular (i.m.) injection with 10^9 SRBC after treatment for 2 weeks. The response was measured 12 days after a second i.m. injection which was given 4 weeks after priming. See Fig. 1 for further explanation.

whereas the formation of immunological memory and the secondary response itself were apparently not affected.

Regulation by specific antibody

Antigen presentation at the start of the secondary response might be different from the same process during a primary response due to the presence of some specific antibody at the initiation of the secondary response.

In order to investigate this hypothesis carp were i.m. injected with SRBC simultaneously with an i.v. administered anti-SRBC serum simulating a "secondary" antigen presentation during a primary response. In preliminary experiments various dosages of carp anti-SRBC serum were tested. Dilutions of 1:10 and 1:100 were inhibitory but a dilution of 1:1000 caused a significant increase in the number of PFC in pronephros and mesonephros compared with control animals which were injected with SRBC and normal carp serum.

As expected oxyTC depressed the primary anti-SRBC response when normal carp serum was injected in combination with the antigen. However, anti-SRBC serum (1:1000) abolished completely the immunosuppressive effect of oxyTC on the primary response in oxyTC-feeded animals. In oxyTC-injected animals the same phenomenon was observed but to a lesser extent (TABLE II).

TABLE II

The effect of antibody on the primary anti-SRBC response in carp

Treatment	Plaque forming cells / 10^6 white cells					
	Spleen		Pronephros		Mesonephros	
	NCS	anti-SRBC	NCS	anti-SRBC	NCS	anti-SRBC
Control	29 ± 4	37 ± 9*	59 ± 7	179 ± 45	29 ± 5	76 ± 23
OxyTC-feeding	4 ± 2	67 ± 28	27 ± 11	151 ± 34	15 ± 6	79 ± 11
OxyTC-injection	2 ± 1	24 ± 5	5	94 ± 31	2 ± 1	34 ± 11

Carps treated for 6 weeks were injected intramuscular with 10^9 SRBC. At the same time the animals were injected intravenously either with 0.5 ml carp anti-SRBC serum (agglutination-titre: 2^{10}), diluted 1:1000 in phosphate buffered saline (PBS) or with 0.5 ml normal carp serum (NCS, diluted 1:1000 in PBS). The number of plaque forming cells in spleen, pronephros and mesonephros was determined 12 days after injection. *Arithmetic mean ± 1 S.E. (n=4).

It is concluded that the differential oxyTC sensitivity of primary and secondary immune responses is probably a reflection of differences in antigen presentation in which specific antibody plays an important role. However, other factors (macrophages, suppressor cells) might be involved too since anti-SRBC serum can not fully restore the primary anti-SRBC response in oxyTC-injected animals.

DISCUSSION

Our findings that serum immunoglobulin levels were reduced by 40-75% after oxyTC treatment (TABLE I) whereas the antibiotic had no effect on the secondary anti-SRBC response (Fig. 2) are apparently in contradiction with one another. One may assume that under natural circumstances the vast majority of serum immunoglobulin is produced as consequence of secondary responses against so-called "environmental antigens" entering through epithelial barriers. In oxyTC treated animals these environmental antigens (as far as drug sensitive bacteria are concerned) are eliminated by the antibiotic, causing diminished antigenic load and consequently reduced immunoglobulin levels. A comparable situation exists in germfree and specific pathogen free mice were low immunoglobulin levels were observed compared with conventional mice (Sell and Fahey, 1964; Van Snick and Masson, 1980). The secondary anti-SRBC response was evoked by a high antigen dose while secondary responses against environmental antigens are probably caused by low antigen doses. The influence of antigen dose in a secondary response will be discussed in more detail when dealing with the regulation of the immune response.

A discrepancy between immunoglobulin levels was observed when comparing the rocket and ELISA technique (TABLE I). This difference may be caused by the use of 2 different antisera for these techniques. Moreover, in rocket electrophoresis only precipitated complexes are visualized while in ELISA the affinity of the antiserum for the antigen is important.

The immunosuppressive effect of oxyTC on a primary anti-SRBC response as measured by PFC numbers confirmed our earlier results with the rosette assay (Rijkers et al., 1980a). However, under the experimental conditions during this study, secondary anti-SRBC responses were not inhibited by oxyTC. It appeared that high doses of passively transfered specific antibody inhibit the primary anti-SRBC response (feed back inhibition) whereas low antibody doses have a stimulating effect (TABLE I). The same phenomenon has been encountered in mice (Möller and Wigzell, 1965) and chickens (Morgan and Tempelis, 1977).

Following a primary injection with SRBC cell interaction is necessary for the development of a proper immune response. SRBC is generally accepted as a "T-dependent" antigen in mammals (Greaves et al., 1974) but also in fish (Sailendri, 1973). Non-lymphoid cells such as monocytes, macrophages and dendritic cells are involved in the regulation of the immune response and memory formation (Unanue, 1975; Van Rooijen, 1980). It has been shown that antibiotics interfere both quantitatively and

quantitatively with monocytes (Kreutzmann, 1977) and macrophages (Rhodes and Hsu, 1974; Alexander, 1975). These are interesting observations because macrophages play an essential role in antigen presentation (Mosier and Coppleson, 1968). Differences in antigen presentation during primary and secondary responses are caused by the formation of immune complexes. These complexes are an important factor in the regulation of memory formation and the secondary response itself. Depending on the antigen:antibody ratio and the antibody class involved immune complexes have an immunostimulating or an immunosuppressive effect (Eardley and Tempelis, 1975; Gordon and Murgita, 1975; Klaus, 1978). We have shown that a primary anti-SRBC response is inhibited most clearly by oxyTC injections (Fig. 1.). As a consequence the antigen:antibody ratio in immune complexes will be different for control, oxyTC-feeded and oxyTC-injected animals which may result in differences in memory formation. In addition, a second antigen injection in control and oxyTC-treated animals will result in immune complexes with a different antigen:antibody ratio. We have shown that a second injection with a high antigen dose evoked a secondary response which was not affected by oxyTC (Fig. 2.). However, a conclusion that all secondary responses are oxyTC insensitive is premature. More refined immunization schedules and antibiotic treatment regimens, as well as information on antigen trapping and persistence are needed to clarify the role of oxyTC in memory formation and secondary immune responses in fish.

On base of the data discussed above we propose the following model to explain the effect of oxyTC upon the immune response:

During a primary response oxyTC reduces antigen presentation by non-lymphoid cells to a suboptimal level resulting in a depressed humoral response. When a "secondary" antigen presentation is simulated by injecting specific antibody, antigen is presented in a supraoptimal way. The final antigen processing remains at an optimal level even after oxyTC interference. Therefore oxyTC has no inhibiting effect on normal or simulated secondary responses. Another possibility is an adverse effect of oxyTC upon cell interaction between lymphoid cells. During the primary response cell interaction might be a prerequisite for differentiation of B-like cell into plasma cells. However, during the secondary response another type of antigen presentation permits a T-independent B cell response. If this is true, oxyTC has no effect upon the secondary response.

In conclusion a careful use of the drug oxyTC is recommended since oxyTC exerts an immunosuppressive effect under certain conditions. On the other hand the data presented here have shown that this drug can be a valuable tool for studying the regulation of the humoral immune response in lower vertebrates.

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