Natural adaptive immune responses in humans against Toxoplasma gondii and Herpes simplex virus type I
Meek, B.

Citation for published version (APA):
Meek, B. (2002). Natural adaptive immune responses in humans against Toxoplasma gondii and Herpes simplex virus type I
Natural and adaptive immune responses in humans against *Toxoplasma gondii* and Herpes simplex virus type 1

Bob Meek
Natural and adaptive immune responses in humans against
*Toxoplasma gondii* and Herpes simplex virus type 1

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van van de Rector Magnificus
prof. mr. P.F. van der Heijden
ten overstaan van
een door het college voor promoties ingestelde commissie,
in het openbaar te verdedigen
in de Aula van de Universiteit
op woensdag 25 september 2002,
te 10.00 uur

door

Bob Meek
geboren te Groningen
Promotiecommissie

Promotores: Prof. dr. M.D. de Smet
Prof. dr. P.T.V.M. de Jong

Co-promotor: Dr. R. Peek

Overige leden: Prof. dr. M.L. Kapsenberg
Prof. dr. F.G.M. Kroese
Prof. dr. S.J.H. van Deventer
Prof. dr. J. Dankert
Dr. P.K. Das
Prof. dr. R.W. Sauerwein

Faculteit der Geneeskunde.

© Bob Meek, Amsterdam, The Netherlands, 2002. All rights reserved. No part of this book may be reproduced in any form or by any means without permission of the author.


The research presented in this thesis was carried out at the Netherlands Ophthalmic Research Institute, an institute of the Royal Dutch Academy of Sciences.

Cover-design by Greetje Meek.

Publication of the thesis was made possible by the financial support of Visio, Stichting Bartiméus, Landelijke Stichting voor Blinden en Slechtzienden, Abbott Diagnostics Nederland and the University of Amsterdam.

Printed by: Ipskamp Enschede.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Abbreviations</td>
<td>4</td>
</tr>
<tr>
<td>Nederlandse samenvatting</td>
<td>5</td>
</tr>
<tr>
<td>Chapter 1: IgA antibodies to Toxoplasma gondii in human tears</td>
<td>11</td>
</tr>
<tr>
<td>Chapter 2: Protein disulfide isomerase of <em>Toxoplasma gondii</em> is targeted by mucosal IgA antibodies in humans</td>
<td>21</td>
</tr>
<tr>
<td>Chapter 3: Conserved regions of protein disulfide isomerase are targeted by natural IgA antibodies in humans</td>
<td>29</td>
</tr>
<tr>
<td>Chapter 4: Herpes Simplex Virus Infection of the Human Eye Induces a Compartmentalized Virus-Specific B cell Response</td>
<td>41</td>
</tr>
<tr>
<td>Chapter 5: The ocular humoral immune response in health and disease</td>
<td>51</td>
</tr>
<tr>
<td>Reference List Chapters 1-5</td>
<td>75</td>
</tr>
<tr>
<td>Chapter 6: Dissecting the IgM antibody response during the acute and latent phase of toxoplasmosis.</td>
<td>89</td>
</tr>
<tr>
<td>Chapter 7: IgM recognition of recombinant <em>Toxoplasma gondii</em> antigens by sera of acutely or latently infected humans</td>
<td>99</td>
</tr>
<tr>
<td>Reference List Chapters 6 and 7</td>
<td>109</td>
</tr>
</tbody>
</table>
List of Abbreviations

aa = amino acids
ACAI DD = anterior chamber associated immune deviation
Agg = agglutination assay
AH = aqueous humour
AI = acutely infected
AID = activation-induced cytidine deaminase
AP = ammonium sulphate precipitation
APC = antigen presenting cell
ARN = acute retinal necrosis syndrome
as = anti-sense
AS = ammonium sulphate
ASC = antibody secreting cell
BBB = blood brain barrier
BCR = B cell antigen receptor
BOB = blood ocular barrier
CALT/EALT/LDALT/NALT = conjunctiva/eye/lacrimal drainage/nasal associated lymphoid tissue
CC/CXCL = CC/CXC chemokine ligand
CC/CXCR = CC/CXC chemokine receptor
CDR = complementary determining region
CFT = complement fixation test
CLN = cervical lymph node
CM = cell-mediated immunity
CMIS = common mucosal immune system
CMV = cytomegalovirus
CNR = clinically non-relevant
CSF = cerebro-spinal fluid
DC = dendritic cell
Ds IgX = double sandwich IgX (X = IgM, A, or G)
ELISA = enzyme immuno-assay
EIA = enzyme immuno-assay
ELISA = enzyme-linked immunosorbent assay
ER = endoplasmatic reticulum
ESA = excreted/secreted antigens
Fas-L = Fas ligand
Fc = constant domain of heavy chain
FDC = follicular dendritic cell
GC = germinal center
gG = glycoprotein G
GM-CSF = granulocyte-monocyte colony-stimulating factor
GPI = glycosylphosphatidylinositol
Gra = granule
GST = glutathione S transferase
GWC = Goldmann-Witmer coefficient
HEV = high endothelial venule
HLA = human leukocyte antigen
HHV = human Herpes virus
HSV = Herpes simplex virus
IB = immunoblot
ICAM = intercellular adhesion molecule
IDR = immunodominant region
IEF = iso-electric focusing
IF = immunofluorescence
IFN = interferon
IgA/IgG/IgM = immunoglobin A/G/M
IgV = variable domain of immunoglobin
IL = interleukin
IOF = intraocular fluid
J = joining chain
LAP = latency associated peptide
LAT = latency associated transcription
LFA = leukocyte function-associated antigen
LG = lacrimal gland
LI = latently infected
LN = lymph node
LP = lamina propria
LP-DC = lamina propria dendritic cells
LPS = lipopolysaccharide
M = microfold
MadCAM = mucosal addressin cell adhesion molecule
MHC = major histocompatibility complex
MIS = mucosal immune system
MLN = mesenteric lymph node
n = native
NR = not reduced
o = ocular
OT = ocular toxoplasmosis
PC = phosphorylcholine
PCR = polymerase chain reaction
PDI = protein disulfide isomerase
PP = Peyers patches
PtC = phosphatidylcholine
r = recombinant
R = reduced
RAG = recombination activating gene
RF = rheumatoid factor
RIP = radio immunoprecipitation
ROP = rhothryp
RT = reverse transcriptase
s = sense
SAG = surface antigen
SC = secretory component
SDF-1 = stromal-cell derived factor-1
SDS-PAGE = sodium dodecyl sulphate – polyacrylamide gel electrophoresis
Ser = serum
SF = Sabin-Feldman assay
sFas-L = soluble Fas ligand
sIgA = secretory IgA
SN = seronegative
SP = surface-proteins
STAT = signal transducer and activator of transcription
TCR = T cell receptor
TdT = terminal deoxyxide transferase
TECK = thymus-expressed chemokine
Tg = Toxoplasma gondii
Th = T helper
TI = T cell independent
TLR = Toll-like receptor
TP = total protein
VCAM = vascular cell adhesion molecule-1
VDJ segment = variable-diversity-joining segment
VZV = Varicella zoster virus
Acknowledgements

‘Thank you’ to all those inspiring people who encouraged me to keep going on these past 6 years, especially RON (the constructor), moeije (for you, nice drawings!), Harro (paranymf exterior), Vincent (paranymf interior), pa (food-supplies and fast machines), Lidy (ken-je-die-nog?), Dave (Speijer or be speijerd) and Jaap Willem (ramoned).

Those foreign toxo-enthusiastics I met and/or had valuable contacts with: Dr. Velge-Roussel, Remy Magné (and wife+kids!), Alain Jacquet, Michèle Haumont and Dominique Soldati.

I would also like to express my gratitude to my thesis-advisors Marc de Smet (for enthusiasm) and Paulus de Jong (for structure), to the thesis committee-members, all the volunteers/patients (sometimes: victims) who were willing to donate tears, and, most importantly, coffee + Sly and Larry.

Bob.
Nederlandse samenvatting

Aangeboren en adapterende immuunresponsen bij mensen gericht tegen *Toxoplasma gondii* en Herpes simplex type 1

Hoofdstuk 1: Detectie van anti-toxoplasma IgA antilichamen in trane van mensen.

*Toxoplasma gondii* is een algemeen voorkomende parasiet in Nederland en veel mensen worden vroeg of laat geïnfecteerd. In de meeste gevallen zal dit hooguit gepaard gaan met een griepachtig gevoel, aangezien in gezonde mensen een actieve cel-gemedieerde en antilichaam gemedieerde (humorale) immuunrespons ervoor zorgt dat de snel delende *T. gondii* tachyzoït zich terug trekt in een weefsel cyste. Daarbij ondervindt de parasiet een transformatie van tachyzoït tot een langzaam groeiende bradyzoït. Aangezien de bradyzoït voor een belangrijk deel uit andere eiwitten bestaat en alleen binnen een cyste voorkomt, zal deze buiten bereik van het immuunsysteem blijven. Ook de cyste zelf geeft niet of nauwelijks aanleiding tot een immuunrespons, en het gevolg is dat men voor de rest van zijn leven passief drager zal zijn van de parasiet (‘latent geïnfecteerd’), die echter wel elk moment in staat is tot reactivatie, i.e. transformatie tot tachyzoït. Indien men gezoond is, oftewel ‘immuun-competent’, zal dit uiterst zeldzaam gebeuren, tenzij de parasiet kans ziet het oog binnen te komen, wat resulteert in oculaire toxoplasmose (OT). Aangezien cel-gemedieerde immuunresponsen in het oog onderdrukt zijn, geeft OT vaker aanleiding tot reactivaties dan systemisch dragerschap. Bij elke reactivatie wordt een gedeelte van de retina verwoest. Wanneer daarbij het centrum van de gele vlek, de fovea, betrokken is, dan kan dat leiden tot blindheid. Elke reactivatie resulteert in een scherp begrensd litteken. In vergelijking met het totaal aantal geïnfecteerden, komt OT relatief zelden voor.

Ook onder immuun-gecompromiteerde omstandigheden (AIDS en transplantatie patiënten) is met name de cel-gemediërde immuniteit aangedaan en zal een residente parasiet kunnen reactiveren. Daarnaast kunnen complicaties onstaan als *T. gondii* een aanstaande moeder infecteert, waarbij de kans bestaat dat de parasiet transplacentaal tevens in de foetus komt. Afhankelijk van het stadium van de zwangerschap kan congentiale infectie leiden tot spontane abortus, hydrocephalus en/of OT.

*T. gondii* infecteert de mens via de darmwand en veroorzaakt een mucosale immuunrespons die o.a. leidt tot productie van anti-toxoplasma gesecreteerd IgA (‘secretory’ IgA- slgA). Bij proefdieren is aangetoond dat orale infectie met *T. gondii* resulteert in een sterke activatie van het mucosaal immuun systeem (MIS), waarbij anti-toxoplasma slgA kan worden aangetoond in allerlei mucosale weefsels buiten de darmen. De traanklier vormt ook een onderdeel van het MIS. Omdat *T. gondii* zeer algemeen voorkomt in Nederland, uiteindelijk wordt 70-80% van de populatie geïnfecteerd, komen mensen waarschijnlijk met enige regelmaat in contact met de parasiet, waarbij telkens een mucosale immuunrespons kan ontstaan. Het is dus mogelijk dat anti-toxoplasma slgA aan te tonen is in trane van gezonde mensen. Om dat uit te zoeken, werd van een groot aantal gezonde volwassenen traanvocht en bloed afgenomen. Uitgezocht werd of anti-toxoplasma IgA antilichamen aan te tonen waren met behulp van de immunoblot-methode en een water-oplosbaar extract van *T. gondii* tachyzoïten.

Van de bemonsterde mensen bleek 81% anti-toxoplasma IgA antilichamen in hun tranen te hebben, terwijl op basis van aanwezigheid van anti-toxoplasma IgG in het bloed maar 23% van de mensen latent geïnfecteerd leken te zijn. Er was geen relatie tussen de aanwezigheid van IgA in traanvocht en het latent geïnfecteerd zijn, en opmerkelijk was dat het IgA antilichaam herkennings-patroon op een immunoblot vaak beperkt bleef tot vier antigenen met een molekuul gewicht van 74, 70, 49 en 34 kDa. Herkenning van het 49 kDa antigeen was zeer intens...
en algemeen. Het totale anti-toxoplasma IgA antilichaampatroon in tranenvocht bleef opmerkelijk stabiel gedurende een periode van 1 jaar. Uit ureum-elutie analyse bleek verder dat de aviditeit van de anti-toxoplasma IgA antilichamen gelijkwaardig was aan serum IgG antilichamen. Om te bepalen of de anti-toxoplasma IgA antilichamen gericht zijn tegen oppervlakte antigenen van de parasiet, werd tranenvocht geïncubeerd met intacte, gezuiverde *T. gondii* tachyzoïten. Na de parasieten afgedraaid te hebben, werd het tranenvocht (supernataant) vervolgens getest op immunoblot, en uit dit experiment bleek dat de anti-49 en 74 kDa IgA antilichamen gericht zijn tegen epitopen die zich aan de oppervlakte van de parasiet bevinden. IgA antilichamen gericht tegen *T. gondii* zijn dus zeer algemeen aanwezig in tranen van gezonde mensen. Deze resultaten kunnen echter geen duidelijk antwoord geven op de vraag of deze IgA antilichamen het gevolg zijn van een geïnduceerde mucosale immuunrespons tegen *T. gondii*, of dat ze het aangeboren antilichaam repertoire vertegenwoordigen.

**Hoofdstuk 2:** Identificatie van het 49 kDa eiwit dat frequent herkend wordt door IgA in tranen: protein disulfide isomerase (PDI).

Om een eerste stap te zetten in het beantwoorden van bovengestelde vraag, werd het opmerkelijk vaak en intens herkende 49 kDa antigen gezuiverd met behulp van ammonium-sulfat precipitatie, met trypsin behandeld en geschikt gemaakt voor analyse met massaspectrometrie. Uit peptide-fragment analyse met behulp van MALDI-TOF analyse bleek dat het om een op dat moment nog niet gekarakteriseerd eiwit betrof. Daarom werd een van de peptides vervolgens gefragmenteerd in een Q-TOF, en door de afgeleide aminozuur-volgorde te vergelijken met gegevens in een database voor DNA-fragmenten (ESTs), kon het 49 kDa antigen geïdentificeerd worden als protein disulfide isomerase (PDI). PDI is een eukaryotisch enzym dat voorkomt in het endoplasmatisch reticulum van cellen en hier vooral disulfide bruggen van nieuw gesynthetiseerde eiwitten aanlegt en modificeert. Met behulp van de geïdentificeerde PDI-EST, werd het RNA en DNA coderend voor *T. gondii* PDI volledig gekarakteriseerd, waarna *T. gondii* PDI tot expressie werd gebracht in *E.coli*. Dit recombinante PDI bleek ook herkend te kunnen worden door IgA antilichamen in tranen. PDI is een evolutionair geconserveerde enzym en komt voor bij alle eukaryoten. Deze observatie past binnen het profiel van een aangeboren antilichaam-respons, die vaak een auto-immuun component heeft.

**Hoofdstuk 3: **Geconserveerde gebieden binnen PDI worden herkend door aangeboren IgA antilichamen in mensen.

Naast tranen van volwassenen, bleken anti-PDI antilichamen ook aanwezig te zijn in tranen van babies en in moedermelk. Het resultaat van de preabsorptie experimenten wordt in dit hoofdstuk bevestigd met immunofluorescentie (IF): IgA antilichamen in tranenvocht lieten een opmerkelijk vlekkerig patroon zien op de buitenkant van niet-gefixeerde intacte parasieten. Overigens werd dit alleen waargenomen als tranen gebruikt werden van mensen die een sterk anti-PDI signaal hadden op *T. gondii* immunoblot, dat een veel gevoeliger detectie-methode is dan IF. Doordat recombinant PDI ook herkend werd door IgA, was het mogelijk om een antilichaam-epitop analyse uit te voeren. Hiervoor werd een reeks stapsgewijs ingekorte PDI mutanten tot expressie gebracht in *E.coli*, gezuiverd en geblot. Deze mutanten verschillen 8-10 aminozuren in lengte, wat ongeveer de grootte is van een antilichaam epitop. Tranen van diverse volwassenen, kinderen en babies werden vervolgens geïncubeerd met een serie immunoblot strips die elk een PDI mutant bevatten. Uit analyse bleek dat traan IgA voornamelijk gericht was tegen twee geconserveerde gebieden binnen PDI, waaronder het op thioredoxine-gelijkend domein. Dit domein vertegenwoordigt het enzymatisch gedeelte van PDI, en is zeer geconserveerde binnen PDIs van vermoedelijk alle eukaryoten. Zoals daarom verwacht kon worden, waren traan IgA antilichamen ook in staat om PDIs van de malaria parasiet *Plasmodium falciparum* en mens te herkennen. Dit in tegenstelling tot anti-PDI antilichamen opgewekt in konijn tegen
recombinant *T. gondii* PDI. Deze opgewekte, conventionele antilichamen waren gericht tegen epitopen in de gebieden van PDI die 'soort-specifiek' zijn, en daarmee wordt voorkomen dat deze antilichamen kunnen binden aan 'eigen' PDI. Hoewel de anti-PDI IgA antilichamen ook in staat zullen zijn tot herkenning van 'eigen' PDI, zal dit waarschijnlijk niet tot problemen leiden omdat IgA geproduceerd in de lamina propria van mucosa trans-epitheliaal wordt uitgescheiden in het lumen. Om terug te komen op de vraag of anti-PDI IgA het gevolg is van een opgewekte/conventionele mucosale immuunrespons of is aangeboren, werd vastgesteld dat deze antilichamen aangeboren zijn. Hoewel er voor de epitope-analyse op basis van literatuur-gegevens over aangeboren antilichamen al sterke aanwijzingen waren dat ze aangeboren zijn, immers de anti-PDI IgA titer was constant - al aanwezig in baby-tranen - en PDI is een geconserveerd eiwit, geeft de IgA epitope-analyse het onomstotelijke bewijs. Het geeft ook het bestaan aan van B cellen in mucosa (in dit geval de traanklier) die het aangeboren antilichaam repertoire moeten produceren. Die zijn tot op heden niet gevonden in de onderzochte mucosale weefsen, maar de traanklier is nooit onderzocht.

**Hoofdstuk 4:** Infectie van het humane oog door Herpes simplex virus type 1 induceert een gecompartmentaliseerde virus-specifieke B cel respons.

Intraoculaire infectie door Herpes virus type 1 (HSV-1) kan een zeer ernstige vorm van uveitis veroorzaken, acute retinale necrosis (ARN), en kan leiden leiden tot blindheid van het aangedane oog. Hoewel onder normale omstandigheden het lumen van het oog gevrijwaard is van cellen, hetgeen onderdeel is van de 'immuun-privilege' van het oog, wordt het oog bij ARN geïnfiltreerd door allerlei immuun-competente cellen, zoals macrofagen, B en T cellen. Dit leidt o.a. tot een intraoculaire humorale respons tegen HSV, die wordt gebruikt voor het bepalen van de zogenaamde Goldmann-Wittmer coëfficiënt (GWc). Een GWc waarde >3 wordt beschouwd als een laboratorium-diagnostische bevestiging van ARN. Opmerkelijk genoeg zijn er weinig details bekend over deze intraoculaire humorale immuun-respons. In deze studie is dmv gepaarde serum - intraoculair vloeistof monsters van ARN patiënten met een GWc >3 de anti-HSV humore respons tot in detail gekarakteriseerd. De verschillen in herkennings van antigenen door anti-HSV-1 IgG antilichamen in serum en oogvloeistof waren zeer groot op immunobieks met een HSV-1 partikel extract. Dit geeft aan dat de antilichaam-respons tegen HSV-1 sterk gecompartmentaliseerd is. Uit epitope-analyse met stapsgewijs ingekorte HSV-1 glycoprotein G eiwitten (gG1), bleek dat ook als in beide compartimenten anti-gG1 IgG antilichamen aangetoond kunnen worden, zelfs de epitope verdeling tussen de compartimenten opmerkelijk verschilt. De resultaten van deze studie laten zeer duidelijk zien dat er bij een HSV-1 geïnduceerde uveitis een sterk gecompartmentaliseerde humorale respons op gang komt, gericht tegen het virus.

**Hoofdstuk 5:** De oculaire humorale immuun-response in gezondheid en ziekte.

In dit hoofdstuk wordt een overzicht gegeven van beschikbare immunologische kennis die de resultaten uit de hoofdstukken 1 t/m 4 ondersteunen en in een breder kader kunnen plaatsen. Het immuun-systeem van het oog kan op basis van anatomie onderverdeeld worden in twee verschillende compartimenten: het oogoppervlak en het inwendige van het oog. Terwijl ze beide tot doel hebben pathogenen te bestrijden zonder het oog schade te berokkenen, zijn deze immunologische compartimenten zeer verschillend georganiseerd.

Het oogoppervlak is het domein van het mucosaal immuunssysteem, waarvan bekend is dat het onafhankelijk opereert van het inwendige immuun-systeem. Dit wordt weerspiegeld in de dominantie van slgA klasse antilichamen in mucosale excreta en IgG klasse antilichamen in bloed. Bij de mens is niet veel bekend van de aangeboren component binnen het totaal aan slgA dat geproduceerd en uitgescheiden wordt in mucosale structuren. Door de analyse van de slgA respons tegen PDI, zoals beschreven in **hoofdstuk 3**, zijn er sterke aanwijzingen dat er zich in de traan- en
Nederlandse samenvatting

melkklier van de mens B cellen moeten bevinden die aangeboren antilichamen produceren. Van het inwendige van het oog is bekend dat cel-gemediëerde immuunresponsen actief onderdrukt worden, terwijl humorale responsen tot op zekere hoogte toegestaan worden bij intra-oculaire infecties. Uit onderzoek naar de intraoculaire IgG respons tegen de pathogenen *T.gondii* en Herpes simplex virus type 1 blijkt dat het oculaire milieu het ontstaan van een uniek antilichaam repertoire veroorzaakt. Recent onderzoek naar het ontstaan van lokale humorale responsen in hersenen bij proefdieren, en de verschillende typen en eigenschappen van ‘geheugen’ B cellen bij mensen kunnen een intraoculaire humorale respons ten dele verklaren.

Er worden voorstellen aangedragen voor verder onderzoek naar het ontstaan van locale antilichaam responsen in- en op het oog.

**Hoofdstuk 6:** Ontleding van de IgM antilichaam respons gedurende de acute en latente fase van toxoplasmosis.

Zoals aangegeven in de samenvatting van hoofdstuk 1, is toxoplasmosis een bekende risico-factor voor zwangere vrouwen die nog niet geïnfecteerd zijn geweest door *T.gondii* (seronegatief). Als er een vermoeden bestaat van een infectie gedurende de zwangerschap, dan kan worden onderzocht of er sprake is van een recente seroconversie. Maatgevend voor veel diagnostische laboratoria is naast de anti-toxoplasma IgG titer, de aan- of afwezigheid van anti-toxoplasma IgM antilichamen in het bloed. Normaal gesproken zijn anti-toxoplasma IgM antilichamen tot hooguit een jaar na een infectie detecteerbaar. Een IgM test kan, in combinatie met bepaling van anti-toxoplasma IgG titer, over het algemeen voldoende uitsluitge geven of er sprake is van recente infectie door *T.gondii*. Echter, een algemeen probleem binnen de serologie van toxoplasmosis is het voorkomen van klinisch non-relevante IgM ('clinical non-relevant' - CNR) antilichamen in het bloed van sommige mensen, d.w.z. dat IgM antilichamen nog in het bloed aanwezig zijn terwijl de persoon al jaren geleden geïnfecteerd is. Er kan een serologisch beeld ontstaan van iemand die recent geïnfecteerd is: een positieve IgM titer tegen *T.gondii* dat samengaat met een relatief lage IgG titer. Zodra het vermoeden bestaat dat de seroconversie heeft plaatsgevonden gedurende het eerste of tweede trimester van de zwangerschap, dan luidt in sommige landen het advies om over te gaan tot abortus, gezien de ernstige gevolgen die de parasiet kan hebben op de ongeboren vrucht in deze fases. CNR IgM antilichamen hebben daarom ook geleid tot abortussen op twijfelachtige indicatie. Mede daarom is in deze studie de gevoeligheid onderzocht voor CNR IgM antilichamen van algemeen verkrijgbare, op ELISAs gebaseerde anti-toxoplasma IgM bepalingen, zoals de Toxo ISAGA IgM, Platelia Toxo IgM, en de Vidas Toxo IgM. De sera die gebruikt werden, hadden alleen een CNR IgM titer in de Abbott Imx Toxo IgM test. Door gebruik te maken van de IgM immunoblot methode werd ook de specificiteit onderzocht van de CNR IgM antilichamen, en vergeleken met IgM antilichamen in sera van mensen die recentelijk geïnfecteerd waren (‘acutely infected’ - AI).

Uit het onderzoek naar de commercieel verkrijgbare testen bleek dat van de 19 sera met CNR antilichamen er maar zes ook als positief aangeduid werden in de ISAGA test, vergeleken met 16, respectievelijk 17 in Vidas IgM en Platelia Toxo IgM bepalingen. Opmerkelijk genoeg kon op basis van de intensiteit van het (vermoedelijk) anti-SAG1 IgM signaal op immunoblot met niet-gereducedeerde antigenen een duidelijk onderscheid worden gemaakt tussen CNR IgM en AI IgM.

Uit deze studie blijkt dat iedere commercieel verkrijgbare test gevoelig is voor CNR IgM, en zou bij twijfel uitstekend gebruik gemaakt kunnen worden van de IgM immunoblot methode. Een ander duidelijk resultaat is dat CNR IgM antilichamen specifiek zijn voor dezelfde toxoplasma antigenen als AI IgM antilichamen. Dat houdt in dat het moeilijk zal zijn om op basis van ELISA een anti-toxoplasma IgM test te ontwikkelen die niet gevoelig zal zijn voor CNR IgM antilichamen.
Hoofdstuk 7: Herkenning van recombinante antigenen van *Toxoplasma gondii* door IgM in sera van acuut en latent geïnfecteerde mensen.

Aangezien het met de IgM immunoblot methode mogelijk is onderscheid te maken tussen CNR en AI IgM, wordt met deze studie verder ingegaan op het mogelijk implementeren van de IgM immunoblot als werkbare, reproduceerbare test. Daarvoor zou het toxoplasma lysaat als basis voor de immunoblot vervangen moeten worden door recombinant geproduceerde equivalenten. Uit de vorige studie met niet-gereducierde antigenen op immunoblot is gebleken dat vooral op basis van herkenning van een oppervlakte antigeen op 31 kDa, vermoedelijk SAG1, in combinatie met herkenning van een 55 kDa antigeen een goed onderscheid gemaakt kan worden tussen CNR en AI anti-toxoplasma IgM.

Met natief SAG1, gezuiverd uit een *T.gondii* tachyzoït extract, werd bevestigd dat het signaal op 31 kDa het resultaat was van herkenning van SAG1 door IgM antilichamen. Recombinant Sag1, geproduceerd in de gist *Pichia Pastoris*, werd echter niet herkend, terwijl van deze recombinant bekend is dat hij wel gebonden kan worden door anti-SAG1 IgG antilichamen. Het 55 kDa antigeen van *T.gondii* kon worden geïdentificeerd als Rop1. Dit laatste was opmerkelijk, omdat uit een preabsorptie experiment was gebleken dat zowel de anti-31 kDa antilichamen als de anti-55 kDa antilichamen konden binden aan de buitenkant van de intacte parasiet. Dit bevestigde een algemeenheid van IgM antilichamen; ze zijn vooral gericht tegen oppervlakte antigenen van pathogenen. Echter Rop1 komt normaal gesproken helemaal niet voor op het oppervlakte van de parasiet. Met immuunfluorescentie en monoklonalen specifiek voor Rop1 kon toch het resultaat van het preabsorptie experiment bevestigd worden: in een klein percentage van de intacte parasieten zit Rop1 toch op het oppervlakte van de parasiet. Recombinant Rop1 op immunoblot werd wel herkend door AI IgM antilichamen, maar helaas bleken veel van de CNR IgM sera ook lage titers tegen dit recombinante Rop1 te hebben.

Uit deze studie blijkt eens te meer dat anti-Sag1 IgM antilichamen puur gericht zijn tegen disulfide brug afhankelijke, conformationele epitopen op dit antigeen en dat het moeilijk is de conformatie van natief SAG1 te reproduceren. Recombinant Rop1 wordt wel herkend, maar er zullen extra recombinante antigenen nodig zijn om een IgM immunoblot bepaling te ontwikkelen die onderscheid kan maken tussen CNR and AI anti-*T.gondii* IgM antilichamen.
Chapter 1

IgA antibodies to Toxoplasma gondii in human tears

Published in Investigative Ophthalmic and Visual Sciences, 2000

B. Meek, V.N.A. Klaren, N.J. van Haeringen, A. Kijlstra and R. Peek

Abstract

Purpose: To investigate whether mucosal immune responses directed against the ubiquitous parasite *Toxoplasma gondii* can be detected in tears of healthy humans.

Methods: Non-stimulated tears and blood was obtained from 62 healthy humans (mean age: 35 ± 10 [SD] years). Serum anti-*T. gondii* immunoglobulin titers were determined by Sabin-Feldman (SF) dye test. Western blot analysis was used to compare the anti-*T. gondii* repertoire in tears and serum, and antibody avidity was determined by urea elution. Diluted tear and serum samples were incubated with intact parasites to determine whether the antibodies found in tears and serum are capable of binding to surface exposed antigens of *T. gondii*.

Results: Eighty-one percent of the individuals tested had an anti-*T. gondii* IgA response in their tears, whereas only 23% had evidence of systemic immunity against the parasite. There was no apparent relation between chronic infection and presence of anti-*T. gondii* IgA in tears. Characteristically, the antigens recognized by the IgA antibodies in tears were often limited to at least one of four antigens with a MW of 74, 70, 49, and 34 kDa. The avidity of the anti-*T. gondii* IgA antibodies in tears was similar to the avidity of serum IgG antibodies. IgA antibodies directed against the 49- and 74-kDa antigens recognized epitopes exposed on the surface of the parasite.

Conclusion: A major finding of this study is that tears of many individuals, chronically infected or not, contain IgA antibodies against *T. gondii*. It is not known whether these frequently observed antibody responses are the result of common mucosal immune responses against *T. gondii* or represent the natural antibody repertoire.
Chapter 1

Introduction

Toxoplasma gondii is an obligate, intracellular parasite of vertebrates, including man. Infections by T. gondii are usually asymptomatic in immunocompetent humans, but serious or even lethal complications may occur in neonates or in immunocompromised patients. The gastrointestinal tract is the port of entry of the parasite [1]. The first immunologic barrier encountered by the parasite is the mucosal immune system (MIS). The humoral component of this barrier is represented by secretory IgA (sIgA), which interferes with the ability of pathogenic microorganisms, such as T. gondii, to adhere to the mucosal surface, thereby preventing systemic infection.

Epidemiological studies have shown that the rate of infection by T. gondii in the Dutch population increases gradually with age, resulting in an incidence rate of approximately 76% in people over 75 years of age (L.M. Kortbeek, personal communication) [2]. According to these epidemiological data approximately 1% of the population experiences a seroconversion each year, which presumes a continuous exposure to T. gondii. Every successful systemic infection has probably been preceded by a failing response of the MIS.

Mack and McLeod demonstrated that protective anti-T. gondii sIgA is present in whey of acutely infected and chronically infected women [3], indicating that acute infection indeed coincides with a common mucosal immune (CMIS) response. Animal experiments have also shown that a CMIS response is induced during infection with T. gondii cysts [4].

In general, seroconversion and/or detection of pathogen-specific IgM responses is a good marker for acute infection. However, preimmune sera are often not available and, in the case of IgM serology of toxoplasmosis, misinterpretation is possible because of the existence of natural IgM in sera of otherwise seronegative individuals [5,6] and long-lived IgM antibodies in sera of chronically infected individuals [7]. The simultaneous detection of anti-toxoplasma IgA in mucosal excreta may be helpful in these particular cases. Especially tear fluid is a possible candidate, because the concentration of sIgA in tears is high [8,9], and collection of tears is easy and non-invasive. Oral immunization experiments have demonstrated that antigen-specific sIgA can be detected in tears [10], indicating that B cells primed in the gut also extravasate into the lacrimal gland and that the lacrimal gland is an effector organ of the CMIS [9]. In contrast to the results of Mack and McLeod [3], others have demonstrated that the presence of toxoplasma-specific IgA antibodies in mucosal excreta is not confined to acutely or chronically infected individuals, but can be detected in saliva of seronegative individuals as well [11,12]. This discrepancy could be related to differences in seroprevalence of T. gondii in the geographic areas involved, and also indicates that T. gondii may induce CMIS responses more frequently than expected on the basis of the seroconversion data.

The anti-T. gondii antibody content of tears was determined in order to investigate whether anti-T. gondii immune responses occur in chronically infected or noninfected humans. Because the seroprevalence of toxoplasma in The Netherlands is relatively high, T. gondii specific antibodies should be detectable in tear samples. To distinguish extravasated from locally produced antibodies, the antigen specificity, isotype, and avidity of anti-T. gondii antibodies in tears was compared with that of serum antibodies. We also determined whether the antibodies found in tears are capable of binding to surface antigens exposed on the intact parasite. The results of this study demonstrate that 81% of the volunteers tested had anti-T. gondii IgA directed against a limited number of (surface) antigens, indicating that mucosal immune responses directed against this parasite are present in a high percentage of the normal Dutch population. Many volunteers had IgA antibodies against the parasite in their tears without showing evidence of having experienced a systemic infection.
Material and Methods

Volunteers and sample collection: Sixty-two healthy persons (age 35 ±10 [SD] years, 28 men and 34 females) participated in this study. They were recruited from laboratory personnel and their relatives. From each person, 10 μl tear fluid was collected from each eye by using glass-capillaries (Assistent, Karl Hecht, Sondheim, Germany), with minimal stimulation of the tear flow [8]. A blood sample was collected by venipuncture. Serum and tears were stored at -20 °C, until use. Of 6 volunteers tear fluid was collected more than once within a period of 16 months. Informed consent was obtained from all participants before entry into this study. The study was conducted in accordance with the Declaration of Helsinki.

Serology: Anti- T. gondii antibody titers of sera were determined by the Sabin-Feldman dye test (SF) [13]. This test was performed at the Department of Microbiology of the Academic Medical Centre in Amsterdam, a reference laboratory for the serological diagnosis of toxoplasmosis in The Netherlands.

Antigen preparation: All animal procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All steps were performed at room temperature, except when stated otherwise.

Antigen preparation was performed as described elsewhere [14]. Briefly, T. gondii tachyzoites of the RH strain were injected intraperitoneally into Swiss mice (average weight: 25-30 g, Harlan laboratories, Horst, The Netherlands). The animals were sacrificed 48 hours after injection. Tachyzoites were collected by repeated flushing of the abdominal cavity with phosphate-buffered saline (PBS, pH 7.4). Tachyzoites were pelleted at 800 g and the pellet was resuspended in 1 ml PBS. To remove contaminating mouse macrophages and to free the tachyzoites from the macrophages, the suspension was forced through a 27.5 gauge needle three times. The tachyzoites were separated from cellular debris by centrifugation, the pellet was resuspended in red blood cell lysis buffer (165 mM NH₄Cl, 10 mM KHCO₃, 1 mM EDTA [pH 7.4]), washed once with PBS, and finally resuspended in a small volume of PBS. The number of parasites in the suspension was determined and this suspension was frozen at -20 °C. After 3 freeze-thaw cycles, the tachyzoite suspension was sonicated (eight times for 15 seconds 30 kHz microprobe; Soniprep 150; MSE, Loughborough, GB). The suspension was kept on ice during the procedure. After the sonication, the suspension was centrifuged at 14400g for 30 minutes at 4 °C. The protein concentration of the supernatant (lysate) was measured using the Bradford assay with bovine serum albumin as a standard. The lysate was frozen in small aliquots containing 200 μg protein and kept at -70 °C until use.

SDS-PAGE and western blotting: SDS-PAGE was performed as described previously [15]. Briefly, 200 μg of the lysate (equal to 7.7x10⁶ tachyzoites) was suspended in SDS-PAGE sample buffer (with 5% β-mercaptoethanol) to a final volume of 200 μl, boiled for 2 minutes, and loaded onto 13% SDS-PAGE gels. A broad range marker (Bio-Rad, Hercules, CA) was included. After electrophoresis, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) overnight. Transferred proteins and markers were visualized using the ponceau-red dye staining. Binding capacity of the blots was saturated by incubation with Tris-buffered saline (TBS: 50 mM TrisHCl, 150 mM NaCl, pH 10) containing 0.5% Tween-20 and 2% nonfat powder milk.

Immunostaining of peroxidase-conjugated antibodies: Except when stated otherwise, all samples and conjugates were diluted in TBS containing 0.5% Tween-20 and 0.03% nonfat powder milk (TBS-T). All incubations were performed with a multiscreen apparatus (Mini-Protean II, Bio-Rad). Isotype-specific antibodies conjugated to peroxidase were obtained from Dako (Glostrup, Denmark). The conjugates were incubated with the blots for 90 min. All incubations were performed at room temperature.
When 3,3 diaminobenzidine tetrahydrochloride (DAB) was used as substrate, tear fluid and serum were diluted 1:25 and 1:50, respectively. The dilutions were incubated with the blots overnight. As positive controls, an anti-\textit{T. gondii} IgG-containing serum and a mouse monoclonal antibody against a 30-kDa tachyzoite membrane protein (BioGenex, San Remon, CA) were used. The diluted samples were tested for the presence of anti-\textit{T. gondii} IgG and IgA in serum and tear fluid, and anti-\textit{T. gondii} IgM in serum only. Peroxidase-conjugated isotype specific antibodies (Dako) were diluted 1:1000. DAB substrate was prepared according to the manufacturer’s descriptions (ICN Biomedicals, Zoetermeer, The Netherlands).

When a chemiluminescence substrate was used, tear fluids and sera could generally be tested at higher dilutions (1:200 and 1:600 respectively). The samples and positive controls were incubated with the blots for 90 minutes. The peroxidase-conjugated anti-human IgA and anti-human IgG (Dako) were diluted 1:5000 and 1:10,000, respectively. Chemiluminescence substrate was prepared according to the manufacturers instructions (ECL, Amersham-Phar-macia Biotech, Essex, UK). The membranes were incubated with the chemiluminescence substrate for 1 minute, wrapped in plastic, and exposed to x-ray film.

\textbf{Avidity test:} Tear and serum samples were diluted and incubated with the blots in triplicate. After incubation, the lanes previously incubated with a sample were simultaneously rinsed three times for 5 minutes, either with 5 M Urea in TBS-T, 2.5 M Urea in TBS-T, or TBS-T without Urea. All lanes were subsequently rinsed for 5 minutes with TBS-T. Bound antibodies were stained by chemiluminescence. The avidity of serum antibodies was also determined using a \textit{T.gondii} IgG avidity assay according to the manufacturer’s instructions (Labsystems, Helsinki, Finland).

\textbf{Preincubation with intact parasites:} The parasites were purified according to the protocol described, resuspended in TBS, and counted. Tear and serum samples were diluted in TBS, mixed with 50 μl PBS containing 2 x 10⁷ purified parasites and incubated for 30 minutes. During the incubation, the suspension was shaken carefully. The parasites were pelleted (800g, 5 minutes) after the incubation period, and the supernatants were split into halves. One half was immediately incubated with the blots (fraction 1), the other half was absorbed again with the same amount of fresh parasites for 30 minutes. After centrifugation, this double-absorbed supernatant was incubated with the blots as well (fraction 2). As negative control, samples were treated similarly, but mixed with buffer alone instead of parasites.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Tear antibody staining patterns. (A): Volunteer 10: An example of a typical tear IgA staining pattern showing the 4 \textit{T. gondii} antigens most frequently recognized by tear IgA. Arrows: antigens of the standard staining pattern with molecular weights (MW in kilodaltons) of approximately 74, 70, 49, and 34 kDa. Staining of anti-\textit{T.gondii} IgG was observed in 16/62 samples tested, of which 13 were a weak copy of the IgA staining pattern, as seen in the IgG lane of volunteer 10. (B): Volunteer 50: A relatively intense anti-70-kDa response sometimes obscured the anti-74-kDa signal (indicated by an arrow in the IgA lane).}
\end{figure}
IgA antibodies to *T. gondii* in human tears

**Figure 2:** Summarized results of tear IgA staining. Diluted tear samples were incubated for Western blot analysis, and bound antibodies were visualized by DAB. Molecular weight (MW in kilodaltons) and trace density of each band was calculated. For each antigen of the standard staining pattern, the trace densities of the bands of each volunteer were enumerated and divided by band, providing the total density-to-band ratio. Tears of many volunteers contained IgA immunoglobulins that stained antigens with a different MW than those of the standard staining pattern. These unique bands were assigned to groups according to their MW (for instance, a band with a MW of 90 kDa was assigned to the group of bands ranging from 120 ↔74 kDa). Trace densities of all bands in each group were enumerated as well and divided by total number of volunteers showing bands in that range. Each bar thus represents the ratio of total density of all bands in a particular group to total number of volunteers having bands in that group (density to band ratio). Total number of volunteers having bands in each group is indicated on top of each bar.

of parasites (control). Before the fractions were incubated with the blots, Tween-20 was added to a final concentration of 0.5%. Bound antibodies were stained by chemiluminescence.

The blots and films were scanned and analysed by computer (Imagemaster software; Pharmacia, Upsula, Sweden). Molecular weight and trace density (OD x mm) of each band were calculated, after correction for background staining. DAB-stained bands with an OD of 0.01 or less were excluded, except when stated otherwise.

**Results**

**Anti-*T. gondii* antibodies in tears:** To investigate the MI responses against *T. gondii* in healthy humans, we determined the secretion of specific antibodies at one of the readily accessible effector sites of the CMIS: the lacrimal gland. A sensitive Western blot analysis technique was used to detect and compare parasite-specific antibody repertoires in tears and paired serum samples. Anti-*T. gondii* IgA antibodies were present in most tear samples tested. Fifty-one of 62 tear samples (81%) had anti-*T. gondii* IgA antibodies that stained at least one band on blots containing a protein extract of purified parasites. Most of these IgA antibodies were specific for a limited number of antigens. The four most frequently recognized antigens were designated as antigens of the standard tear fluid staining pattern (Fig. 1). To objectively determine the antibody repertoire of the subjects, the staining patterns on the blots were scanned, and the trace density of each band was calculated (Fig. 2). This standard staining pattern was characterized by bands at 74 kDa (28 samples), at 70 kDa (42 samples), at 49 kDa (47 samples), and at 34 kDa (19 samples). The monoclonal antibody specific for SAG1/P30 stained at the same height as the 34-kDa band of the standard staining pattern, suggesting that IgA antibodies in tears recognize this antigen. It should be noted that in some cases only a single broad band was observed at the 74- and 70-kDa heights, suggesting that a strong signal at 70 kDa may have obscured the anti-74-kDa staining (example Fig. 1, volunteer 50). These results were scored as an anti-70 kDa response.

Anti-*T. gondii* IgG could be detected in tears of 16 volunteers. In most cases (13/16) the antigens that were recognized by tear IgG had the same molecular weights as antigens stained by IgA. These antibodies were always accompanied
by a positive tear IgA response (volunteer 10 in Fig. 1, volunteers 5 and 19 in Fig. 4).

To determine whether the secretion of anti-\textit{T. gondii} IgA antibodies in tears is a continuous or a transient phenomenon, possibly related to the frequency of processing of the parasites by the MI system, several consecutive tear samples were collected from six volunteers over a period of 16 months. After anti-\textit{T. gondii} antibody profiles were analyzed from Western blots, it appeared that the IgA response designated as the standard staining pattern remained clearly detectable in tears throughout this period (Fig. 3). This was also seen for individually unique bands as, for example, a 63-kDa antigen stained by tear IgA of volunteer 8 (arrowheads Fig. 3). Other antigens were stained at only one or two time points by IgA (Fig. 3).

\textbf{Serology:} According to the SF dye test 14 (23\%) of 62 volunteers had circulating anti-\textit{T. gondii} antibodies, indicating a chronic infection. The SF titers were relatively low, ranging from 2 to 32, indicating that the SF-positive volunteers had not been infected recently [16]. To further validate our Western blot analysis method, we compared SF data with the staining of anti-\textit{T. gondii} antibodies in serum (Fig. 4). It was necessary to include bands with an OD of 0.01 to differentiate between SF titer positive and negative sera. With this criterion, anti-\textit{T. gondii} IgG was detectable in 46 of the 62 sera. Eleven of 14 SF titer-positive sera could be differentiated from SF titer-negative sera by having IgG staining patterns on the blots with more than seven bands with an OD of 0.01 or more, including at least one band with an OD higher than 0.01. Three sera with a positive result in the SF dye test did not meet these criteria, whereas one of the sera negative in the SF test conformed to the criteria. The sera that did not meet these criteria had a clear SAG1/P30 band on blots containing non-reduced antigens, in contrast to the SF-negative sera (data not shown).

Staining of IgA (16/62) and IgM (29/62) antibodies was observed, but was not related to the SF dye test results.

\begin{table}[ht]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{SF titer} & \textbf{Anti-\textit{T. gondii} IgA in tears} & & \\
 & \textbf{Present} & \textbf{Not present} & \textbf{Total} \\
\hline
Positive & 12 (85) & 2 (15) & 14 \\
Negative & 39 (81) & 9 (19) & 48 \\
Total & 51 (82) & 11 (18) & 62 \\
\hline
\end{tabular}
\caption{Anti-\textit{T. gondii} IgA antibodies in tears and chronic infection.}
\end{table}

Anti-\textit{T. gondii} IgA in tears was considered present when at least one band with an OD of 0.02 or more was detected. There was no relation between chronic infection (SF-positive or SF-negative individuals) and the presence of anti-\textit{T. gondii} IgA in the tears of the individuals (P=0.52, Fisher’s Exact Test). Data are number (%) of individuals.
Volunteers were divided into groups on the basis of the presence or absence of a tear IgA staining pattern and/or SF titer. A representative example of each group is shown. Left: SF-positive volunteer 19 (SF titer, 1:16) Right SF-negative volunteer 5. Occasionally, the 49-kDa antigen was recognized by antibodies in tears as well as serum (arrowhead). A, G, and M represent IgA, IgG, and IgM antibodies, respectively; MW, molecular weight (in kilodaltons).

Comparison of anti-toxoplasma antibodies in tears and sera: When the antigens of the standard tear fluid staining pattern were compared to the antigens that were recognized by serum IgG of SF-positive sera, the antigen at 49 kDa was only occasionally stained by serum IgG (not shown). No relation could be found between chronic infection and the presence of anti-*T. gondii* IgA in the tears of the individuals (Fisher's exact test, Table 1), which further substantiates the different immunologic compartments from which these immunoglobulins have originated: systemic versus mucosal.

To assess the avidity of the antibodies, serum and tear antibodies bound to lysate antigens on Western blot were exposed to two different concentrations of the chaotropic agent urea [17]. Serum anti-*T. gondii* antibodies of volunteer 23 had an avidity percentage of 76%, and therefore served as positive control, whereas the serum antibodies of volunteer 4 had a borderline, or intermediate, avidity of 28%. This method was very similar to the enzyme-linked immunosorbent assay (ELISA) method used by Jenum *et al.* [16] and, in contrast to ELISA, allowed us to compare the avidity of antibodies at the level of the individual antigens. When compared, the avidity of most of the anti-toxoplasma IgA antibodies in tears for their antigen was similar to the avidity of most of the serum IgG antibodies of volunteer 4 (Fig. 5, *arrowheads*). Antibodies with the highest avidity were found in serum of volunteer 23, and these stained antigens 35, 34, and 28 kDa (Fig. 5, *arrows*). In that respect, the avidity of IgA immunoglobulins in tears for their antigens was found to be intermediate.

In preventing *T. gondii* from binding to the surfaces of (epithelial) cells during the process of infection, mucosal IgA antibodies should be able to bind to exposed parts of surface antigens of the intact parasite. To determine whether IgA from tears has this capability diluted tear and serum-samples of both SF-positive and -negative volunteers were depleted from IgA antibodies specific for surface exposed epitopes by two consecutive incubations with an excess of intact purified parasites. The result of this depletion was monitored by Western blot analysis of depleted tears and sera (Fig. 6). This analysis revealed that IgA antibodies directed against the 49- and 74-kDa antigens were largely removed from the tear IgA staining profile (Fig. 6: Compare the tear IgA lane C with lane 1 of each volunteer, and lane C with lane 1 of volunteer 4 respectively). The level of tear IgA antibodies directed against several other antigens was not affected by the incubation with *T. gondii* tachyzoites. Also, in the diluted sera the intensity of several bands was diminished as a result of the depletion (Fig. 6: Compare the serum IgG lane C with lane 2 of volunteers 4
Chapter 1

Figure 5: Avidity of tear IgA and serum IgG antibodies. The avidity of tear IgA and serum IgG for their antigens was tested by exposing the antibody-antigen complexes formed after incubation of diluted tears and sera with the toxoplasma antigens on the blots, to two dissociating concentrations of urea. Volunteer 4: a tear IgA-positive, SF-negative individual; volunteer 23: a tear IgA-positive, SF-positive individual. Urea concentrations are indicated above each lane (in molarity). Arrows: antibodies with a high avidity for a specific antigen (found only in serum); arrowheads: antibodies with an intermediate avidity. MW, molecular weight (in kilodaltons).

and 9), whereas the staining of other bands was unaffected. No apparent differences between tear IgA staining profiles after the first and the second incubation with parasites were noted, suggesting that all IgA antibodies capable of binding to the surface of the parasite were captured during the first incubation (Fig. 6; Compare the tear IgA lanes 1 and lanes 2.). Remarkably, the level of tear IgA antibodies that stained the 34-kDa antigen at the same height as the major membrane antigen of tachyzoites, P30/SAG1, was not affected by the depletion procedure. In contrast, a clear decrease in signal intensity at the height of 34 kDa was seen using the paired serum sample (Fig. 6: Compare the serum IgG lane C with lane 2 of volunteer 4 and 9). This suggests that if these antibodies are specific for the P30/SAG1 antigen, they are directed against an epitope that is not exposed on the native protein. These data strongly suggest that a substantial part of the anti-T.gondii tear IgA response is directed against surface antigens of the parasite, including the frequently found immunoglobulins specific for the 74 and 49-kDa antigens.

Discussion

The major finding of our study is that a clear anti-T.gondii IgA response could be detected in a high number (51/62) of tear-samples obtained from healthy volunteers. The anti-T.gondii antibodies in the tears of the volunteers recognized only a limited number of antigens on Western blot, resulting in a very similar pattern of antigen recognition between individuals. This typical antigen recognition pattern, referred to as the standard staining pattern, showed a response against 74, 70, 49, and 34-kDa antigens of T.gondii. The detection of these bands was not associated with age, the flow rate during sampling or the IgA concentration of the tear samples (data not shown). Another important result of this study is that there was no relation between the presence of anti-T.gondii IgA in tears and chronic infection. Tears of the volunteers that showed an intense staining of the high-molecular-weight antigens of the standard staining pattern, also had a tear IgG response against the 74 kDa, 70 kDa, and 49 kDa antigens. Mucosal secretions often contain IgG antibodies that are probably the result of MIS responses as well [18].

The avidity of the anti-T.gondii IgA antibodies in tears was intermediate compared with most IgG antibodies specific for T. gondii in sera from chronically infected individuals. This could be expected of antibodies in mucosal excreta that originate either from natural or specific MIS responses [18,19]. Assuming that cross-reactive antibodies have a low avidity for T.gondii antigens, it seems unlikely that the stainings observed are caused by cross-reactive antibodies. By incubating diluted tears with intact parasites to identify those antibodies with an affinity for surface
exposed antigens, we found a marked reduction in signals specific for the 49- and 74-kDa antigens from the tear IgA staining profile. Clearly, these immunoglobulins are capable of binding to antigens on the surface of the parasites.

We have established the infection status of the volunteers with the SF dye test, as this test is known to be both very specific and sensitive [20]. There is a good association between IgG anti-\(T.gondii\) staining profiles on immunoblots and dye test titers [21], which is less, however, at low SF dye test titers. At low titers, sera cannot be diluted extensively, which limits the specificity of the immunoblot as the risk of staining of natural and cross-reactive antibodies is increased. The sensitivity is limited as well because reduction of toxoplasma antigens lowers the immunogenicity of the major membrane antigen of \(T. gondii\), SAG1/P30, and at low concentrations of specific antibodies this antigen is no longer stained. This may explain why three of the SF dye test volunteers with positive results and one of the seronegative volunteers did not fulfill the criteria.

The sIgA found in mucosal secretions is composed of specific (conventional) antibodies and so-called natural polyreactive antibodies [18]. On the basis of our results, it is not possible to discriminate whether these antibodies are part of the natural antibody repertoire of each individual or originate from CMIS responses.

An argument in favor of a natural origin is that others have demonstrated that natural IgG and IgM antibodies specific for \(T. gondii\) generally occur in sera of individuals not previously infected with the parasite [5,6]. Furthermore, the longitudinal analysis of the volunteers showed that there is not much variation with time in \(T. gondii\) antigens recognized by tear IgA.

Tears are known to contain naturally occurring IgA antibodies directed against various common bacterial and viral pathogens, such as \(Staphylococcus epidermidis\), \(Streptococcus mutans\), influenza virus, and rhinovirus [22]. These pathogens are able to chronically infect mucosa, which is essential for their transmission, and probably chronically stimulate the MIS as well. \(T.gondii\), however, encounters the mucosa and its immune system at the initiation of the acute phase of disease and establishes a chronic infection elsewhere. If the antibodies are the result of a specific response, then \(T.gondii\) has to be omnipresent in the environment to trigger the immunological memory of the MIS continuously, similar to the bacterial and viral pathogens mentioned above. However, there are no current data available to support this notion.

In agreement with a specific origin of the anti-\(T.gondii\) IgA observed in tears is the remarkably similar anti-\(T.gondii\) sIgA staining patterns of whey from breast milk of infected mothers [3]. The antibodies present in whey were capable

---

**Figure 6:** Anti-\(T.gondii\) IgA antibodies in tears are directed against surface-exposed antigens. Diluted tear and serum samples were incubated with either PBS or intact \(T.gondii\) parasites. After 30 min the parasites and the immunoglobulins attached to them were removed by centrifugation. Part of the supernatant was used for Western blot analysis (lane I); the remainder was subjected to a second extraction with fresh parasites before Western blot analysis (lane 2, PBS [control] lane C). Arrow: bands in which intensity was affected by the depletion procedure. MW, molecular weight (in kilodaltons).
of preventing tachyzoites from infecting epithelial cells \textit{in vitro} [3], which is in accordance with our results that some of the IgA antibodies in tears were directed against surface exposed antigens of \textit{T. gondii}. In contrast, the seronegative controls participating in the study of Mack and McLeod [3] did not have anti-\textit{T. gondii} slgA in their whey, whereas this study and the results of Hajeer \textit{et al.} [11] clearly established the presence of anti toxoplasma antibodies in tears and saliva in a high percentage of the seronegative individuals tested. The cause of this discrepancy could be differences in prevalence of \textit{T. gondii} in the specific geographic areas involved [23]. Loyola \textit{et al.} [12] detected anti toxoplasma antibodies in only 20\% of the saliva samples tested, although a high percentage of the volunteers had anti toxoplasma IgG antibodies in their serum, indicating a high prevalence of \textit{T. gondii}. Because an ELISA was used for the detection of the anti toxoplasma antibodies, these differences are most likely caused by the use of different methods.

The IgA antibodies are probably specific for tachyzoites, or a stage that is an intermediate between \textit{T. gondii} bradyzoites-sporozoites and tachyzoites. In the study reported by Mack and McLeod [3], every acute infection coincided with IgA antibodies in whey specific for SAG1/P30, the major tachyzoite membrane protein, suggesting that the response was directed against tachyzoites. Also, in experiments in which mice were fed cysts, the CMIS response was dominated by an anti-SAG1 response [4]. In contrast, the slgA found in whey of chronically infected mothers, as well as the IgA response in tears described in this study, predominantly recognized a 49- and 46-kDa antigen, respectively. Hajeer \textit{et al}. did not find this distinction in IgA staining patterns between chronically infected and acutely infected individuals [11]. However, no information was provided regarding the intensities of the bands between the different groups tested, probably because a distinction could already be made between acutely infected individuals and the other groups based on the presence of specific IgG and IgM in saliva.

In the cases of acute infection mentioned earlier [3,4], the tachyzoites are responsible for the vigorous mucosal anti-SAG1 response and the subsequent infection. To prevent systemic infection, the MIS has to intercept the parasite before it develops into tachyzoites. Therefore, the humoral responses detectable in mucosal secretions of seronegative and chronically infected individuals could be directed against the luminal stages, or intermediates between the luminal stages and tachyzoites of \textit{T. gondii} [24]. This may explain the differences observed between these staining patterns. It has been demonstrated that oral vaccination can result in detection of specific IgA in tears, in the absence of a detectable systemic response [10]. Therefore, the detection of only a CMIS response is not a marker for systemic infection, but may indicate recent contact with \textit{T. gondii} antigens. Whether the MIS is capable of preventing viable parasites from systemic infection, remains to be investigated.

In conclusion, anti-\textit{T. gondii} IgA antibodies specific for surface exposed antigens of \textit{T. gondii} were frequently found in tears of healthy volunteers. There was no apparent relation between the presence of anti-\textit{T. gondii} IgA in tears and chronic infection. On the basis of our results it is not possible to determine whether the frequently observed antibodies originate from CMIS responses against \textit{T. gondii} or represent a part of the natural antibody repertoire. However, the seroprevalence of \textit{T. gondii} in The Netherlands, the similarities with slgA staining patterns of whey of recently infected mothers, and the existence of the CMIS, suggest that the antibodies in tears are the result of a CMIS response. Experiments determining the sequences encoding the 49- and 74-kDa surface exposed antigens of tachyzoites, recognized by IgA antibodies in tears, are ongoing.
Chapter 2

Protein disulfide isomerase of *Toxoplasma gondii* is targeted by mucosal IgA antibodies in humans.

Published in FEBS letters, 2002

Bob Meek, Jaap Willem Back, Vincent N. A. Klaren, Dave Speijer and Ron Peek.

Abstract

Mass spectrometric analysis identified a 49 kDa antigen from *T. gondii* as protein disulfide isomerase (PDI). This antigen is generally recognized by IgA in tears of healthy humans. We determined the complete open reading frame and expressed PDI recombinantly. Recombinant PDI was recognized by IgA in human tears known to contain antibodies specific for the 49 kDa antigen.

High expression level and similarity to other protozoan PDIs suggest that *T. gondii* PDI might be a suitable target for recently described anti/protozoan drugs. PDI specific antibodies clearly constitute part of the mucosal antibody repertoire possibly involved in defence against parasites.
Chapter 2

Introduction

Toxoplasma gondii is a protozoan parasite that has the remarkable ability to infect a wide variety of mammalian species and birds [1]. In humans, infection can cause serious complications like systemic, ocular and congenital toxoplasmosis [1,25].

To prevent toxoplasmosis, several immunological mechanisms are available of which the mucosal system forms the first line of defense. In this context, our recent finding that 80% of individuals have anti-\textit{T.gondii} IgA antibodies in their tears is of interest [26]. The presence of these IgAs was independent of systemic immunity against \textit{T.gondii} and anti-49 kDa IgA antibodies in human tears could be pre-absorbed by incubation with intact, live \textit{T.gondii} tachyzoites, indicating that the antigen is present on the exterior of the parasite [26]. Tears are continuously secreted by the lacrimal gland, an effecter organ of the common mucosal immune system (CMIS), and contain high concentrations of IgA antibodies [8]. Besides constituting the continuous immunologic barrier of the ocular surface and conjunctiva against pathogens during the day, tears are continuously drained by the nasolacrimal ducts, implying that tear IgA antibodies protect epithelial layers of these ducts, the upper respiratory tract, throat and oesophagus. These anti-toxoplasma antibodies might be part of the continuous mucosal defense against protozoan parasites, either as a natural adaptation to co-existence with protozoan parasites or due to regular mucosal contact with parasitic antigens. Interestingly, a 49 kDa antigen was prominently recognized by IgA antibodies in tears [26]. Identification of this antigen will give indications whether these antibodies are actively involved in defense against \textit{T.gondii}, and whether targeting of the antigen offers new opportunities in prevention of toxoplasmosis.

Material and Methods

Volunteers and sample collection: Tear fluid was collected from eyes of 4 healthy adults (V1 to V4), using glass-capillaries [8](Assistent, Karl Hecht, Sondheim, Germany). Tears were stored at \(-20^\circ\text{C}\), until use. This study was conducted in accordance with the Declaration of Helsinki.

Parasites: \textit{Toxoplasma gondii} tachyzoites of the RH strain were propagated \textit{in vitro} in RK13 cells in RPMI medium with 3\% fetal calf serum (Invitrogen). Alternatively, RH strain tachyzoites were propagated \textit{in vivo} in Swiss mice. Parasites were filter purified using filters with a pore-size of 3 \(\mu\text{m}\) supplemented with a pre-filter (Millipore). Following two washes with phosphate buffered saline (PBS, pH 7.4), parasites were resuspended in PBS containing protease-inhibitors (Complete EDTA free, Roche) and kept at \(-20^\circ\text{C}\) until use. Following freeze/thawing, parasite suspensions were sonicated on ice (8 x 15 sec, 30 kHz microprobe, Soniprep 150, MSE, Loughborough, UK), and insoluble material was removed by centrifugation (16000g, 20 min, 4\(^\circ\text{C}\)). The water-soluble extract of \textit{T.gondii} parasites (lysate) was frozen in small aliquots.

Ammonium sulfate precipitation: Ammonium sulfate (AS) was added stepwise within 15 minutes to 10 ml of \textit{Toxoplasma} lysate to saturation percentages of 26, 38, 51, 64, 78, and 100\%. At each of the percentages, the suspension was gently stirred for another 20 minutes. Following centrifugation for 20 minutes at 10000xg and \(4^\circ\text{C}\), supernatants were used for the next precipitation step, while the pellet was dissolved in TE buffer (10 mM Tris [pH 8], 1 mM EDTA) containing protease inhibitors. Ten \(\mu\text{l}\) of each fraction was loaded on a gel, size-fractionated, and subsequently blotted.

SDS-PAGE, Western blotting and detection of peroxidase (PO) conjugated antibodies: Procedures were performed as described earlier [15,26,26]. \(\beta\)-Mercaptoethanol (\(\beta\)-ME) was added to a final concentration of 5\% prior to electrophoresis. Following electrophoresis, protein preparations were transferred to polyvinylidifluoride (PVDF) membranes (Millipore). Bound antibodies were visualized by chemiluminescence and
exposed to X-ray film.

**Mass spectrometry:** The 49 kDa antigen, one of the few antigens in the pellet of the 100% AS fraction, was cut from the gel after staining and destaining with bio-safe Coomassie stain (Bio Rad) according to the manufacturer’s instructions. For MALDI analysis, protein-containing gel slices were S-alkylated with iodoacetamide, digested with trypsin (Roche Molecular Biochemicals, sequencing grade), and extracted according to Shevchenko et al. [27]. Only peptides eluted with 20 mM NH$_4$HCO$_3$ were used in the analysis. After drying in a vacuum centrifuge, peptides were dissolved in 10 µl of a solution containing 1% formic acid and 60% acetonitrile. Eluted peptides were mixed 1:1 (v/v) with a solution containing 52 mM α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich Chemie BV) in 50% ethanol/50% acetonitrile. Prior to dissolving, the α-cyano-4-hydroxycinnamic acid was washed briefly with acetone. The mixture was spotted on target and allowed to dry at room temperature. Reflectron MALDI-TOF spectra were acquired on a Micromass Tof Spec HT (Wythenshawe, UK). The resulting peptide spectra were used to search ABC Non-Redundant Protein Database release 20000301 (Advanced Biomedical Computing Center, Frederick, MD) (http://www-fbsc.ncifcrf.gov/). For further MSMS analysis, a few µl of several peptide solutions was concentrated on a C18 ZipTip (Millipore), eluted in 5 µl 60% acetonitrile/1% HCOOH and analysed on a Micromass Q-TOF mass spectrometer. Low-energy collision-induced dissociation (CID) experiments were performed using argon as a collision gas. Homology searches with fragmented peptides were performed using the BLASTx program with default settings and dbEST database (at http://www.ncbi.nlm.nih.gov:80/blast/Blast.cgi and http://www.ncbi.nlm.nih.gov/dbEST/).

**Primers (s: sense, as: anti-sense):** The following primers were based on *T. gondii* EST dBEST ID# 1374495: PDI 1, 5’-GGGTCCGTTGCTTCTTCTTC-3’ (as); PDI 3, 5’-AACGTGCAAAGAAACTCCG-3’ (s); PDI 5, 5’-GGGACAGGCTCAGACTTC-3’ (as); PDI 6, 5’TACGCAGGTCCTCCAGGA-3’ (as). Primers used to amplify full-length cDNA and genomic sequence: PDI 23, 5’-GAGACATATGGAGGAGTAAGGATTCTGACT-3’ (s); PDI 24 5’-AGACTCGAGTTACAGTTCTTCACCCTT-3’ (as). Other primers: PDI 7, 5’-GAGACCCGTTTTTTTTTTTTTTTV-3’ (with V = A, C or G); PDI 11, 5’-TAAGGTAGATGGCAACCGAG-3’ (s); PDI 22, 5’-GAGACCATGGAAGAGTGCCTGCAAAGGAAACCCGACT-3’ (s).

**Cloning of PCR products, RT, and RACE:** PCR products were either ligated into the pGEM-T Easy cloning vector as described by the manufacturer (Promega), or digested and ligated into expression vector pRP261, a derivative of vector pGEX-3X (Amersham Biosciences).

Rapid amplification of unknown cDNA ends (RACE) was performed as described [28], with minor modifications. RNA was extracted from *T. gondii* tachyzoites using RNAzol reagent (Campro Scientific, Hilversum, The Netherlands). cDNA was synthesized using 1 µg RNA and Superscript II reverse transcriptase (RT)(Invitrogen). RNA was subsequently removed by RNase H treatment (Promega, Leiden, The Netherlands). For 5’ RACE, primer PDI 1 was used for first strand synthesis, followed by dA tailing by TdT (Invitrogen). Second strand cDNA was synthesized using primer PDI 7 (s). Semi-nested PCR reactions were carried out with primers PDI 7 (s) and PDI 6. Primers PDI 3 and PDI 7 (as) were used for 3’ RACE. Four independent clones from both the 5’ and 3’ RACE procedure were sequenced, using pGEM-T Easy vector specific primers (SP6 and -40) and PDI 7, PDI 11, PDI 1 and PDI 6.

**Sequencing and analysis of nucleotide and protein sequences:** All sequencing was performed on an Applied Biosystems Prism 310 dye terminator fluorescent-based genetic analyser (PE Applied Biosystems, Warrington, UK). Homology searches were done using the BLASTx program with default settings. Multiple sequence alignments were performed using the CLUSTAL W program with default settings (at http://www.ebi.ac.uk/clustalw/).
Splice sites in genomic sequences were predicted using the Fgenesm program (version FGENES-M 1.5.0, http://genomic.sanger.ac.uk/gf/gf.shtml). Signal P program was used for analysis of potential signal peptide cleavage sites (version 1.1, at http://www.cbs.dtu.dk/services/SignalP/).

**Northern blotting, Southern blotting and autoradiography:** These were performed as described earlier [29], with minor modifications.

Northern blots containing 2 and 10 μg size-separated *T. gondii* RNA were incubated with [α-32P]dCTP labeled probes specific for SAG1 (~1 kb fragment) and the PDI-EST (~300 bp fragment).

**Construction of the PDI expression vectors:** To release the fragment encoding the 5’ part of PDI, the 5’RACE-pGEM-T Easy construct was digested with *Bst*XI (Invitrogen), followed by deletion of the 3’ overhang using Klenow enzyme (Roche) and digestion with *Bsu*36 (New England Biolabs). The 3’RACE-pGEM-T Easy construct was digested with *NcoI*, its 5’ overhang filled by Klenow, followed by digestion with *Bsu*36. The fragment encoding 5’ PDI was ligated into this digested vector, resulting in a full-length PDI-pGEM-T Easy construct. Upon digestion of this latter construct with *NcoI* and *SpeI*, the resulting fragment with the PDI open reading frame was transferred to pRP261. The 3’ RACE-pGEM-T Easy construct was digested with *Mae*II (New England Biolabs) and Klenow (Roche) filled. Following purification, the 3’ RACE product was released by digestion with *SpeI*. This fragment was ligated into pRP261, previously digested with *SmaI* and *SpeI*, and will be referred to as 3’ rPDI.

**Expression, purification, and detection of recombinant proteins coupled to GST:** All proteins were expressed in *E. coli* strain BL21 according to standard procedures [29]. Expression was induced by adding isopropyl-β-D-thiogalactopyranoside (1 mM) at 30°C. Glutathione S Transferase (GST), with or without fusion protein, was purified using Glutathione Sepharose 4B beads according to the manufacturer’s instructions (Amersham Biosciences). For protein gels, beads were directly solubilized in sample buffer. The amount of each recombinant protein used for immunoblots was normalized based on intensity of Coomassie staining on gel and staining with anti-GST antibody on immunoblot. For the detection of GST and GST coupled proteins, antibodies were removed by incubation of blots in
Figure 1: Identification and characterization of the 49 kDa antigen of *T. gondii*.

(A): Ammonium sulfate was added to saturation percentages indicated above the lanes and precipitated proteins were analyzed. Ponceau red staining patterns on blots (PR) are shown, as well as IgA staining patterns of strips incubated with tear sample V1 (IB). Tear samples of volunteer V1 were used for this experiment, because they predominantly contained IgA antibodies for the 49 kDa antigen. (B): A triply protonated tryptic peptide of m/z 580.6 was fragmented from which a deconvoluted MSMS spectrum with a partial sequence tag, ISQFFDDVEA (569.3), was constructed. This tag was used to screen databases and matched with dBEST entry ID# 1374495, an EST of 300 bp identified as part of the message encoding *T. gondii* protein disulfide isomerase (PDI). X-axis: mass to charge ratio, Y axis: relative intensity. (C): Northern blots containing size-separated *T. gondii* RNA were incubated with labelled probes specific for SAG1 and the PDI-EST. The ribosomal RNA bands of *T. gondii* run at ~3400 (28S) and ~1850 (18S) nt, while SAG1 mRNA is about 1500 nt [39]. Shown are the lanes representing 10 µg (SAG1) and 2 µg RNA (PDI). Blots were exposed to film for 5 hours. PDI mRNA is estimated to be about 2000 nt in length. (D): *T. gondii* DNA was digested with the restriction enzymes indicated above each lane. Southern blots were probed with a labelled PDI cDNA fragment of 1350 bp. EcoRI digestion resulted in 3 fragments. Since PDI cDNA has two EcoRI sites, this pattern indicates a single copy gene for PDI. (E): Positions of introns in genes encoding PDI of *P. falciparum* and *T. gondii* are indicated by arrowheads. The position in the Trx domain of the first intron in the coding sequence is identical to the single intron of *P. falciparum*. *T. gondii* is unique in having a second intron at nt position 815. Light-gray boxes represent Trx-like domains with CGHC motif, while a Trx-like domain with CGYC motif at *T. gondii* aa positions 380-383 is indicated by a dark-gray box. Recombinant proteins were expressed as glutathione-S-transferase (GST) fusion proteins. The position of the peptide identified by mass-spectrometry is given.

erase buffer (62.5 mM Tris [pH 6.8], 100 mM β-ME, 2% (w/v) SDS) for 1 hour at 70°C. Subsequently, blots were blocked for 1 hour and incubated with anti-GST-PO (diluted 10,000x, Sigma).

**PDI specific antibodies**: A rabbit previously selected for being non-responsive on a *T. gondii* lysate immunoblot was immunized once s.c. and i.m. with 500 µg of the purified PDI-GST fusion-protein, using Specol (ID-DLO, Lelystad, The Netherlands) as adjuvant. Plasma was stored at −20°C until use.

**Results and Discussion**

**Fractional precipitation and mass spectrometry**: The 49 kDa antigen was partially purified from an extract of *T. gondii* by ammonium sulfate (AS) precipitation. Western blot analysis of the AS fractions showed that the 49 kDa antigen precipitated most efficiently at 100% saturation with only few other *T. gondii* proteins, as these had been precipitated at 78% saturation (see figure 1A). To exclude the possibility that the major band at 49 kDa in this fraction consisted of multiple proteins, a gel fragment containing this band was loaded onto a protein gel with a different acrylamide/bisacrylamide ratio. Coomassie staining revealed a single band that was sliced out and used for mass spectrometric analysis. Upon digestion with trypsin, resulting peptides were analysed by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF). Because the peptide mass fingerprint did not identify the 49 kDa protein, selected peptides were analysed by collision induced dissociation on an ESI-Q-TOF. Some of the peptides matched with dBEST entry ID# 1374495 (figure 1B and 1E). This EST of 300 bp encodes part of protein disulfide isomerase (PDI) of *T. gondii*, a protein not yet described for this parasite.
Chapter 2

Protein disulfide isomerases (PDIs) belong to the superfamily of thioredoxins (Trx's), a group of oxidoreductases characterized by one or more thioredoxin-like boxes with a CXXC motif [30]. One of the main functions of oxidoreductase is facilitating de novo formation of disulfide bridges between cysteines (oxidation), and/or rearranging existing disulfide bridges (isomerization). Besides acting as oxidoreductase, PDI also assists in protein folding, functions as a chaperone, and forms a subunit of prolyl-4-hydroxylase and triacylglycerol transfer proteins [30]. All major surface antigens of *Toxoplasma gondii* (SAG1) and related proteins, have high numbers of intramolecular disulfide bonds [31], which might explain the high expression level of PDI observed in *T. gondii* (see below).

**Characterization of the cDNA encoding *T. gondii* PDI:** The full length cDNA sequence of PDI was obtained by 5' and 3' rapid amplification of cDNA ends (RACE) using primers based on dBEST entry 1D #1374495. This resulted in cDNA products of approximately 1100 bp (5' RACE) and 600 bp (3' RACE). Northern blot analysis of total *T. gondii* RNA with a PDI cDNA fragment showed a single transcript of about 2 kb (figure 1C), abundantly present (compare with signal of the SAG1 message) and roughly matching the combined length of the 5' and 3' RACE products.

Sequence analysis showed that *Toxoplasma* PDI contained the two Trx-like domains with active site sequence motif CXXC characteristic for PDIs (indicated in figure 1E; accession number AJ306291). 28 tryptic peptide peaks present in the MALDI spectrum, including all predominantly peaks, matched to the sequence obtained, yielding a sequence coverage of 60%, confirming the 49 kDa protein as PDI.

*T. gondii* PDI differs from all other known eukaryotic PDI sequences in an amino acid substitution of the consensus histidine (H) by a tyrosine (Y) in the C-terminal Trx-like domain. This H→Y substitution was confirmed by sequencing DNA from two different sources of *T. gondii* RH strains (data not shown). In addition to Trx-like domains other functional elements were predicted (see figure 1E), including a hydrophobic N-terminal signal sequence of 25 amino acids and a C-terminal ER-retention signal (GEEL).

The replacement of H by Y in one of the *Toxoplasma* CXXC boxes is unique for eukaryotic PDIs described so far. It is not likely to affect the oxidoreductase function of PDI. Besides being a neutral substitution, it is not unique to Trx-like proteins in general, as one of the oxidoreductases of *E.coli*, DsbC, also has Y in its CXXC box. However, compared with DsbA, an oxidoreductase containing a 'classic' box, the function of DsbC is shifted towards isomerization [32], so this might hold true for PDI of *Toxoplasma*.

Based on amino-acid similarities, PDI of *Toxoplasma* most closely resembles PDI of other members of the family of protozoan parasites like *Plasmodium falciparum* [33]and, somewhat less, *Cryptosporidium parvum* [34]. For alignments of *T. gondii* PDI with closely related PDIs and associated features, see Chapter 3 figure 4.

**Characterization of the genomic sequence encoding *T. gondii* PDI:** Analysis of a genomic fragment containing the complete coding sequence of *T. gondii* PDI (accession number AJ312317) showed the presence of two introns at bp positions 303 and 815 (figure 1E). The position in the Trx domain of the first intron in the coding sequence is identical to the single intron of *P. falciparum* [33], the second intron is unique for *T. gondii*. The genomic sequence encoding PDI of *C. parvum* does not contain introns (see accession number U48261). Southern blotting demonstrated a major fragment of approximately 3 kb with EcoRI digested chromosomal *T. gondii* DNA (figure 1D) indicating a single locus for PDI in the *T. gondii* genome.

**Recognition of recombinant PDI by IgA in human tears:** Full length *T. gondii* PDI and its C-terminal part were expressed as GST fusion protein (figure 1E, rPDI and 3'rPDI, resp.) in *E.coli*, purified and blotted. Both recombinant PDI proteins were recognized by IgA antibodies in tears of individuals known to have anti-49 kDa antibodies (figure 2A). This recognition was not due to the GST part of the recombinant protein as none of the
T. gondii PDI is recognized by IgA

volunteers had IgA antibodies recognizing GST protein alone (see second panel figure 2A). Furthermore, rabbits challenged with recombinant PDI generate IgG antibodies reacting with the 49 kDa antigen, now identified as PDI, on a T. gondii lysate blot (figure 2B).

As mentioned, PDI acts in the endoplasmic reticulum (ER). However, the binding of PDI specific IgA antibodies to the exterior of parasites clearly demonstrated the presence of PDI on the surface [26]. There are examples of an extracellular location of PDI, despite the presence of a carboxyterminal ER retention signal: e.g. on the surface of lymphocytes, involved in adhesion of Human Immunodeficiency Virus [35], and on the surface of platelets, mediating adhesion to integrin [36]. It is possible that T. gondii PDI exits the ER bound to its targets (e.g. SAG1 and related proteins, TgAma-1 [37,38]) functioning as chaperone for these membrane proteins. Together with proteins known to be involved in host cell adhesion like SAG3 and TgAma-1 [37,38], PDI may also participate in adhesion to host cells during the initial phase of infection. Although PDIs of protozoan parasites resemble their human equivalents, compounds inhibiting growth of Plasmodium selectively target PDI of P. falciparum [33]. These compounds are screened for anti-malarial capacity, and may also be of use in treatment of Toxoplasmosis, especially in the light of a possible adhesion function for PDI.

With respect to its function, PDI can be regarded as a conserved protein. This suggests that these anti-

T. gondii PDI antibodies constitute part of the natural antibody memory repertoire of humans, which might have evolved to cope with co-existing protozoan parasites. To confirm that these anti-PDI antibodies are natural antibodies, experiments are performed to characterize the regions on PDI they recognize. Other mucosal sites will be analysed to determine whether these antibodies are unique for the lacrimal gland or are a general characteristic of the CMIS.

Figure 2: Recognition of recombinant and native PDI by IgA in tears and immune serum.

(A): IgA antibodies in diluted tear samples of volunteers were incubated with blot strips containing either soluble T. gondii lysate proteins (Lysate), purified GST (GST, ~28 kDa) protein, GST coupled to 3' part of PDI (3' rPDI, ~45 kDa) and full-length PDI (rPDI, ~77 kDa). Positions of native PDI (~49 kDa) on the Lysate strips, and recombinant proteins are marked (>). IgA in tears recognized rPDI and 3' rPDI. Two exposures are shown (10 and 30 min). The anti-GST staining patterns were obtained after GST and 3' rPDI containing immunoblot strips were ‘erased’. Vol. No. indicates volunteer number 1 to 4. Tear samples were diluted 200x.

(B): The antibody specifically generated against recombinant PDI stained a 49 kDa antigen on lysate blot. [+ ] indicates post-immune serum following immunization with PDI and [- ] pre-immune serum. Rabbit serum was diluted 20,000x.
Chapter 3

Conserved regions of protein disulfide isomerase are targeted by natural IgA antibodies in humans.

To be published in International Immunology

Bob Meek, Jaap Willem Back, Vincent N. A. Klaren, Dave Speijer and Ron Peek.

Summary
Secretory IgA (sIgA) antibodies in human tears and milk were found to recognize protein disulfide isomerase (PDI) on a Toxoplasma gondii (Tg) lysate immunoblot (IB). These antibodies were already detectable in tears of infants. To determine the epitope containing regions on PDI, we generated truncated versions of recombinant PDI that differ 8-10 amino acids in length. By IB, it was found that the sIgA epitopes were confined to conserved regions of PDIs, including the functionally essential thioredoxin like domain. This suggested the capacity of sIgA to react with PDI of other species, which was confirmed by recognition of human PDI by IgA in tears. In contrast, anti-Tg PDI antibodies generated by immunization were not able to cross-react. Binding to thioredoxin like domain on IB could be gradually abrogated by incubation with peptide constituting the same domain. By consecutive investigation of function of the protein targeted by sIgA, presence of antibody in relation with age and analysis the epitope constituting regions on PDI we demonstrate that sIgA directed against PDI are self-reactive natural antibodies. Furthermore, analysis of antibody epitopes on an antigen is a useful method to distinguish conventional, affinity maturated antibodies from natural antibodies. The presence at early age and continuity of anti-PDI sIgA in relation with age suggests the existence of B cells secreting germ-line encoded antibodies in human mucosa outside of the gut. Overall, the PDI specific antibodies clearly are part of the natural antibody repertoire suggesting an active role for these antibodies in the innate defence against pathogens.
Chapter 3

Introduction
The humoral component of the mucosal immune system (MIS) mainly consists of secretory IgA and IgM antibodies. The most important functions of these antibodies are 'immune exclusion' and 'immune elimination', achieved by binding to surface antigens on potential pathogens. 'Immune exclusion' refers to prevention of adherence to epithelial cells and subsequent infection, while 'immune elimination' involves pathogen clearance in the lamina propria, and neutralization of intracellular viruses [18,40,41]. Both conventional (monospecific) and natural (polyspecific) antibodies are important for optimal protection against pathogens [18,42].

Conventional antibodies are produced by B2 cells and are affinity matured upon exposure to specific antigens at inductive sites of the MIS, such as the Peyers Patches (PP) in the ileum, the mesenteric lymph nodes (MLNs) and the tonsils. Once induced, mature B cells migrate to effector sites that can be located distant from the inductive site [43-45]. Whether specific IgA can be detected at mucosal surfaces apart from those close to an inductive site depends on immunogenicity and quantity of the antigen(s) presented at these site(s) [43,45]. Natural antibodies can be found in any mucosal secretion in man and mice. Characteristically, natural antibodies target phylogenetically conserved structures of commensals, pathogens and auto-antigens [19,46,47]. Most murine B cells secreting natural antibodies at mucosal sites express their antibodies in or near germline configuration [48]. In humans, there does not seem to be a well defined population of B cells secreting IgA antibodies in germline configuration [49-52], although not all mucosal sites have been studied. Nevertheless, natural polyreactive IgA have been demonstrated in human mucosal secretions [19,53]. General features of both natural and conventional antibodies recognizing proteins are summarized in table 1.

In a recent study we found that IgAs produced by the human lacrimal gland predominantly recognize 49, 70 and 74 kDa antigens of Toxoplasma gondii [26]. The 49 kDa antigen was identified as protein disulfide isomerase (PDI) [54], an essential enzyme that functions in the endoplasmic reticulum of eukaryotic cells [30]. T.gondii is prevalent in North-Western Europe [23], where the previous studies were conducted, resulting in a gradual increase in prevalence of anti-toxoplasma serum IgG with age [2,23]. Toxoplasmosis is usually self-limiting and benign in immunocompetent individuals, but may cause serious complications in immunocompromised patients and neonates [23]. Penetration of the gut epithelial barrier by T.gondii is required to initiate systemic infection, and is usually accompanied by a vigorous common mucosal secretory IgA response in humans and mice [3,4]. This suggests that the anti-toxoplasma IgA in tears are the result of occasional encounters with T.gondii, and implicates that they are conventional antibodies. On the other hand, the anti-toxoplasma IgA staining pattern on immunoblot remained remarkably stable in time [26] and PDI is a highly conserved protein [30], implying a natural origin of these antibodies. Since PDI has both species specific as well as highly conserved regions, we hypothesize that conventional antibodies are preferentially directed against T.gondii-specific, non-‘self’ regions, while natural antibodies will primarily recognize conserved regions of T.gondii PDI, presumably being moulded by ‘self’ antigens.

The relation between age and presence of these antibodies in tears was determined to see whether they confirmed to the characteristics of conventional or natural antibodies as summarized in table 1. Next, we tested our hypothesis by identifying the protein regions involved in antibody recognition and determined whether there was cross-reactivity

<table>
<thead>
<tr>
<th>Protein Function</th>
<th>Species specific function</th>
<th>conventional Ab</th>
<th>natural Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>[19,53,56,68-70]</td>
<td>Conserved function</td>
<td>conventional Ab</td>
<td>natural Ab</td>
</tr>
<tr>
<td>Age2</td>
<td>Relation with age</td>
<td>conventional Ab</td>
<td>natural Ab</td>
</tr>
<tr>
<td>[23,56,57,71]</td>
<td>No relation with age</td>
<td>conventional Ab</td>
<td>natural Ab</td>
</tr>
</tbody>
</table>

Table 1.
*Based on dynamics of natural IgM and conventional IgM titers.

30
Conserved regions of PDI are targeted by IgA

with PDI from other species. Our findings strongly support an innate origin for these antibodies. To our knowledge, this is the first report of a detailed analysis of a natural antibody response in man.

Methods

Volunteers and sample collection

Tear fluid was collected from eyes of healthy volunteers with glass-capillaries (Assistent, Karl Hecht, Sondheim, Germany)[8]. Spontaneously produced tears were collected from infants by their parents. Tears were stored at -20°C, until use. Paired tear fluid and milk samples were obtained from 3 women at different stages of lactation. Tears of adults and infants were diluted 200x and 150x, respectively, except when stated otherwise. Human milk was diluted 25x. This study was conducted in accordance with the Declaration of Helsinki.

Parasites

Toxoplasma gondii tachyzoites of the RH strain were propagated in vitro under normal cell culture conditions in RK13 cells in RPMI medium supplemented with 3% fetal calf serum. Plasmodium falciparum schizonts and gametocytes were provided by H.Eling (Academic Hospital Nijmegen, Nijmegen, The Netherlands).

Immunofluorescence (IF)

IF was performed as described earlier [31], except that parasites were not fixated. Briefly, 10^5 filter-purified parasites were double-labelled by incubation with tears and a mouse monoclonal antibody against SAG1 (HyTest, Turku, Finland), diluted 500x, in PBS containing 2% bovine serum albumine, at 4°C for 30 min. Following washes, parasites were incubated with anti-human IgA conjugated to fluorescein-isothiocyanate (FITC), diluted 50x, and anti-mouse Fab conjugated to indocarbocyanine (Cy3), diluted 750x, for 30 min. Following washing and drying, labelled parasites were embedded in Vectashield (Vector Laboratories, Burlingame, CA) and visualized using a laser scanning microscope (Zeiss LSM 410, Carl Zeiss Microscopie, Göttingen, Germany).

Preparation of antigens

T. gondii tachyzoites were filter purified using filters with a pore-size of 3 µm supplemented with a pre-filter. P. falciparum parasites were provided as pellets, and resuspended in PBS with protease-inhibitors before use. Human embryonic kidney cells extract derived from cell line 293T was provided by C. Neefjes (NORI, Amsterdam, The Netherlands). Parasites were processed as described [26]. The extracts of P. falciparum and the human cell line were concentrated 8-10 times to allow detection of PDI.

SDS-PAGE, Western blotting and detection of peroxidase (PO) conjugated antibodies

Procedures were done as described earlier [15,26]. β-mercaptoethanol was added to a concentration of 5% prior to electrophoresis. Following this, proteins were transferred to polyvinylidifluoride (PVDF) membranes. Membranes were blocked with Tris-buffered saline (TBS: 50 mM Tris [pH 10], 150 mM NaCl), containing 0.5% Tween 20 and 2% non-fat milk powder.

All samples and conjugates were diluted in TBS containing 0.5% Tween 20 and 0.03% non-fat milk powder (TBS-T). A PO conjugated polyclonal antibody preparation specific for human secretory component was diluted 4,000x. Bound antibodies were visualized by chemiluminescence, according to the manufacturers’ descriptions (ECL, Amersham Biosciences, Essex, UK) and exposed to X-ray film.

Primers and peptides

For primers, see Table 2. Synthetic peptide FYAPWCYGHCK-COOH was manufactured commercially (Pepsca Systems, Lelystad, The Netherlands).

PCR products were ligated into the pGEM-T Easy cloning vector as described by the manufacturer (Promega,
Madison, WI), or digested and ligated into expression vector pRP261, a derivative of vector pGEX-3X (Amersham Biosciences).

**Construction of the PDI expression vectors (figure 2)**

Construction of vectors expressing full length and PDI Δ2 has been described previously [54]. The PDI Δ1 mutant was obtained after EcoRI digestion of full-length PDI in pRP261, and subsequent ligation (figure 2). To determine the location of the antibody-binding regions in PDI Δ2, the fragment encoding PDI Δ2 was split in two parts, named PDI Δ3 and PDI Δ4. PDI Δ3 was generated by digestion of pRP261-Δ2 with Sall and NcoI, followed by treatment with 1 U S1 nuclease in 100 mM NaCl for 30 min at 30°C, and subsequent ligation. The vector expressing PDI Δ4 was generated by digestion with Sall, Klenow filling, and further digestion with Smal.

For finemapping of the Δ4 region, truncated versions of PDI Δ4 were constructed by cloning NcoI and EcoRI digested PCR products, obtained with PDI 12 as forward primer and PDI 13 to 21 as reverse primers into pRP261 (see above).

**Expression, purification, and detection of recombinant proteins coupled to GST**

All proteins were expressed in *E.coli* strain BL21 according to standard procedures [29]. Expression was induced by adding isopropyl-β-D-thiogalactopyranoside (1 mM) at 30°C. GST fusion proteins were purified using Glutathione Sepharose 4B beads according to the manufacturer’s instructions (Amersham Biosciences). For protein gels, beads were directly solubilized in sample buffer [15]. The amount of recombinant protein used for immunoblots was normalized based on intensity of Coomassie gelstaining and staining with anti-GST antibody on blot. For the detection of GST fusion proteins, antibodies were removed using erase buffer (62.5 mM Tris [pH 6.8], 100 mM β-mercaptoethanol, 2% (w/v) SDS) for 1 hour at 70°C. Subsequently, blots were blocked for 1 hour and incubated with anti-GST-PO (diluted 10,000x). Visualization by chemiluminescence was done as described above.

**Results**

**Anti-*T.gondii* IgA in tears and milk samples**

Different mucosal samples were analyzed to determine whether the secretion of PDI specific IgA antibodies was a phenomenon restricted to the lacrimal gland or a more general feature of the CMIS. The latter seems to be the case as PDI specific IgA was detectable in both milk and tear samples of lactating women (figure 1, M2 and M3), although human milk had to be used at low dilutions (25x) compared with tears (200x).
Conserved regions of PDI are targeted by IgA

Figure 1: Tear and milk antibody staining patterns on T.gondii lysate blots.
To determine whether secretion of anti-PDI antibodies was correlated with age or mucosal site, tear samples of siblings D1-3 (aged 6, 9, 11 years), infants B1-2 (aged 3, 5 months), mothers M 1-3, father F and volunteers V1-7, human milk samples (Milk) and serum sample (Ser) were incubated on T.gondii lysate blots. The serum sample used was a pool of 4 sera obtained from patients recently infected by T.gondii. All samples were stained for anti-toxoplasma IgA, except for the ‘Ser’ and ‘SC’ lanes where samples were stained for anti-toxoplasma IgG and anti-secretory (SC) IgA antibodies, respectively. The antibody specifically generated against recombinant PDI stained a 49 kDa antigen, as expected (α-PDI). Sample M1 and V7 were diluted 500x, Ser: 1000x.

In an earlier analysis of tears, we only included adults. In order to shed light on the natural or conventional origin of anti-T.gondii IgA’s, we analyzed tears of children for whom it was highly unlikely that they already were exposed to T.gondii (prevalence of anti-toxoplasma IgG is very low in these age-groups [2]). Anti-T.gondii IgA staining patterns from tears obtained from children and infants were compared with those of adults (figure 1). We readily detected T.gondii PDI specific IgA antibodies in tears of young children (D1, D2 and D3, aged 6, 9, 11 years) as well as in tears of infants fed exclusively on human milk (B1 and B2, 3 and 5 months of age, respectively). The presence of sIgA in infant tears is not surprising as infants already produce (natural) sIgA antibodies within 3 days postpartum [55].

Unexpectedly, three sisters of 6, 9 and 11 years of age displayed an almost identical IgA staining pattern of total T.gondii extract blots (figure 1, D1-3), with both clear differences and similarities in comparison to parental IgA staining patterns (figure 1, M1 and F). In addition, the tear IgA staining pattern of the infant brother (figure 1, B1) not only differed markedly from all his siblings, it also retained its simplicity even after long exposures; only PDI and the other common constituents of tear IgA staining patterns comprising a T.gondii 70/74 kDa antigen doublet [26], being recognized by IgA. The paucity of antigens recognized and the predominance of anti-PDI antibodies in tears was confirmed with a tear sample from an unrelated infant (figure 1, B2).

Finally, we confirmed that anti-PDI antibodies are of the secretory IgA class using a polyclonal antibody specific for secretory component (SC, figure 1, V7).

Expression and analysis of regions involved in epitope formation
In view of the high percentage (about 80%) of volunteers with anti-T.gondii PDI IgA in their tears [26], it became important to determine whether different PDI regions were recognized by IgA’s in tears of different individuals. Assuming natural antibodies are not specific for T.gondii PDI, their ability to cross-react with PDIs of other species would imply that natural antibodies should be mainly directed against conserved regions of PDI. To identify T.gondii PDI regions involved in IgA epitope recognition, mature PDI lacking the N-terminal signal sequence was expressed.
as a recombinant glutathione-S-transferase (GST)-fusion protein (figure 2). In addition, series of PDI mutants truncated at either the N-terminal and/or C-terminal end were constructed. Strips of western blots with recombinant PDI were incubated with tears from individuals at dilutions resulting in recognition of PDI on a *T. gondii* lysate western blot. Tear IgA from all individuals tested recognized the full length PDI and various large fragments of PDI (PDIΔ1, PDIΔ2 and PDIΔ4) with exception of the region between aa 402-471 (PDIΔ3, see figures 2 and 3).

To further delineate the epitope(s) constituting regions within PDIΔ4 (aa 266-400) several truncated proteins were produced having their C-terminal end within this region (PDIΔ4, Δ10-12). As shown in figure 3 (left panels) all but one sample (F) failed to bind to PDIΔ10, indicating that the regions involved in IgA epitope formation were located at the C-terminal end of PDIΔ4, and not in the common sequences of PDIΔ1 and PDIΔ4. Importantly, the region present in PDIΔ4 and absent in PDIΔ10 (aa 351-400) contains the thioredoxin-like domain, a motif highly conserved in eukaryotic PDI sequences [30].

Additional truncated PDI proteins (PDIΔ5-Δ10), differing only a few amino acids in length (figure 2), allowed much more precise localization of regions essential for IgA epitope formation. Two major epitopes were delimited within this region, represented in the 9 aa difference between PDIΔ6-PDIΔ7 (aa 375-384) and the 8 aa difference between PDIΔ9-PDIΔ10 (aa 351-359, figure 3). The sequence between PDIΔ6-PDIΔ7 contains the thioredoxin-like domain with the H→Y amino acid substitution [54], while the sequence between PDIΔ9-PDIΔ10 is a more hydrophobic region with a VKVVG motif. This region is also particularly well conserved between *T. gondii* and various known human PDI sequences (compare entries XM 053104.1, BC 000425.1, XM 016522.2, AL356378.17, U75886.1, and figure 4). The sibling analysis depicted in figure 3 (D1, D2) demonstrated that the thioredoxin-like domain is the main IgA epitope within PDIΔ2 for the two sisters. The newborn brother (B1) did not display IgA reactivity against this region but, similar to the mother (M1), relied on the VKVVG motif present between PDIΔ9-PDIΔ10 (aa 351-359).

These results show that healthy human subjects produce secretory IgA recognizing at least three different highly conserved regions on *T. gondii* PDI, including the thioredoxin-like domains.
Conserved regions of PDI are targeted by IgA

Figure 3: Recognition of truncated versions of recombinant *T.gondii* PDI by IgA in tears.

Each sample contained IgA that recognized PDI Δ2. Subsequently, samples were incubated with strips containing stepwise truncated (±90 aa) versions of PDI Δ2: PDI Δ4 and Δ10 to Δ13 (see figure 3). All samples that recognized PDI Δ4 only, were subjected to additional fine-mapping with stepwise truncated (±8 aa) versions of PDI Δ4: PDI Δ5 to Δ9 (see figure 3). Strips were stained for GST after the PDI specific antibodies were removed (upper panels marked α-GST). Fine mapping demonstrated that PDI Δ6 and Δ9 contain regions essential for binding of IgA antibodies. In cases of PDI Δ9 recognition, PDI Δ8 signals were always considerably weaker than PDI Δ7 and Δ9 signals. Subject ID and dilutions of tears used correspond with figure 1.

**IgA in tears recognizes thioredoxin-like domains and cross-react with human and *P.falciparum* proteins**

To confirm recognition of thioredoxin-like domains by IgA in tears, tear samples of V1 and V7 were incubated with blots of truncated PDI with (PDI Δ6) and without (PDI Δ7) a thioredoxin-like domain. Subject V1 was already shown to recognize only PDI Δ6 in this region and not the slightly smaller truncated versions lacking this domain (Figure 3). Subject V7 had an anti-PDI staining pattern similar to M1 and B1 (Figure 3), recognizing PDI Δ9 containing the
VKVVV motif. This motif is present in both PDI Δ6 and Δ7.

Before incubation, peptide FYAPWCGHCK representing the thioredoxin-like domain was added in various concentrations. In both cases, increasing amounts of peptide resulted in a marked decrease in recognition of PDI Δ6, while recognition of the other PDI versions was hardly affected (figure 5A). This confirmed that IgA in tears from subject V1 recognized the thioredoxin-like domain and indicated that these particular antibodies form part of the repertoire of subject V7 as well. The PDI Δ7 recognition of subject V7 and the ‘full’ recognition of subject V1 remained intense despite the absence of a contribution by the thioredoxin-like domain. It seemed that a reduction in binding to the thioredoxin domain is compensated by an increase in binding to the VKVVV motif. Possibly, binding to these different nearby IgA epitopes in PDI is mutually exclusive.
Conserved regions of PDI are targeted by IgA

Figure 4: Clustal W (1.81) multiple sequence alignment of T.gondii, P.falciparum, C.parvum, H.sapiens PDIs. Overall, the protein has the highest similarity to PDI from other species of the phylum Apicomplexa: Plasmodium falciparum (AJ250363) and Cryptosporidium parvum (U48261), with 210/444 and 171/448 identities (id's), respectively, followed by PDI homologues of Ostertagia ostertagi (AJ419174), Humicola insolens (P55059) and Onchocerca volvulus (U12440). There is considerable homology with human PDI (P55, P07237) as well: 163/453 id's. Of the 5 domains of PDIs denoted A-B-B'-A'-C (102-60-68-104-16 aminoacids), the A domains characteristically display the highest similarity with other PDIs, mainly due to conservation of the thioredoxin-like domains [33,59]. The alignment pattern of the A' domain is similar to the overall alignment, with T.gondii PDI (AJ306291) being most homologues with A' domains of the above mentioned protozoan family members (56/104 and 59/104 id's, resp.) and human PDI (53/105 id's). Remarkably, the A domain of T.gondii displays more homology with PDIs of O.ostertagi (62/102 id's), Caenorhabditis briggsae (AJ005807, 61/102 id's), Schistosoma mansoni (S34275, 61/100 id's) and C.elegans (S71862, 57/102 id's) than with P.falciparum (54/101 id's). In contrast to the A regions, both B regions of T.gondii have only limited homology with B regions of PDI of P.falciparum (17/58 and 29/61 id's, respectively).

A and A' regions, [--------], and [-----] of Human PDI according to [72].

More importantly, recognition of the highly conserved thioredoxin-like domains by subjects V1 and V7 implies species cross-reactive capabilities. To study cross-reactivity, we compared the reactivity of tear IgA with the reactivity of two polyclonal antibody preparations directed against T.gondii PDI and bovine PDI, the latter known to cross-react with human PDI. The anti-T.gondii PDI polyclonal generated in rabbit revealed bands at expected heights in our preparations of tachyzoites and recombinant T.gondii PDI, but not in the human cell line extract or the P.falciparum extract (fig 5B).

The polyclonal against bovine PDI reacted with a 55 kDa antigen in the human cell extract but not with antigen in the other preparations. In marked contrast, the IgA staining patterns of infant B1 and volunteer V1 on blots containing human or plasmodium extracts were remarkably similar in their complexity (figure 5B). The patterns had bands at expected heights for human, T.gondii, and P.falciparum PDI (figure 5B). A third sample, V7, did not show such a complex staining pattern on strips with human or plasmodium extract, but also stained bands at expected heights for the various PDIs (results not shown).

**T.gondii PDI is a surface-antigen**

To confirm our previous finding that anti-toxoplasma IgA are capable of binding to the parasite surface and thus be functional in defence against T.gondii (figure 6A), intact parasites were incubated with anti-SAG1 antibodies, specific for the major membrane protein of T.gondii [31], and diluted tears of five individuals. While the anti-SAG1 antibody gave a homogeneous fluorescent pattern contouring all parasites, IgA antibodies in tears gave a coarse fluorescence of most parasites and a homogeneous fluorescence of a minority (figure 6B, using sample V4). There were differences in fluorescence patterns between tear samples tested; sample V1 only displayed a coarse pattern, weaker and more apically localized (not shown). Two other incubations did not give detectable fluorescence patterns. In general, fluorescence was only observed with tear samples showing brisk recognition of PDI.

Although suggesting a possible function, surface-staining of IgA does not help to distinguish between conventional and natural antibodies as conventional IgM (and most likely also slgA) responses are directed against surface-exposed epitopes of antigens [56], while natural slgA are capable of binding to the surface of bacteria [48], and, as shown here, protozoan parasites.

37
Figure 5: Recognition of a thioredoxin-like domain and analysis of cross-reactivity of tear IgA antibodies. (A): Tears of subjects V1 and V7 were incubated with Western blots containing full length PDI and two truncated versions with (PDI Δ6) and without (PDI Δ7) the thioredoxin-like domain. Before incubation peptide FYAPWCGHKCK was added in the amounts indicated. Strips were stained for GST after the PDI specific antibodies were removed (lower panel marked α-GST). Please note that the positions of the bands in this figure do not reflect the actual MW of the proteins, as small blot-strips were used in an attempt to minimize volumes required. Subject ID corresponds with figure 1. (B): Strips with Plasmodium schizont extract (pl), human embryonic kidney cell extract (hu), PDI Δ2 (Δ2), and T. gondii lysate (tg) were incubated with diluted tears of B1 and V1 and polyclonals specific for bovine and T. gondii PDI. The tg strips were taken from another blot. Strips incubated with tears from subject B1 were incubated with the polyclonal specific for bovine PDI after tear antibodies were removed. Even upon long exposure anti-bovine and T. gondii PDI polyclonals showed no cross-reactivity. Cross-reaction by IgA in tears occurred despite considerably lower anti-PDI concentrations: tears were diluted 100x compared with 4000x and 20,000x for bovine and T. gondii PDI, respectively.

Discussion
In a recent study we identified Toxoplasma gondii protein disulfide isomerase (PDI) as the major antigen recognized by tear IgA's [54]. Now we demonstrate that anti-PDI secretory IgA are present in different mucosal secretions (tears and milk) from adult healthy human subjects, and tears from young children and infants. It is highly unlikely that these infants were exposed to T. gondii when nursed and there were no symptoms of an acquired infection of the mothers during gestation. Previous findings already pointed out that there is no relation between the presence of these IgA antibodies in tears and chronic infection by T. gondii, i.e. no correlation with presence or absence of T. gondii antibodies in serum. The fact that human milk also contains anti-PDI antibodies confirms that human milk is also a rich source of natural sIgA antibodies [19,53]. Clearly, antibodies with specificity for PDI form part of the secretory IgA repertoire responsible for the primary protection of nursed infants and probably remain present in tears throughout life [26], stressing their importance.

PDI was one of the few antigens recognized by lacrimal IgA in infants and adults. The paucity of antigens recognized by natural antibodies in infants as compared to older children and adults, has been observed previously in natural IgM repertoires [57]. Apparently IgA anti-PDI antibodies are secreted by a select group of B cells naturally activated early in MIS development. As anti-PDI antibodies are continuously secreted irrespective of age [26], these B cells apparently remain present throughout life, a property shared with natural IgM secreting B cells in mice [57]. Remarkably, 3 siblings had similar anti-toxoplasma IgA staining patterns, including anti-PDI. Although autoimmune
Conserved regions of PDI are targeted by IgA

Figure 6: Tear antibody staining patterns of intact parasites.
(A): Diluted tear samples of 2 individuals were incubated twice with PBS [-], or intact parasites, [+] as described earlier [26]. The IgA staining pattern after the second extraction is shown. (B): Intact parasites were double stained by incubation with tears of V4, known to have a brisk anti-49 kDa titer, and a monoclonal antibody specific for the major surface antigen of T. gondii, SAG1. Staining of parasites by IgA in tears and anti-SAG1 antibodies was analyzed using a laser scanning microscope. Tear IgA and anti-SAG1 patterns are superimposed in ‘Overlay’. Red in the tear α-IgA/FITC and SAG1/CY3 pictures indicated saturated pixels. In ‘Overlay’, green indicates predominant tear IgA staining, red - predominant SAG1 staining and yellow – colocalization of tear IgA and SAG1 staining. Tears of V4 were diluted 25x. Bar: 2 μm.

Detailed analysis of amino acid sequences recognized by anti-PDI antibodies showed at least two conserved regions involved, including a thioredoxin-like domain of PDI. This domain is highly conserved among PDIs [30,33,59][figure 4], suggesting that anti-T. gondii PDI IgA antibodies could react with PDI from other species. This was demonstrated with extracts of the closely related Plasmodium falciparum and cultured human cells. The other region of 8 amino acids usually contains at least 6 conserved residues, the VKVVVxxN motif. This region is conserved between T. gondii and human PDI sequences (figure 4). The conformational flexibility of natural antibodies could allow cross-reactivity with PDIs that have moderately diverged [60,61]. That conserved regions within a conserved protein are recognized, allowing interspecies cross-reactivity, establishes these anti-PDI antibodies as so-called natural antibodies and exponents of the innate immune system (see figure 5). In contrast, forced immunization with recombinant T. gondii PDI did not generate antibodies that are capable of cross-reaction, which indicates that these antibodies are primarily directed against species-specific regions of PDI (‘B’ regions figure 4).

Table 3 summarizes our findings and those obtained by others regarding characteristics of natural antibodies directed against protein antigens. Although analysis of polyreactivity has been used convincingly to characterize sets of antibodies [19], this is the first report in which delineation of epitopes has been used for characterization of a particular (natural) antibody.

PDI is a multi-functional enzyme. It belongs to the superfamily of thioredoxins, functioning primarily as an ER oxidoreductase of eukaryotic cells. It facilitates de novo formation of disulfide bridges between cysteines (oxidation) and/or rearranges existing disulfide bridges (isomerization) in secretory and cell-surface proteins [30]. All
major surface antigens of *T. gondii*, SAG1, SAG3, and SAG1 related proteins have high numbers of intramolecular disulfide bonds [31], which might explain the high expression level of *T. gondii* PDI.

Although its intracellular localization suggests that PDI is unavailable for binding by secretory IgA antibodies in human mucosal secretions, there are indications that PDI has functions apart from its role in the ER. Despite its ER retention signal, PDI is found on the surface of several cell types: on thyroid cells, where it is involved in shedding of the TSH receptor ectodomain [62]; on platelets, where PDI mediates adhesion to integrin [36]; on lymphocytes, involved in adhesion of HIV [35], and on hepatocytes [63]. Our results demonstrate that also *T. gondii* expresses PDI on its cell surface as well. Like PDI on the membrane of human cells, *T. gondii* PDI may contribute to adhesion processes, e.g. to host cells during infection, be involved in maintenance of structure and function of surface antigens, and/or the concomitant shedding of surface antigens, such as SAG1 [64]. Importantly, this would make mucosal anti-PDI IgAs function against *T. gondii* in the innate, first line defence immune reaction. Although there are strong indications that tear IgA also recognizes *P. falciparum* PDI, it is not known whether PDI is exposed on the surface of this parasite as well.

Natural antibodies are usually cross-reactive and recognize non-self antigens of pathogens and self-related antigens [18,19,46,60], like the anti-PDI antibodies described here. It is possible that the anti-PDI antibodies may be selected for their ability to bind to self-PDI, as both the thioredoxin-like domain and the VKVxVxxN motif are conserved between PDI's. There are several models to explain natural antibody repertoire selection [57,58,65], but only one seems compatible with our results: anti-PDI secretory IgA could represent the evolutionary conserved germline repertoire of antibodies [19,57], and form part of the 'natural immune memory' [66,67] or 'immunological homunculus' [58]. These antibodies might be selected for their ability to bind to evolutionary conserved self-antigens having the potential to be (ab)used by pathogens to adhere to cells and initiate infection, as e.g. PDI by HIV in adherence to lymphocytes [35]. This implies that secretion of anti-PDI IgA's could be due to their ability to bind to both 'self'- and 'nonself'-PDI, including *T. gondii* PDI. The production of these antibodies appears to be largely confined to mucosal surfaces where dimeric IgA is rapidly secreted, thereby restricting their autoreactive potential.

*T. gondii* PDI was identified as major target for naturally occurring human IgA antibodies secreted at mucosal sites. The epitopes on PDI recognized by these natural antibodies are evolutionary conserved, suggesting they have evolved to participate in the innate immunity against protozoan parasites and perhaps other eukaryotic pathogens as well. Whether PDI can be regarded as a primitive invasion protein that can be neutralized by primitive (natural) IgA remains to be investigated. Our results suggest that the human lacrimal gland, along with the mammary gland, is one of the few mucosal sites with predominant secretion of natural antibodies. This should be confirmed by detailed analysis of plasma cells present at these sites.
Chapter 4

Herpes Simplex Virus Infection of the Human Eye Induces a Compartmentalized Virus-Specific B cell Response

Submitted

Ron Peek, Georges M.G.M. Verjans and Bob Meek

Abstract

Intra-ocular infection with herpes simplex virus (HSV) can cause uveitis, a potentially sight-threatening disease. The disease is characterized by an ocular infiltration of inflammatory cells like macrophages, B and T cells. The characteristics of the local humoral and cellular immune responses elicited upon intra-ocular HSV infection are poorly understood. The local herpes virus-specific antibody production, routinely used for confirmation of a clinical diagnosis of herpetic uveitis, has never been analyzed in detail. In the present study we have analyzed the humoral immune response against HSV type 1 (HSV-1) in paired samples of intra-ocular fluid and serum of patients with intra-ocular herpes virus infection. In addition, the B cell epitope distribution on a single HSV-1 type-specific antigen, glycoprotein G, was compared for these paired samples. The results presented in this study indicate that inflamed eyes of patients with HSV-induced uveitis display a compartmentalized B cell response directed to the triggering virus.
Introduction

Infection with herpes simplex virus type 1 (HSV-1) is widespread in the human population and a significant health concern. The clinical expression of HSV-1 related disease varies and ranges from self-limiting illness to fatal disease. Ocular complications include infection of the eyelid, conjunctiva, cornea and retina. HSV infection of the eye is the most common infective cause of blindness in developed countries, mainly due to its recurrent nature [73,74]. Although HSV-2 infection has also been implicated in ocular disease, especially in younger patients [75-77] most cases of orofacial herpes infection are attributable to infection with HSV-1 [73,78,79].

Inflammation of the human eye elicited upon herpetic infections is characterized by the influx of mononuclear inflammatory cells. The cell infiltrate is mainly composed of T cells, whereas limited numbers of macrophages and plasma cells are predominantly found in the affected ocular tissues [80-83]. However, the local microenvironment strongly influences the expression of immunity to local antigens, a phenomenon known as "immune privilege". Various anatomical, cell bound and soluble factors contribute to the immune privilege of the eye resulting in impaired T cell responses (reviewed in [84] and [85]). The humoral immune response remains intact and HSV-specific antibodies can be detected in intraocular fluids [86]. In addition to detection of viral DNA by PCR, the relative titers of these locally produced virus-specific antibodies are routinely used for confirmation of a suspected clinical diagnosis of ocular HSV infection. However, the specificity and functional properties of these virus-specific intra-ocular antibodies remain enigmatic [87]. Recently, we have analyzed the specificity of intra-ocular antibodies of uveitis patients with an ocular infection caused by the protozoan parasite Toxoplasma gondii. Marked differences between the intra-ocular and systemic T.gondii-specific IgG response were detected. Some antigens were preferentially recognized by serum or ocular-derived antibodies, suggesting a non-random distribution of IgG producing plasma cells between the ocular and systemic compartment [88,89]. Besides differences in clinical presentation between HSV- and T.gondii-induced uveitis the life cycles of these two pathogens differs considerably. While T.gondii has a complex life cycle involving several stages that uniquely express numerous antigens, HSV displays only a very limited variability in antigens. Therefore, egress and presentation of their antigens intra-ocularly and the subsequent induction of a local immune response may differ between both uveitis entities.

In the present study, the HSV-specific intra-ocular humoral immune response in eyes of patients with HSV-induced uveitis was characterized. The IgG antibody repertoires from paired serum and ocular fluid (IOF) samples were analyzed by immunoblotting of a total HSV-1 extract. Additionally, the B cell response to the HSV-1 typespecific antigen glycoprotein G (gG1) and a series of C-terminally truncated mutants of gG1 was studied by IgG epitope mapping. The data presented show that affected eyes of patients with HSV-induced uveitis contain antibodies with different specificities than the serum. This difference was not only observed for different HSV-1 antigens, but also for the distribution of B cell epitopes on gG1. To our knowledge this is the first report on a compartmentalized B cell response in humans during viral infection.

Material & Methods

Patient materials: The patients’ samples analyzed in this study were sent to the Netherlands Ophthalmic Research institute by ophthalmologists for confirmation of a clinical diagnosis of herpes-virus-induced uveitis. All patients were sampled during an acute episode of uveitis and did not receive pre-operative anti-viral or immunosuppressive treatment. Serum and IOF samples, either aqueous humor from the anterior chamber or vitreous fluid from the vitreous body, were obtained from patients during therapeutic pars plana vitrectomy or paracentesis for diagnostic purposes. The IOF and serum were routinely tested for IgG antibody against T. gondii. Herpes simplex
Intraocular B cell response induced by HSV infection

<table>
<thead>
<tr>
<th>Pt. Nr.</th>
<th>sex</th>
<th>Age (yrs)</th>
<th>IgG (mg/ml)</th>
<th>Anti-HSV IgG relative titer</th>
<th>HSV</th>
<th>HSV-1 PCR</th>
<th>EIA assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Serum</td>
<td>IOF</td>
<td>Serum</td>
<td>IOF</td>
<td>GWc</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>M</td>
<td>15.90</td>
<td>0.13</td>
<td>14679</td>
<td>6683</td>
<td>56</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>24.10</td>
<td>0.50</td>
<td>30980</td>
<td>108819</td>
<td>1691</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>21.10</td>
<td>0.87</td>
<td>18962</td>
<td>3676</td>
<td>19</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>20.52</td>
<td>0.34</td>
<td>2048°</td>
<td>4096°</td>
<td>53</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>15.89</td>
<td>0.94</td>
<td>4096°</td>
<td>4096°</td>
<td>9</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>22.30</td>
<td>31.86</td>
<td>8192°°</td>
<td>191072°°</td>
<td>9</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>19.30</td>
<td>1.31</td>
<td>20464</td>
<td>8930</td>
<td>6</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>17.70</td>
<td>6.40</td>
<td>1024°°</td>
<td>4096°</td>
<td>8</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>15.80</td>
<td>0.29</td>
<td>10168</td>
<td>7513</td>
<td>40</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>16.20</td>
<td>0.13</td>
<td>1735</td>
<td>176</td>
<td>13</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>24.10</td>
<td>0.81</td>
<td>8192°°</td>
<td>512°</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>7.80</td>
<td>0.02</td>
<td>10640</td>
<td>2555</td>
<td>9</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>F</td>
<td>15.70</td>
<td>0.13</td>
<td>2048°°</td>
<td>256°</td>
<td>15</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>15.50</td>
<td>0.01</td>
<td>29816</td>
<td>249</td>
<td>13</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>17.10</td>
<td>0.75</td>
<td>21253</td>
<td>12742</td>
<td>14</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>M</td>
<td>12.10</td>
<td>2.44</td>
<td>256°°</td>
<td>512°</td>
<td>10</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>F</td>
<td>15.40</td>
<td>0.54</td>
<td>10893</td>
<td>3298</td>
<td>9</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>M</td>
<td>9.29</td>
<td>0.02</td>
<td>8192</td>
<td>128</td>
<td>7</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 1: Patient data. The HSV Goldmann-Wittmer coefficient (HSV GWc) is a measure for specific anti-HSV intraocular antibody and is expressed as the quotient of the relative amounts of HSV-specific antibody, corrected for total IgG, in the IOF sample and serum (see materials and methods). Anti-HSV IgG antibody titers were determined by HSV-specific ELISA, or by an immunofluorescence assay (indicated by °). Note that the IOF sample of patient 18 was PCR positive for HSV-2.

Intraocular antibody production was determined by calculation of the Goldmann-Wittmer coefficient (GWc), which is the quotient of the relative amounts of anti-pathogen antibodies in the eye and serum and is calculated as follows: (antibody titer ocular fluid/total IgG ocular fluid) : (antibody titer serum/total IgG serum) [86]. A GWc exceeding 3 is considered as evidence for intra-ocular pathogen-specific antibody production [87]. The IOF samples were tested for the presence of HSV-1 DNA by a gG1-specific PCR as described previously [90]. Paired samples of 16 patients with a GWc for HSV exceeding 3 were selected for this study (see Table 1). Sera were examined for HSV type-specific antibodies using the Gull HSV-1 IgG and HSV-2 IgG EIA assays (Gull Laboratories, Salt Lake City, Utah) and were performed according the manufacturer’s guidelines. The assays are based on plates coated with affinity-purified gG1 from HSV-1 or gG2 from HSV-2. Sera were assigned HSV-1 or/and HSV-2 specific according to the manufacturer’s criteria. Paired samples of patients with a confirmed clinical diagnosis of ocular toxoplasmosis or intra-ocular VZV infection were used as disease controls. Additionally, sera of healthy controls with serologically proven prior infection with either HSV-1 (n=5), HSV-2 (n=5), or both serotypes (n=4) were included. The present study was performed according the Declaration of Helsinki and informed consent was obtained.

SDS-PAGE and Western blot analysis: A commercially available purified HSV-1 extract (MacIntyre strain; ABI, Columbia, Maryland) was used to prepare immunoblots containing total HSV-1 proteins. Proteins were size fractionated by electrophoresis on 10% SDS polyacrylamide slab gels and transferred to PVDF membranes.
(Immobilon-P, 0.45 μm pore size; Millipore, Bedford, MA). Transferred proteins and markers were visualized using ponceau red dye staining. Protein binding capacity of the blots was saturated by incubation with Tris-buffered saline (TBS: 50 mM Tris-HCl and 150 mM NaCl [pH 10]) containing 0.5% Tween-20 and 2% nonfat powdered milk (blocking buffer; Biorad, Hercules, CA). Blot strips were incubated with patients’ samples’ dilutions 1:500 (except when stated otherwise) in TBS containing 0.5% Tween-20 and 0.03% nonfat powdered milk (TBS-T). Paired IOD and serum samples were always tested on adjacent blot strips from the same gel allowing precise alignment afterwards. After washing in TBS-T strips were incubated with horseradish peroxidase (HRPO)-conjugated goat-anti-human-IgG (DAKO, Glostrup, Denmark) for one hour. After washing in TBS-T strips were soaked in detection reagents (ECL, Pharmacia, UK) and covered with a polyethylene sheet. Exposures for various time intervals to X-ray film were made. Following analysis of the patients’ samples, blots were stripped from antibody by incubation with 62.5 mM Tris pH 6.8, 2% SDS and 100 mM β-mercaptoethanol for 30 min at 70°C. Blots were again blocked and re-probed with horseradish peroxidase-conjugated anti-GST mAb (Biotech, Buckinghamshire, UK) to visualize the quantity and integrity of the recombinant glutathione S-transferase (GST)~gG1 fusion proteins.

**Construction of the HSV-1 glycoprotein G expression plasmid and deletion mutants:** The open reading frame (ORF) of HSV-1 gG was amplified from a purified HSV-1 extract (ABI, Columbia, Maryland) using *Pfu* DNA polymerase (Stratagene, La Jolla, California) with the addition of 5% DMSO and equimolar amounts of dGTP and 7-deaza-2'-dGTP to circumvent the formation of secondary structures due to the high G+C content of the target sequence [91]. The sequences of the primers for amplifying the complete ORF (amino acid (aa) 1-238) were: 5’GAGACCATGGGCCGCGGCGCAT3’ (primer 1) and 5’GAGAATTCTACCCGCGTTCGGACGGC3’. This cloning procedure created an *NcoI* site at the 5’ end covering the initiation codon and introduced an amino acid substitution at position 2 (Ser→Ala). For constructing the C-terminal deletion clones primer 1 was used in combination with 5’GAGAATTCCCTTGAGGTAGTGGCGC3’ (ΔC1; aa1-165), 5’GAGAATTCCCTTGCGGACGGTGGTTTGT3’ (ΔC3; aa1-125), 5’GAGAATTCCCTGGGAGGGTGCTCA3’ (ΔC4; aa1-105), 5’GAGAATTCCCTCCTCGCTTCTCTCTCT3’ (ΔC5; aa1-85), 5’GAGAATTCCGCTGGCTGATGGCGGT3’ (ΔC6; aa1-65), 5’GAGAATTCTGAGGGACGGACCCG3’ (ΔC7; aa1-45) and 5’GAGAATTCAACCCCGATACACCGA3’ (ΔC8; aa1-25). PCR fragments were gel purified, digested with *Neol/EcoRI* and ligated into a derivative of pGEX-3X plasmid (Pharmacia, Uppsala, Sweden) to produce GST-gG1 fusion proteins.

**Expression and purification of recombinant proteins:** Cultures of *Escherichia coli* strain DH5α transformed with GST-HSV-1 expression constructs were grown at 37°C to an OD₆₀₀ of 1. Expression of fusion protein was induced by the addition of isopropyl-β-D-galactopyranoside to a concentration of 1 mM. After 2h at 30°C bacteria were pelleted, resuspended in PBS containing protease inhibitors (Complete, Boehringer Mannheim, Germany) and lysed by repeated freezing and thawing. After sonication the extract was treated with DNase I (50 μg/ml) for 20 min at 37°C. Insoluble material was removed by centrifugation and the supernatant containing the recombinant protein was filtered through a 0.45 μm filter. To purify the recombinant protein, the cleared extract was incubated with glutathione agarose beads (Pharmacia) for 1h at room temperature. After extensive washing with PBS the glutathione agarose beads were resuspended in SDS-sample buffer. The concentration of the purified recombinant protein was determined by analysis of a serial dilution on protein gels stained by Coomassie, using BSA as a standard,
and the signal obtained with the anti-GST mAb.

**Absorption of gG1-specific antibody:** Patient sera were diluted 1:2000 in TBS-T and incubated with blot strips containing 25 μg of recombinant GST-gG1 or control blot strips containing *E. coli* protein. To saturate all protein binding sites, the strips were soaked in blocking buffer for 1 h. Blot strips were incubated for 1 h with diluted serum in 500 μl wash buffer. Part of this pre-absorbed serum was used for a second and third round of depletion. After each round of absorption part of the diluted serum was incubated with blot strips containing recombinant gG1 protein and blot strips containing size fractionated total HSV-1 protein. To determine the exact position of the various glycosylated forms of native gG1 on the blots strips containing total HSV-1 protein, an anti-gG1 mAb was used (clone LP10; kindly provided by A.C. Minson). Bound antibodies were visualized by incubation with HRPO-conjugated secondary antibodies followed by chemiluminescence.

**Results**

**Differential HSV-1 antigen recognition by IgG in paired serum and IOF samples:** The 18 patients included in this study had a clinical picture of herpetic uveitis. The patients 1-10 and 12-17 had an intraocular antibody synthesis against HSV, but not to VZV, CMV or *T. gondii* (Table 1). Control patients 11 and 18 had a VZV- or HSV-2-induced uveitis, respectively. On the basis of clinical and laboratory data the ocular lesions were typed as HSV-induced uveitis. The seroprevalence to HSV-1 and -2 was determined using gG-based HSV type-specific commercial assays. Whereas all patients were seropositive for HSV-1, the sera of patients 2 and 6-8 also contained antibodies against HSV-2 (Table 1). Paired serum and IOF samples of 10 of these patients were analyzed for the presence of anti-HSV-1 IgG by Western blotting, using a purified extract of total HSV-1 proteins. A diverse banding pattern was observed on the immunoblots (Fig. 1). In addition to major antigens of mainly high molecular weight, which were efficiently recognized by most of the samples, minor antigenic viral proteins of various molecular weights were observed. Large differences in antigen recognition were detected between sera of individual patients. More interestingly, the paired serum and IOF samples of all patients with a positive GWc for HSV displayed clear differences in antigen recognition (Fig. 1). For example, several high molecular weight antigens are intensely stained by IgG from the ocular compartment of patient 2, but hardly visible in the corresponding serum lane. In some patients particular antigens appeared to be recognized in one compartment only, as for example the antigen running at ~100 kDa in the ocular compartment of patient 10, and the ~80 and ~50 kDa antigens in the corresponding serum lane. There were no particular antigens consistently recognized by the patient samples. Also patient 18, with a confirmed diagnosis of intra-ocular HSV-2 infection and no detectable anti-HSV-1 IgG in either serum or IOF (Table 1),

Figure 1: Immunoblot analysis of a size fractionated total HSV-1 protein extract with paired serum (s) and ocular fluid (o) samples of herpetic uveitis patients.

Patients 1-10 and 18 suffered from a HSV-1 or -2-induced uveitis, respectively. Patient 11 was diagnosed with an intra-ocular VZV infection. Numbers on the left represent molecular weight markers (in kDa).
showed a differential antigen recognition profile between the serum and the IOF (Fig. 1). As disease controls several patients with a negative GWc (<3) for HSV-specific IgG and suffering from intra-ocular infection due to *T. gondii* or VZV were tested. While some of these patients did have HSV-1-specific IgG in either serum or both compartments (Fig. 1, patient 11 and data not shown) no differences in antigen recognition were observed. These results demonstrate that the HSV-1 antibody response is compartmentalized, but does not provide any information on the epitopes of a single HSV antigen recognized by antibodies in these compartments.

**Recognition of a single HSV antigen by IgG in serum and IOF samples:** To further characterize the HSV-1-specific intra-ocular humoral immune response as compared to the serum, the IgG epitopes of a single defined HSV-1 antigen were studied in detail. For this purpose gG1 was selected. The humoral immune response to this virus envelope protein is devoid of serologic cross-reactivity between the HSV serotypes or other human herpes viruses [92-94]. Furthermore, human IgG serum epitopes of gG1 have been identified [95]. The complete gG1 ORF was amplified by PCR and cloned in a bacterial expression vector allowing production of a recombinant glutathione (GST)-gG1 fusion protein. Based on the amino acid sequence, the calculated molecular mass of gG1 is about 27 kDa. In contrast to bacterial expressed gG1, the native protein is differentially glycosylated in HSV-1-infected cells, leading to several products with apparent molecular masses between 38 and 50 kDa [93,95]. This indicates that a major part of native HSV-1 gG is composed of carbohydrate residues that, in addition or in combination with the primary amino acid sequence, might also be targeted by the IgG antibody response. To validate the use of the (nonglycosylated) GST-gG1 recombinant fusion protein for the analysis of IgG epitopes, a patient serum with a high anti-gG1 IgG titer (patient 9) and a patient serum with a low titer (patient 4; results not shown) were incubated with solid-phase bound GST-gG1. The efficiency of absorption was monitored by incubating the non-absorbed and absorbed fractions of the patient sera on immunoblot strips containing either recombinant GST-gG1 or an extract of total HSV-1 proteins. As shown in Fig. 2, a single pre-incubation of diluted serum of patient 9 with solid phase bound recombinant fusion protein reduced the reactivity with recombinant GST-gG1 considerably (compare lanes 1 and 2), while additional absorptions completely abolished recognition of the recombinant protein (lanes 3 and 4). These same pre-absorbed fractions were tested in parallel with an anti-gG1 mAb (lane 5) on blot strips containing total HSV-1 protein extract (lanes 5-9). These analyses demonstrated that the antibodies against the various forms of native gG1 are efficiently and specifically absorbed from the diluted serum (compare lane 6 with 7-9). Control pre-incubations of diluted serum with solid phase bound *E. coli* proteins did not show a specific absorption (lanes 10-17). Similar results were obtained with serum of patient 4 (not shown). These data suggest that the IgG immune response to gG1 is mainly directed against the primary amino acid sequence and not against the extensive post-translational
Intraocular B cell response induced by HSV infection

Figure 2: Depletion of serum-derived HSV-1 glycoprotein G (gG1)-specific antibodies by solid phase bound recombinant GST-gG1.

Diluted serum of patient 9 was sequentially incubated with blot strips containing 25 μg of GST-HSV-1 gG fusion protein. After incubation part of the diluted serum was tested on blot strips containing recombinant protein (lanes 2-4) or total HSV-1 protein (lanes 7-9). In lane 5 the gG1-specific mAb was used and in lanes 1 and 6 reaction of the non-absorbed serum is shown. The control experiment is shown at the right (lanes 10-17), where the serum was sequentially incubated with blot strips containing solid phase bound E.coli proteins. Arrowheads point to the various glycosylated forms of gG1. The molecular weight markers (in kDa) are indicated. Note that the recombinant fusion protein is highly susceptible to proteolytic degradation.

modifications. Furthermore, this demonstrated that the GST-gG1 fusion protein is suitable to identify regions of gG1 involved in IgG epitope formation.

To exclude the possibility that HSV-2-specific IgG cross-reacts with gG1, the sera of 4 healthy HSV-2 seropositive controls and the paired serum and IOF of a patient with HSV-2 induced uveitis (patient 18; Fig. 1 and Table 1), were tested for reactivity to GST-gG1. In concordance with other studies [92-94], the HSV-2 specific samples did not show any cross-reactivity with gG1 (data not shown).

To determine the gG1-specific IgG response in serum and IOF, paired samples of 17 patients were tested by immunoblotting with purified GST-gG1 (Fig. 3). All patients with ocular HSV infection had gG1-specific antibodies in their serum, although in some patients the signal was relatively weak (patients 4-6). However, in the IOF sample of 6 patients (3, 4, 7, 12, 14 and 15) no IgG against GST-gG1 could be detected, not even after overexposure (data not shown).

Mapping of B cell epitope regions of gG1 in paired serum and IOF samples: In order to define the gG1 epitopes recognized by antibodies in the patients’ samples, 8 C-terminally truncated gG1 deletion mutants were generated (designated as ΔC1-ΔC8) and expressed as GST fusion proteins (Fig. 4). The mutant GST-gG1 fusion proteins and GST alone were purified and used for immunoblots containing similar amounts of each protein. From the panel of paired samples shown in Fig. 3, seven patients were selected that had gG1-specific IgG in both compartments (patients 1, 2, 8-10, 13 and 16). To minimize the volume of ocular fluid required, the8 C-terminal GST-gG1 mutants and GST protein were blotted onto small immunoblot strips. Protein sequences involved in B cell epitope formation were identified by a discrete change in the intensity of the chemiluminescent signal of two subsequent mutant proteins (Fig. 5). As expected, the gG1-specific mAb LP10 displayed only a single epitope region between gG1 aa 65 (ΔC6) and aa 45 (ΔC7) (Fig. 5, panel B). The low molecular weight protein consistently

Figure 3: Immunoblot analysis of purified recombinant GST-gG1 fusion protein with paired serum (s) and ocular (o) patient samples.

The position of the full-length fusion protein is indicated at the left. Part of the blot was stained with an anti-GST monoclonal antibody (GST). The position of the molecular weight markers (in kDa) is shown at the left.
recognized in mutants GST-gG1 ΔC1-ΔC6 is most likely a gG1-derived degradation product. Interestingly, major differences in reactivity to the truncated gG1 proteins were observed within and between paired serum and IOF samples of 4 out of 7 patients analyzed. The serum of patient 1 (panel C) was still fully reactive with ΔC4 but showed less intense staining of ΔC5, indicating that the region between ΔC4 and ΔC5 is involved in epitope formation. Further truncation to gG1 aa 65 (ΔC6) completely abolished recognition by the serum of this patient. In contrast, the paired IOF (panel D) displayed only a single epitope region between gG1 aa 125 and 105 (ΔC3-ΔC4). The serum of patient 2 revealed the recognition of two epitope regions (between ΔC2-ΔC3 and ΔC3-ΔC4), while the IOF of this

Figure 4: Schematic representation of the native gG1 (top) and recombinant GST-gG1 fusion proteins.

The positions of the signal sequence (signal), the immuno-dominant region (IDR) and the putative transmembrane region (TMR) are indicated. Mutants ΔC1 – ΔC8 were generated by PCR amplification. The numbers refer to the position of the amino acids of gG1.
Figure 5: Mapping of B cell epitopes on HSV-1 glycoprotein G.

Blot strips with similar amounts of different mutant GST-gG1 proteins (ΔC1-ΔC8) or GST were incubated with patient samples (panels C-P). Panel B was incubated with a gG1-specific mAb and panel A with an anti-GST mAb. Arrowheads indicate discrete changes in signal intensity between two subsequent deletion clones. Note that of each blot several exposures for various time intervals were made but a single, most informative exposure is shown.

Patient (panel F) did not have a major epitope in these regions but displayed only one epitope region between gG1 aa 105-85 (ΔC4-ΔC5). The serum of patient 10 (panel K) recognized three epitopes in the region between gG1 aa 165-85. The paired ocular fluid had no major epitopes in this region but depended on sequences between aa 85-45 for full recognition of gG1. The IOF sample of this patient showed a similar reactivity pattern as the gG1-specific mAb LP10 (panel B), recognizing an epitope located between gG1 aa 65-45 (panel L). Both samples of patient 16 (panels O and P) depended on sequences between gG1 aa 85 and 65 (ΔC5-ΔC6) for epitope formation, while the serum displayed an additional epitope region between gG1 aa 105 and 85. For the paired samples of patients 8, 9 and 13 no differences in epitope recognition were observed (panels G-J, M and N).

Discussion

In the present study we have analyzed the HSV-specific IgG repertoire in the ocular compartment of patients with herpetic uveitis and compared this with the systemic compartment. In the patients tested for reactivity to total HSV-1 protein extract, several antigens were predominantly or exclusively recognized in one compartment. Ocular inflammation can disrupt the blood-eye barrier, leading to influx of serum proteins like immunoglobulins. This phenomenon may account for the recognition of HSV-1 proteins by IOF samples of patients with non-HSV-induced uveitis (e.g. patient 11 suffering from a VZV-induced uveitis). However, these patients will have a GWc less than 3 for HSV, indicating that the observed anti-HSV antibodies in the IOF are not produced locally but probably are extravasated from the systemic compartment. The differential recognition of various HSV-1 antigens by patients with a GWc exceeding 3, strongly suggests a compartmentalized HSV-1-specific B cell response. Within this cohort, sera of 4 patients contained IgG against both HSV serotypes, suggesting that some differences in intensity of antigen recognition observed in either compartment might be the result of cross-reactive antibodies. Antibodies against HSV-2 are known to cross-react with HSV-1 proteins [96] and the paired serum and IOF sample of a patient with HSV-2-induced uveitis (patient 18) tested by immunoblot indeed confirmed this. However, also these anti-HSV-2 antibodies clearly demonstrated compartmentalization of the antibody response. To further exclude the possibility of HSV cross-reactivity and to study the compartmentalized B cell response in more detail, the analyses were extended by mapping the IgG epitopes of a single HSV-1 antigen: gG1. In concordance with previous studies [92-94], our data show that gG1 is not recognized by sera of HSV-2 seropositive donors or an HSV-2 positive ocular sample, illustrating that gG1 enables the analysis of HSV-1 type-specific B cell responses. Furthermore, our results suggest that the gG1-specific IgG response is mainly directed towards the primary amino acid sequence and not to the extensive post translational modifications of gG1. Therefore, the HSV-1 gG fusion protein is suitable to map the major linear epitopes of this antigen.

Paired serum and IOF samples of 16 patients with HSV-induced uveitis were analyzed for reactivity to recombinant GST-gG1. Whereas all patients had gG1-specific antibodies in their serum, 7 IOF samples did not contain detectable amounts of gG1-specific IgG. Because these IOF samples did contain antibodies against several other HSV-1 antigens when tested on blots containing total native HSV-1 protein, this again demonstrated a compartmentalized B
cell response in these patients. Among the 7 patients with anti-gG1 reactivity in both compartments, 4 showed marked differences in IgG epitope distribution on gG1 between the IOF and the serum sample. Although the other 3 patients appeared to recognize the same regions of gG1 in both compartments, this does not exclude the possibility that also these patients display differences in IgG epitopes between both compartments. The 20 aa deletions between sequential GST-gG1 deletion mutants may contain more than one B cell epitope. Furthermore, the technique employed to identify epitopes was based on discrete changes in intensity of the chemiluminescent signal and will probably not detect minor epitope regions. The immunodominant region of gG1 identified in this study maps in the central part of the protein between aa 65–125 and overlaps with the region delimited by aa 112-127 identified using gG1-specific synthetic peptides [95].

In autoimmune diseases local oligoclonal IgG production has been reported for the synovial tissues of patients with rheumatoid arthritis [97], salivary glands of patients with Sjögren’s syndrome [98,99] and cerebrospinal fluid of patients with multiple sclerosis [100]. In contrast to the current study however, the antigens recognized by these locally produced antibodies in autoimmune disease were not defined.

A possible cause that may be responsible for compartmentalized antibody responses could be that several pathogens have various stages during their life cycle that display marked differences in antigenic composition. These differences may be responsible for a compartmentalized humoral immune response against complex eukaryotic pathogens like T. gondii [88,89], but are unlikely to account for the observed compartmentalized antibody response against pathogens like HSV, that have a limited set of invariant antigenic components.

The uveitis patients analyzed in our study are likely to have acquired HSV-1 prior to development of ocular disease [101]. This implies that mainly memory B cells are triggered in the draining LN of ocular fluid and subsequently infiltrate the eye. It has been demonstrated that memory B cells do not depend on secondary LN or spleen to develop into plasma cells, but do require complementary and activated T helper cells. The ocular compartment contains several Th2 cell associated cytokines that support B cell responses and differentiation [102-105]. Ocular inflammation in patients with HSV-induced uveitis is commonly associated with the ocular infiltration of T cells directed to the triggering agent, hence the entire process of activation and differentiation into plasma cells could take place in the eye, depending on availability of specific antigen. It is not known if B cells are able to cross the blood-retina barrier without prior instruction in LN. However, in vitro studies with retinal pigment epithelial cells have shown that these cells respond to infection by secreting cytokines and expression of adhesion molecules [106], which might support chemotaxis and entry of lymphocytes into the ocular compartment.

In this paper we present evidence for a compartmentalized B cell response in eyes of patients suffering from an HSV-induced uveitis. This phenomenon is not restricted to viral infections, since analogous results have recently been observed in patients infected with the protozoan parasite T. gondii [89]. The type of immune cells activated, the combined activity of local immunoregulatory molecules and the antigenic load are likely to be the key factors to determine the intra-ocular differentiation of B cells into plasma cells upon infection with either HSV or T. gondii. HSV-specific IgG synthesized within the affected eye may have a beneficial role in clearing the virus from the eye (e.g. virus neutralizing activity) or exert a pathogenic effect on ocular cells by the induction of antibody dependent cell-mediated cytotoxicity. Future studies are mandatory to unravel the role of these antibodies in HSV-induced uveitis.
Chapter 5

The ocular humoral immune response in health and disease.

Submitted

Bob Meck, Dave Speijer, Paulus T.V.M. de Jong, Marc de Smet and Ron Peek

Short introduction
The ocular humoral immune system is divided anatomically in two distinct compartments, the ocular surface and the intraocular cavity. While cooperating to prevent infection, these immunologic compartments are very differently organized.

The ocular surface is the domain of the mucosal immune system and known to function largely isolated from the systemic immune system. This is reflected by the respective predominance of secretory IgA in mucosal secretions as compared to IgG in systemic circulation. Surprisingly little information is available on the innate, natural component of the secretory IgA response in humans, an issue that can be addressed provided the proper methods are used. By analyzing the epitope-constituting regions of a commonly found secretory IgA specific for a *Toxoplasma gondii* protein, we demonstrate directly the existence of natural antibodies in human tears and provide evidence for the existence of natural antibody producing B cells in human mucosa.

The intraocular environment is known to actively suppress cell-mediated immune responses, while humoral responses seem to be allowed to a certain extent under pro-inflammatory conditions. Following analysis of the local humoral immune response against the intraocular pathogens *Toxoplasma gondii* and Herpes simplex type 1, it appeared that the intraocular environment sustains an antibody repertoire that is completely different from the repertoire in the systemic circulation. Apparently, immune privilege of the intraocular compartment operates to such an extent that it functions independently as well.

This review will present our current knowledge on the subject, starting with basic anatomy and a description of the immunocompetent cells found in these compartments, followed by a short introduction of the pathogens and extensive analysis of site-specific antibody responses.
Table of contents

Short introduction ........................................................................................................51

Table of contents ........................................................................................................52

1 Immunologic compartments of the eye .......................................................................53
   1.1 External ocular compartment ..............................................................................53
      1.1.1 Anatomic description .................................................................................53
      1.1.2 Immunocompetent cells in the lacrimal gland and drainage system ................53
   1.2 Internal ocular compartment ..............................................................................54
      1.2.1 Anatomic description .................................................................................54
      1.2.2 Immunocompetent cells in the eye ...............................................................55
      1.2.3 Changes observed upon intraocular infection or inflammation ................55

2 Model pathogens in the eye .......................................................................................56
   2.1 *Toxoplasma gondii* and Herpes simplex virus ..................................................56
      2.1.1 Life cycle and prevalence of *Toxoplasma gondii* ......................................56
      2.1.2 Immunopathogenesis of *Toxoplasma gondii* ...........................................56
      2.1.3 Life cycle and prevalence of Herpes simplex virus .....................................57
      2.1.4 Immunopathogenesis of Herpes simplex viruses .......................................57

3 Characteristics and consequences of a compartmentalized immune response ..........57
   3.1 Antibodies are the result of somatic recombination and/or hypermutation events during B cell development .................................................................57
   3.2 Antibodies at the ocular surface .........................................................................59
      3.2.1 IgA produced by the lacrimal gland ............................................................59
      3.2.2 The natural IgA repertoire contains autoreactive antibodies .................61
      3.2.3 Factors inducing natural sIgA responses ..................................................62
      3.2.4 B cells responsible for the natural (sIgA) antibody repertoire in mice and man 63
      3.2.5 Recruitment of B cells at mucosal sites .....................................................64
      3.2.6 Initiation and continuation of natural IgA ASC .......................................65
   3.3 Intraocular antibody production .........................................................................67
      3.3.1 Gra2 of *Toxoplasma gondii* .................................................................68
      3.3.2 Glycoprotein G of Herpes simplex type I ...............................................68
      3.3.4 Immune privilege .....................................................................................69
      3.3.5 Conditions/models for local antibody responses .......................................71
      3.3.6 Which model is applicable to the eye? – Relation with antigen presentation 73

4 Future prospects .......................................................................................................74
1 Immunologic compartments of the eye

1.1 External ocular compartment

1.1.1 Anatomic description

The lacrimal gland (LG), conjunctiva, and associated structures form part of the mucosal immune system (MIS), and each component has characteristics of MIS tissues. Three components of the human eye associated lymphoid tissue (EALT) have been described so far: the LG itself, conjunctival associated lymphoid tissue (CALT), and lacrimal drainage associated lymphoid tissue (LDALT), which comprises the lacrimal sacs, naso-lacrimal ducts and surrounding tissues.

The human LG is consists of a major gland that is separated in an orbital and palpebral lobe, and accessory glands, the glands of Krause and Wolfring [107]. These glands are organized in encapsulated lobules, dispersed in the superotemporal anterior orbital fat and the conjunctival fornices [107,108]. The major gland has a tubuloacinaiar structure, while the accessory glands have a tubular structure [108,109]. In the mouse, the exo-orbital gland is located between the eye and ear, while the intra-orbital gland is located in the orbital fat [110].

In humans, tear fluid produced by the LG is collected by the ipsilateral lacrimal sac and drained into the inferior meatus of the nose via its lacrimal duct, which is located in an osseous structure between the maxillary and the lacrimal bones (figure 1). The lacrimal sac and duct are covered by a double to quadruple layer of epithelial cells with microvilli, supported by a lamina propria. Solitary and grouped goblet cells can be found throughout this epithelium, as well as serous glands with excretory ducts into the sac [111,112]. Conjunctival tissue covers the eyelids (palpebral conjuntiva) and the ocular surface (bulbar conjuntiva) to the level of the limbus. It consists of an epithelial layer with goblet cells and a stromal layer that is divided in an adenoid (deep) and a fibrous (deep) layer [113,114].

1.1.2 Immunocompetent cells in the lacrimal gland and drainage system

An exhaustive study of the immunoarchitecture of human LG demonstrated that the LG contains many antibody secreting cells (ASCs) that stain mainly positive for IgA, confirming the predominant secretion of secretory IgA (sIgA) in tears. In addition, some B cells, dendritic cells (DCs), macrophages and many T cells were observed. The ASCs are mainly dispersed in the interstitial connective tissue. Small lymphocytic foci consisting of T and B cells are also found in the interstitium and probably represent primary B cell follicles, while secondary follicle-like structures with germinal centers (GC) were only occasionally observed [108,111]. These follicles develop in response to antigens; primary follicles mostly contain naïve B cells, while secondary follicles actively generate antigen-adapted B cells (see section 3.1).

In the LG of conventionally reared mice most of the IgA⁺ B cells belong to the B1a subtype, i.e. B220low and CD5⁺
B1a cells are the prototypic natural antibody producing B cells (see sections 3.1 and 3.2.4). The LG T cell population is diverse and consists of T cells that are associated with adaptive immune responses, CD4+αβTCR+ cells and CD8+ TCRαβ+ cells, and cells that are representatives of the innate immune system: CD8+ TCRγδ+ cells and TCRαβ+ NK 1.1 cells [110]. Lymphatic vessels are present in LGs from man and mice that drain to the preauricular and cervical lymph nodes (CLN) [115,116].

Analyses of conjunctiva and lacrimal draining canaliculi obtained from human cadavers revealed that the conjunctiva has a lymphoid layer consisting of T cells and IgA+ B cells below the epithelial layer. Like the LG acinar cells, these epithelial cells express secretory component (SC), indicating that dimeric IgA is actively secreted. Intraepithelial lymphocytes were observed between epithelial cells. The stromal layer of the conjunctiva seems to contain more lymphoid follicles than LG, suggesting that, importantly, that MIS responses can be generated locally. Supporting an active role for these follicles is their location near or along high endothelial venules (HEVs). However, no GC like structures or M cells (microfold cells, columnar epithelial cells involved in passing antigens to underlying antigen-presenting cells), were seen [113,114]. The basic structure of the conjunctiva extends into the lacrimal canaliculi, sac, and nasolacrimal duct. Together, they represent the lacrimal drainage associated lymphoid tissue (LDALT). However, more structures resembling secondary B cell follicles, and follicles covered by M-like cells were found in these latter three structures [112]. Interestingly, MHC class II+ DC-like cells [112] and/or macrophages [111] were occasionally observed in the epithelium of the lacrimal canaliculi and sac. Whether DC like cells are present in the conjunctiva remains to be determined.

Thus, recent anatomical data clearly demonstrate that the human lacrimal system is part of the MIS, possessing both efferent and afferent pathways. These collectively can be identified as eye associated lymphoid tissue (EALT). A minor cautionary note may be the overall old age of individuals sampled. Importantly, when the eye lids are closed and in the absence of tear flow, considerable amounts polymorphonuclear neutrophils can be found on the corneal surface [117,118].

1.2 Internal ocular compartment
1.2.1 Anatomical description

The eyeball is subdivided in two segments separated by the iris and posterior zonular surface (figure 2). The anterior segment consists of two parts; the section between the cornea endothelium and the iris is the anterior chamber and contains aqueous humor, while the space between the iris and the lens plus zonular fibers is called the posterior chamber. The posterior segment is bordered by the lens and retina/choroid, and is occupied by the vitreous [113].

Figure 2: Diagram of the human eye

AC = anterior chamber
(from: 'The eye: Basic sciences in practice' by Forrester et al. Copyright WB Saunders company LTD).
Intraocular fluid (IOF) drainage occurs through two pathways: via Schlemm’s canal and the trabecular meshwork to the venous plexus (major pathway), and via the ciliary body to the suprachoroidal space (minor pathway), the uveoscleral route. From the suprachoroidal space IOF either drains through vortex veins, or flows through the sclera into the conjunctiva and lymphatics.

Analogous to the blood-brain barrier, the blood-ocular barrier (BOB) prevents and controls in- and efflux of cells and molecules from the systemic circulation. This contributes to the general suppression of immune responses observed in mice against intra-ocular antigens (immune privilege). In the posterior chamber, the retinal pigment epithelial cells (RPE) and the retinal vascular endothelium represent the BOB. Anteriorly, the BOB is kept intact by ciliary body epithelium, posterior iris, corneal stroma and the (avascular) cornea. The blood vessels in the iris stroma do not show characteristics of BOB vessels, but are apparently tight enough not to allow direct access of cells and molecules to the anterior compartment under normal circumstances [119].

### 1.2.2 Immunocompetent cells in the eye

Under normal conditions hardly any T, B, or ASCs can be detected in the human eye, including the iris [120,121]. Granulocytes are absent in the normal eye. Rodent studies have demonstrated that intraocular tissues, like iris, ciliary body and choroid harbor a dense network of potential antigen presenting cells such as macrophages and MHC class II+ dendritic cells [122]. In contrast, the density of CD1a+ DCs in the human retina is low. However, there is a clear ‘network’ of CD68+ macrophages of which the majority is HLA-DR+, and, importantly, an extensive network of CD45+ and HLA-DR+ microglial cells [121,123,124]. These share a common monocytic precursor with macrophages. The cells associated with blood vessels may represent perivascular cells, while those in the nerve fiber and ganglion cell layer are parenchymal microglia [121,125]. The fact that in normal human retina obtained from donor-eyes the CD45+ cells were already strongly MHC class II positive [121] may be related to post-mortem conditions, as quiescent microglial cells are reported to have limited MHC class II expression [125].

### 1.2.3 Changes observed upon intraocular infection or inflammation.

Vitrectomy or retinal biopsy specimens obtained from individuals during intraocular infection with *Toxoplasma gondii* or Herpes simplex virus show infiltration of T lymphocytes, predominantly CD4+ T helper cells with a T helper 0 cytokine secretion profile [126,127]. These T cells are able to proliferate upon stimulation with pathogen-specific antigen, and do not show autoimmune reactivity. Early studies demonstrated that B lymphocytes and/or plasmacells can be detected in the vitreous during infections with pathogens such as *T. gondii* [80,83]. A few recent studies confirmed that B cells do occur, but form a minor component of the infiltrating lymphocytes [81,82]. Interestingly, the percentage of B cells among infiltrating lymphocytes is increased in samples obtained from eyes graded as relatively mildly inflammation. This led to the interesting observation that ‘B cells are increased in ocular tissues during relatively inactive cases of uveitis’ [82]. This may also be a question of duration of the inflammation, as chronic ocular inflammation switches from a predominantly T helper to a B cell mediated process. B lymphocytes are also found in cryosections of retinas from patients suffering from sympathetic ophthalmia and in iris biopsy specimens from patients with unspecified uveitis, although generally most lymphocytes are again T cells [120,128]. In this latter study, the presence of LFA-1 (CD11a/CD18) and ICAM-1 (CD58) was observed on infiltrating lymphocytes and endothelium of HEV-like vessels [120].
2 Model pathogens in the eye

2.1 Toxoplasma gondii and Herpes simplex virus

These two pathogens are important causes of intraocular infections worldwide. Besides being responsible for an incurable, potentially blinding disease, these pathogens have several other aspects in common. They both induce mucosal immune responses, albeit at regionally different sites, cause systemic infections, and induce a compartmentalized immune response in the eye characterized by elevated pathogen-specific intraocular antibody titers. Both pathogens will be briefly introduced in the following sections.

2.1.1 Life cycle and prevalence of Toxoplasma gondii

Toxoplasma gondii, a protozoan parasite, is a common cause of intraocular inflammation, usually located in the posterior part of the eye [25,129]. The presence of the parasite in the eye can be the result of congenital or acquired infection. In the first case, infection occurs at the fetal stage as the parasite crosses the placental barrier upon infection of a seronegative mother, in the latter case the parasite is acquired later in life [23,129]. Infection can result from consumption of meat contaminated with tissue cysts, the product of asexual multiplication in warm-blooded animals. Infection can also occur from co-incident ingestion of oocysts, which are the result of sexual multiplication of T.gondii in feline and contaminate the environment via stool [1]. A recent multicentre survey among seroconverted pregnant women indicated that inadequately cooked or cured meat is the main risk factor for T.gondii infection [130].

2.1.2 Immunopathogenesis of Toxoplasma gondii

In the fetus, parasites seem to target neuronal tissue, and toxoplasmosis limited to the eye (OT) is one of the mildest forms of congenital infection [23,129]. Acquired infection probably only occasionally results in OT in most developed nations, but in some areas of Brazil it is the main mode of human transmission [131,132]. How the parasite ends up in the eye during acquired infection is still a matter of speculation. One hypothesis states that T.gondii tachyzoites (figure 3) enter the eye by co-incidence in cells that have to be replenished in the eye regularly, such as macrophages and DCs. When acquired later in life, systemic infection by the parasite is preceded by a mucosal immune response, as evidenced in experimental infection of laboratory animals [4]and the presence of slgA specific for the major surface antigen of T.gondii, Sag1, in human milk of recently seroconverted individuals [3]. T.gondii Sag1 appears to be the immunodominant antigen for slgA responses in both animals and humans. The CMIS response is followed by a vigorous cellular and humoral immune response, which forces encystment of the parasite, and elimination of free tachyzoites. Mouse experiments and human studies have demonstrated that both B and T cell responses, along with pro-inflammatory cytokine IFN-γ release, are essential for induction and maintenance of T.gondii encystment [133-136]. Of crucial importance is the fact that during encystment the antigenic composition of the parasite changes while the immune response remains directed mainly against antigens present on the surface of extracellular tachyzoites [24,137]. Perhaps due to the preoccupation with tachyzoite antigens, in combination with
limited immunogenicity of bradyzoite antigens, hardly any immune response develops against the intracellular bradyzoite or cyst antigens. The parasite remains viable in immune-silent cysts and characteristically reverts to tachyzoite at varying intervals, a significant feature of OT [25,129]. These reactivations are countered by a hefty inflammatory response, and this combination results in the gradual enlargement of the chorioretinal scars due to lysis of retinal cells, resulting in progressive loss of vision. Due to lack of appropriate animal models of OT, it is still a matter of debate which factor(s) trigger reactivation. There are no drugs available that eradicate the parasite, especially the metabolically inactive bradyzoites.

2.1.3 Life cycle and prevalence of Herpes simplex virus
Eight herpes viruses are recognized so far, of which Herpes simplex virus type 1 (HSV-1, or HHV-1), HSV-2 (HHV-2), Varicella zoster virus (VZV, HHV-3) and cytomegalovirus (HHV-5) are the major causes of ocular disease [73]. Other herpes viruses that occasionally cause ocular disease are Epstein-Barr virus (HHV-4), HHV-6 and HHV-8. Systemic infection by herpes viruses is usually initiated by mucosal contact with contaminated lesions and/or mucosal secretions of a person that actively sheds virus.

2.1.4 Immunopathogenesis of Herpes simplex viruses
Herpes simplex viruses are neurotropic pathogens, and infection of the eye can be manifested as blepharitis, keratitis or iridocyclitis. Less frequently the retina is infected resulting in acute retinal necrosis syndrome (ARN), a rapidly progressive blinding disease. Three types of Herpes viruses, HSV-1, HSV-2 and Varicella-zoster virus, are responsible for the majority of the posterior intraocular infections that result in diagnostically valuable intraocular production of virus-specific antibodies. Herpes induced keratitis results from pathogen-specific inflammatory responses in the corneal stroma [138], which are also accompanied by a virus specific secretory IgA response [139], presumably initiated by EALT. Which of the herpes types predominates in ARN depends on geographic location and age of the patient [73]. In the Netherlands, HSV-1 is the predominant type responsible for ARN [personal observation Ron Peek, personal communication George Verjans, Erasmus MC, Rotterdam]. Similar to T.gondii induced disease, HSV has a high recurrence rate and these recurrences are typically due to viruses of the same type (usually the same strain). Instead of occupying an immuno-silent cyst like T.gondii, HSV resides in the cell bodies of sensory neuronal cells like the trigeminal ganglia during the latent phase of disease. Generally it is assumed that latently infected neurons remain undetected by the immune system because they hardly express viral genes, except for the latency-associated LAT genes. However, recent findings in mice suggest that in some neurons all viral genes are expressed, and that such neurons are surrounded by inflammatory cells [140]. Analogous to T.gondii, herpetic infection can be either acquired before birth (congenital infection) or later in life, and there is no cure for HSV infection. The main difference is that most people get infected with HSV at an early age (<30 years) [141], while seroconversion percentages due to T.gondii infection increase more gradually throughout life [2]. This difference can be attributed to easier transmittance of HSV through mucosal secretions (saliva etc), compared with consumption of contaminated, undercooked meat or vegetables. As with acquired OT, the intraocular infection in cases of retinitis and ARN are preceded by a systemic infection, unless infection already occurred at the fetal stage.

3 Characteristics and consequences of a compartmentalized immune response
3.1 Antibodies are the result of somatic recombination and/or hypermutation events during B cell development
Before describing in detail about antibodies secreted in tears and IOF in sections 3.2 and 3.3, these two processes that
Chapter 5

occur during B cell development have to be discussed. Both processes fundamentally influence the specificity of the B cell receptor (BCR), or surface immunoglobulin, and thus the antibody that will be produced by the B cell upon maturation into an ASC. The specificity of the BCR essentially determines whether a B cell will develop into a conventional or a natural ASC.

The BCR is composed of a heavy and a light chain, of which the heavy chain is encoded by constant (Fc), variable (V), diversity (D) and joining (J) segments, while the light chain is made of a V and a J segment. The D, J and V segments are joined at the pro-B cell stage of the developing B cell in fetal liver and bone-marrow in a process called somatic recombination [142], mediated by the V(D)J recombinase complex that, amongst others, contains the recombination activating gene (RAG) 1 and 2 products. The specificity of each immunoglobulin is determined by the V, D and J segments combination, collectively called the immunoglobulin variable segment (IgV). Three regions within the variable segment encode parts of the immunoglobulin that directly interact with the antigen; the complementary determining regions, or CDRs. The V region constitutes CDR 1 and 2, while the joining of the V, (D) and J segments forms the third region (CDR 3). Most of the variation among antibodies occurs in CDR3, which is even further extended by a process called junctional diversification. This involves random addition and removal of nucleotides from the various segments before assembly, amongst others by the enzyme terminal dideoxy transferase (TdT). Both conventional and natural B cells have successfully gone through the somatic recombination process. At this early stage, B cells express a mixture of \( \mu \) and \( \delta \) class BCRs. Natural IgM in serum of humans and mice is produced by IgM ASCs in germline-configuration, which, ideally, contain little or no junctional diversification (so-called germline configuration). B cells that develop in fetal liver of mice and man appear to have a restricted use of V segments [143,144], thereby limiting the natural IgM antibody repertoire [145].

Conventional B cells migrate to inductive sites of the mucosal immune system, such as Peyers patches in the ileum, Mesenteric Lymph Node (MLN), adenoid, tonsils and Nasal Associated Lymphoid Tissue (NALT). As soon as the BCR has demonstrated affinity for an antigen and cognate T cell help is present, the process of affinity maturation is initiated. Affinity maturation involves somatic hypermutation in germinal centers of secondary lymphoid follicles during which the nucleotide sequences encoding the antigen binding domains of the BCR, CDR 1-3, are altered in an attempt to heighten antigen-affinity and decrease antibody-flexibility [61]. It requires prolonged exposure to antigens presented by follicular dendritic cells (FDCs) within inductive sites. FDCs are specialized DCs that express CD40L along with various Fc and complement receptors that are used to present intact antigens aggregated with antibodies and/or complement to B cells. Characteristically, FDCs are MHC class II negative [146]. The local cytokine milieu provided by stromal cells and FDCs, direct cell-cell contact with cognate T helper cells by TCR-MHC and co-stimulatory molecules such as CD40-CD40 ligand and CD80/CD86-CD28, and sustained presence of antigen allows the expression of proteins that are involved in affinity maturation, such as activation induced cytidine deaminase (AID) and error-prone DNA polymerases [147-149]. Due to the action of these enzymes, the CDR regions are altered. If successful, the B cell will leave the inductor site, possibly already class-switched to IgA and committed to dimeric IgA production as indicated by J chain production and migrate to effector sites like the LG, mature into an IgA ASC upon interaction with cognate T cells and start producing dimeric IgA.

Although in mice natural B cells have been demonstrated in PP [150], natural B cells are probably not capable of affinity maturation to the extent of conventional B cells. Natural B cells are able to class-switch, which allows them to mature into natural IgA ASC's and participate in the production of natural sIgA at mucosal surfaces. This process is not dependent on TCR-MHC interaction with cognate T cells.

In human mucosa, only the B cell population of the salivary gland and the gut lamina propria has been
The ocular humoral immune response in health and disease

3.2 Antibodies at the ocular surface

The outer eye is protected from mechanical and pathogen-induced damage by mechanical barriers and various soluble factors. The eyelid and the blink reflex form mechanical barriers and prevent respectively absorbance of macromolecules and adherence of foreign objects to the eye. Non-specific soluble anti-bacterial factors that occur abundantly in tears include lysozyme, lactoferrin and defensins (reviewed in [22] and [151]). Of the various immunoglobulin classes that can be detected in ocular tears, sIgA is by far the most abundant, with equal distribution of both subclasses [152]. Until recently, sIgA was thought to contribute only to the specific ocular immune response, but this view needs revision. Recent data demonstrate that sIgA antibodies secreted at mucosal surfaces consist of both monospecific conventional antibodies (as a result of somatic hypermutation) and natural antibodies. Natural antibodies are usually cross-reactive, hence ‘polyspecific’ or ‘less-specific’. They recognize non-self antigens of pathogens as well as self-related antigens [18,19,46,60], which can be partly attributed to the enhanced flexibility of antigen-binding regions [61]. We demonstrated that these natural antibodies can be distinguished from specific antibodies by antibody epitope analysis (see next section).

3.2.1 IgA produced by the lacrimal gland

As already suggested by the immunohistochemical data, lacrimal fluid of many species contains predominantly IgA antibodies. Compared with other antibody classes and other mucosal sites the proportion of IgA in the LG is substantially higher, indicating that the LG selectively retains IgA positive B cells, and/or strongly supports IgA class switching. Although not much is known about the B cell populations present in the human LG, a general idea can be obtained by analysis of sIgA specificities in tears.

It can be assumed that the antibodies secreted at any human mucosal surface are a mixture of monospecific and polyspecific IgA antibodies, but it is very difficult to estimate ratios. For the LG, it is known that immunogen- or pathogen-specific antibodies can be detected in tears following mucosal immunization via the conjunctival, nasal or oral route [10]. Clearly, affinity matured B cells producing monospecific antibodies are able to find their way to the LG, and this phenomenon has been used as proof for the existence of the common mucosal immune system (CMIS) in humans. Tears generally contain antibodies directed against a range of common bacterial, viral and parasitic pathogens, such as *Staphylococcus epidermidis*, *Streptococcus mutans*, herpes simplex virus, influenza virus, rhinovirus and *Toxoplasma gondii* [9,22,26].

Since large groups of individuals have antibodies with specificities for these pathogens in their tears, they are confusingly categorized as ‘naturally occurring’ IgA antibodies. These antibodies could either be the result of specific CMIS responses, as most of these pathogens are able to chronically colonize mucosa, or represent natural antibodies. For example, Herpes virus induced keratitis is associated with increased secretion of virus-specific IgA in tears, which might very well be due to a specific MIS response via EALT, but keratitis-negative individuals usually have a ‘background’ anti-HSV titer [139,153] that most likely can be attributed to natural antibodies.

In case of *T. gondii*, this parasite does not chronically infect mucosa, but is a frequent cause of systemic infection based on seroconversion data [2]. Therefore, the frequent presence of lacrimal anti-*T. gondii* IgA antibodies may suggest that *T. gondii* in its frequent attempts to pass the mucosal barrier of the gut induces a mucosal IgA response. However, a longitudinal study of the anti-toxoplasma IgA antibodies demonstrated that both titer and composition...
hardly varied over time [26], which is a characteristic of natural IgM titers in serum [57,145]. A remarkable property of natural IgM titers is that they are quickly restored following selective depletion, which indicates that the natural IgM specificity palette in serum is tightly controlled [57].

There are only a few experimental approaches available that might establish the origin of particular antibody responses. The most direct approach would be isolation and characterization of the antibody secreting cells [110], but human LG material is very difficult to obtain. Another approach could be to characterize the polyspecificity of a set of antibodies, or antigen-binding properties of particular antibodies, by selection of antibodies for a specific antigen and subsequent elution and incubation with a range of other antigens [18,19]. In these experiments, monospecific antibodies are not expected to bind to other antigens. A disadvantage of this method is that it requires a large amount of antibodies from tears, which is difficult to obtain from any species. Nevertheless, both methods have been used successfully in other settings and by exploiting the results from these studies we adapted an alternative, sensitive approach to determine the origin of particular antibody responses not requiring a vast amount of human material (see table 1, conventional versus natural antibodies) [154]. By analyzing the epitope containing regions of protein disulfide isomerase (PDI), an antigen commonly recognized by anti-\textit{T. gondii} antibodies in tears (see figure 4), we found that IgA recognized exclusively conserved regions of this protein in human tears [155]. This observation also implies that these antibodies should be able to recognize PDI of other species, which we indeed confirmed experimentally. As these antibodies recognize conserved stretches of this protein, already produced by infants, we concluded that the IgA antibodies consistently observed in tears must be natural antibodies. In comparison, forced immunization by intra-muscular injection of recombinant PDI along with adjuvant does result in anti-PDI specific
Figure 4: Analysis of Protein Disulfide Isomerase epitopes recognized by secretory IgA.
(Upper panel): Blots containing size-fractionated T.gondii lysate proteins were incubated with tears of two volunteers (V1 and V2) and two infants (B1 and B2, 3 and 6 months of age), and human milk (Mi). The band stained at 49 kDa by lacrimal fluid and milk sIgA is protein disulfide isomerase (PDI) [54]. The open reading frame encoding T.gondii PDI was cloned into a glutathion-S-transferase (GST) expression vector. The GST moiety allows easy purification of fusion proteins. Immunization of a rabbit with recombinant PDI resulted in the induction of anti-49 kDa antibodies (see insert). Recombinant GST-PDI fusion protein was also recognized by tear sIgA, and using truncated versions A5 through A10 it was possible to determine the epitope containing regions of PDI. Deltas 5 through A10 constitute the regions of PDI that are most conserved among PDIIs of different species, and include the thioredoxin-like domain [72]. This domain is essential for the enzymatic oxidation/isomerization activity of PDI [30]. Note that the truncated proteins differ only 8-10 amino acids in size, while B cell epitopes generally range between 10-15 amino acids. (Lower panels): Following immunoblotting, IgA in samples of volunteers recognized two major epitopes. Epitope region 1 contains a characteristic VKVTVV motif that is moderately conserved among PDIIs (V1), and epitope region 2 involves the highly conserved thioredoxin like domain (V2).

IgG, but these antibodies do not recognize PDI of other species and have other epitopic specifications. This indicates that antibodies generated by forced immunization are directed against species-specific regions of PDI [155]. The method used strongly relies on the recognition by particular antibodies of linearized (recombinant) antigens. Provided this requirement is met, the method can be used to analyze any ‘naturally occurring’ antibody. Their existence implies the presence of a distinct population of natural antibody secreting B cells in the human lacrimal gland.

The following sections will try to describe the phenotype of these B cells, based on research carried out in mice regarding B1 and B1-like cells, and compare these findings with the limited data on natural antibody responses in human mucosa. First we will discuss recent experiments that might explain how IgA ASCs are specifically retained in the lacrimal gland, and speculate on factors that initiate and drive the secretion of natural antibodies.

3.2.2 The natural IgA repertoire contains autoreactive antibodies

Few studies have analyzed the cross-reactivity of natural IgA in human mucosal secretions. They demonstrated that a (high) percentage of the natural secretory IgA antibodies recognize self-antigens [19,53], similar to the natural IgM repertoire in serum which is known to recognize self-antigens [46,57]. In general, the avidity of self-recognition by natural IgM is low and does not lead to autoimmunity. However, in some autoimmune diseases, there is a general elevation of autoimmune IgM levels, for example in rheumatoid arthritis. Elevated levels of IgG and IgM antibodies

<table>
<thead>
<tr>
<th>Protein Function [19,53,54,56,68-70]</th>
<th>Species specific function</th>
<th>Conserved function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age [23,26,56,57,71,155]</td>
<td>Relation with age</td>
<td>No relation with age</td>
</tr>
<tr>
<td>Epitope containing Regions [155]</td>
<td>Species specific stretches</td>
<td>Conserved stretches</td>
</tr>
</tbody>
</table>

Table 1: What are the characteristics that distinguish conventional from natural antibodies?
that bind the Fc portion of IgG are responsible for the so-called rheumatoid factor [156,157]. It should be noted that IgA deficiency is associated in man with autoimmune disease in man, very likely due to a compensatory increase in IgM levels and the enhanced complement binding capacity of IgM as compared to IgA.

There are two potential problems associated with production of local autoantibodies recognizing auto-antigens. The first is that cross-linking of surface Ig might lead to auto-activation/maturation of the B cell, a process known as type 2 T cell independent (TI-2) antibody response. Type 2 TI antibody responses have proven to operate against cytopathic viruses with a highly structured surface coating, such as Vesicular Stomatitis Virus (VSV) in mice and human polio virus [158], and can be induced artificially by haptenated antigens. Type 1 TI B cell responses follow aspecific cross-linking of the surface-Ig by LPS.

There are mouse-models in which the presence of high concentrations of natural autoantibodies results in autoimmunity [157]. For instance, mice transgenic for an IgM directed against red blood cells develop hemolytic anemia [159]. However, it is not just the ‘specificity’ of the natural antibody or surface localization of the self-protein that determines whether autoimmunity will develop. In mice transgenic for Thy1 expressed on the surface-of lymphocytes and Thy-1 specific natural IgM, the production of anti-Thy 1 IgM is not inhibited (so B cells are not tolerized or deleted in bone-marrow). The titer even proved to be dependent on the expression of surface Thy-1 [160]. Although it is not known whether PDI is found on the surface of stromal, epithelial, or other cells in the LG or conjunctival lamina propria, this protein can be present on the cell surface of hepatocytes, lymphocytes and platelets [35,36,63], thus being able to bind anti-PDI antibodies.

Because type 2 TI antibody responses require a strictly organized pattern of surface-antigens that has never been observed on eukaryotic cells (‘self’), it does not apply to the PDI and anti-PDI antibody combination. The requirement for type 2 TI responses is met only for highly structured viral coats of certain viruses, like polio, while surface proteins on eukaryotic cells never acquire a structured organization. As only ‘non-self’ is sometimes characterized by structured surface coating, this combined with specificity of the BCR seems to be a safe selection criterion for type 2 TI responses [158].

The other possible problem is that a T cell dependent antibody response is induced that leads to local production of antibodies. This has been observed in a particular form of breast cancer, in which tumor-regression is correlated with the local production of anti-actin (self) antibodies and, probably, unusual expression of actin on the surface of cancer cells, allowing accumulation of antibodies aggregated to these cells [161], thereby marking them for destruction by granulocytes and NK cells and activated scavenger cells like macrophages and DCs.

If PDI were present on cells in the lamina propria of LG, this does not per se imply the development of autoimmune disease. Dimeric IgA is rapidly discarded through the secretory pathway, and hardly activates complement.

Another potential function of polyreactive slgA antibodies, suggested by Murakami and Honjo, could be clearance of denatured self-antigens through the SC pathway [47]. This possibility remains to be investigated.

3.2.3 Factors inducing natural slgA responses

What induces natural slgA production in mucosa? Plausible answers came from analyses of gut commensals, as both commensals in man and mice are covered with slgA [48,162]. Moreover, immunoblots and ELISAs with bacterial extracts also show an intense slgA staining pattern and signal [163,164]. In addition, the significant reduction of total slgA secreted in germfree mice can be reversed by introduction of a set of colonizing bacteria [163,164]. Therefore, the answer may be that the development and composition of the commensal population selects B cells with affinity for bacterial antigens, presumably from the peritoneum and pleural cavity. There is tendency to focus on particular
bacteria that can be held responsible for the induction of natural antibody secretion, for example in mice gram+ segmented filamentous bacteria [164]. With regard to the anti-PDI antibodies; PDI is a eukaryotic enzyme, suggesting that perhaps fungi attempting to colonize mucosa may have to be included as inducers of natural slgA responses. IgA secreted in human tears or saliva is known to react with bacteria or bacterial components, and these can readily be detected at a very early age [55,165]. Thus, colonization of the mucosa may be the main drive for natural slgA production in humans as well. However, even under germfree conditions a low titer of slgA is detected in mice that seems not to be directed against bacteria [166]; there may be other factors involved in secretion of these antibodies. An interesting concept is that certain germline antibodies, like the anti-phosphorylcholine (anti-PC) and anti-phosphatidylcholine (anti-PC) antibodies are secreted by default because of their proven evolutionary effectiveness against infection. This might explain why, for instance, anti-PDI antibodies can be found in both tears of babies and human milk. This concept seems to be consistent with the natural IgM repertoire [57,145], and may very well be applicable to the natural slgA repertoire as well.

Unfortunately, most of the experimental evidence is derived from the gut, as little is known of the natural antibody responses at other mucosal sites, which are likely to be differently organized. In order to investigate this, a comparative phenotypic analysis of B cells should be made between intestinal and ocular surface associated mucosa.

3.2.4 B cells responsible for the natural (slgA) antibody repertoire in mice and man

In contrast to humans, mice have well characterized sets of B cells that are associated with secretion of natural or specific antibodies in serum and mucosal sites. The already mentioned B1 population in mice, held responsible for production of natural IgM and slgA, can be subdivided into B1a and B1b cells based on the presence or absence of CD5, a marker that is always and exclusively linked with production of natural antibodies in mice and man. A general distinction between the two B1 and the B2 cell populations is based on the location from which they migrate to effector tissues in the adult mouse. B1 cells can be found in the peritoneal and pleural cavity, and recent data suggest that the peritoneal B1 population fluxes from the peritoneal cavity via the omentum and MLN to the gut lamina propria and vice versa [48,167,168]. In contrast, following primary activation at induction sites of the MIS, B2 cells generally circulate to the spleen and secondary lymph nodes, like the MLN, before they finally migrate to MIS effector sites and complete their maturation. In man, CD5+ B cells can be found in peritoneal and pleural cavities, but the population is relatively small [169,170]. There are a few surface-markers that allow distinction between murine B1 and B2 cells: B1 cells in the peritoneum characteristically are IgM high, IgD low, B220 low and Mac1+ to low, while naïve B2 cells are IgM low, IgD high, B220 high and Mac1+. B1 cells keep the Mac1 marker after class-switching, but it is lost during final maturation into an ASC [171]. Unfortunately, markers that separate B cells in relation to the antibodies they produce have so far not been found in other species, including man.

Another difference between B1a and B2 cells may be found in their temporal development: B1a cells develop only early in ontogeny from fetal omentum and liver, and are responsible for the primary antibody production following birth. This population is not expanded after birth, but the self-replenishing capacity keeps the B1a population intact during life, which might explain the stability of the natural antibody repertoire. In contrast, B2 cells are derived from bone-marrow and this population develops after birth. The B1b cells that enrich the peritoneal population during life might be derived from pro-B cells whose surface Ig proved to have low self-avidity. Pro-B cells with high avidity are clonally deleted at this stage. Recent findings suggest that B cells expressing self-reactive BCRs and secreting autoreactive antibodies, can also have escaped clonal deletion by co-expression of a second, non-self reactive BCR [172,173], although this requires compromising the principle of allelic exclusion that normally ensures expression of
only a single BCR. In addition, expression levels of the BCR seems to be important, as activation of B1 cells is highly dependent on BCR signaling [173]. Whether B1a CD5+ cells can be generated during life, hence whether they can be regarded as a lineage separate from B2 cells, remains a matter of vivid debate [67,174]. CD4 expression during B cell development may be a marker that separates B1a cells from B2 cells, as fetal and neonatal pro-B cells do not display CD4, while pro-B cells isolated from adult bone marrow generally express CD4 [175]. Interestingly, a new developmental pathway for B cells has been observed in μMT (μ heavy chain) KO mice lacking expression of IgM and IgD. This mutation blocks development of most B cells, including the peritoneal B cells, at an early pro-B cell stage, except for a small population of B cells that develop into IgA ASCs responsible for IgA production in serum and gut in these μMT mice [166]. The secretion of slgA by these B cells is correlated with the presence of commensals, rudimentary PP and (M)LN, while IgV sequences of isolated B cells show the entire spectrum with germline configuration, N-additions, and somatic (hyper-) mutations. This suggests that B cells derived from this developmental pathway are also able to adapt their antibodies. Next to fetal and adult B1 cells, the existence of these B cells seems to support the theory of independent B lineages.

In summary, current data indicate that 'low avidity' self-reactive B cells acquire at least a B1 like phenotype and are directed to/collection in the murine peritoneum, from where they may be available for participation in producing natural slgA. In humans, slgA present in human milk, saliva and tears was demonstrated to have self-reactivity, and the B cells responsible might very well be acquired from the self-reactive population present in the nearby pleural cavity. Important for this review is the type and specificity of germline IgM and IgA antibodies generally associated with early B1a cells in mice: the IgM component has proven to be functionally active in the defense against bacterial and viral pathogens [176]. Classic examples are antibodies with specificities for PrC (autoantibody) and PC [157]. Apparently, certain VDJ combinations result in an antibody with an antibiotic-like anti-pathogen specificity, and are capable of affecting broad ranges of pathogens. This general resistance to pathogens that may have developed in evolution is commonly referred to as innate humoral memory [67,174], and the anti-PDI antibodies might very well be part of this memory because PDI can act as an adhesion molecule for prokaryotic and eukaryotic pathogens. Antibodies, such as anti-PDI slgA, are likely to be produced by B1a cells. However, studies that have compared B cells from mucosa of corresponding tissues of humans and mice have shown clear differences between these two species. It is therefore important to remain cautious with interspecies extrapolations. Although their existence has been suggested by others [19], B1 like cells secreting antibodies in germ-line or only slightly altered configurations have so far not been demonstrated in mucosal tissues of humans. This might be related to the high antigenic load of the gut and perhaps to a more strongly developed adaptive antibody generating machinery. Mature B cells with the potential to secrete antibodies in germ-line configuration have only been found in adult peripheral blood [177,178], fetal spleen and fetal liver [179]. In children, IgA ASCs isolated from the gut already have mutation percentages similar to adults [180]. In addition, the self-replenishing peritoneal B cell population, largely CD5+, seems to be much smaller in man [169], and recent immunohistochemical analyses of gut LP lymphocytes did not show any CD5+ B cells [181]. The contribution of peritoneal and/or pleural B cells to the secretion of IgA at mucosal sites might thus be rather limited in humans. However, especially mucosal sites with a lower antigenic load, such as the ocular surface, have not yet been analyzed in detail.

3.2.5 Recruitment of B cells at mucosal sites

Remarkably, the density of IgA positive B cells in the LG is as high as in the colon, and much higher than in the salivary and mammary gland [182]. The high density in the colon is most likely related to the extensive population of
commensals and/or prolonged exposure to food-antigens [182], both known to be inducers of (natural) IgA responses. Whether most of the IgA ASCs in LG originate from immunologic exposure in the conjunctiva and/or lacrimal drainage system, but the anatomical data of EALT do not show a very extensive set of primary or secondary follicles that can be held responsible for local antigen-adapted IgA ASCs. Hence, it is unlikely that conjunctival exposure is responsible for all IgA LG ASC. It is more likely that they are recruited from distant mucosal sites and/or the pool of natural antibody producing B cells. Surprisingly little is known about the expression of adhesion molecules and chemokines on endothelial cells and immunocompetent cells in LG that might explain the selective retention of IgA ASC in the human or mouse LG. In the murine LG, a low level of expression of VCAM-1 on blood vessels has been observed in normal tissue, that is upregulated during inflammation. The MIS associated adhesion molecule MAdCAM-1 was not detected [183], but this protein and its cognate molecule integrin α4β7, present on lymphocytes has never been observed on HEVs, or cells from mucosal tissues or associated lymph nodes (LN) of the upper airway tract and throat [182]. In man, the expression of adhesion molecules on LG lymphocytes is no different from lymphocytes in the mesenteric LN, with prominent expression of L-selectin [183], an adhesion molecule that is highly expressed on naïve lymphocytes bound for secondary LNs. The role of specific adhesion molecules in directing lymphocytes to mucosal sites remains to be investigated. Some lymphocytes in LDALT have the α4β7 integrin on their surface [112], which also has been demonstrated on CD8⁺ intra-epithelial lymphocytes in the lamina propria of the gut [184]. It is hypothesized that expression of this integrin is induced locally by TGF-β, a cytokine that is produced by stromal cells in the murine gut [185]. CD8⁺ lymphocytes (see section 1.1.2), as well as expression of TGF-β 1 and 2 has been detected in the LG [186].

Chemokines are also involved in regionalization of the MIS response. TECK, or thymus-expressed chemokine (CCL25) selectively recruits B cells already committed to IgA production and is mainly expressed in the small intestinal mucosa, but not in lungs or salivary gland [187,188]. Regionalized expression of TECK corresponds with expression on lymphocytes of the ligand CCR9 [187]. There are probably chemokine and/or adhesion ligand/receptor sets that are selective for the upper respiratory tract and LDALT which have yet to be discovered. It is also possible that recruitment of B cells to the LG is not restricted by regional boundaries. IgA ASCs also show excellent chemotactic responses to SDF1-α (CXCL12), compared with IgG and IgM ASC cells [187]. The receptor of SDF1-α, CXCR4, is commonly present on leukocytes. SDF1-α is produced at many mucosal sites [189], but whether it is present in the LG remains unknown. Expression of this chemokine in combination with production of TGF-β, a stimulatory cytokine for IgA class-switching, by LG cells might be sufficient for selective terminal differentiation of IgA⁺ B cells into ASCs. It is important to realize that the process described above involves recruitment of B cells already committed to IgA production. This might very well be applicable to conventional (B2) B cells as these are committed for IgA upon departure from the mucosal inductor site, but whether this will be valid for natural B cells depends on how and where natural B(1) cells become committed to IgA production. Of interest here is that B1b cells in the peritoneum already show Fc-α expression [190,191]. Other factors that have proven to be selectively involved in the differentiation of peritoneal B1 cells into IgA ASC in the gut LP are IL-15 and IL-5, produced by epithelial cells and activated T helper 2 cells, respectively [191].

3.2.6 Initiation and continuation of natural IgA ASC
An intriguing question is how natural B cells end-up in mucosal organs such as the lacrimal gland and how the internal milieu allows them to mature into IgA ASCs. Again, this issue has been most extensively studied in mice. As
mentioned, there appear to be two critical selection areas involved in natural IgA production in the gut: the mesenteric lymph node and the lamina propria [48,167,168]. Perhaps a similar route applies to the human LG, involving the draining lymph node of LG and natural antibody producing cells from the pleural cavity, a site related to the peritoneum in so far as it stores natural antibody producing B(1) cells.

Recent studies in mice have indicated that the entire process of B cell differentiation resulting in production of dimeric IgA can take place in the lamina propria (LP) of the gut without involvement of Peyers patch-like structures or the mesenteric lymph node. In this process, stromal cells, bacterial LPS and, most likely, LP dendritic cells are able to provide an entire spectrum of signals allowing immature IgM+B cells to switch to the IgA class [185]. LP dendritic cells (LP-DC) appear capable of crossing the epithelial barrier without breaking the junctional seal and directly sample the luminal content, which probably results in local presentation of bacterial antigens [192]. This might be an important selection criterion for retention/maturation of immature B cells and provides an explanation for how natural slgA antibodies are continuously selected and produced, as commensal composition will not change drastically over time. Aspecific stimulation of lamina propria B cells by LPS may further contribute to the maintenance of an entire spectrum of natural slgA antibodies [164]. This process appears to function largely independently of T cells, as TCR β/δ KO mice still produce IgA in the murine gut [163]. In contrast, slgA responses induced by mucosal immunization are dependent on T cells and appear to be short lived compared with subcutaneous or intramuscular immunizations, indicating a limited capacity of the MIS to generate memory responses [164]. Therefore, maintenance of both natural and conventional antibody responses may require regular exposure to antigens and, perhaps, aspecific stimulators like LPS. For natural antibodies with anti-bacterial specificities this entire process requires proximity of a bacterial
The ocular humoral immune response in health and disease

Figure 5: Glycoprotein G (gG) of HSV-1 is differentially recognized by IgG in serum and ocular fluid. Intraocular fluid (o) and serum (s) were collected from a patient suffering from HSV-induced ARN. An extract of HSV-1 was subjected to SDS-PAGE and subsequently blotted. HSV-1 blot strips were incubated with paired intra-ocular fluid and serum samples. (A): There are both similarities and clear differences in individual HSV antigens recognized by anti-HSV IgG in intraocular fluid and serum compartments. To determine whether these differences can only be observed in recognition of intact antigens, or may even be extended to antigen epitope regions, gG1 was expressed recombinantly and purified as a GST-fusion protein. This is the only protein that allows definite serological distinction between HSV-1 and HSV-2, but is only a minor component of the humoral response against HSV, hence not always strongly recognized by IgG. (B): Using purified recombinant gG1 N-terminally fused to Glutathion-S-Transferase (GST), this patient proved to have gG1 specific antibodies in both compartments and was a suitable candidate for analysis of epitope-containing regions. (C): A series of C-terminally truncated GST-gG1 was produced, purified and blotted in approximately equimolar quantities. (D): Blots-strips containing GST-gG1 Δ1 through 8 were incubated with paired patient samples as indicated above the figures and stained for IgG. Epitope regions were recognized by abrupt decreases in IgG staining intensity in consecutive truncations of gG1 (marked with △). Previous experiments already indicated that there is a immuno-dominant region (IDR, between amino acids 85 - 145) predominantly recognized by antibodies [95], and this region is indeed exclusively targeted by serum IgG, as demonstrated by the stepwise reduction in signal from Δ2 to Δ5. In contrast, ocular IgG clearly recognized a different part of gG1 and bound to sequences between Δ5 and Δ7 (amino acids 45 -85). Clearly, the compartmentalization of the humoral response against HSV is extended to the epitope-level.

3.3 Intraocular antibody production. For decades the existence of compartmentalized humoral immune responses in the eye has been used to confirm the clinical diagnosis of an intraocular infection by pathogens such as T. gondii, Herpes viruses (HSV-1, HSV-2, VZV) and Borrelia. Local production of antibodies is confirmed by a high Goldmann-Wittmer coefficient (GWc), which is the ratio of the local pathogen-specific titer x total systemic antibody titer over the systemic specific x total local antibody titer. A coefficient of over 3 is considered proof of a specific intraocular infection [77,86,87,193]. During an initial intraocular infection affecting the posterior chamber calculation of the GWc may be the only reliable method to confirm or establish a clinical diagnosis. Until recently, surprisingly little information was available regarding the specificity of the antibodies involved. Our group has successfully addressed this issue by analyzing the IgG antibody responses following intraocular infection by T. gondii and HSV-1 [89,194]. We subsequently provided evidence for selective maintenance of a particular set of antibody secreting cells within the eye, possibly related to its status of immune privilege. Before summarizing the results and discussing possible immunologic mechanism(s) behind compartmentalized immune responses in immune population, which may represent a difference with the LG. However, bacterial and viral organisms are detectable in human tears and conjunctival sacs (reviewed by [9,22], indicating that the LG-MIS is not at all a sterile environment. Especially at night, when production of tears is markedly decreased [118], pathogens on the ocular surface may even directly access the LG. This may explain the existence of primary and occasional secondary lymphoid follicles in the LG, as well as the presence and function of dendritic cells within the LG. Whether the LP-DC system also operates in LG, LDALT, and/or mammary glands remains to be investigated.
privileged structures like the eye, we will first discuss the antigens that allowed us to study compartmentalization at the antibody-epitope level. The pathogens from which these antigens are derived have been described in sections 2.1.1 and 2.2.1.

3.3.1 Gra2 of *Toxoplasma gondii*

Gra2 is one of the few proteins expressed by both the tachyzoite and the bradyzoite stage of *T. gondii*. Gra2 is located in the dense granules of tachyzoites (figure 3). Dense granules release their content within 20 minutes following infection and the Gra proteins are involved in the formation of the parasitic vacuole following infection of a cell. Gra2 forms an integrated membrane-associated protein complex with at least two other Gra proteins, Gra4 and 6 [195]. This intravacuolar membrane complex probably serves to enhance the contact between the cytoplasm of the host-cell and the parasitic vacuole and might be involved in the exchange of metabolites across the vacuolar surface [196].

3.3.2 Glycoprotein G of Herpes simplex type I

Glycoprotein G (gG) is one of the surface mantle proteins of HSV, and therefore is usually the subject of antibody responses. Importantly, it is the only protein of HSV-1 and its closest relative HSV-2 that allows serotyping [93]. It is the only protein suitable for analysis of compartmentalized antibody responses specific for HSV-1 infection.

3.3.3 Analysis of local antibody production

Using immunoblotting of an extract of *T. gondii* tachyzoites or HSV-1 virions, we directly demonstrated that there are differences in the overall composition of antibodies between the two compartments. As can be seen in the example given in figure 5A, the anti-pathogen IgG content of serum and ocular fluid differs radically in patients with an established GWc of 3 or more. In cases with a GWc lower than 2, there are no differences in pathogen-specific IgG content, and the intraocular pathogen-specific titer can be largely attributed to extravasation of antibodies from the
The ocular humoral immune response in health and disease

Figure 6: Analysis of epitope containing regions recognized by anti-Gra2 IgG in serum and ocular fluid (modified from [89]. Copyright 2001, the American Association of Immunologists, Inc.).

(A): An example of the compartmentalized immune response against Toxoplasma gondii is found in the analysis of anti-Gra2 antibody response of a patient with posterior uveitis. Using recombinant T. gondii-Gra2 fused with GST, this patient proved to have anti-Gra2 antibodies in both serum and ocular fluid. (B, C): Following delineation of the major epitope-containing regions using GST-Gra2 AA through ΔC, the region between ΔB and ΔC proved to be the major target. Especially ocular fluid intensely recognized full length Gra2 to ΔB, while also ΔC was still recognized, indicating that there is a minor antibody epitope between amino acid 1 and 36. There were no serum antibodies that recognized ΔC. Therefore, Gra2 ΔB was C-terminally truncated according to the scheme shown in (B), purified and blotted. Note that the differences in amino acid number between the various truncated versions of Gra2 ΔB is in the antibody epitope range (10-15 aa). Paired serum and ocular fluid were incubated with the various blot-strips with ΔB, ΔB1 to ΔB10 and ΔC. The anti-gG1 antibodies in intraocular fluid and serum of this patient proved to have one region in common (between ΔB1 and ΔB2), while the second region recognized was unique compartment (marked with ▲).

serum compartment. In these latter cases, the pathogen-specific serum titers are usually high. Further evidence of a compartmentalized immune response was provided by the analysis of epitope containing regions of single antigens. Immunogenic proteins of each pathogen, Gra2 and gG, were selected for these analyses. By sequentially truncating the recombinant Gra2 and gG1, we demonstrated that humoral immune responses against specific antigens differ at the level of epitopes between the two compartments (see figure 5, gG1 and figure 6, Gra2). Using IEF-immunoblotting, a similar difference in pathogen-specific IgG staining patterns between liquor and serum in human neuroborreliosis was observed, directly proving compartmentalized immune responses [197]. However, differences at the epitope level were not investigated in more detail.

In autoimmune diseases, local oligoclonal, compartmentalized IgG production has been reported for the rheumatoid synovial tissues [97], salivary glands in Sjögren’s syndrome [98] [99] and the cerebrospinal fluid (CSF) of patients with multiple sclerosis [100,198]. In some patients with multiple sclerosis the locally produced antibodies proved to be high affinity anti-DNA antibodies, a common target of natural antibodies [199]. However, in most of the other studies the antigens recognized by locally produced antibodies were not investigated or could not be defined.

It is not known whether natural IgM or IgG can be detected in IOF. There are no specific studies addressing this issue. However, the observation that laboratory analysis of IOF is usually not hampered by background staining due to cross-reacting antibodies indicates that natural antibody titers will be low to nil. As the titers of particular natural antibodies are generally low in serum, this will prevent intra-ocular accumulation due to extravasation, hence detection.

3.3.4 Immune privilege

The fact that pathogen-specific antibodies are locally produced following intra-ocular inflammation is well established and, as mentioned earlier, is used for laboratory diagnostic purposes. These intraocularly produced antibodies may play an essential role in immunoprotection, as cell-mediated immunity (CMI) is suppressed in the eye. This suppression of cell-mediated innate and specific immunity, and the preservation of humoral immunity, is a trait of structures like the eye, testis and brain. How this ‘immune privilege’ status is maintained will be briefly described in this section.
CMII suppression is achieved by soluble and membrane-bound factors. Prominent soluble factors found in IOF of mice that provide CMII (down-)regulatory effects on T cells are the neuropeptides α-melanocyte stimulating factor [200], vasoactive intestinal peptide [201], and substance P [201]. For an overview of immune-regulatory neuropeptides and cytokines found in IOF, see [84]. IOFs also contain TGF-β2 [102], a cytokine well known for its T helper 2 skewing capacity following induction of antigen specific immune responses, and its inhibitory effect on T cell activation and differentiation [85]. In an attempt to determine how the intra-ocular milieu affects immune-competent cells as soon as they enter the eye, DCs derived from immature monocytes were incubated in vitro with human aqueous humor (figure 7). Using differential display and RT-PCR, we demonstrated that exponents of CMII (STAT1, a regulator of IFN-γ signaling) and innate immunity (Toll-like receptor 4) are downregulated in DCs exposed to aqueous humor.

Studies that focus on the mechanism responsible for the phenomenon of anterior chamber associated immune deviation (ACAID) indicate that TGF-β2 probably acts primarily on antigen presenting cells, hence on the afferent immune responses [104]. Peritoneal macrophages incubated with this cytokine in combination with a specific antigen are able to downregulate systemic Th1 responses towards the antigen. This is mediated by CD8+ suppressor cells, a cell-type known to be ultimately responsible for ACAID [202]. However, under normal circumstances, intraocular TGF-β2 is inactive or latent [203]. The mechanism of activation of latent TGF-β2 in the eye is not known, though it may parallel TGF-β1 activation. Latency of TGF-β1 is most often achieved by covalent coupling to the large or small latency associated peptide (LAP), by association with the protease inhibitor and normal serum component α2-macroglobulin, or by binding to various TGF binding proteins [204]. Association with α2-macroglobulin can impose latency of TGF-β2, but α2-macroglobulin is normally absent in IOF. Besides acid activation of latent TGF-β2, which may be not be relevant under physiological conditions, the LAP associated form can be activated by exposure to various proteolytic enzymes such as plasmin. Interestingly, thrombospondin-1 and IgG are able to release TGF-β1 from the LAP complex by inducing a conformational change in the complex [204]. Further investigations need to be conducted to elucidate the effect of thrombospondin, present in the normal eye [205], and IgG on the activation of latent TGF-β2.

Several cell types can be held responsible for the continuous production of the soluble factors mentioned above, among which microglial and RPE cells [106,119,206,207]. Because MHC class I expression on cells in the eye is limited, cytotoxic immune responses are mediated by NK cells rather than cytotoxic CD8+ T cells. In IOF of rabbits, both active TGF-β2 and macrophage migration inhibitory factor inhibit NK mediated cell-lysis [208]. Neutrophils are also inhibited by soluble factors in IOF, amongst others by TGF-β [209].

The most highly expressed and extensively studied membrane-bound factor capable of influencing CMII responses in immune privileged sites is the Fas-L receptor (CD95-L) and its cognate ligand, Fas protein (CD95). Fas is present on most T cells, NK cells, and granulocytes that enter the eye. Interaction with Fas-L causes apoptosis of these cells. Fas-L is expressed on various structures in the eye, such as the cornea, iris, ciliary body, and retina, and on T cells and macrophages [210,211]. Both membrane-bound and soluble forms of Fas-L exist (sFas-L), and sFas-L has affinity for fibronectin, a component of the extracellular matrix [212]. Interestingly, while in ARN patients the expression of surface bound Fas and FasL seems to be decreased [213], the levels of sFasL in IOF of uveitis patients are increased [214]. Although sFasL is not as potent as FasL in apoptosis induction, it may have an equally important regulatory role [215].
RNA was extracted according to standard procedures. Differential display was used to analyze differences in expression pattern, which is an ideal method to identify changes in relevant gene transcripts in spite of the minimal amounts of RNA available [227]. (A): Two transcripts were markedly downregulated following AH exposure, and were identified to encode STAT1 (signal transducer and activator of transcription 1) and TLR4 (Toll-like receptor 4). (B): The down-regulation of the STAT1 transcript was confirmed with RT-PCR with β-actin as control. Both down-regulation of STAT1 and TLR4 under the influence of aqueous humour is consistent with the concept of moderation of eye immune responses as STAT1 is a major transducer within the IFN-γ signaling complex [228], and down-regulation makes DCs less sensitive for the major T helper 1 cytokine IFN-γ. TLR-4 is the receptor for lipopolysaccharides (LPS) and an important component of the innate immunity regulated by IFN-γ [229,230].

Next to Fas-L/Fas, the p55 TNF-α receptor interaction has also been demonstrated to be important in immune regulation. It is important in rapid control of intra-ocular spreading of intracranially administered rabies virus in mouse. This limiting effect was attributed to infiltration of T-lymphocytes and neutrophils, and this apparent temporal/local breach of immune privilege seems to be mediated by expression of the TNF-α receptor [216].

Clearly, the factors responsible for immune privilege strongly inhibit both innate and adaptive CMI, but CMI is allowed to operate within a limited time frame following acute infection by pathogens. Beyond this limited period, only antibodies are available to clear infections and limit the spread of pathogens. This probably is the main function of intra-ocular antibodies. The proposed capacity to activate TGF-β adds to the important role of intraocular antibodies.

3.3.5 Conditions/models for local antibody responses
Several mechanisms may lead to compartmentalized antibody responses as observed during the ocular pathogenesis of T. gondii and HSV. We will discuss three models.

Model I: Antigen-specific B cells enter the compartment and mature locally into specific antibody secreting cells. Analogous to inflammatory responses in the eye, similar responses in the brain are also accompanied by the influx of large numbers of T cells and, to a lesser extent B cells and ASCs. Recently, the antigen-specific intra-cerebral antibody responses have been analyzed in rats following intra-thecal antigen inoculation. Compared to serum, the
intra-thecal antigen-specific antibody response was detected earlier and displayed a different B cells repertoire [217]. Thus, antigen-specific B cells along with cognate T cells are able to cross the BBB, home in to sites where antigen was originally deposited and mature locally into ASCs. These infiltrating B and T cells are most likely stimulated in the cervical lymph node (CLN), the major draining LN of CSF, either by migrated antigen presenting cells (APCs) that carry fragments of the deposited antigen and/or by APCs that have picked up the antigen in the CLN. Because the brain has extended its immune privileged milieu to the draining CLN, immune responses will be skewed towards T helper 2 dependent antibody responses [218]. Analogous to the brain, the eye is considered as an immune privileged site and does not contain classical draining lymphatic organs. However, studies in mice have demonstrated that IOF is partly collected in the submandibular LN (SLN) and specific T cell expansion has been observed in the SLN following injection of peptide in the posterior chamber, strongly suggesting a functional link between the eye and the SLN [219]. Thus, a similar set of components supporting a compartmentalized humoral immune response exists for the eye and the CNS. While there is no direct evidence for the association of a local LN with the eye in humans, intraocular tumors frequently involve the SLN and CLN.

Model II: Memory B cells responding to ocular T lymphocyte associated cytokines become specific antibody secreting cells.

In contrast to studies on experimental mouse models, patients suffering from an intraocular infection due to HSV or T.gondii have acquired these pathogens prior to development of ocular disease [89,194]. This implies that mainly memory B cells are triggered systemically and subsequently infiltrate the eye. The memory B cell population in humans, generally characterized by CD27 and CD40 expression, is composed of phenotypically and functionally different subtypes that can be distinguished based on expression of CD80, an essential co-stimulatory molecule. The CD80+ population, capable of antigen presentation and activation of T helper cells without pre-activation, continuously circulates throughout the body and possesses an activated phenotype as defined by the expression of CD11b (Mac1). This is the integrin family adhesion molecule/type 3 complement receptor, which may reflect the preferential migration tendency of these cells [Bar-Or]. In contrast to normal CD19+ adult B cells, memory B cells do not depend on secondary LN or activated T helper cells for activation, and rapidly start secreting antibody upon CD40-CD40L interaction [220,221]. As discussed, the ocular compartment contains several T helper 2 lymphocyte associated cytokines, like IL-4, IL-6 and IL-10 and TGF-B2 that support B cell responses and differentiation [102,103,105,202]. Ocular inflammation in patients with HSV or T.gondii-induced uveitis is commonly associated with ocular infiltration by T cells headed for the triggering pathogen [126,127]. The entire process of activation and differentiation into ASCs could thus take place in the eye, depending on the availability of specific antigenic epitopes. In vitro studies with RPE cells and microvascular endothelial cells have shown that these cells respond to infection by secretion of cytokines and expression of adhesion molecules that support chemotaxis and entry of lymphocytes into the ocular compartment [106,119,222], which might be the in vivo trigger for memory B cells to cross the blood-retina barrier.

Model III: Antigen-specific memory B cells encounter such a high load of antigen in the specific compartment that they locally turn into specific ASCs.

A third possibility arises from studies of T cell independent B cell responses against viral particles [158]. The viral structure of HSV does not seem to allow direct T helper independent B cell responses, and primary B cell responses against HSV are mainly complement dependent [223]. But conditions for a local secondary response might be different. During HSV-induced acute retinal necrosis, the viral load might be sufficient to cause surface Ig cross-
linking and subsequent virus-specific memory B cell differentiation into ASCs. This would allow rapid establishment of protective intra-ocular IgG titers without need for (perhaps) rare encounters between specific B cells and primed T helper cells.

3.3.6 Which model is applicable to the eye? - Relation with antigen presentation

Depending on intraocular conditions, several models may be applicable. In cases of ARN and T. gondii induced chorioretinitis inflammation is preceded by systemic infection. This makes model II the most likely candidate, because during low-grade inflammations a relative increase of B lymphocytes compared to T cells has been observed among the infiltrating lymphocytes. Memory B cells are capable of rapidly initiating secretion of IgG antibodies, and possibly activating TGF-β2 in the process. In order to induce final differentiation into ASCs, production of specific antibodies may be preceded by antigen presentation by memory B cells to select the cognate CD4+ memory T helper cells. Resident microglial cells and/or perivascular cells may also be able to function as APC and activate B and T cells. Under inflammatory conditions, microglial cells in the brain rapidly upregulate chemokine production, MHC class II, CD40, CD80, CD86 and CD45, thus having the capability of being potent antigen presenting cells [206]. However, microglial cells isolated from the human retina do not appear to be very potent APCs under pro-inflammatory conditions; exposure to IFN-γ and LPS seems to induce production of IL 10, which in turn downregulates expression of MHC class II and co-stimulatory molecules. Freshly isolated microglial cells are capable of pinocytosis [123], which suggest that they are good phagocytes. Although they probably function principally as clearing cells [224], removing cell-debris, parenchymal microglia cells could be involved in the initial phase of an intraocular immune response. Other candidates for APCs are the perivascular cells [125] and DCs. Although the DC population in the retina is not very extensive, this could be related to their high turn-over rates and/or rapid flux from the intraocular environment to the draining LN.

Migration to the eye of immunocompetent cells may just simply be a general response of circulating, mostly memory, B and T cells to inflammatory markers on blood-vessel endothelial cells in the retina near the source of inflammation. Although entrance of immunocompetent cells might result in a local breach of the immune privilege, several soluble (including IgG itself and sFasL) as well as cell-bound factors will remain available to restrain CMI and innate responses.

Because the load of viral and parasitic particles can be very high locally upon lysis of an infected cell or cyst, directly activated memory B cells may be an additional source of antibodies, as suggested in model III.

It remains to be explained how B cells in the eye secrete antibody clones differing from their systemic counterpart. Our results demonstrate that this even extends to the epitope level of single antigens, even with pathogens like HSV-1 that have an overall very simple antigenic ‘make-up’ when compared with the multiple stage parasite T. gondii. The heterogeneity of the memory B cell population based on CD80 expression and migratory behavior may be partially responsible for the overall differences in antibody staining patterns observed between compartments. Not every B cell with a particular BCR specificity will differentiate into the CD80 and CD11b+ memory type, explaining why some B cell clones occur only in the eye or in serum. Also, differences in availability of antigens in the eye compared with the ‘blood-compartment’ will contribute to these overall differences in staining patterns, which is especially applicable to T. gondii.

However, it does not explain the differences observed in epitopes recognized within single antigens like HSV-gG1. This suggests de novo generation of B cell clones as described in model I. It is conceivable that with Gra2 the systemic humoral response will be directed against free Gra2 released by parasites during infection, while intraocular
Gra2 will initially only be available complexed with other antigens as part of a tubule network in the tissue-cyst [195]. As a result of this, different regions of Gra2 might become exposed and available for binding by antibodies. In combination with the supposed leakage of antigens from tissue cysts, drainage of intra-ocular fluid to local LN and presentation of complexed Gra2 by FDCs could result in production of new B memory cell clones. The same might hold true for gG1; the initial humoral response will be directed against the viral particles, while in the chronic stage gG1 might be available as single antigen in membranes of succumbed infected cells. It does suggest that the intra-ocular IgG production has to be preceded by a systemic IgM response, which could remain below threshold levels of diagnostic assays because of its low abundance.

Furthermore, it is also possible that the intra-ocular milieu induces differences in peptides expressed on MHC class II, thereby selecting different sets of B and T cells. This would fit in with both models I and II, and the combination of these two models may be responsible for the differential epitope recognition observed.

4 Future prospects
This review provides an overview of the immunologic defense of the eye in humans, and substantiates that both the internal and the external eye compartments function largely independently of the systemic immune system, either because of immune privilege or as part of the mucosal immune system. Clearly, many issues are a matter of speculation and can only be settled by future research efforts. For instance, it would be challenging to unravel the immunological framework supporting clonal diversity of antibody responses against single antigens. This would require a model in which both chronic intraocular infection and local antibody production can be established. Not only is the time-frame of intraocular Ab production of importance, but also the localization of the B cells and entrance of immunocompetent cells in the eye during both active and latent phase of ocular disease are crucial. To study this requires a model in which both chronic ocular disease and intraocular antibody production can be established. As the retina of pigs closely resembles the human retina [225] this may be an appropriate model. T.gondii can infect pigs, but retinal involvement remains to be confirmed.

Even more intriguing issues have to be addressed in case of slgA production in the LG, as it is not known how immunocompetent cells enter the LG, and whether natural antibody producing B cells really exist in human mucosa associated organs like the LG. The first issue can be addressed initially in mice [110], in which GALT already has received a lot of attention. Insights about the existence natural B cells in human mucosa can only be obtained by analysis of resident B cells in the human LG.
Reference List Chapters 1 - 5


125. Williams, K., X. Alvarez, and A. A. Lackner. 2001. Central nervous system perivascular cells are immunoregulatory cells that connect the CNS with the peripheral immune system. *Glia* 36:156-164.


Reference list chapters 1-5


171. Kamata, T., F. Nogaki, S. Fagarasan et al. 2000. Increased frequency of surface IgA-positive plasma cells in the intestinal lamina propria and decreased IgA excretion in hyper IgA (HIGA) mice, a murine model of IgA nephropathy with hyperserum IgA. J.Immunol. 165:1387-1394.


Reference list chapters 1-5


Chapter 6
Dissecting the IgM antibody response during the acute and latent phase of toxoplasmosis

Published in: Diagnostic Microbiology and Infectious Diseases, 2001

Bob Meek, Tom van Gool, Henk Gilis and Ron Peek.

Abstract
A major problem in anti-toxoplasma IgM serology is the occurrence of clinically non-relevant (CNR) IgM responses. The susceptibility for CNR IgM of the Toxo ISAGA IgM, Platelia Toxo IgM and Vidas Toxo IgM assays was determined using sera with a CNR IgM titer in the Abbott IMx Toxo IgM assay. The specificity of CNR-IgM antibodies was determined by immunoblotting, and compared with IgM antibodies in sera of acutely infected (AI) individuals.

Only 6/19 samples were found positive in the ISAGA IgM, compared with 16/19 with the VIDAS IgM and 17/19 with the Platelia Toxo IgM. Staining intensity of non-reduced SAG1 by IgM antibodies allowed a clear distinction between CNR IgM and AI IgM antibodies.

Any IgM assay is susceptible to CNR IgM antibodies. In cases of doubt, immunoblotting using non-reduced T. gondii antigens is of value as confirmation method. Because CNR IgM antibodies are specific for toxoplasma, it will be difficult to improve IgM ELISAs.
**Chapter 6**

**Introduction**

*Toxoplasma gondii* (*T. gondii*) is a protozoan parasite capable of infecting many vertebrate species, including humans. The symptoms of disease in immuno-competent humans are usually benign and self-limiting. In contrast, in immuno-compromised individuals toxoplasmosis is a major opportunistic infection that may lead to toxoplastic encephalitis. Primary infection during pregnancy may cause serious complications or even death in congenitally infected children [1].

Anti-toxoplasma IgM antibodies are considered a reliable marker for acute infection. A major problem of anti-toxoplasma IgM serology is the occurrence of clinically non-relevant (CNR) IgM responses [2-4]. A positive result in an IgM test due to CNR IgM antibodies suggests acute toxoplasmosis while the patient actually is in the latent stage of toxoplasma infection. Detection of these CNR anti-toxoplasma IgM antibodies during pregnancy has led to unnecessary distress and even abortion [5].

Surprisingly, very little attention has been paid to the characterization of these CNR IgM responses. Anti-toxoplasma IgM kits frequently used for routine diagnostic purposes and confirmatory testing, such as the Abbott IMx Toxo IgM, Toxo ISAGA IgM, Platelia Toxo IgM and Vidas Toxo IgM, have never been evaluated with sera containing CNR IgM antibodies. In this study sera were used from latently infected individuals with an established CNR IgM titer in the Abbott IMx Toxo IgM test to determine and compare the susceptibility of the Toxo ISAGA IgM, Platelia Toxo IgM and Vidas Toxo IgM kits for these antibodies. Immunoblotting was performed to characterize CNR-IgM antibodies and to compare their specificity with those of IgM antibodies in sera of acutely infected individuals.

**Material & Methods**

**Sera:** Serum samples of 54 cases were submitted to the Academic Medical Center for routine examination for toxoplasmosis. Sera were stored at -20°C. Sera of immuno-compromised patients, including AIDS patients, were excluded.

**Serologic tests:** All sera were examined for anti-toxoplasma specific antibodies by the Sabin-Feldman dye test (SF) [6]. Sera were examined for specific IgM by immunoblotting and with the following assays: Abbott IMx Toxo IgM (IM-x Toxo IgM, Abbott Laboratories, Abbott Park, Ill.), Toxo ISAGA IgM (ISAGA IgM, bioMérieux, Lyon, France), Platelia Toxo IgM (Platelia Toxo IgM Sanofi Diagnostics Pasteur, Marnes la Coquette, France), bioMérieux VIDAS Toxo IgM (VIDAS Toxo IgM, bioMérieux, Lyon, France). All commercially available kits were performed according to the instructions given by the manufacturers. Sera with indexes or ODs between the cut-off values were considered equivocal/borderline. For the SF dye test the cut-off value was 1 IU/mL. For the IgM assays these values were an OD of 0.600 for the IMx Toxo IgM kit, an index of 9 for the ISAGA IgM kit (borderline index 6-8), an OD of ≥ 100% of the cut-off serum for the Platelia Toxo IgM kit (~ 0.350), and an OD of 0.500 for the VIDAS IgM kit. Sera with values greater than or equal to the cut off value were considered positive. If anti-toxoplasma IgM antibodies were detected in the first sample, then sequential samples were obtained within 3-4 weeks and analyzed with the SF dye test.

Sera were divided into 4 groups on the basis of the results of the dye test and anti-toxoplasma IgM assays.

**AI group:** serum samples taken from 21 cases with acute toxoplasmosis (AI), characterized by an IgG titer ≥ 250 IU/ml in the SF dye test and/or a three fold increase of the SF dye test titer in the follow up sample taken 3-4 weeks later, and positive results in the IMx IgM and ISAGA IgM assays.

**CNR group:** serum samples from 19 persons who were latently infected with toxoplasma as characterized by
Dissecting the CNR IgM response

a low and stable SF titer between 6 and 63 IU/ml in two serum samples taken three weeks apart and a clinically non-relevant (CNR) IgM titer in the IMx IgM assay.

LI group: serum samples of 7 latently infected (LI) individuals with negative results in the IMx IgM and ISAGA IgM assays and a low SF titer between 6 and 63 IU/ml.

SN group: serum samples from 7 subjects with negative results in the IMx IgM and ISAGA IgM assays, and the SF dye test.

Antigen preparation: Antigen preparation was performed as described [7,8]. *T. gondii* tachyzoites of the RH strain were propagated in Swiss mice (Harlan laboratories, Horst, The Netherlands). The animals were sacrificed 48 hours post-injection and tachyzoites were collected and purified. Following freeze/thawing, the tachyzoite suspension was sonicated on ice (8 x 15 sec 30 kHz microprobe, Soniprep 150, MSE, Loughborough, GB), and insoluble material was removed by centrifugation. The sonicate was frozen in small aliquots and kept at −70°C until use.

SDS-PAGE and Western blotting:

SDS-PAGE was performed according to [8,9]. Briefly, 200 μg of the sonicate was loaded onto 10% SDS-PAGE gels and transferred to polyvinylidifluoride (PVDF) membranes (Millipore Corporation, Bedford, MA) after electrophoresis. To reduce toxoplasma lysate antigens, β-mercaptoethanol was added to a final concentration of 5% prior to electrophoresis. Membranes were blocked with Tris-buffered saline (TBS: 50 mM TrisHCl, 150 mM NaCl, pH 10) containing 0.5% Tween20 and 2% non-fat milk powder.

**Immunostaining of peroxidase conjugated antibodies:** Except when stated otherwise, all samples and conjugates were diluted in TBS containing 0.5% Tween20 and 0.03% non-fat milk powder (TBS-T). All incubations were done using a multiscreeen apparatus (Mini-Protean II, Bio-Rad Laboratories, Hercules, CA). Isotype specific antibodies conjugated to peroxidase were obtained from DAKO (Glostrup, Denmark). The conjugates were diluted 1:5000, and incubated with the blots for 75 min. All incubations were performed at room temperature.

Serum samples were diluted 50x, incubated with the blots for 75 min, and tested for the presence of anti-*T. gondii* IgM. As a positive control, a serum containing anti-glycosylphosphatidyl-inositol (GPI) IgM antibodies was aliquoted and included on each immunoblot in a 50x dilution. A mouse monoclonal antibody against SAG1 (HyTest, Turku, Finland) was also included, diluted 250x/1000x on immunoblots containing R or NR antigens, respectively. Chemiluminescent substrate was prepared according to the manufacturers' descriptions (ECL, Amersham Pharmacia Biotech, Essex, UK). The membranes were incubated with the chemiluminescent substrate for 1 min, wrapped in plastic and used to expose X-ray film (Fuji Super RX, Fuji Photo Film Co., Tokyo, Japan) for 5 min, 3 min, 1 min, and 30 sec.

**Evaluation of the IgM staining patterns:** Serum samples of the categories AI and CNR were always tested together, allowing direct comparison of the staining patterns. The films exposed for 1 minute allowed a good distinction between staining patterns of AI and CNR, and subsequently was selected for the evaluation. The intensity of each band was scored in relation to the positive control serum included on each blot.

A ‘++’ score was assigned to intensely stained bands on the films exposed for 1 minute, a ‘+’ score was assigned to clearly stained bands, and a ‘±’ score was assigned to bands that were well identifiable, but weakly stained on the films exposed for 1 minute.
Chapter 6

Results

Evaluation of the IgM kits: Serum samples of 19 latently infected individuals proven to have clinically non-relevant (CNR) anti-toxoplasma IgM titers in the Abbott Toxo IMx Toxo IgM test were analyzed using the ISAGA IgM, Platelia Toxo IgM and VIDA S Toxo IgM kits (table 1).

In the CNR group, 7 out of 19, 17/19 and 16/19 serum samples had a positive result in the ISAGA IgM, Platelia Toxo IgM, and VIDAS Toxo IgM assays, respectively. Importantly, none of the samples containing CNR IgM antibodies was negative in all three assays (table 1), and only 2 CNR samples had a negative result in 2 assays. In the AI group, all samples were positive by the commercially available IgM tests. All samples in the LI and SN groups had a negative result in every IgM assay (table 1).

IgM antibody profiles analyzed by immunoblotting: To determine which antigens were recognized by the CNR IgM antibodies and to assess the diagnostic value of IgM immunoblotting in discrimination between CNR and AI IgM, the panels of sera were tested on blots containing size-fractionated reduced or non-reduced *T. gondii* tachyzoite antigens.

Upon initial analysis, it appeared that CNR IgM antibodies recognized the same antigens as the AI IgM antibodies. Therefore the comparison of antigens stained by CNR IgM versus AI IgM antibodies was focused on the antigens most often recognized by AI IgM antibodies. As shown in figure 1, IgM antibodies in AI samples recognized antigens with a molecular weight of 4-5 kDa, 23-25 kDa, 28-30 kDa, 32 kDa, 35 kDa and 55 kDa on blots with reduced antigens, and 4-5 kDa, 20 kDa, 30 kDa, 35 kDa, 38 kDa and 55 kDa on blots with non-reduced antigens. The antigen running at 4-5 kDa was identified as the glycosylphosphatidyl-inositol (GPI) anchor, while the antigens at 32 kDa (reduced) and 30 kDa (non-reduced) were identified as SAG1 (not shown).

CNR IgM antibodies most often recognized the GPI anchor of *T. gondii*: 68%, followed by SAG1: 58% reduced / 42% non-reduced. AI IgM antibodies also had a preference for the GPI anchor and the SAG1 antigen: at least 95% of the samples contained IgM antibodies that stained the GPI anchor and SAG1 (reduced/non-reduced).

IgM antibodies in our panel of control sera occasionally did bind antigens associated with acute infection (table 1), and antigens with a molecular weight of more than 80 kDa.

Importanty, there was a marked difference in the intensity of staining of the GPI anchor between LI and AI individuals, despite the overlap in titers in to the commercially available IgM assays. If the [+ ] and [++] scores on films exposed for 1 minute for the GPI anchor were considered, only 11% of the CNR samples (2/19) clearly stained this antigen versus 81% of the AI individuals. A similar, even more striking result was obtained with the SAG1 antigen; none of the CNR samples displayed intense staining of this antigen, versus 76% (reduced) and 100% (non-reduced) of the AI individuals. This latter difference in intensity of staining by IgM of reduced SAG1 versus non-reduced SAG1 also demonstrated that IgM antibodies in AI sera preferably stained non-reduced SAG1 (figure 1: AI 12 and 17), while this preference was never seen in the CNR group.

In combination with clear staining of the GPI anchor and the SAG1 antigens staining of a 55 kDa antigen by AI IgM was also frequently found (50%), irrespective of antigen reduction. The same accounted for the 23-25 kDa antigen(s) on reduced blots; 41% of the AI showed this particular combination. None of these combinatorial stainings were observed in the CNR group (table 1).
Dissecting the CNR IgM response

Table 1: Overview serological data and analysis of the IgM patterns

<table>
<thead>
<tr>
<th>Group</th>
<th>Nr</th>
<th>SF (EU/ml)</th>
<th>IM x M</th>
<th>ISAGA- M</th>
<th>Platelia M</th>
<th>Vidas-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>2</td>
<td>1000</td>
<td>23.50</td>
<td>12</td>
<td>1.74</td>
<td>7.34</td>
</tr>
<tr>
<td>A1</td>
<td>2</td>
<td>1000</td>
<td>8.68</td>
<td>12</td>
<td>1.57</td>
<td>8.68</td>
</tr>
<tr>
<td>A1</td>
<td>3</td>
<td>1000</td>
<td>7.76</td>
<td>12</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>A1</td>
<td>4</td>
<td>1000</td>
<td>6.55</td>
<td>12</td>
<td>1.17</td>
<td>1.15</td>
</tr>
<tr>
<td>A1</td>
<td>5</td>
<td>250</td>
<td>6.50</td>
<td>12</td>
<td>1.64</td>
<td>5.6</td>
</tr>
<tr>
<td>A1</td>
<td>6</td>
<td>500</td>
<td>6.48</td>
<td>12</td>
<td>1.62</td>
<td>7.38</td>
</tr>
<tr>
<td>A1</td>
<td>7</td>
<td>4000</td>
<td>6.33</td>
<td>12</td>
<td>1.63</td>
<td>5.8</td>
</tr>
<tr>
<td>A1</td>
<td>8</td>
<td>500</td>
<td>5.94</td>
<td>12</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>A1</td>
<td>9</td>
<td>250</td>
<td>5.63</td>
<td>12</td>
<td>1.66</td>
<td>3.87</td>
</tr>
<tr>
<td>A1</td>
<td>10</td>
<td>250</td>
<td>5.61</td>
<td>12</td>
<td>1.57</td>
<td>5.27</td>
</tr>
<tr>
<td>A1</td>
<td>11</td>
<td>500</td>
<td>5.13</td>
<td>12</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>A1</td>
<td>12</td>
<td>250</td>
<td>5.09</td>
<td>12</td>
<td>1.20</td>
<td>6.78</td>
</tr>
<tr>
<td>A1</td>
<td>13</td>
<td>250</td>
<td>4.63</td>
<td>12</td>
<td>1.70</td>
<td>3.89</td>
</tr>
<tr>
<td>A1</td>
<td>14</td>
<td>250</td>
<td>4.52</td>
<td>12</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>A1</td>
<td>15</td>
<td>1000</td>
<td>4.49</td>
<td>12</td>
<td>1.55</td>
<td>4.05</td>
</tr>
<tr>
<td>A1</td>
<td>16</td>
<td>4000</td>
<td>4.31</td>
<td>12</td>
<td>1.72</td>
<td>3.43</td>
</tr>
<tr>
<td>A1</td>
<td>17</td>
<td>4000</td>
<td>4.31</td>
<td>12</td>
<td>1.70</td>
<td>4.56</td>
</tr>
<tr>
<td>A1</td>
<td>18</td>
<td>250</td>
<td>4.12</td>
<td>12</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>A1</td>
<td>19</td>
<td>500</td>
<td>2.64</td>
<td>12</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>A1</td>
<td>20</td>
<td>250</td>
<td>2.40</td>
<td>12</td>
<td>1.21</td>
<td>6.62</td>
</tr>
<tr>
<td>A1</td>
<td>21</td>
<td>2000</td>
<td>1.29</td>
<td>12</td>
<td>1.64</td>
<td>1.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Nr</th>
<th>SF (EU/ml)</th>
<th>IM x M</th>
<th>ISAGA- M</th>
<th>Platelia M</th>
<th>Vidas-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNR  2</td>
<td>2</td>
<td>64</td>
<td>2.22</td>
<td>12</td>
<td>1.04</td>
<td>1.44</td>
</tr>
<tr>
<td>CNR  3</td>
<td>2</td>
<td>64</td>
<td>1.98</td>
<td>12</td>
<td>1.54</td>
<td>2.54</td>
</tr>
<tr>
<td>CNR  4</td>
<td>3</td>
<td>32</td>
<td>1.86</td>
<td>12</td>
<td>0.86</td>
<td>0.98</td>
</tr>
<tr>
<td>CNR  5</td>
<td>3</td>
<td>32</td>
<td>1.80</td>
<td>12</td>
<td>1.76</td>
<td>2.72</td>
</tr>
<tr>
<td>CNR  6</td>
<td>3</td>
<td>32</td>
<td>1.42</td>
<td>12</td>
<td>0.90</td>
<td>1.35</td>
</tr>
<tr>
<td>CNR  7</td>
<td>3</td>
<td>32</td>
<td>1.20</td>
<td>6</td>
<td>0.40</td>
<td>0.55</td>
</tr>
<tr>
<td>CNR  8</td>
<td>3</td>
<td>32</td>
<td>1.17</td>
<td>3</td>
<td>0.34</td>
<td>0.97</td>
</tr>
<tr>
<td>CNR  9</td>
<td>3</td>
<td>16</td>
<td>1.13</td>
<td>6</td>
<td>0.36</td>
<td>1.03</td>
</tr>
<tr>
<td>CNR  10</td>
<td>3</td>
<td>32</td>
<td>1.12</td>
<td>7</td>
<td>0.26</td>
<td>0.78</td>
</tr>
<tr>
<td>CNR  11</td>
<td>3</td>
<td>16</td>
<td>0.99</td>
<td>9</td>
<td>1.00</td>
<td>0.73</td>
</tr>
<tr>
<td>CNR  12</td>
<td>3</td>
<td>8</td>
<td>0.99</td>
<td>8</td>
<td>0.11</td>
<td>0.14</td>
</tr>
<tr>
<td>CNR  13</td>
<td>3</td>
<td>4</td>
<td>0.92</td>
<td>3</td>
<td>0.50</td>
<td>0.40</td>
</tr>
<tr>
<td>CNR  14</td>
<td>3</td>
<td>64</td>
<td>0.84</td>
<td>3</td>
<td>0.53</td>
<td>1.03</td>
</tr>
<tr>
<td>CNR  15</td>
<td>3</td>
<td>32</td>
<td>0.83</td>
<td>3</td>
<td>0.61</td>
<td>0.68</td>
</tr>
<tr>
<td>CNR  16</td>
<td>3</td>
<td>32</td>
<td>0.75</td>
<td>8</td>
<td>1.13</td>
<td>0.74</td>
</tr>
<tr>
<td>CNR  17</td>
<td>3</td>
<td>32</td>
<td>0.74</td>
<td>3</td>
<td>0.37</td>
<td>0.91</td>
</tr>
<tr>
<td>CNR  18</td>
<td>3</td>
<td>16</td>
<td>0.72</td>
<td>6</td>
<td>0.42</td>
<td>0.36</td>
</tr>
<tr>
<td>CNR  19</td>
<td>3</td>
<td>16</td>
<td>0.70</td>
<td>7</td>
<td>0.48</td>
<td>0.85</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Nr</th>
<th>SF (EU/ml)</th>
<th>IM x M</th>
<th>ISAGA- M</th>
<th>Platelia M</th>
<th>Vidas-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>LI</td>
<td>2</td>
<td>2</td>
<td>0.32</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LI</td>
<td>3</td>
<td>4</td>
<td>0.25</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LI</td>
<td>4</td>
<td>8</td>
<td>0.24</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LI</td>
<td>5</td>
<td>8</td>
<td>0.19</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LI</td>
<td>6</td>
<td>16</td>
<td>0.17</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LI</td>
<td>7</td>
<td>8</td>
<td>0.15</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SN</td>
<td>2</td>
<td>0</td>
<td>0.13</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SN</td>
<td>3</td>
<td>0</td>
<td>0.13</td>
<td>3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SN</td>
<td>4</td>
<td>0</td>
<td>0.11</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SN</td>
<td>5</td>
<td>0</td>
<td>0.11</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SN</td>
<td>6</td>
<td>0</td>
<td>0.10</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SN</td>
<td>7</td>
<td>0</td>
<td>0.10</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

AI = Acutely Infected individuals, CNR = Individuals with a clinically non-relevant IgM response, LI = Latently Infected individuals, SN=Seronegative individuals.

IgM titers of the CNR group are corrected for IgM rheumatoid factor.
IgM patterns, reduced antigens (R) | IgM patterns, non-reduced antigens (NR)

<table>
<thead>
<tr>
<th>Exposure</th>
<th>MW (kD)</th>
<th>AI</th>
<th>LI IgM [+]</th>
<th>LI IgM [-]</th>
<th>SN</th>
<th>MW (kDa)</th>
<th>AI</th>
<th>LI IgM [+]</th>
<th>LI IgM [-]</th>
<th>SN</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 min A</td>
<td>55 &gt; 80</td>
<td>35 &gt;</td>
<td>SAG1 &gt; 28-30</td>
<td>20 &gt;</td>
<td>GPI &gt;</td>
<td>55 &gt;</td>
<td>30 &gt;</td>
<td>SAG1 &gt; 20 &gt;</td>
<td>GPI &gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 7 12 17 4 20 5 19 14 3 4 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 min B</td>
<td>55 &gt; 80</td>
<td>35 &gt;</td>
<td>SAG1 &gt; 28-30</td>
<td>20 &gt;</td>
<td>GPI &gt;</td>
<td>55 &gt;</td>
<td>30 &gt;</td>
<td>SAG1 &gt; 20 &gt;</td>
<td>GPI &gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 7 12 17 4 20 5 19 14 3 4 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1: IgM staining patterns of acutely and latently infected patients and seronegative individuals.

AI, CNR, LI, and SN individuals were incubated on blots containing either R or NR antigens of *T. gondii*, and IgM reactivity was demonstrated using chemiluminescence as described in Material and Methods. The staining patterns after exposure-periods of 3 (A, C) and 1 (B, D) minutes are shown. The numbers of the individuals, corresponding with those in table 1, are depicted in between. The antigens most frequently recognized by IgM are indicated in the columns labeled A – D (>). R and NR SAG1 run at 32 kDa and 30 kDa, respectively. The GPI anchor runs at 4-5 kDa.

Reduction of the antigens did not only affect the mobility of several antigens but frequently also the immunogenicity (for example serum AI 12; SAG1, and serum AI 17; SAG1 and the 20 kDa antigen, figure 1). As expected, the immunogenicity of the GPI anchor, having no disulfide bonds, was not affected by antigen reduction.

It was not possible to establish a relation between the IgM staining profiles of AI and CNR samples and the (‘false-positive’) results in the commercially available IgM tests.

Discussion

Our study shows that next to the IMx Toxo IgM, also the ISAGA IgM, Platelia Toxo IgM and Vidas Toxo IgM assays are susceptible for clinically non-relevant (CNR) anti-toxoplasma IgM antibodies.

All commercially available IgM kits used in this study are well evaluated, though with variable results [4,5,10-13]. Of interest is that only in one study a group was included known to contain CNR IgM antibodies [4]. The ISAGA IgM assay did show the best performance with the panel of 19 CNR samples: only 6/19 (32%) samples were found positive, compared with 16/19 (84%) with the VIDAS IgM and 17/19 (89%) with the Platelia Toxo IgM. Seven samples in the CNR-IgM group did have an ISAGA IgM index between 6-8. We followed the recommendations of the manufacturer by regarding these sera as unequivocal, with a need to repeat the examination.
All samples used in this study were negative for rheumatoid factor, and immunocapture assays like the ISAGA IgM, VIDAS Toxo IgM and Platelia IgM are not sensitive for anti-nuclear antibodies. Therefore, the differences in performance of commercially available assays with samples containing CNR IgM antibodies are not related to factors known to cause "false-positive" reactions. More likely, these differences are due to differences in antigenic composition and preparation of the antigens.

To determine which of the specific toxoplasma antigen(s) are involved in these false-positive reactions, immunoblotting was performed. IgM staining patterns of CNR IgM were compared with those of IgM antibodies in sera of acutely infected (AI) individuals. CNR IgM antibodies clearly recognized the same antigens as those most often stained by AI IgM antibodies, i.e. GPI anchor and SAG1, followed by the 35 and 55 kDa antigens. Many early studies have identified the GPI anchor and SAG1 as being the main targets of the IgM antibodies during acute infection [14-18]. Importantly, the CNR IgM staining patterns did not constitute as many different antigens and the staining of these antigens was not as intense as those from AI individuals. This was especially apparent when the antigens were not reduced. In addition, there were antigens exclusively stained by AI sera, namely the 23-25 kDa antigens (reduced) and the 38 kDa antigen (non-reduced). Due to these quantitative differences a clear distinction could be made between CNR and AI IgM staining patterns, which clearly demonstrates the diagnostic value of IgM immunoblotting as a confirmatory test. Although it has been reported that CNR or "long-lived" IgM antibodies recognize the same antigens as AI IgM antibodies [19], that study missed the very clear quantitative differences IgM staining patterns between the two groups.

It was not possible to determine a direct relation between the CNR and AI IgM staining patterns on immunoblot and anti-toxoplasma titer in any of the commercially available assays, even though there was overlap in CNR and AI IgM titers. For instance, both the Platelia Toxo IgM and the VIDAS Toxo IgM assays are antibody capture ELISAs specifically designed to detect antibodies specific for SAG1, but there was no relation between the titer in these assays and the intensity of staining of SAG1 on immunoblots. A possible explanation for these discrepancies could be that in some cases most of the antibodies are able to recognize SAG1 in its native conformation only, while antigens are denatured on immunoblot. It is remarkable that the ISAGA IgM assay is capable of ruling out most of the CNR samples proven to contain toxoplasma specific antibodies, while on the other hand it is regarded as being very sensitive [20,21].

Occasionally the antigens recognized by AI and CNR IgM were bound by IgM antibodies in our panel of control serum samples as well (though weakly), and more frequently recognized antigens with a molecular weight of more than 80 kDa. These IgM antibodies probably originate from the naturally occurring antibody repertoire [10,22].

In general, identification of *T. gondii* proteins on the basis of their molecular weight on immunoblot is not very accurate. Recognition of a 35 kDa antigen by antibodies has been reported before [23] and this antigen could be SRS3 (P35), while the 38 kDa antigen recognized by several AI sera could be SAG3 (P43) [24]. Reduction of the latter antigen virtually abolished recognition by IgM which would be consistent with the properties of the structurally related major membrane protein SAG1 [25,26]. The 23-25 kDa antigens recognized only by AI IgM could be SAG2 (P22) and/or P23 [24]. The identity of the 28-30 kDa and 55 kDa bands is not clear, but these proteins have parasite surface exposed epitopes, because IgM specific for these antigens were efficiently depleted by pre-incubation experiments with intact parasites (unpublished observation).

In recent studies the diagnostic value of IgM immunoblotting using other preparations of *T. gondii* antigens has been investigated [27,28]. However, none studied the IgM antibody reactivity against non-reduced lysate antigens or sera of latently infected individuals with a CNR IgM titer [27]. In view of the conformation dependent
immunogenicity of several important T. gondii antigens the use of only reduced antigens by these studies is remarkable.

The results presented suggest that the CNR IgM antibodies in sera of latently infected individuals could be due to persistence of specific anti-toxoplasma IgM antibodies, many years after the acute phase. Most anti-toxoplasma IgM antibody studies have demonstrated that IgM can persist for 3 months to one year after acute infection [3]. The fact that IgM antibodies remain detectable for many years after infection has not been reported before, and seems to be a unique phenomenon for toxoplasmosis. The course of the anti-toxoplasma IgM levels in CNR sera probably follows the levels of the matured IgG antibodies. This is substantiated by the stability of the anti-T.gondii IgM levels parallel to the anti-T.gondii IgG levels and suggests that the plasma cells producing these IgM antibodies have missed the antibody class-switching process, but may have gone through the affinity maturation event. Although matured antibodies are generally of the IgG class, recent evidence suggests that matured IgM antibodies may exist as well [29].

This study demonstrates that any IgM assay is susceptible to CNR IgM antibodies. In case of doubt, the immunoblot method using non-reduced T.gondii antigens can be of value as a confirmation method. Especially staining intensity of non-reduced SAG1 by IgM antibodies allowed the distinction between CNR IgM and AI IgM antibodies. In order to have an objective cut-off value with the immunoblot, a small panel of IgM positive samples of latently infected individuals should be tested in parallel with samples of interest. This prerequisite clearly limits the use of IgM immunoblotting in routine screening of sera for the diagnosis of acute acquired toxoplasma infection. Any assay should take care not to focus on the reactivity against the highly immunogenic GPI anchor. In contrast to SAG1, this antigen can be stained intensely by both CNR IgM and AI IgM antibodies, while not every AI serum contains IgM antibodies specific for the GPI anchor. Because CNR IgM antibodies are specific for toxoplasma, it will be difficult to improve IgM ELISAs with defined, recombinant toxoplasma antigens.
In table 1 we compared our results obtained with IgM immunoblotting [31] with those obtained by others using a similar preparation of *T. gondii*. Only a few studies were comparable in their usage of a lysate as their source of parasite-antigen, and, remarkably, none has studied the IgM antibody reactivity against non-reduced antigens. Most of these studies reported a low molecular weight antigen of 4 to 6 kDa, and an antigen of 32 to 35 kDa as being the primary targets for the IgM antibodies, which corresponds to the GPI anchor and SAG1. Differences in molecular weights of the antigens reported, or lack of staining of the 35 kDa antigen, can be attributed to minor differences in the protein-electrophoresis and sensitivity of the detection methods used. In contrast to the other studies, only the staining of the 55 kDa surface antigen was markedly accentuated in our study, especially because staining of this antigen was also observed using higher dilutions of the sera. More recent studies have investigated the diagnostic value of IgM immunoblotting using other preparations of *T. gondii* antigens (table 2) [27,28]. None of these and the other studies described in table 1 and 2 tested non-reduced preparations and/or sera of latently infected individuals with a CNR IgM titer. The negative effects of reduction on the immunogenicity of antigens and CNR IgM responses specific for *T. gondii* were already documented at the time [32]. Our study has demonstrated that both points have to be taken into account in order to develop a confirmation assay that is better in discriminating between AI and CNR IgM responses than commercially available IgM assays specific for *T. gondii*.

Table 1: Comparison of molecular weights (in kDa) of major and minor bands found in this study with those reported by others, using similar immunoblot methodology.

<table>
<thead>
<tr>
<th>Reference:</th>
<th>Number of sera</th>
<th>Major bands</th>
<th>Al IgM Minor bands</th>
<th>Other bands</th>
<th>Number of sera</th>
<th>Major bands</th>
<th>CNR IgM Minor bands</th>
<th>Other bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>[17]</td>
<td>19</td>
<td>6, 19</td>
<td></td>
<td></td>
<td>19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[15]</td>
<td>4</td>
<td>6, 22, 32</td>
<td>45, 66</td>
<td></td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[18]</td>
<td>2</td>
<td>35, 50</td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[30]</td>
<td>1</td>
<td>6, 25, 35</td>
<td>14, 30, 40, 50</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[14]</td>
<td>17</td>
<td>4, 35</td>
<td>27-30.8</td>
<td></td>
<td>17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduced</td>
<td>22</td>
<td>4.5, 32</td>
<td>23-25, 35, 55</td>
<td>28-30</td>
<td>19</td>
<td></td>
<td>4.5, 32</td>
<td>35, 55</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>% sera containing IgM reactive to a particular band</th>
<th>&gt; 80%</th>
<th>30-80%</th>
<th>&lt; 30%</th>
<th>&gt; 80%</th>
<th>30-80%</th>
<th>&lt; 30%</th>
</tr>
</thead>
</table>

Table 2: Overview current data on IgM IB, patients and/or sera

Abbreviations: ?: not clear, Agg=agglutination assay, CFT=complement fixation test, ESA=excreted/secreted antigens, DsIgX=double sandwich IgX, IFA=immunofluorescence assay, Rf=rheumatoid factor, RIP=radio-immuno precipitation, s=Ser, SP=surface proteins. All sera were from patients who were serologically and/or clinically proven AI, LI, and congenitally infected [33]. BAG, Lich, Germany. Platelia Toxo IgM, Sanofi Diagnostics Pasteur, Marnes la Coquette, France. Organon, Boxtel, The Netherlands. According to the Palo Alto Medical Foundation serological profile [5]. Results obtained with the 40% ammonium sulphate precipitable fraction. Results of patients with a high IgM titer. Occasional bands with the following MW were found: 20/50/70/75/ 80/88 kDa. Labsystems, Helsinki, Finland.
## Chapter 6, addendum

<table>
<thead>
<tr>
<th>Reference:</th>
<th>Number of subjects:</th>
<th>Infection status of the subjects analysed:</th>
<th>Criteria used for the establishment of the infection status:</th>
<th>Serological tests used to establish the infection status:</th>
<th>Immunoblot details:</th>
<th>MW of the antigens recognized by IgM, in relation to the infection of the subject:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AI</td>
<td>CNR</td>
<td>SN</td>
<td></td>
<td></td>
<td>Strain Antigen preparation.</td>
</tr>
<tr>
<td>Ehlrich et al. [17]</td>
<td>37</td>
<td>19</td>
<td>9</td>
<td>9</td>
<td>AI: positive in SF and dsIgM±</td>
<td>SF, DslgM [34]</td>
</tr>
<tr>
<td>Partanen et al. [18]</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>AI: verified by lymph node biopsy</td>
<td>IgM EIA±, IgM EIA±</td>
</tr>
<tr>
<td>Partanen et al. [30]</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td>AI: accidental infection with T. gondii tachyzoites via direct blood-ascites contact</td>
<td>IgG EIA±, IgM EIA±</td>
</tr>
<tr>
<td>Herbrink et al. [36]</td>
<td>18</td>
<td>111 sera</td>
<td>115 sera</td>
<td>Prospective analysis with 115 sera</td>
<td>AI: clinical signs of toxoplasmosis and 4-fold increase of IgG</td>
<td>IgG ELISA, IgM ELISA, DslgM [37], DslgM±</td>
</tr>
<tr>
<td>Decoster et al. [38]</td>
<td>610</td>
<td>139 s</td>
<td>432 s</td>
<td>140 s</td>
<td>AI: Detection of IgM in at least two of three tests</td>
<td>IgM EIA</td>
</tr>
<tr>
<td>Romans et al. [41]</td>
<td>763 sera</td>
<td>Prospective analysis with 763 sera</td>
<td>AI: presence of IgM and IgG, with seroconversion or a rapid increase in titer(s)</td>
<td>IgM ELISA, DslgM [37]</td>
<td>RH</td>
<td>Sonicate</td>
</tr>
<tr>
<td>Verhofstede et al. [19]</td>
<td>80</td>
<td>20</td>
<td>30</td>
<td>30</td>
<td>AI: rise in IgG, dslgM±</td>
<td>Indirect IgG Elisa, DslgM±</td>
</tr>
<tr>
<td>Gross et al. [27]</td>
<td>144</td>
<td>10</td>
<td>10</td>
<td>pool</td>
<td>pool</td>
<td>AI: a) SF or IFA titer &gt;1:1000, CFT titer &gt;1:10, rising titer(s), and IgM [±], b) PCR [±], c) mouse inoculation test [±], d) histological identification of T. gondii, or e) clinical improvement after treatment.</td>
</tr>
</tbody>
</table>

*Note: AI, CNR, SN, and R/N refer to different columns or categories.*
Chapter 7

IgM recognition of recombinant *Toxoplasma gondii* antigens by sera of acutely or latently infected humans.

To be published in Diagnostic Microbiology and Infectious Diseases

Bob Meek, Robert Jan Diepersloot, Tom van Gool, Dave Speijer and Ron Peek.

Abstract

Clinical non-relevant (CNR) IgM specific for *Toxoplasma gondii* is responsible for false-positive results in commercially available IgM assays. Using IgM immunoblotting, it is possible to distinguish between IgM in sera of acutely infected (AI) patients and CNR IgM. Especially the combination of staining of a 55 and 30 kD antigen in *T.gondii* lysate proved useful in this respect. The 55 kD antigen was identified as Rop1, while the 30 kD antigen was confirmed to be Sag1. However, the use of recombinant antigens instead of lysates for diagnostic assays would improve reproducibility. IgM recognized recombinant Rop1, but most CNR sera also had low anti-Rop1 titers. Although purified native Sag1 separated AI and CNR sera very well on immunoblot, IgM did not recognize recombinant Sag1 at all. Clearly, it is difficult to produce a recombinant Sag1 that can be recognized by IgM. Recombinant Rop1 might be suitable as one of the recombinant antigens in an IgM immunoblot assay, but has to be combined with at least one other immunogenic antigen.
Chapter 7

Introduction

*Toxoplasma gondii* (Tg) is a ubiquitous protozoan parasite that infects many individuals worldwide. Although the symptoms of infection are usually benign, especially Tg infection during pregnancy can cause life-threatening complications to the fetus. Tg serology is essential to determine whether a pregnant woman is experiencing seroconversion. Detection of anti-Tg IgM is one of the hallmarks of seroconversion [42]. However, one of the problems of Tg serology is the existence of clinically non-relevant (CNR) Tg specific IgM antibodies that persist in sera of individuals who have been infected many years ago [2-4,31]. Our previous experiments demonstrated that many commercially available IgM tests give positive results with well-defined sera containing CNR IgM antibodies [31]. In combination with low IgG titers, sera with CNR IgM are easily confused with those from acutely infected (AI) individuals, and have caused unwarranted concerns and terminations of pregnancy [5]. It has also been demonstrated that CNR IgM antibodies recognize the same antigens as those frequently recognized by IgM in sera of AI individuals [19,31], making it very difficult to devise a toxoplasma antibody test based on specific antigens that will distinguish CNR from AI sera.

Interestingly, using standard immunoblotting (IB) of non-reduced Tg antigens it is possible to distinguish CNR from AI sera based on differences in intensity of recognition of a 30 kD antigen, most likely Sag1. In addition, a combination of intense recognition of the 30 kD antigen and one other antigen, either a GPI anchor or an unidentified 55 kD antigen, proved very useful in the distinction between CNR and AI sera [31].

Sag1 is one of the first antigens recognized by IgM during acute infection, along with the GPI anchor [15,17]. Detection of Sag1 specific IgM is regarded as clinically relevant, and an anti-Sag1 IgM specific ELISA is available [43]. Although our previous results indicated that CNR IgM antibodies recognize Sag1 as well, IgM IB is clearly not as sensitive to anti-Sag1 CNR IgM as commercially available assays. Therefore, IgM IB should be used as a confirmation assay in cases of doubt. However, the use of an incompletely defined Tg lysate as antigen source may cause reproducibility problems in anti-Tg IgM staining patterns due to batch differences, while maintenance of Tg parasites is laborious and expensive. To establish IgM IB as a confirmation assay, it is important to use a standard set of well-defined recombinantly produced antigens.

The objective of this study was to test whether a selected set of immunoblotted recombinant antigens would result in an easy and reproducible confirmation assay for the serology of toxoplasmosis. Identification and cloning of the Tg 55 kD antigen may result in a very useful candidate for an IgM IB assay. In addition, recent expression techniques has allowed the production of purified, recombinant Sag1 recognized by IgG antibodies in human sera [44], indicating that recombinant Sag1 protein has adopted a conformation required for recognition by antibodies. This preparation of recombinant Sag1 together with a recombinant version of the 55 kD antigen and IgM IB may allow the important distinction between sera containing CNR or AI IgM. Before exploring this, it is important to exclude that in some cases other Tg antigens with an approximate molecular weight (MW) of 30 kD are also involved in the anti-Tg IgM response. Moreover, it is not known whether IgM specific for free GPI anchor is also able to recognize GPI-anchored Sag1. Therefore, it is critical to determine if recognition of the 30 kD antigen on immunoblot is solely due to Sag1.

Material & Methods

**Sera:** All sera used in this study were submitted for routine toxoplasmosis screening. Sera were stored at 20°C. Sera of patients suffering from AIDS or sera of immuno-compromised patients were excluded.

**Antigen preparation:** Antigen preparation was performed as described elsewhere [8]. The protein concentration of the supernatant (lysate/sonicate) was measured using the Bradford assay with BSA as a standard.

100
Recombinant antigens recognized by IgM

The sonicate was frozen in small aliquots containing 200 μg protein (equal to 7.7x10⁶ tachyzoites) and kept at -70°C until use.

**Primers and cloning of PCR products:** Primers used for amplification of open reading frames encoding Rop1 by PCR and Gra2 by RT-PCR: Rop-FW 5’GAGACCATGGTACTTTCTGAGCCACAAATGGA3’, Rop-RV 5’GAGAGAATTCTTGCGATCCATCATCCT3’, Gra-FW 5’GAGAGGATCCCATGTTCGCCGTAAAACATG3’ and Gra-RV 5’GAGAGGTACCTTACTGCGAAAAGTCTGGGA3’.

PCR products were ligated into an appropriately digested expression vector pRP261, a derivative of vector pGEX-3X (Amersham-Pharmacia).

**Immunoadfinity purification of native SAG1 and production of recombinant SAG1:** Native SAG1 was immunoadfinity purified as described earlier [45]. Recombinant SAG1 produced in *Pichia pastoris* was kindly provided by Alain Jacquet [44].

**Expression and purification of recombinant GST-Rop1, GST-Gra2 and GST:** All proteins were expressed in *E.coli* strain BL21. Before expression induction, BL21 were grown until OD₆₀₀ : 0.8-1 at 37°C. Expression was induced by isopropyl-β-D-thiogalactopyranoside (1 mM) for 2 hours at 30°C. Upon centrifugation at 3500g for 10 min, cells were resuspended in PBS (pH 7.4) with protease inhibitors (Complete, Boehringer), 1 mM EDTA, 1 mM dithiotreitol and 1% (v/v) Triton X-100. Next, the suspension was freeze/thawed three times and sonicated eight times for 15 sec (Soniprep 150) on ice. Insoluble components were removed by centrifugation (16000g, 10 min, 4°C), and subsequent filtration (0.45 μm). GST fusion proteins were purified and eluted from the supernatant using Glutathione Sepharose 4B beads according to the manufacturer’s instructions (Amersham-Pharmacia). The amount of each recombinant protein used for immunoblots was normalized based on intensity of Coomassie staining on gel and staining with anti-GST antibody (Sigma) on immunoblot (see below).

**SDS-PAGE and Western blotting:** SDS-PAGE was performed as described [9]. 200 μg of the sonicate or 10 μg of purified protein was suspended in SDS-PAGE sample buffer (without reducing agent) to a final volume of 200 μl, boiled for 2 minutes, and loaded onto 10% SDS-PAGE gels. A broad range marker (Bio-Rad Laboratories, Hercules, CA) was included. After electrophoresis, proteins were transferred to polyvinylidifluoride (PVDF) membranes (Millipore) overnight. Transferred proteins and markers were visualised using Ponceau-red dye staining. Blocking buffer consisted of Tris-buffered saline (TBS: 50 mM TrisHCl, 150 mM NaCl, pH 10) containing 0.5% Tween20 and 2% non-fat milk powder.

**Immunostaining of peroxidase conjugated antibodies:** Except when stated otherwise, all samples and conjugates were diluted in TBS containing 0.5% Tween20 and 0.03% non-fat milk powder (TBS-T). All incubations were done using a multiscreen apparatus (Mini-Protean II, Bio-Rad Laboratories). Isotype specific antibodies conjugated to peroxidase were obtained from DAKO (DAKO, Glostrup, Denmark). The conjugates were diluted 1:5000. All incubations were performed at room-temperature for 75 min.

The serum samples of the patients were diluted 50x (unless stated otherwise), incubated, and tested for the presence of anti-*T.gondii* IgM. A mouse monoclonal antibody against SAG1 (HyTest, Turku, Finland) was diluted 1000x, monoclonal Tg49 specific for Rop1 (kindly provided by Dominique Soldati, Heidelberg) was diluted 500x, Gra2 specific monoclonal (gift from Marie-France Cesbron-Delauw, Grenoble) was diluted 10000x. Chemiluminescent substrate was prepared according to the manufacturers descriptions (ECL, Amersham Life Science, Amersham Pharmacia Biotech, Essex, UK). The membranes were incubated with chemiluminescent substrate for 1 min, and exposed to X-ray film for various time-intervals.
Chapter 7

Figure 1: The 55 kDa antigen, SAG1/P30, and the GPI anchor are surface exposed antigens. Heat inactivated sera of AI 1 and CNR 5 were diluted as indicated, and mixed with a serum that contained IgM antibodies recognizing a 45 kDa T. gondii antigen (diluted 1/1000). The diluted sera were mixed sequentially (lanes labeled ‘1st’ and ‘2nd’, see method section) with either 2x10⁷ parasites in PBS or PBS only. Control lanes represent IgM staining patterns after two incubations with PBS only.

Preincubation with intact parasites: Parasites were purified as described above, resuspended in TBS, and counted. Serum samples were heated for 30 minutes at 56°C to inactivate complement, diluted in TBS, mixed with 50 µl PBS containing 2x10⁷ purified parasites and incubated for 30 min under gentle rotation. Parasites were pelleted (800xg, 5 min) after incubation and supernatants were split: half was immediately incubated with blots (fraction 1), the rest was incubated once more for 30 min with fresh parasites. After centrifugation, this double absorbed supernatant was incubated with the blots as well (fraction 2). As negative control, samples were treated similarly, but mixed with buffer without parasites (c). Before fractions were incubated with blots, Tween-20 and milk powder were added to final concentrations of 0.5% and 0.03%, respectively. Bound antibodies were detected by chemiluminescence.

Preincubations with purified recombinant Gra2, Rop1 and GST were essentially performed as described for intact parasites; 2 µg recombinant protein was used for each incubation.

Immunofluorescence (IF): IF was performed as described [26], except parasites were not fixed. In short, 10⁵ filter-purified, freshly egressed parasites were incubated with mouse monoclonal antibodies against SAG1 (HyTest, Turku, Finland) and Rop1 (Tg49), diluted 500x and 250x, respectively, in PBS containing 2% BSA, at 4°C for 30 min. Following 4 washes, parasites were incubated with an anti-mouse Fab conjugated to indocarbocyanine (Cy3) (Jackson ImmunoResearch Laboratories, West Grove, PA), diluted 750x, for 30 min. Following washing and drying, labelled parasites were embedded in Vectashield (Vector Laboratories, Burlingame, CA) and visualized using a fluorescence microscope (Leica DMRE/RD, Leica, Wetzlar, Germany) equipped with a camera. Alternatively, parasites were dried on slides and fixed with either aceton (-20°C) or 3% paraformaldehyde, 0.05% glutaraldehyde at room-temperature as described [46,47,47]. Fixed parasites were exposed to the same monoclonals.

Elution of IgM antibodies: Fragments of lysate blots containing either GPI anchor or antigens with MWs ranging between 28-32 kDa were incubated with sera
Recombinant antigens recognized by IgM

Figure 3: A monoclonal specific for Rop1 stains the surface of a minority of parasites. Intact (A-C) and fixated (D-F) parasites were incubated with anti-Rop, and analyzed with immunofluorescence. Panel B is a brightfield image of parasites in panel A. Panel C is an overlay of A and B. Panel E shows the nuclear staining of the parasites in panel D by Hoechst 33258. Panel F is an overlay of D and E, demonstrating apical Rop1 staining of fixated parasites, which was observed irrespective of fixation procedure and treatment with 0.2% Triton X100. Bar = 2 μm.

Results

The 55 kDa antigen is Rop1: IgM antibodies are usually directed against surface antigens, therefore preabsorption using intact parasites was performed to determine if the 55 kDa antigen recognized predominantly by AI sera, has surface-exposed epitopes. As shown in figure 1 preabsorption indeed resulted in disappearance of the 55 kDa band, demonstrating that this antigen is present on the surface of tachyzoites. A monoclonal specific for Rop1 also stained an antigen at 55 kDa, so, being a candidate-antigen, Rop1 was cloned and expressed as glutathione-S-transferase (GST)-fusion protein in E.coli. In addition to full length GST-Rop1 (~85 kDa), expression and purification resulted in various smaller truncated forms in the range of 70-83 kDa (not shown). Clearly, GST-Rop1 is highly susceptible to proteolytic degradation, as described earlier, possibly due to its unusual charge distribution [46,48]. Sera from acutely and latently infected individuals known to contain anti-55 kDa IgM were incubated successively with 2 μg of the purified recombinant Rop1. In each case, this resulted in gradual decrease and final disappearance of 55 kDa antigen staining from the anti-toxoplasma IgM staining patterns (figure 2), identifying these frequently found IgM antibodies in sera of acutely infected individuals as anti-Rop1 antibodies.

Rop1 is not specifically recognized as a surface-antigen [49]. However, immunofluorescence experiments using the monoclonal specific for Rop1 on intact parasites indeed showed a homogeneous surface staining of approximately 5% of the parasites (figure 3 A-B-C). Once fixated, all parasites demonstrated the expected apical...
staining pattern following incubation with the anti-Rop1 monoclonal (figure 3 D-E-F; [49]). Homogeneous staining of fixated parasites was not observed.

**Potentially confounding factors:** SAG1 and the GPI anchor are known to be major targets of the AI and CNR anti-toxoplasma IgM antibodies in humans. As SAG1 is a GPI-anchored membrane protein we wondered whether IgM antibodies specific for the GPI anchor contribute to the intense signal at 30 kDa. By elution of IgM antibodies recognizing either GPI anchor or SAG1 from blots, and subsequent testing of these eluates on a lysate blot, it was demonstrated that these antibodies only recognized the antigen they were initially eluted from (figure 4). The 30 kDa signal may not only result from SAG1 recognition, as the Gra2 specific monoclonal stained an antigen at 30 kDa as well, although Gra2 is supposed to run at approximately 28 kDa. The monoclonal proved to be Gra2 specific as recognition was efficiently abrogated following preabsorption with purified, recombinant GST-Gra2 (Figure 5).

**Recognition of a panel of recombinant T. gondii antigens by IgM:** Based on earlier results [31] and the experiments described above, a number of antigens were selected for testing with a panel of extensively characterized sera from AI and CNR individuals on immunoblot. First, it was of interest to determine with this panel of sera if recombinant Rop1 recognition could distinguish between AI and CNR sera. Secondly, we wanted to dissect the IgM signal at 30 kDa that allowed distinction between AI and CNR individuals on T. gondii lysate IB. Thus this panel of sera was tested on blots containing purified, native SAG1, recombinant Rop1, recombinant GST-Gra2 (figure 6), recombinant Sag1 and GST alone (not shown).

Recognition of recombinant Rop1 by AI and CNR IgM coincided with recognition of the 55 kDa antigen on a toxoplasma lysate immunoblot using shorter exposures (≤ 10 min, figure 6, compare upper and middle panel). Longer

![Figure 4: IgM antibodies recognizing GPI anchor do not cross-react with SAG1.](image)

IgM antibodies in serum of AI 5 strongly recognize GPI anchor and SAG1 (lane A). Serum diluted 200x, incubated with either a toxoplasma lysate blot containing antigens ranging in MW from 28 to 38 kDa (a.o. SAG1, B) or a blot containing antigens of up to 6 kDa (GPI anchor, C) was tested upon elution, using the conditions indicated (see method section) and neutralized. Samples were applied to a whole lysate blot and stained for IgM antibodies (lanes B'; SAG1 and C'; GPI anchor, respectively).

![Figure 5: Gra2 and SAG1 comigrate.](image)

The specificity of Gra2 staining was investigated by sequential incubation of both monoclonals with recombinant GST-Gra2. Monoclonal specific staining on a toxoplasma lysate blot following preabsorption is shown: anti-Gra2: lanes 2 and 3, anti-SAG1: lane 3 (only shown after two rounds of preabsorption rounds). Controls are sequential incubations with GST (lanes 4) or with PBS (lanes 1).
Recombinant antigens recognized by IgM

Figure 6: A Comparison of purified antigen and *T. gondii* lysate recognition by Al and CNR IgM.

Diluted sera were applied to blots containing purified recombinant (GST-Rop1 and GST-Gra2) and native (nSAG1) Toxoplasma proteins or total lysates (*T. gondii* lysate). Besides full-length protein (~83 kDa), expression of Rop1 resulted in various truncated versions of Rop1, ranging mainly between 70 and 83 kDa, hence the ladder of IgM bands in the rRop1 lanes.

Exposures (≥ 60 min) revealed that many samples showed an anti-Rop1 staining IgM pattern that coincided with recognition of the 55 kDa antigen, except for samples CNR 7 and CNR 12 (figure 6, compare second panel above and middle panel).

Most of the Al sera with an intense signal at 30 kDa also intensely recognized native SAG1, while none of the sera displayed strong recognition of recombinant Gra2 (figure 6, compare lower three panels). Three sera showed a weaker anti-SAG1 (Al 4, Al 10 and CNR 7) signal than expected, which may be due to recognition of a third antigen at approximately 30 kDa, while one sample had an overall weak response to the purified proteins (Al 5). In contrast, CNR sera never intensely recognized native SAG1. Except for 1 sample (CNR10, figure 6), recognition of native SAG1 by CNR IgM was as could be expected on the basis of IgM patterns on lysate immunoblot. CNR IgM never bound recombinant Gra2. None of the sera displayed reactivity on immunoblot against GST, or recombinant SAG1 (results not shown).

Discussion

In this study we tested whether a selected set of immunoblotted recombinant antigens would result in an easy and reproducible confirmation assay for toxoplasmosis serology. Immunoblotting is one of the few available methods allowing a clear distinction between CNR and Al IgM. The 55 kDa antigen preferentially recognized by IgM in Al sera [31], has now been identified as Rop1. This protein belongs to a group of antigens discharged from the rhoptries during Tg invasion and is involved in the formation of the parasitophorous vacuole [50]. Samples from both groups with a clear signal at 55 kDa on Tg lysate immunoblot indeed recognized recombinant Rop1. On longer exposure many other serum samples displayed reactivity against rec Rop1, especially in the CNR IgM group, despite the
absence of a signal at 55 kDa in the IgM staining patterns analyzed. These anti-Rop1 antibodies might have been missed in our earlier analysis [31], as the exposures used were too short to allow detection of low titers of anti-Rop1 IgM.

Surprisingly, IgM antibodies specific for Rop1 were preabsorbed using intact parasites, while Rop1 is not known to be a surface antigen. Our finding was supported by surface staining of a minority of the intact parasites by the Rop1 monoclonal. Experiments with fixed parasites demonstrated exclusive anti-Rop1 staining of the anterior part of the parasite as described earlier [49,51]. Studies that describe localization and function of Rop1 during infection generally have used fixed cells, which might explain how surface staining was missed previously. The function of Rop1 at the parasites surface remains enigmatic.

Rop1 (P66) is a known target of IgM antibodies during acute infection, and has been selected for an anti-Tg IgM ELISA based on recombinant antigens [52]. Our results indicate that Rop1 as single antigen is not suitable for diagnostic assays, as anti-Rop1 antibodies may cause persistent backgrounds due to intense recognition by CNR IgM. In combination with other recombinant antigens, however, Rop1 may still be valuable in an IgM IB assay as CNR IgM generally recognizes only 1 antigen intensely.

As expected, the signal at 30 kDa observed in previous experiments can, in most cases, be attributed to anti-Sag1 IgM. Surprisingly, Gra2 turned out to be a potential confounding antigen, despite its predicted lower molecular weight. The observation that Gra2 and Sag1 comigrate might be a peculiarity of the tricine gels. Although anti-Gra2 antibodies constitute part of the anti-Tg IgG repertoire and recombinant Gra2 is well recognized by IgG [53], recombinant Gra2 was hardly recognized by IgM and could thus be excluded as potential confounder. This confirms earlier findings that Gra2 is not a major target of anti-Tg IgM antibodies [52].

Another potential contribution to the signal at 30 kDa could stem from anti-GPI anchor IgM. In a Tg lysate, Sag1 is known to be GPI anchored [54,55]. Two glycoforms of the Tg GPI anchor have been described, of which type B contains the unique and immunogenic glycosylated N-acetylgalactosamine side chain [56]. Using monoclonals, at least two epitopes have been identified on the GPI anchor that are sensitive to periodate and phosphatidylinositol specific phospholipase C treatment [15,57]. These GPI specific monoclonals do not stain membrane antigens on a lysate immunoblot [57], indicating that these epitopes are not available when the GPI anchor is coupled to a membrane antigen. This does not exclude that a polyclonal (human) anti-GPI response could generate cross-reactive antibodies. Nevertheless, our results indicate that also during a polyclonal response anti-GPI antibodies do not stain Sag1.

Following exclusion of confounding factors, we finally tested a recombinant version of Sag1 that is recognized by IgG antibodies in sera of latently infected individuals [44]. This indicates that at least part of the recombinant Sag1 is assembled in the proper conformation for IgG recognition. Unfortunately, IgM did not recognize this recombinant Sag1. Other attempts to incorporate recombinant Sag1 in IgM diagnostic assays have failed probably due to the complex structure of native Sag1 [52]. Importantly, recognition by IgM strongly relies on protein conformation, which is dependent on proper disulfide bridges [45]. The conformation of Sag1 results from a fine-tuned assembly interplay by several Tg chaperones and oxidoreductases. Although there is a report that mentions recognition by AI IgM of cloned Sag1 [58], it is difficult to express properly folded Sag1 in the large amounts required for standardized diagnostic assays.

IgM in two sera appeared to recognize another antigen at 30 kDa next to Sag1. A possible candidate that we have not tested is the P35 antigen that runs also at approximately 31 kDa under non-reduced conditions [24]. Recombinant P35 performed well in an anti-Tg IgM ELISA [52,59] and could be an alternative to Sag1 to be tested in an IgM IB assay.
Potentially important for the next generation of anti-Tg IgM assays are attempts to produce the GPI anchor in vitro [60]. Our previous results argue against the use of the GPI anchor as a single antigen in an assay, but in combination with other cloned antigens it will certainly be of value.

Recombinant Rop1 might be suitable as one of the recombinant antigens in an IgM immunoblot assay, but has to be combined with at least one other, more immunogenic antigen like Sag1. Although immunoblotted native Sag1 can distinguish very well between Al and CNR sera, it clearly is difficult to make recombinant Sag1 recognizable by IgM. Promising alternatives are recombinant P35 and synthetic GPI anchor.
Referenc e List Chapters 6 and 7


110


Reference list chapters 6 and 7


57. Tomavo, S., G. Couvreur, M. A. Leriche *et al.* 1994. Immunolocalization and characterization of the low molecular weight antigen (4-5 kDa) of Toxoplasma gondii that elicits an early IgM response upon primary infection. *Parasitology* 108:139-145.


