Natural adaptive immune responses in humans against Toxoplasma gondii and Herpes simplex virus type I
Meek, B.
Chapter 1

IgA antibodies to Toxoplasma gondii in human tears

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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.
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 immuno-compromised 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 venupuncture. 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.
Chapter 1

Figure 1: Tear antibody staining patterns.
(A): Volunteer 10: An example of a typical tear IgA staining pattern showing the 4 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-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 IgA response sometimes obscured the anti-74-kDa signal (indicated by an arrow in the IgA lane).

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-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-T. gondii IgG and IgA in serum and tear fluid, and anti-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-Pharmacia Biotech, Essex, UK). The membranes were incubated with the chemiluminescence substrate for 1 minute, wrapped in plastic, and exposed to x-ray film.

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 T.gondii IgG avidity assay according to the manufacturer’s instructions (Labsystems, Helsinki, Finland).

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 10^7 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.
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 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, Uppsala, 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**

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.
Figure 3: Anti-toxoplasma IgA antibodies remain detectable for at least 16 months. Tear samples were collected from volunteers 8 and 9 at 12 months (2) and 16 months (3) after the first sample (1), and tested for the presence of anti-*T. gondii* IgA. IgA antibodies specific for the 74-, 70-, 49-, and/or 34-kDa antigens (the standard staining pattern, indicated by arrows) remained detectable in tears throughout this period. This also accounts for several other unique bands (arrowheads), whereas other bands could be found only at one or two time points. MW, molecular weight (in kilodaltons).

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-*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 M I system, several consecutive tear samples were collected from six volunteers over a period of 16 months. After anti-*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). **Serology:** According to the SF dye test 14 (23%) of 62 volunteers had circulating anti-*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-*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-*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.

Table 1: Anti-*T. gondii* IgA antibodies in tears and chronic infection.

<table>
<thead>
<tr>
<th>Anti-<em>T. gondii</em> IgA in tears</th>
<th>SF titer</th>
<th>Presen t</th>
<th>Not present</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td><strong>Positiv e</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>12 (85)</td>
<td>2 (15)</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Negative</td>
<td>39 (81)</td>
<td>9 (19)</td>
<td></td>
<td>48</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>51 (82)</td>
<td>11 (18)</td>
<td></td>
<td>62</td>
</tr>
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</table>

Anti-*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-*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...
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-\textit{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-\textit{T. gondii} IgA response could be detected in a high number (51/62) of tear-samples obtained from healthy volunteers. The anti-\textit{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 \textit{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-\textit{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-\textit{T. gondii} IgA antibodies in tears was intermediate compared with most IgG antibodies specific for \textit{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 \textit{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-\textit{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 \textit{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 \textit{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 \textit{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 \textit{Staphylococcus epidermidis}, \textit{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. \textit{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 \textit{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-\textit{T.gondii} IgA observed in tears is the remarkably similar anti-\textit{T.gondii} sIgA staining patterns of whey from breast milk of infected mothers [3]. The antibodies present in whey were capable

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**Figure 6:** Anti-\textit{T.gondii} IgA antibodies in tears are directed against surface-exposed antigens. Diluted tear and serum samples were incubated with either PBS or intact \textit{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 1); 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 *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 *T. gondii*. In contrast, the seronegative controls participating in the study of Mack and McLeod [3] did not have anti- *T. gondii* slgA in their whey, whereas this study and the results of Hajeer *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 *T. gondii* in the specific geographic areas involved [23]. Loyola *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 *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 *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 *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 *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 *T. gondii* antigens. Whether the MIS is capable of preventing viable parasites from systemic infection, remains to be investigated.

In conclusion, anti-*T. gondii* IgA antibodies specific for surface exposed antigens of *T. gondii* were frequently found in tears of healthy volunteers. There was no apparent relation between the presence of anti-*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 *T. gondii* or represent a part of the natural antibody repertoire. However, the seroprevalence of *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 1