Detection and Characterization of Circulating Antigens in Acute Experimental Infections of Mice with Four Different Strains of Toxoplasma gondii*

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Summary

Acute infections with four different strains of *Toxoplasma gondii,* all of them being highly pathogenic for mice, were provoked by intraperitoneal injection of 2.2 x 10^7 trophozoites. The times of the appearance of circulating antigens in the sera of the infected mice were determined and the amounts of these antigens were measured by an enzyme-linked immunosorbent assay. The molecular weight of the circulating antigens was determined by gel filtration and by Western blot following PAGE. The isoelectric points of these antigens were determined by immunoblotting after isoelectric focusing.

Circulating antigens were detectable up to ng amounts/ml serum from the 1st day p.i. onwards. The circulating antigens consisted of a number of proteins with molecular weights of > 10^4, 300, 65, 25, and < 5 kd. The isoelectric points of these proteins were situated between pH 3.5 and pH 7. Time of appearance and structure of the circulating antigens were very similar in all four *Toxoplasma* strains. Circulating antigens are apparently generated regularly in experimental acute *Toxoplasma* infections, and lysis of parasite cells appears to be the major mechanism of their formation.

Zusammenfassung

Durch intraperitoneale Injektion von je 22 Millionen Trophozoiten von vier verschiedenen, hochpathogenen *Toxoplasma gondii*-Stämmen wurden in Mäusen akute Infektionen ausgelöst. Der Zeitpunkt des Auftretens und die Menge von zirkulierenden Antigenen in den Seren der Tiere wurden mittels eines ELISA bestimmt. Das Molekulargewicht der zirkulierenden Antigene wurde mittels einer Gelfiltration und eines Immunoblots nach einer PAGE gemessen, die Isoelektrischen Punkte der Antigenmoleküle wurden mit Hilfe eines Immunoblots nach einer Isoelektrischen Focussierung bestimmt.

Zirkulierende Antigene konnten in ng-Mengen/ml Serum ab dem ersten Tag nach der Infektion nachgewiesen werden. Die zirkulierenden Antigene bestanden aus einer Anzahl von Proteinen mit Molekulargewichten von > 10^4, 300, 65, 25 und < 5 kD. Die Isoelektrischen Punkte dieser Proteine lagen zwischen pH 3,5 und 7. Der Zeitpunkt des Auftretens ebenso wie wesentliche molekularbiologische Eigenschaften der zirkulierenden Antigene aller vier Stämme waren sehr ähnlich. Zirkulierende Antigene scheinen bei experimentellen akuten Toxoplasma-Infektionen regelmäßig gebildet zu werden, wobei die Lyse der Parasitenzellen den wichtigsten Mechanismus zur Antigen-Freisetzung darstellt.

Introduction

The appearance of circulating antigens (cags) of *Toxoplasma gondii* in sera of mice is a characteristic of experimentally induced toxoplasmosis (1, 4, 13, 15, 17). Although the experimental designs and methods applied in prior studies (4, 15) were similar to a certain extent, there are strange inconsistencies in the findings published concerning the biochemical and biological properties of the cags, which may in part be due to parasite strains with different virulence properties. Thus, we investigated and compared quantities and some structural characteristics of the cags detectable in the sera of mice experimentally infected with virulent strains of *Toxoplasma gondii*.

Materials and Methods

Four strains of *Toxoplasma gondii* (BK, RH, T, and 928; for characteristics see Table 1), were maintained by serial passage in the peritoneal cavities of mice. After harvesting on day 2 p.i. or day 3 p.i., respectively (see Table 1), the parasites were washed three times in physiological saline and counted. 2.2×10^7 cells of each strain (with less than 0.1% mouse cells) were inoculated into the peritoneal cavity of 30 mice (female; 20 g; Him: OF1 (Swiss) SPF; source: Versuchstierzucht Himberg, Austria) (day 0). At intervals of 24 h, five mice were anesthetized, bled by heart puncture and killed. This procedure was carried out as long as living mice were available. Infections with each of the four strains were lethal for the mice within five days. The serum samples of between 0.2 and 0.8 ml/mouse were stored at —70 °C. Negative control sera were obtained by heart puncture from non-infected mice.

On day 2 (strains RH and T) or day 3 p.i. (strains 928 and BK), the parasites were collected from the peritoneal cavities of the bled mice. Specimens were checked to contain less than 0.1% mouse cells, washed three times in physiological saline, and suspended in distilled water to give a final concentration of 1×10^9 cells/ml. These suspensions were

Table 1. Characteristics of the *Toxoplasma* strains RH, BK, T, 928

Strain	Origin	Time of maturity	Date of isolation	Author/source
RH	Human brain	43 h	1939	(14)
BK	Human ventri-cular fluid	68 h	1948	(3)
T	Unknown	43 h	before 1981	Dr. *Thalhammer,* Univ. Vienna
928	Pig lung	68 h	1961	Dr. *Overdulve,* pers. comm.

stored at –70 °C. After thawing, the lysates were mixed, sonicated for 30 s, and centrifuged at 1700 g for 20 min. The supernatant, pure *Toxoplasma* antigen in a concentration of 0.7 mg/ml (Protein Assay, Bio-Rad Laboratories Ges.m.b.H., Vienna), was used as a positive control and as a standard for titration throughout the further procedures.

The sera of the infected mice and the control sera were tested and titrated in an enzyme-linked immunosorbent assay for *Toxoplasma* antigens (cags-ELISA), which had been set up as follows: serocluster EIA plates (Costar, Cambridge, MA) were coated with 5 µg of F$_{(ab)2}$-fragments of human IgG antibodies diluted in 100 µl 0.1 M carbonate buffer pH 9.6 (IgG preparation from a pool serum with high *anti-Toxoplasma* antibody titres (IFAT: 1 : 1000, CFT: 1 : 40) by affinity chromatography on protein-A sepharose according to the manufacturer's recommendation (Pharmacia Ges.m.b.H., Vienna); F$_{(ab)2}$-preparation according to (12), specificity of F$_{(ab)2}$-parts to *Toxoplasma gondii;* see Fig. 1). After blocking free binding sites with supplemented ELISA buffer (116 mM phosphate-buffered saline, pH 7.2 (PBS) + 0.05% Tween 20 + 2% bovine serum albumin), the sera, all diluted 1 : 22 in ELISA buffer, the control antigen, diluted to different concentrations in ELISA buffer, and plain buffer were added to the wells and incubated for 18 h at 4 °C. Then, affinity-purified rabbit IgG antibodies against *Toxoplasma gondii* were added (for specificity see Fig. 1). After an incubation of 2 h at 37°C, conjugate (goat anti-rabbit IgG/PO, Polysciences Inc., Washington, PA; diluted 1 : 1000 in ELISA buffer) was added; our substrate was 0.005% H$_2$O$_2$ with 5-aminosalicylic acid (EGA-Chemie, Steinheim, FRG), purified according to (5). Tests were read after 20 min, and extinction values were determined at 450 nm by the use of a photometer.
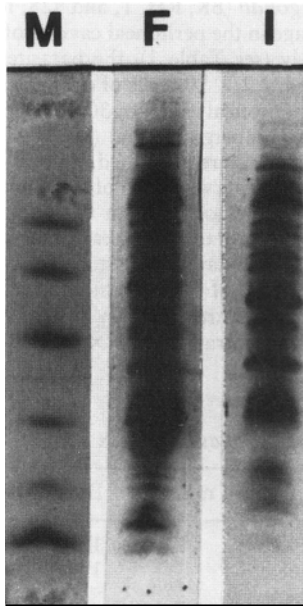

Fig. 1. Western blot of the human *anti-Toxoplasma* F$_{(ab)2}$-parts (F) and rabbit anti-Toxo-*plasma* IgG (I) used in the ELISA for cag detection. The antibodies were tested against an antigen obtained by lysis of *Toxoplasma* trophozoites after SDS-PAGE under reducing conditions. M: marker proteins; from top: 94, 67, 43, 30, 20.1, 14.4 kd.

Thereafter, the ten sera of each strain from days 2 and 3 were pooled separately, and these mixtures were used as sources of strain-specific cags. The sera of the non-infected mice were also pooled and used as negative controls. As a first step, these pool sera as well as the *Toxoplasma* antigen were separated by gel filtration. 100 µl of sera were applied to a 10/30 column of Superose 6 HR (Pharmacia Ges.m.b.H.). As an eluant, PBS with 0.1% Tween 80 was used with a constant flow of 6 ml x h^{-1}. Fractions of 1 ml were collected and frozen at — 70 °C until tested for their antigen content in the cags-ELISA. For determining the a.m.u. of the fractions collected, commercially available marker proteins (Pharmacia Ges.m.b.H) were co-filtrated.
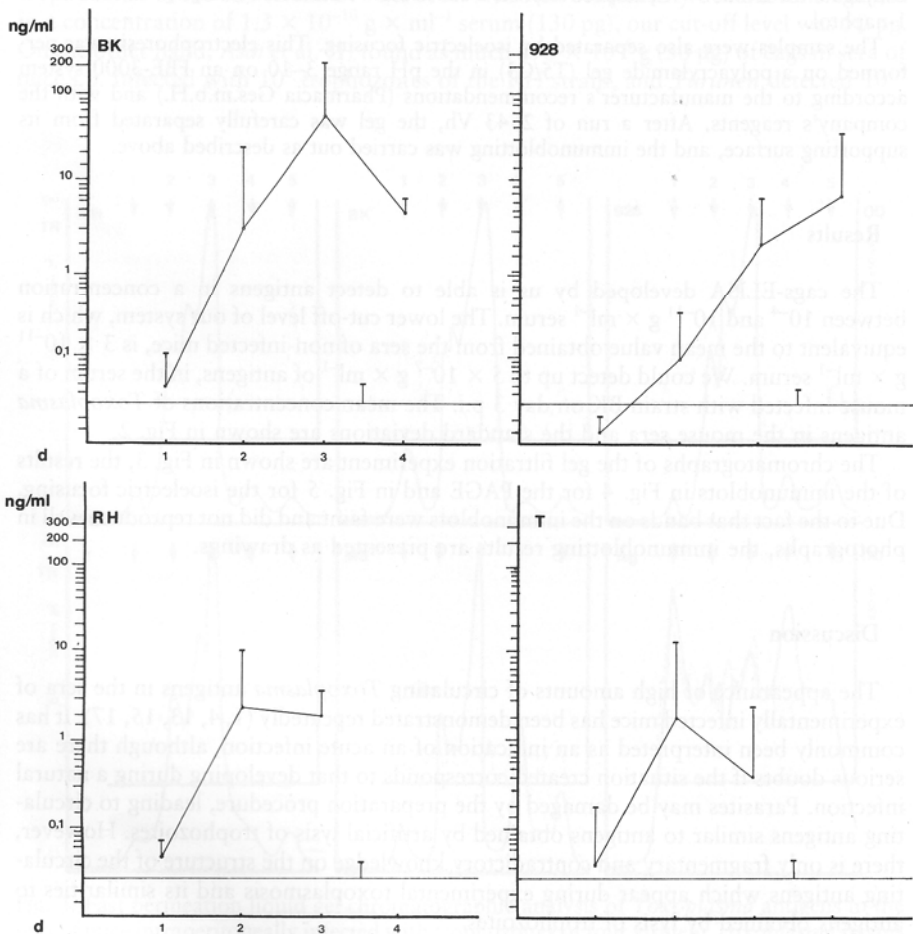
Fig. 2. Amounts of circulating *Toxoplasma* antigens in the sera of mice after intraperitoneal inoculation of 2.2 x 10^7 trophozoites of four virulent strains (BK, RH, T, 928) measured by an ELISA and calculated by comparison with a *Toxoplasma* antigen obtained by lysis of trophozoites. Values are means + standard deviations (five animals each). The line at 30 pg is the cut-off level determined by testing sera of non-infected mice.

Then, the pool sera and the antigen were separated in PAGE followed by immunoblotting. 10 µl of the samples and commercially available marker proteins were mixed with 10 µl sample buffer (90 mM Tris, 80 mM boric acid, 2.5 mM Na_2EDTA) and applied to a 4/30 slab gel and run on a GE 2/4 vertical system according to the manufacturer's recommendations (Pharmacia Ges.m.b.H.). After a run of 650 Vh (125 V constant, about 5 h) the gel was soaked in transfer buffer (25 mM Tris, 195 mM glycine, pH 8.3 + 20% methanol) for 30 min. Thereafter, the proteins were blotted to a nitrocellulose membrane by a 7 V x cm^{-1} gradient overnight (325 Vh) using a Bio-Rad transfer chamber. The membrane was cut into two pieces; piece one containing the marker proteins was stained with amidoblack (staining: 10 min in 65 ml a.d. + 25 ml methanol + 10 ml acetic acid + 0.1 g amidoblack; destaining: 2 x 10 min in 65 ml a.d. + 25 ml methanol + 10 ml acetic acid). The other part was soaked in ELISA buffer, incubated with the rabbit IgG antibodies used in the cags-ELISA and in the conjugate for 2 h at 37 °C, respectively. As a substrate we used 0.01% H_2O_2 with 4-chloro1-naphtol.

The samples were also separated by isoelectric focusing. This electrophoresis was performed on a polyacrylamide gel (T5/C3) in the pH range 3-10 on an FBE-3000 system according to the manufacturer's recommendations (Pharmacia Ges.m.b.H.) and with the company's reagents. After a run of 2543 Vh, the gel was carefully separated from its supporting surface, and the immunoblotting was carried out as described above.

Results

The cags-ELISA developed by us is able to detect antigens in a concentration between 10^{-4} and 10^{-11} g x ml^{-1} serum. The lower cut-off level of our system, which is equivalent to the mean value obtained from the sera of non-infected mice, is 3 x 10^{-11} g x ml^{-1} serum. We could detect up to 5 x 10^{-7} g x ml^{-1} of antigens, in the serum of a mouse infected with strain BK on day 3 p.i. The mean concentrations of *Toxoplasma* antigens in the mouse sera and the standard deviations are shown in Fig. 2.

The chromatographs of the gel filtration experiment are shown in Fig. 3, the results of the immunoblots in Fig. 4 for the PAGE and in Fig. 5 for the isoelectric focusing. Due to the fact that bands on the immunoblots were faint and did not reproduce well in photographs, the immunoblotting results are presented as drawings.

Discussion

The appearance of high amounts of circulating *Toxoplasma* antigens in the sera of experimentally infected mice has been demonstrated repeatedly (1, 4, 13, 15, 17). It has commonly been interpreted as an indication of an acute infection, although there are serious doubts if the situation created corresponds to that developing during a natural infection. Parasites may be damaged by the preparation procedure, leading to circulating antigens similar to antigens obtained by artificial lysis of trophozoites. However, there is only fragmentary and contradictory knowledge on the structure of the circulating antigens which appear during experimental toxoplasmosis and its similarities to antigens obtained by lysis of trophozoites.

Our experiments were carried out with the aim of characterizing the cags of different *Toxoplasma* strains with a similar virulence (for characteristics see Table 1 (2)). Due to their very high, though not equally pathogenicity for mice, our infection experiments lasted three or four days only. In all cases, the amounts of antigens in the sera rose from the negative level (3 x 10^{-11} g x ml^{-1}) up to at least 3 x 10^{-9} g x ml^{-1} during the time

of the experiment. The decrease of antigenemia on the last day of the infection experiments with strains RH, T, and BK may be simply attributed to a lower multiplication rate of the parasites in those few mice which had survived until that day. From the group of mice infected with strain 928, which is the strain with the lowest pathogenicity used in our study, almost none of them died before day 4 p.i.

The results of our infection experiments are in good agreement with those of *Brook*s et al. (4), if we consider the dependence of the time of cag appearance on the infection dose. These authors detected cags in the sera of mice from the 2nd day p.i. after i.p. application of 5×10^6 trophozoites of RH strain, whereas an infection with 5×10^3 cells led to antigenemia not before day 6. On the other hand, *Raizman* and *Neva* (13) detected an antigenemia on day 2 p.i. after i.p. infection of mice with only 10^2 trophozoites of the RH strain. *Brooks* et al. (4) could consistently demonstrate antigen in a concentration of 1.3×10^{-10} g x ml^{-1} serum (130 pg), our cut-off level was 50 pg. On the other hand, *Asai* et al. (1) found as much as 3×10^{-5} g (30 µg) of cags in sera of mice i.p. infected with 10^6 trophozoites of the RH strain, and *Turunen* detected 10^{-6}
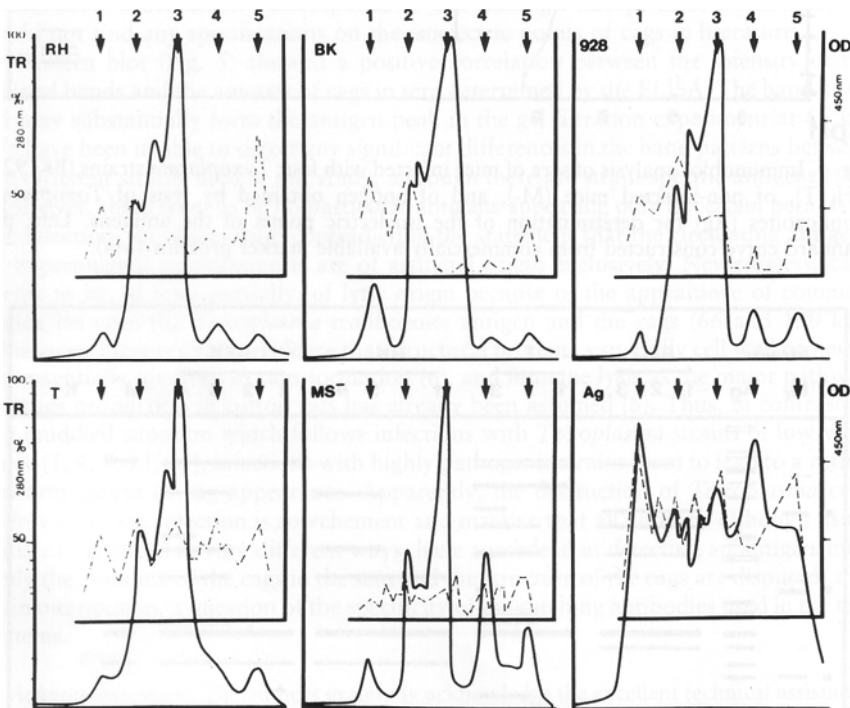

Fig. 3. Fast permeation liquid gel chromatographic analysis of *Toxoplasma* antigens in the sera of mice intraperitoneally infected with trophozoites of four virulent *Toxoplasma* strains (BK, *928*, RH, T). MS-: pool serum of non-infected mice, Ag: antigen obtained by lysis of *Toxoplasma* trophozoites. Left scale + solid line: transmission of the FPLC fractions of sera or antigen at 280 nm, right scale + dotted line: antigen levels in the FPLC fractions of the sera or the antigen measured by a cags-ELISA. Arrows: recurring antigen peaks at > 104, 300, 65, 25, and < 5 kd.
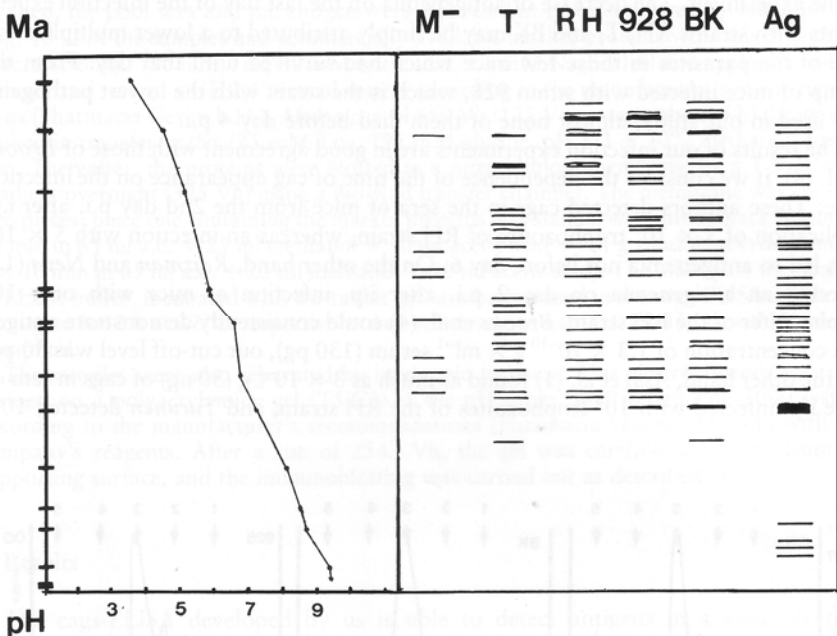
Fig. 4. Immunoblot analysis of sera of mice infected with four *Toxoplasma* strains (BK, 928, RH, T), of non-infected mice (M-), and of antigen obtained by lysis of *Toxoplasma* trophozoites (Ag) for determination of the isoelectric points of the antigens. Left: pH standard curve constructed from commercially available marker proteins (Ma).
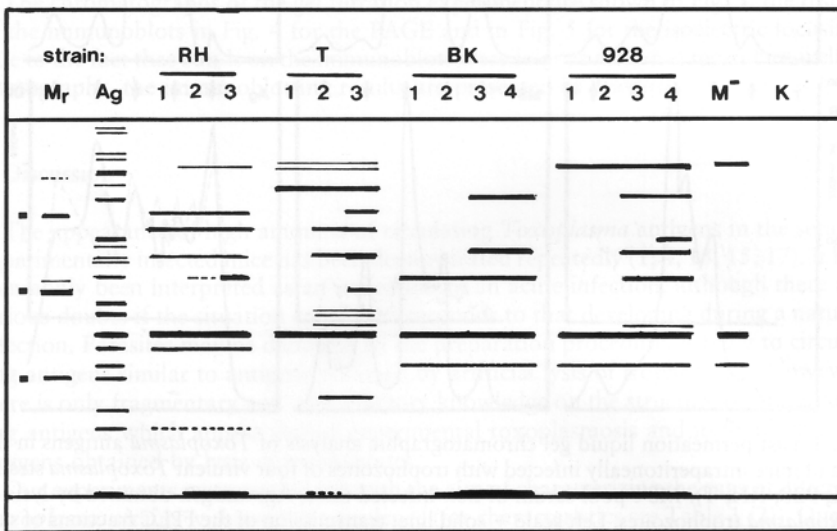

Fig. 5. Immunoblot analysis after PAGE of sera of mice infected with four *Toxoplasma* strains (BK, 928, RH, T), of non-infected mice (M-), of antigen obtained by lysis of *Toxo-plasma* trophozoites (Ag), and of buffer (K). Numbers below strain designation refer to days p.i. on which the sera were obtained. Mr: amido black stained marker proteins, from top: aldolase (150 kd), bovine serum albumin (66 kd), and hen egg albumin (45 kd).

$g \times ml^{-1}$ (16). We could not detect any significant differences in the antigen levels or in the time patterns of antigen appearance between our four *Toxoplasma* strains.

By gel filtration of the specimens and subsequent testing of the fractions, we could demonstrate five distinct antigen peaks (Fig. 3: Nos. 1-5). The molecular weights of these peaks were $> 10^4$ kd (1, void volume), about 300 kd (2), about *65* kd (3), about 25 kd (4), and $<$ 5 kd (5, total volume). Each pool serum which contained antigens showed these five peaks, only the peak heights varied between the strains. A major component of cags in the sera of mice with an a.m.u. of 324 kd has repeatedly been described by several authors (8, 9, 10). We assume that peak 2 is identical with this 324 kd antigen. Interestingly, cags in s.c. infected rabbits consisted of four completely different constituents of > 400, 220, 130, and 45 kd (11), respectively. We could neither detect these antigens nor any strikingly high concentration of a 250 kd component (NTPase) as described by *Asai* et al. (1).

During isoelectric focusing, the cags split into a number of protein bands of different isoelectric points (Fig. 4). Very strong and specific bands could be detected at pI 5.25, 5.10, 4.7, and 4.4. The 324 kd cags constituant had an isoelectric point of 5.28 (9, 10). *Hughes* (8) has described a cags component with a pI between 4.4 and 4.63, an observation which closely corresponds to our findings. Except these statements, we could not find any specifications on the isoelectric points of cags in literature.

Western blot (Fig. 5) showed a positive correlation between the intensity of the stained bands and the amount of cags in sera determined by the ELISA. The band at 66 kd may substantially form the antigen peak in the gel filtration experiment at 65 kd. We have been unable to detect any significant differences in the band patterns between the different strains used in our study, although there are strain-specific antigens (18). Moreover, there seem to be no particular antigens appearing only at certain periods of the infection; an observation contradicting the statement that the circulating antigens of experimental toxoplasmosis are of artificial origin exclusively. Nevertheless, cags seems to be, at least partially, of lytic origin because of the appearance of common bands between the *Toxoplasma* trophozoite antigen and the cags (66 and 100 kd). Moreover, there is strong evidence that structural proteins, especially cell wall proteins, are essentially involved in cags formation (6), and immune lysis as the major pathway for cags production in cotton rats has already been assumed (8). Thus, in contrast to the muddled situation which follows infections with *Toxoplasma* strains of low virulence (1, 4, 7, 11, 15), infections with highly pathogenic strains seem to lead to a rather uniform course of cag appearance. Apparently, the destruction of *Toxoplasma* cells following in i.p. injection is so vehement and massive that all authors, although using antisera produced in very different ways, have succedeed in detecting an antigenemia. Only the amounts of the cags in the sera and the structure of the cags are disputed; this is, in our opinion, a question of the specificity of the catching antibodies used in the test systems.

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