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Experimental Studies on Circulating Antigen of Toxoplasma gondii in Intermediate Hosts: Criteria for Detection and Structural Properties

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Abstract

The present study was performed to demonstrate circulating antigen (cag) of *Toxoplasma gondii* in the sera of orally and intraperitoneally infected rabbits and swine, to determine the time of their appearance after infection, and to characterize the antigenic components of the cag by means of affinity chromatography, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretic blot onto nitrocellulose sheets. Cag, as detected by an indirect ELISA, was found in the sera of both rabbits and swine from week 5 to week 8 after infection. Electrophoretic separation of cag extracted from swine and human sera showed 5 and 8 distinct protein bands, respectively, the molecular weight of which ranged between 25 and 100 kD. After Western blot, 2 of the 5 protein bands of swine-cag (27 and 57 kD) and 3 of the 8 protein bands of human cag (27, 32, and 57 kD) reacted with the anti-Toxoplasma antibody used in the ELISA. On the basis of the data presented, the influence of the dose and mode of infection as well as that of the preparation method of antisera on cag detection is discussed.

Zusammenfassung

Zirkulierendes Antigen von *Toxoplasma gondii* (zAg) wurde in den Seren experimentell oral und intraperitoneal infizierter Kaninchen und Schweine mittels eines indirekten ELISA nachgewiesen und der Zeitraum des Auftretens nach der Infektion festgestellt. Darüber hinaus wurde zAg aus den Schweineseren und einem Humanserum mittels Affinitätschromatographie isoliert und das Molekulargewicht der Proteine durch SDS-Polyacrylamid-Gel-Elektrophorese (SDS-PAGE) und Western Blot ermittelt. ZAg konnte in Schweine- und Kaninchenseren von der 5. bis zur 8. Woche nach der Infektion nachgewiesen werden. Durch elektrophoretische Auftrennung des zAg aus Schweine- und Humanseren in der SDS-PAGE konnten 5 bzw. 8 deutliche Proteinbanden mit Molekulargewichten zwischen 25 und 100 kD identifiziert werden. Nach der Transferierung auf Nitrozellulose reagierten 2 der Banden (MG 27 und 57 kD) des zAg aus Schweineseren und 3 Banden (MG 27, 32 und 57 kD) des zAg aus Humanserum mit dem im

ELISA verwendeten Antikörper gegen *Toxoplasma gondii.* Auf Grund der vorliegenden Ergebnisse wird die Bedeutung des Infektionsmodus, der Infektionsdosis sowie der Verarbeitungsmethode der Antiseren zur Herstellung von Testreagenzien für den Aussagewert des zAg-Nachweises diskutiert.

Introduction

The diagnosis of infection with *Toxoplasma gondii* mainly relies on the detection of specific antibodies in the serum of the holt. A wide range of methods for identification of the various groups of antibodies has been developed for diagnostic purposes. A combination of these laboratory procedures generally allows a definite diagnosis of an acute infection *(Aspöck* and *Flamm,* 1984). Nevertheless, attempts have been made to improve and accelerate the diagnosis of a fresh infection by additional demonstration of circulating *Toxoplasma* antigen (cag). Experiments with intraperitoneally and subcutaneously infected laboratory animals convincingly demonstrated that cag can regularly be detected within the initial, acute stage of infection *(Raizman* and *Neva,* 1975; *Van Knapen* and *Panggabean,* 1977). These findings led to the conclusion that detection of cag is indicative of an active infection *(Van Knapen* and *Panggabean,* 1977; *Van Knapen,* 1984). Thus, a triple test, in particular an ELISA, was developed to determine simultaneously cag and specific IgG and IgM antibodies by testing a single serum sample *(Van Knapen* and *Panggabean,* 1980).

We have performed the ELISA for cag detection according to *Van Knapen* and *Panggabean* (1977) with more than 10 000 sera in the course of toxoplasmosis pregnancy surveillance *(Hassl* et al., 1984). No correlation could be found between the detection of cag and specific antibodies in sera. We, therefore, decided to conduct an experimental study in laboratory animals to clarify the appearance of cag and its relation to the detecting system, particularly to the preparation of antisera and to the mode of infection. Our special interest was focused on the appearance of cag in the sera of animals infected orally according to the natural way of infection. Furthermore, we tried to isolate the cag detected in human and animal serum samples and to separate it subsequently by SDS-PAGE.

Materials and Methods

1. *Infection of animals*

1.1 Rabbits

Ten female outbred SPE rabbits (breed: Iva, CHB), 1.5 to 2 kg of weight, were divided into four groups. Three animals (group 1) were infected with about 3×10^6 oocysts of the Illmitz strain of *Toxoplasma gondii (Edelhofer* et al., 1984) by oral inoculation. The Illmitz strain is a strain of lowgrade virulence for mice and rabbits and was originally isolated from the brain of a pig in 1982. Oocysts were obtained by feeding a cat with brains of infected mice and consecutive isolation of oocysts from faeces. Three rabbits (group 2) were infected with about 3×10^6 trophozoites of the Illmitz strain by oral inoculation, and three animals (group 3) were infected with the same amount of trophozoites by p. inoculation. One animal was kept for control.

The rabbits were regularly bled by cardiopuncture twice a week for ten weeks. Blood samples were centrifuged at 170 g for 10 min and sera were stored at —20°C.

1.2 Swine

Three conventionally raised 10 weeks old swine (breed: Duroc; Niederösterreichische Landes-Landwirtschaftskammer) were tested for antibodies against *Toxoplasma gondii* in an indirect hemagglutination assay (IHA) (Cellognost Toxoplasmosis; Behringwerke, Marburg, FR Germany). As no specific antibodies to *Toxoplasma gondii* were detectable, two of the swine, one male and one female, were infected by oral inoculation of 2 x 10^6 oocysts of the Illmitz strain. One swine was kept for control. The swine were bled by puncture of the jugular vein at days 7, 11, 14, 25, 27, 29, 32, 37, 39, 40, 46, 49, and 55 p. i. Sera were collected and stored as described above.

2. Serological procedures

2.1 ELISA for detection of cag (cag ELISA)

2.1.1 Production of antiserum. Hyperimmune antisera to *Toxoplasma gondii* were obtained by oral infection of two seronegative conventionally raised rabbits, 2.5 kg of weight, with 10 oocysts of the BK strain. Booster inoculations of 10^5 oocysts were given at days 10 and 21 p. i. The rabbits were bled several times after day 46, when antibodies in the IHA test had reached a titre of 1 : 256 000.

2.1.2 Purification of antiserum. The antisera of the rabbits were mixed, dialysed against 0.02 M K_2HP0_4, pH 8, and applied to a column filled with DEAE Affi-Gel Blue (Bio-Rad Lab., Vienna, Austria). IgG separation was performed according to the manufacturer's recommendation. Fractions containing antibodies were pooled, lyophilised and stored at 4 °C.

2.1.3 Preparation of F(ab)$_2$ parts. F(ab)$_2$ parts of purified IgG antibodies were prepared by pepsin digestion as described by *Nisonoff* et al. (1960). The F(ab)$_2$ parts were lyophilised and stored at 4 °C.

2.1.4 Preparation of ELISA conjugate. Purified rabbit antibodies were labelled with horseradish peroxidase (Serva, Heidelberg, FR Germany) by means of the periodate method according to *Nakane* and *Kawaoi* (1977). The conjugate was stored at —20 °C.

2.1.5 ELISA procedure. The ELISA was carried out according to the method of *Van Knapen* and *Panggabean* (1977). In short: Wells of polystyrene plates (Nunc, Kamstrup, Denmark) were coated overnight at 4 °C with 0.1 ml of F(ab)$_2$ parts. The protein content was 23 µg/ml, evaluated by means of a protein assay kit (Bio-Rad Lab.) using a bovine plasma gamma globulin as standard. After three washings with ELISA buffer (0.116 M phosphate-buffered Praline (PBS), pH 7.2 + 0.05% Tween 20 + 2% bovine serum albumin) 0.1 ml of undiluted serum was added to the wells and plates were incubated for 2 h at 37°C. After washing, 0.1 ml of conjugate diluted 1 :50 in ELISA buffer was added. The optimal dilution of the conjugate and the optimal dilution of the F(ab)2 parts were determined in preliminary block titrations. After 2 h of incubation and washing, the substrate (5- aminosalicylic acid [purified for an increased ELISA-sensitivitiy according to *Ellens* and *Gielkens*, 19801 + 0.005% H_20_2) was added. Tests were read after 30 min of incubation and extinction values were determined by a photometer at 450 nm. Positive controls were performed with sera from mice (Him : OF 1 [Swiss] SPF) infected with *Toxoplasma gondii* two days before and with human serum which had been mixed with soluble Toxoplasma antigen. Normal human and mouse sera served as negative controls.

2.2 ELISA for detection of rabbit IgG and IgM antibodies

The antigen for the ELISA was prepared by sonication of trophozoites of the BK strain yielded from the peritoneal exsudate of mice, which had been infected two days before. The lysate was centrifuged at 700 g for 10 min and the supernatant was concentrated and dialysed against PBS. Wells of microtiter plates (Nunc) were coated overnight at 4 °C with 0.1 ml of this soluble antigen, protein content: 25 µg/ml, evaluated as described above. Serum samples, diluted 1 : 10 in ELISA buffer, were applied and plates were incubated for

2 h at 37°C. The specific conjugate used for detection of IgG antibodies was an IgG fraction of goat anti-rabbit IgG/PO (Cappel Lab., Cochranville PA, USA) diluted 1: 1000 in ELISA buffer; for detection of IgM antibodies, an IgG fraction of goat anti-rabbit IgM/PO (US Biochem. Corp., Cleveland OH, USA) diluted 1 : 2000 was used. Addition of substrate and evaluation of results were achieved in the same manner as described for cag-ELISA.

2.3 Other serological tests

The fluorescent antibody test (FAT) was performed according to the recommendations of the Bundesgesundheitsamt of the F.R. of Germany (Bundesgesundheitsblatt, 1976), using a goat anti-swine lg/FITC conjugate (Cappel Lab.).

3. *Affinity chromatography*

Purified rabbit IgG antibodies against *Toxoplasma gondii,* prepared as described above, had been immobilized on CNBr-activated Sepharose 4B (Pharmacia Ges.m.b.H., Vienna). The coupling procedure was performed according to the manufacturer's recommendation. In short: After reswelling the gel and several washings in 1 mM HCI, 5 mg IgG per ml of the gel were coupled by overnight incubation. Remaining active groups were blocked by treatment with 1 M ethanolamine.

To avoid co-elution of non-adsorbed protein, only a small volume of a native serum sample (2% of gel volume), was applied to the gel. PBS was used for sample adsorption as well as for elution of unbound substances. Antigen elution was effected by a pH change from 7.2 to 2.5 using a glycine/FICI buffer (0.1 M glycine). After elution, the pH of the sample was immediately raised to 8 with solid tris (tris[hydroxymethyl]-aminomethane) and dialysed against PBS.

4. Serum samples

Four serum samples *were* adsorbed to the coupled Sepharose: Two sera were from infected swine, both obtained on day 32 p. Two sera were of human origin; one of them was positive in cag-ELISA, the other was negative and did not contain any specific antibodies as determined in FAT, IgM-FAT, IHA and complement fixation test (CFT). The latter was used to control unspecific adsorption of serum onto the Sepharose.

5. TCA *precipitation and SDS-PAGE*

The eluates of affinity chromatography were precipitated with 12% TCA at 4°C. After an interval of 1 h at 0°C die precipitate was collected by centrifugation and washed three times with acetone at —20°C. Acetone was evaporated, the pellets were resuspended and SDS-electrophoresis was performed according to the method of *Lämmli* (1970). In short: A 12.5% separating gel and a 6% stacking gel were used at 8 mA constant current; samples containing 5 µg protein/lane were boiled for 3 min in sample buffer containing 5% mercaptoethanol and applied to the gel. After the electrophoretic Separation one part of the gel was stained in the generally known manner with Coomassie Blue R-250 (Serva, Heidelberg, FR Germany), die other part was used for protein transfer to nitrocellulose (nc) sheets.

6. Electrophoretic blot and ELISA

Gels were soaked in transfer buffer (25mM tris, 195 mM glycine and 20% methanol, pH 8.3) for 30 min and thereafter blotted to ne; a 7 V/cm voltage gradient was applied for 4 h. Nc sheets were soaked in PBS containing 2% bovine serum albumin for 1 h at 37°C to saturate free protein-binding sites. Then the sheets were washed in distilled water and incubated with cag-ELISA conjugate diluted 1 : 50 in ELISA buffer for 1 h at 37°C. After proper washing, the substrate (3 mg chloronaphtol, 1 ml methanol, 5 ml PBS and 2 µl of 30% H_2O_2) was added. The reaction was stopped by washing with distilled water.

Results

1. Detection of cag and demonstration of specific antibodies

The results of the experiments for detection of cag in sera by means of an ELISA are presented in Figs. 1-4: In sera of rabbits infected orally with oocysts of *Toxoplasma gondii*, cag could be demonstrated between the 27th and the 52nd day p. (Fig. 1). In sera of rabbits infected orally with trophozoites, cag was found between days 31 and 52 (Fig. 2). In the serum of one out of two rabbits infected intraperitoneally with trophozoites we found cag on days 38, 45, and 52 p. (Fig. 3). One rabbit infected by the i. p. route died on day 13 after infection. In sera obtained from swine that had been infected orally with oocysts, cag could be demonstrated between days 27 and 46 p. i. (Fig. 4). In sera of non-infected control animals we could not find cag at any time.

Specific antibodies against *Toxoplasma gondii* could be found about three weeks prior to detection of cag. Depending on the mode of infection, IgG and IgM antibodies could be demonstrated first between days 10 and 13. Mean values of antibody titres from rabbit sera determined by indirect ELISA can be seen in Figs. 1-3. The mean values of antibody titres from swine sera measured by FAT are presented in Fig. 4.

2.Structural properties of cag

Figs. 5 and 6 illustrate the results of the SDS-PAGE separation of isolated cag as well as the results of the ELISA on nc sheets. Electrophoretic separation of cag isolated from swine sera shows three major bands with molecular weights of about 85, 57, and 27 kD, two less distinct bands of about 68 and 25 kD, and a number of faint bands. A similar protein pattern could be observed after separation of cag from human serum, but three more bands with estimated molecular weights of 100, 34, and 32 kD could be detected.

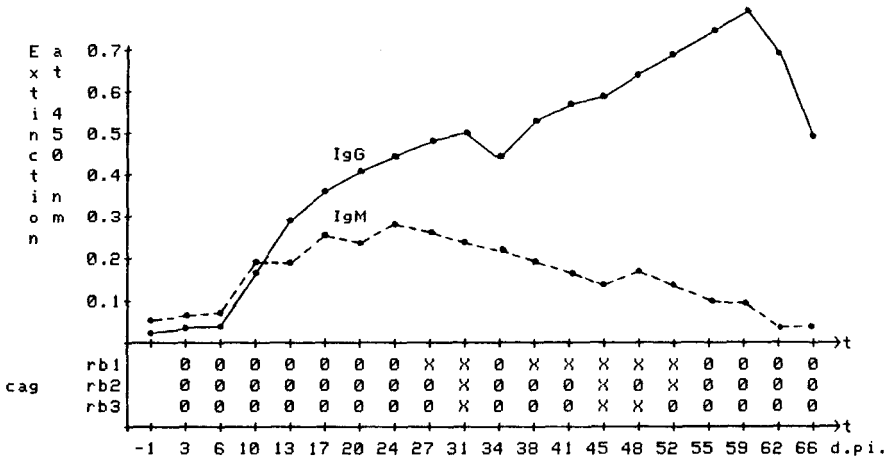

Fig. 1. Course of immune response (mean values) and detection of cag after *oral* infection of rabbits with *oocysts* of *T. gondii;* rb rabbit, x cag detectable, Ø cag not detectable.

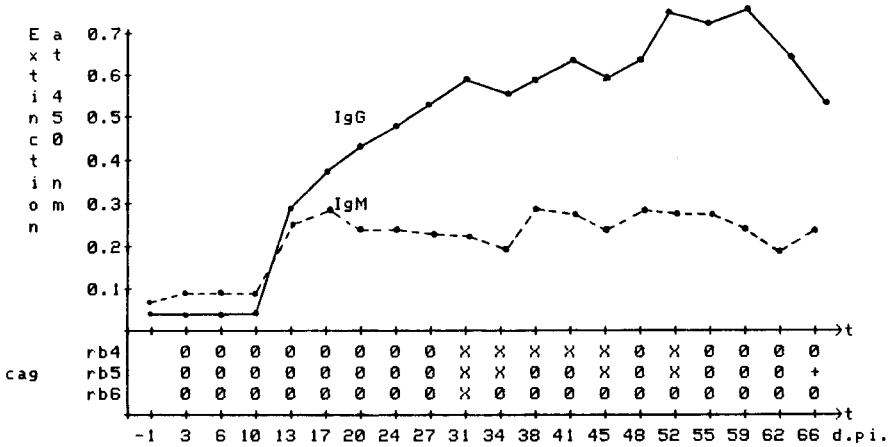

Fig. 2. Course of immune response (mean values) and detection of cag after *oral* infection of rabbits with *trophozoites* of *T. gondii;* rb rabbit, x cag detectable, Ø not detectable, + death of a rabbit.

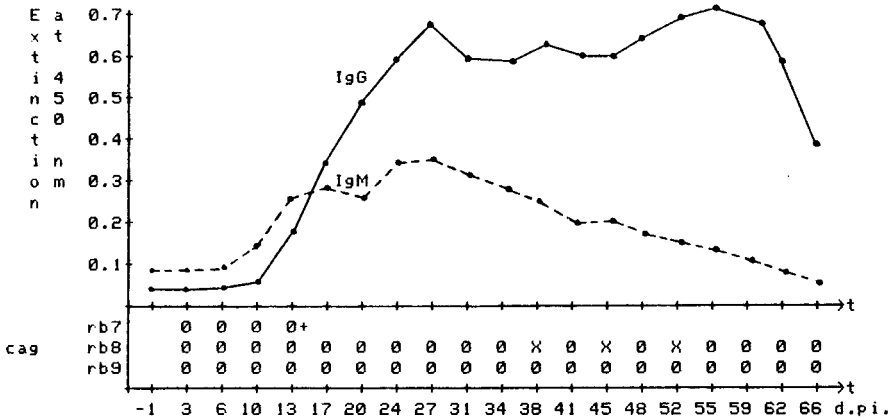

Fig. 3. Course of immune response (mean values) and detection of cag after *intraperitoneal* infection of rabbits with *trophozoites* of *T. gondii;* rb rabbit, x cag detectable, Ø cag not detectable, + death of rabbit.

When blotted on nc, two protein bands of porcine as well as of human origin reacted with the ELISA conjugate. The molecular weights were about 27 and 57 kD. One additional immunoreactive band with a molecular weight of about 32 kd was observed in cag of human origin. No antigenic components could be detected in normal human serum.

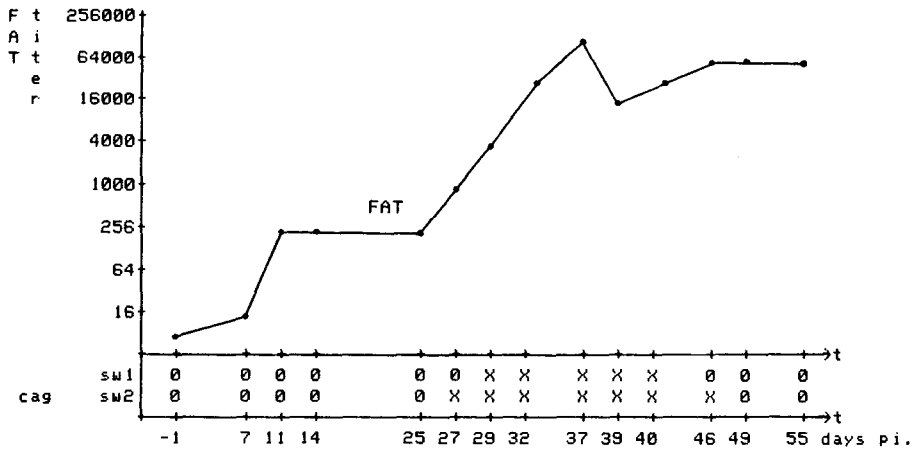

Fig. 4. Course of immune response (mean values) and detection of cag after *oral* infection of swine with *oocysts* of *T. gondii* (antibody titres are reciprocal); sw swine, x cag detectable, Ø cag not detectable.

Discussion

Considering the fact that the detection of cag of *Toxoplasma gondii* was first described as early as 1975 *(Raizman* and *Neva),* only little information on the occurrence of cag as well as on its structure is available at present. This is mainly due to the difficulties of a comparison of results from different authors, both on account of the non-standardized test reagents used and on different detection techniques employed, such as countercurrent electrophoresis and Ouchterlony test *(Raizman* and *Neva,* 1975), ELISA *(Van Knapen* and *Panggabean,* 1977; *Araujo* and *Remington,* 1980; *Ise* et al., 1985; *Lindenschmidt,* 1985), and dot immunoassay *(Brooks* et al., 1985). In previous investigations into the appearance of cag we could not find any correlation between the detection of cag and the rise of antibodies after an acute infection *(Hassl* et al., 1984). This might be due to the ELISA system: we suspect an influence of the infection mode of animals used as donors on the specificity of catching antibodies and ELISA-conjugate. Donor animals infected by the i.m., i.v., or i.p. route *(Raizman* and *Neva,* 1975; *Van Knapen* and *Panggabean,* 1977; *Araujo* and *Remington,* 1980) might give rise to other antibodies than those formed by animals infected orally according to the natural way of infection. Thus, in the present study *we* only used antisera from donors that had been infected orally with oocysts. An ELISA for detection of cag using such antisera was tested under defined conditions. Laboratory animals were infected orally with oocysts and trophozoites and intraperitoneally, with trophozoites for control.

Both groups of orally infected animals showed similar cag patterns regarding the time of appearance and detectability of cag. In control animals infected by the i. p. route, however, cag appearance was delayed and detectable only sporadically. The variation of cag detectability observed in serum samples of the individual animals on different days during the cag survey cannot be sufficiently explained but may be due to

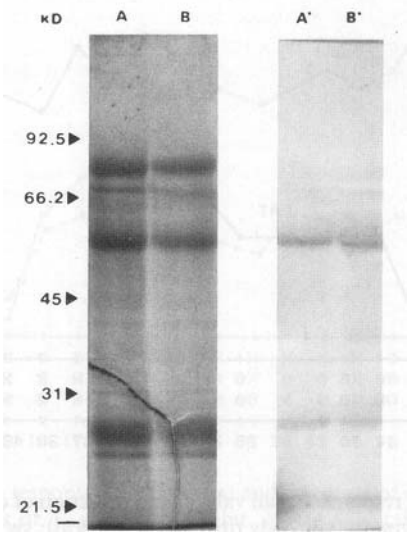

Fig. 5. Cag isolated from swine sera. Left: protein profile of cag after SDS-PAGE; right: immunochemical detection of cag on nc sheets with specific *anti-Toxoplasma* conjugate; kD: molecular weight in kilo dalton; A, A': male swine serum; B, B': female swine serum.

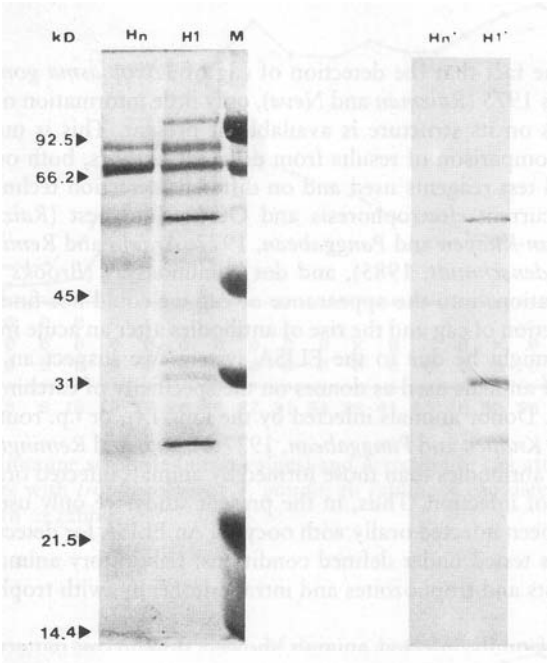

Fig. 6. Cag isolated from human sera. Left: protein profile of cag after SDS-PAGE; right: immunochemical detection of cag on nc sheets with specific *anti-Toxoplasma* conjugate; kD: molecular weight in kilo dalton; Hn, Hn': negative serum; H1, H1': patient serum; M: marker proteins.

variations of the physiological properties of laboratory animals. Unlike the specific antibody response, cag could not be detected before day 27 after infection, this is more than two weeks after the appearance of specific antibodies. Corresponding results were obtained by *Araujo* and *Remington* (1980) who detected cag in some sera from patients with acute toxoplasmosis about three to four weeks after the onset of clinical illness. *Ise* et al. (1985), however, failed to detect cag in the sera of rabbits infected orally with cysts. Yet, a comparison of results obtained by different authors is very problematic, as parasite strain, infection dose, and infection mode seem to influence considerably the detectability of cag *(Brooks* et al., 1985).

The molecular weight analysis of cag by SDS-PAGE revealed five protein bands (85, 68, 57, 27, 25 kD) in porcine serum. Three more bands of cag (100, 34,32 kD) were detectable in human serum. This suggests an influence of the host on the cag formation. Although some molecular weight determinations of cag have been done *(Raizman* and *Neva,* 1975; *Hughes,* 1981; *Hughes* and *Van Knapen,* 1982; *Ise* et al., 1985), no experiments were carried out under reducing conditions up to now. Thus, the molecular weights of protein bands determined by us cannot be compared with those obtained by others.

After immunoblotting on nc, only two of the five protein bands of swine-cag (27 and 57 kD) and three of eight protein bands of human origin (27, 32, and 57 kD) were immunoreactive. The proteins at 100, 85, 68, 34, and 25 kD did not react with the conjugate but have to be considered specific, since cag separated by SDS-PAGE had been previously purified by affinity chromatography. lt is remarkable that one of the protein bands of cag with a molecular weight of 27 kD was immunostained after Western blotting and that a protein band of exactly the same molecular weight has been described to be a constituent of *Toxoplasma* trophozoites *(Handman* and *Remington,* 1980; *Handman* et al., 1980; *Johnson* et al., 1981; *Johnson* et al., 1983; *Partanen* et al., 1983; *Ehrlich* et al., 1983). Further investigations will be necessary to increase the knowledge about cag and to assess the value of cag detection in the diagnosis of acute toxoplasmosis.

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