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Detection of Collagen-cross Reactive Antigenic Components in *Toxoplasma gondii*

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ABSTRACT

Using several immunologic techniques, the reactivity of anti-bovine collagen Type specific antibodies against *Toxoplasma gondii* was examined. Antibodies specific for bovine Type I, II and IV collagen showed relatively high affinity to the parasites. Treatment of *T. gondii* cysts with collagenase, induced cyst wall rupture. Results of the study suggest the presence of collagen cross-reactive component(s) in the parasite. Similarly, the cyst wall outer surface may contain amino acid sequences recognized by collagenase. This paper documents the first report of collagen cross- reactive antigenic components of *T. gondii.*

INTRODUCTION

Cell to cell interaction in tissue organs mediated by extracellular matrix is a generally accepted idea. Extracellular matrix plays an important role in the regulation of cell development, cell differentiation and tissue-specific gene expresion. Pathogens such as *Trypanosoma cruzi* (Quaissi et al. 1988; Quaissi et al. 1986; Velge et al. 1988) and *Leishmania* spp. (Wyler et al. 1985) bind to extracellular matrix in the process of attachment to host cell surface. *Toxoplasma gondii*, an obligate intracellular parasitic protozoan, multiplies in host cells and is protected against host defense reaction by forming parasito-

phorous vacuoles and/or tissue cysts enclosed in elastic and argyrophilic thin membrane. The specificity, however, of the membrane(s) and host cell interaction are poorly understood.

Collagen, a major component of extracellular matrix, occurs either as cross striated fibrils or network-like structure and is composed of repeats of amino acids glycine-hydroxyproline or hydroxylysine. Collagen is essential in the organization, construction and in the development of biological activities in tissues (Timpl 1982). Collagenase, on the other hand, is a specific proteolytic enzyme that cleaves collagen consisting of amino acid sequence R-Pro-X-Gly-Pro in triplex helix, the nick occurring between X- and Gly (Peterkofsky 1982). There are antibodies specific for types of collagen that recognize mainly unfolded chain constituents (Timpl 1982).

In the present study, we analyzed the role of collagen in *T. gondii*-host cell interaction and examined the antigenicity of collagen and presence of collagen-like amino acid sequences in *T. gondii* using immunological tools.

MATERIALS AND METHODS

Male and female ICR mice, 7 weeks of age, were used throughout of the study. Mice were inoculated intraperitoneally with $1x10^2$ *T. gondii* parasites (S-273 strain). On the 35th day post inoculation (p.i.), they were boostered with $1x10^4$ parasite inoculum, and those that survived were used as chronically-infected animals. Under anesthesia with diethylether, tissue cysts from the brain of these chronically-infected mice were isolated using 40% Percoll-NaCl density gradient centrifugation (Cornelisen et al. 1981). Isolated cysts were suspended in phosphate buffered saline (PBS) containing 0.9 mM Ca^{2+} (PBS+Ca) to adjust 2 x 10^2 cysts/ ml. Tachyzoites of *T. gondii* (RH strain) were obtained from infected mouse embryonal cells (MEC) cultured at 37° C in Dulbecco's Minimum Essential Medium containing 10% fetal bovine serum (D-MEM10+FBS).

Type-specific but non-species-specific rabbit anti-bovine collagen Type I, II, III and IV antibodies (LSL Inc. Tokyo, Japan) were diluted in PBS containing 3% bovine serum albumin (BSA-PBS). Collagenase purified from *Clostridium histolyticum* (Seikagaku Co., Tokyo, Japan) was further isolated by HPLC. To confirm the absence of non-specific proteolytic activities in main peak, BSA (protein standard; BIO RAD Co., Richmond, U.S.A.) dissolved in 0.15 M Tris-NaCl-$CaCl_2$ solution supplemented with or without EDTA was incubated with the peak fraction at 37° C for 18 hrs and the protein profile of the digested material were examined using 12.5% SDS-PAGE electrophoresis. The gel pattern visualized by silver stain (Daiichi Pure Chemical Co. LTD. Tokyo, Japan) showed no digested bands. The main peak fraction was dissolved in PBS+Ca at the concentration of 4.5 units/μl.

The location of collagen cross-reactive antigenic components was examined by immunoperoxidase test in MEC inoculated with tachyzoites (RH strain) and in tissue cysts (S-273 strain). On round cover slips (ø15mm), $2x10^5$ tachyzoites were mounted on 2 x 10^5 MEC monolayer and incubated at 37° C for 18 hrs. Cover slips were washed thoroughly in PBS and dried at room temperature. Tissue cysts enriched suspension was mounted onto glass slides and dried at room temperature, stored at -70° C until use. Glass slides coated with cysts or cover slips monolayered with infected MEC were immersed in methanol containing 0.3% H_2O_2 for 15 min to inhibit endogenous peroxidase, and were incubated with BSA-PBS at 4° C, overnight to block non-specific binding. The treated slides were incubated with anti-bovine collagen antibodies in various dilutions at 4° C, overnight washed with PBS containing 0.025% Tween20 and the specimens were incubated overnight at 4° C with HRPO-anti-rabbit IgG, diluted 400 fold in BSA-PBS. The presence of anti-bovine collagen antibodies was visualized by peroxidase reaction using diaminobenzidine-4HCl in 0.1 M Tris-HCl pH 7.4 containing 0.03% H_2O_2. To confirm the antigenic specificity, anti-bovine Type I collagen antibody was absorbed by passing it through an affinity chromatography column. Briefly, one ml of CH-Sepharose beads coupled with bovine Type I collagen was packed in 3 ml disposable polystyrene syringe and washed in 10 ml PBS (pH 7.2), and 10 ml of 0.1 M citrate buffer (pH 3.0) three times. One ml of 50 fold diluted antibody solution was applied onto packed column. Following incubation at 37° C for 30 min, the column was washed in PBS. Fractions that showed the highest absorbance at 280 nm were collected for use in immunohistochemical assay.

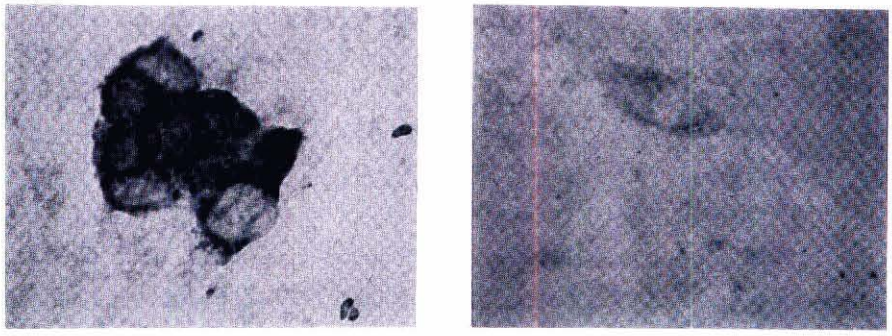
Effect of collagenase on cysts was examined as follows. Nine µl of cysts suspension was mixed with 1 µl of collagenase solution, and the interaction was monitored with a phase contrast microscope at 37° C. Disappearance of the cyst wall and the subsequent, release of parasites were indications of cyst rupture. To determine the specificity of collagenase digestion in bringing about the breaking up of the glycine-hydroxyproline or hydroxylysine amino acid repeats, cyst suspension in 2 mM EDTA-PBS was supplemented with 10 µl collagenase solution. The structural change of the cysts was observed microscopically for 30 min post-incubation.

RESULTS

Immunoperoxidase test done to determine the topographic reactivity of anti-bovine collagen antibodies in MEC-cell culture inoculated with *T. gondii* (RH-strain), showed positive reaction between the parasites (i.e. not with MEC) and anti-bovine Type I, II and IV collagen antibodies (Fig. 1). Parasite reactivity was noted at 400 fold dilution. Anti-bovine Type III collagen antibody had the least reactivity to the parasites. Likewise, anti-bovine Type

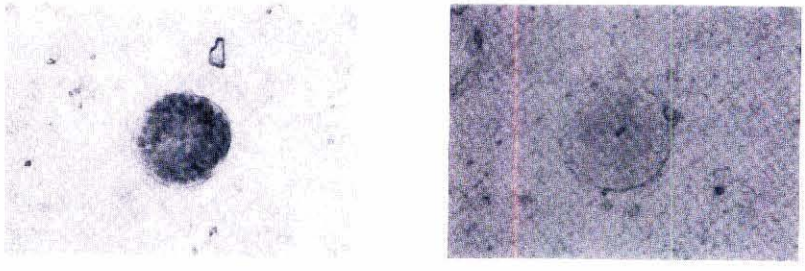
I collagen antibody that were passed through the affinity column had significantly reduced reactivity, compared to the non-treated group. With *T. gondii* (S-273 strain) in tissue cysts incubated with anti-bovine Types I, II and IV collagen antibodies, a positive reaction was likewise, observed but at a titer less than 200 fold dilution (Fig. 2). Parasites and cysts incubated with non-immunized rabbit serum showed negative reaction (data not shown).

Cyst wall rupture resulted from the incubation of tissue cysts with 4.5 units of collagenase. Parasite aggregates released exhibited signs of motility within 3 min post-incubation at 37° C (Fig. 3). Cysts incubated with collagenase supplemented with more than 2mM EDTA sustained the integrity of their cyst walls.



1a 1b

Figure 1. Photomicrographs (Immunoperoxidase Test) of RH strain *T. gondii* in MEC culture incubated with anti-bovine Type II collagen antibody (1a). Note specific reaction with extracellular parasites and parasites inside parasitophorous vacuole (x 400) No specific reaction with anti-bovine Type III collagen antibody (1b). x 200.



2a 2b

Figure 2. Immunoperoxidase reaction of *T.gondii* cysts incubated with anti-bovine Type II collagen antibodies(2a). Parasites but not cyst wall show specific reaction (x400). No specific reaction with anti-bovine Type III collagen antibody (2b). x 400.

3a 3b 3c

Figure 3. Effect of collagenase treatment of parasite cysts at 37° C. Incubation with 4.5 units of collagenase for three (3a) and five minutes (3b). Note cyst wall rupture and parasite release. Cysts incubated with 2 mM EDTA for 10 min (3c) showing intact cyst wall.

DISCUSSION

In the present study, we observed reactivity of anti-bovine Type I, II and IV collagen specific antibodies with *T. gondii* tachyzoites and bradyzoites. Likewise, the passage of Type I collagen antibody through affinity chromatography resulted in marked reduction of its reactivity with the parasite. These results strongly suggest specific reactivity for collagen or its related compo-nents and that tachyzoites and bradyzoites may have similar structural conformation with collagen polypeptides.

While the cyst wall seemed to be lacking of convincing signs of collagenic antigenicity, it showed to be prone to collagenase digestion and its reactivity disappeared with the supplementation of 2 mM EDTA. These observation imply that because collagenase are one of calcium-ion dependent metallobio-molecules, enzymatic cleavage is specific for a particular collagenase activity. Furthermore the amino acid sequence R-Pro-X-Gly-Pro-X, which is less antigenic, constitutes at least a part of the cyst wall and may serve as a barrier against direct attack by antibodies and components of the complement system and may assist in the transport of nutrients, as well.

We have no data in the present study to show the origin of the collagen cross-reaction substance (i.e. parasite or host origin). To date, there are no reports of the activity of collagen or collagen-like substances in protozoan species. Cyst wall of *T. gondii* has been reported to consist of accumulations of parasite substances on the limiting membrane of the cyst (Matsubayashi and Akao 1966), or is likely composed of neuronal cytoskeletal components of the brain (Sims et al. 1988). Likewise, the secretion of some kinds of antigenic components in the process of parasite-host cell penetration and inside parasitophorous vacuole (Cesbro-Delauw et al. 1981; Charif et al. 1990; Kimata and Tanabe 1987; Macleod et al. 1991; Sibley and Krakenbuhl 1988), and the parasite's ability to express Fc receptors (Budzko et al. 1989), C3 binding capacity (Fuhrman and Joiner 1989) and C1q binding substances(Omata et al.

1992) have been reported. To clarify the similarity between collagen cross-reactive antigenic substances and collagen, and the possibility of the parasite's binding capacity to collagen subtypes, further studies are necessary, specifically in the analysis of the amino acid components of collagen cross-reactive substances.

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