Mr 40 000 human colonic epithelial protein expression in colonic mucosa and presence of circulating anti-Mr 40 000 antibodies in cotton top tamarins with spontaneous colitis

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Abstract

Saguinus oedipus, *Callithrix jacchus*, and *Saguinus fuscicollis* are three species of New World monkeys which develop a form of colitis that is similar to human ulcerative colitis. Only *S oedipus*, however, develop colon cancer. We examined intestinal tissues from these animals for the presence of an antigen cross reacting to the Mr 40 000 human colonic epithelial protein that acts as an autoantigen in ulcerative colitis. Using an anti-Mr 40 000 monoclonal antibody (7E₁₂H₁₂, IgM isotype), by an immunoperoxidase assay we showed that all colon specimens from *S oedipus* reacted with 7E₁₂H₁₂; however, the colonic tissue from *C jacchus* and *S fuscicollis* did not. In immunotransblot analysis eluted IgG antibody bound to human ulcerative colitis colon (CCA-IgG) reacted with Mr 40 000 protein(s) present in the extracts of colon from *S oedipus* animals and humans. Small intestinal tissue reacted neither with 7E₁₂H₁₂ nor with CCA-IgG. In *S oedipus*, the Mr 40 000 protein was localised exclusively to colonic epithelial cells. Preincubation of seven *S oedipus* colon specimens with eight of 10 sera from animals with acute or chronic colitis and 0 of four sera from animals without colitis almost completely inhibited the binding of 7E₁₂H₁₂ to the colonic epithelium. Four of these 10 sera inhibited the binding of 7E₁₂H₁₂ to the autologous colon. These results show the presence of circulating autoantibodies in *S oedipus* with colitis against an epitope(s) on Mr 40 000 protein shared by human and *S oedipus* colon.

The study of ulcerative colitis has been greatly hampered by the lack of a suitable experimental animal model for the disease. In recent years the development of spontaneous colitis and superimposed colonic cancer have been described in one species of South American primates (*Saguinus oedipus*, cotton top tamarin).¹⁻³ Interestingly, two other species from the Callitrichidae family (*Callithrix jacchus* and *Saguinus fuscicollis*) also show a similar colitis but have never been reported to develop colon cancer.^{2,4,5} The colitis which apparently develops during the first year or so of life is usually of non-infectious origin,³ chronic, and can show acute exacerbations.⁴ Other similarities to human ulcerative colitis have been observed in studies of *S oedipus* colitis regarding histological features,^{2,6,7} mucin biochemistry,⁸ and lectin histochemistry.⁹⁻¹¹ The mechanisms which cause

the development of colitis in tamarins are unknown.

We recently described an Mr 40 000 protein in human colon that is specifically recognised by colon bound IgG from patients with ulcerative colitis, called CCA-IgG, and not by tissue bound IgG extracted from Crohn's disease affected colon and normal IgG.^{12,13} The Mr 40 000 protein is also recognised by autologous CCA-IgG¹³ and ulcerative colitis serum IgG.^{14,15} These data suggest that the Mr 40 000 colonic protein acts as an autoantigen(s) in ulcerative colitis.¹³⁻¹⁵ The protein has been purified from human colon and murine monoclonal antibodies have been developed.^{16,17} By using one of these monoclonal antibodies the protein has been localised exclusively to human colonic epithelial cells mainly along the basolateral and apical domains of plasma membrane.¹⁶

Because of the similarities observed between tamarin and human colitis, we examined the cross reactivity of the Mr 40 000 human colonic protein to tamarin colon tissue by the immunoperoxidase method using an anti-Mr 40 000 monoclonal antibody. The presence of circulating anti-Mr 40 000 antibodies in tamarins with colitis was investigated by an inhibition immunoperoxidase assay. The immunoreactive antigen(s) in tamarin colon is further analysed by immunotransblot analysis using CCA-IgG extracted from human ulcerative colitis colon.

Methods

All tamarins and marmosets were from the colony housed at the Marmoset Research Center, Oak Ridge Associated Universities, Oak Ridge, Tennessee.

TISSUE SPECIMENS

A total of 66 intestinal tissue specimens from colon (56 specimens) and small bowel (10 specimens) from 24 *S oedipus* (cotton top tamarin), 10 *S fuscicollis* (saddle back tamarins), and nine *C jacchus* (common marmosets) were used for immunoperoxidase experiments. The colonic specimens consisted of multiple samples from right and left colon. The specimens had been obtained at Oak Ridge Associated Universities¹⁸ during endoscopy or at necropsy, fixed in formalin, and embedded in paraffin. Specimens were coded and sent to the Robert Wood Johnson Medical School for the immunoperoxidase assay. Histological interpretations of the specimens were made at Oak Ridge. Tissue

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Figure 1: *Saguinus oedipus* colon from animal with chronic colitis (immunoperoxidase, original magnification $\times 10$). (A) Immunoreactivity of 7E₁₂H₁₂ on tamarin colon. The reactivity is localised only to colonic epithelial cells in the crypts and along the luminal surface. (B) Small intestinal specimen from a tamarin when examined with 7E₁₂H₁₂ monoclonal antibody. No immunoreactivity is seen.

specimens with acute inflammation consisting of mucosal ulcerations, infiltration with polymorphonuclear cells and lymphocytes in the mucosa, and crypt abscess were considered as acute colitis. Chronic colitis was defined as colonic mucosa showing excess inflammatory cellular infiltrates without much epithelial cell destruction.

For immunotransblot experiments, segments of small bowel and colon obtained from two

cotton top tamarins at necropsy were sent. These specimens were prepared following the method previously reported for human colon.¹³ Briefly, specimens were minced, washed in phosphate buffered saline (PBS) containing 2 mmol/l ethylenediamine tetra-acetic acid and 2 mmol/l sodium azide and homogenised with a Polytron. After adding phenylmethylsulphonyl fluoride to a final concentration of 2 mmol/l, the samples were centrifuged at 2000 g for 30 minutes and at 20 000 g for 60 minutes. The resulting protein solution (PBS extract) was used for immunotransblot analysis.

ANTIBODIES

The anti-Mr 40 000 monoclonal antibody (7E₁₂H₁₂, IgM isotype) and an unrelated murine monoclonal antibody of IgM isotype were used in immunoperoxidase experiments. The production and characterisation of the anti-Mr 40 000 monoclonal antibody have been reported previously.¹⁶ For the immunotransblot experiments, tissue bound IgG eluted from two ulcerative colitis colon specimens were used. Colon tissue bound IgG were obtained according to the method previously described.¹³ Colon tissue eluted IgG from a patient with Crohn's colitis and purified normal serum IgG were used as control.

SERA FROM TAMARINS

Fourteen coded sera from cotton top tamarins (*S oedipus*) were sent from Oak Ridge primate centre for the inhibition-immunoperoxidase assay as described below. These included six sera from cotton top tamarins with clinical and histological evidence of acute colitis, four with chronic colitis, and four without colitis.

IMMUNOPEROXIDASE METHOD

Two 5 μ m thick serial sections from each specimen were deparaffinised and rehydrated in decreasing concentrations of ethyl alcohol. The sections were incubated in PBS, pH 7.4, containing 0.3% H₂O₂ for 30 minutes to block the endogenous peroxidase activity and in 1% normal horse serum for two hours to block non-specific binding of the second antibody to the section. Either anti-Mr 40 000 monoclonal antibody or control monoclonal antibody was then added to the sections and incubated overnight. The sections were successively incubated with biotinylated goat antimouse IgM for 60 minutes and with avidin-biotin-peroxidase complex for 90 minutes. 3,3'-diaminobenzidine was then added to show the immune reaction. All the incubations were done at room temperature except for the overnight incubation which was performed at 4°C. Extensive washings in PBS, pH 7.4, were done between each incubation. After dehydration and mounting the slides were observed under a light microscope.

To examine the reactivity of 7E₁₂H₁₂ against carbohydrate moiety, we treated parallel tissue sections with neuraminidase (Sigma) (0.1 unit/section) and periodate (0.1 mol/l solution) before incubation with the monoclonal antibody,

TABLE I Results of immunoperoxidase experiments with 7E₁₂H₁₂ on tamarin intestinal tissues

	No of animals	Sex (M/F)	Mean age (yrs) (range)	No of specimens (colon)*	Mean (SD) reactivity score†
<i>Saguinus oedipus</i> :					
Chronic colitis	10‡	4/6	7·2 (0·4–9·7)	16	1·94 (0·82)§
Acute colitis	13‡	6/7	8·0 (0·4–12·2)	19	1·37 (0·68)
Normal colon	1	1	0·1	1	2
<i>Saguinus fuscicollis</i> :					
Chronic colitis	9	3/6	8·7 (2·5–14·5)	9	0
Normal colon	1	1	9·1	1	0
<i>Callithrix jacchus</i> :					
Chronic colitis	10	5/5	6·2 (0·1–14·3)	10	0

*None of the small intestinal specimens from 10 animals reacted with 7E₁₂H₁₂.
†Expressed as the mean of individual scores (0 to 3+) ranked by two observers (see text) without the knowledge of histological interpretation and the species of animals.
‡Two animals with chronic colitis and one with acute colitis had colon cancer.
§All colon specimens from *S oedipus* species were positive with some variations in the intensity (1+ to 3+); p<0·05 chronic colitis v acute colitis.
||Several deep glands were positive only in two of the 10 specimens.

7E₁₂H₁₂. Subsequent procedure was followed as monoclonal antibody described above.

All the slides were evaluated for the presence of immunoreactivity by two independent observers (KMD and MV) who were not aware of the histological interpretation and the species from which the tissue specimens were obtained. The reactivity was graded taking into account both the intensity of the staining and the number of reactive cells from 0 (no reactivity) to 3 (maximal reactivity). The scores given by the two independent observers never differed in terms of presence or absence of immunoreactivity. Occasional differences (less than 5% of specimens) consisted of one level of reactivity for a few positive specimens. The code was broken (by NC) after completion of the assays.

INHIBITION IMMUNOPEROXIDASE ASSAY

Serial sections of colon from seven *S oedipus* animals (three with chronic colitis and four with acute colitis) were incubated overnight at 4°C with the 14 sera from *S oedipus* animals with colitis (10 sera: six acute and four chronic) and without colitis (four sera) as described above. The sections were washed in PBS and then incubated with 7E₁₂H₁₂ following the same protocol as described above under immunoperoxidase method. Parallel sections were incubated overnight with PBS without the addition of *S oedipus* serum followed by 7E₁₂H₁₂.

IMMUNOTRANSBLOT

The protein solutions from small and large intestine of tamarins and human colon (PBS extracts) were subjected to 10% sodium dodecylsulphate-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose paper.¹³ After blocking with 5% bovine serum albumin for two hours at room temperature the blotted proteins were probed with ¹²⁵I-labelled CCA-IgG and control IgG for 60 minutes. In each experiment human colon PBS extracts were run as positive control. The nitrocellulose strips were washed, dried, and exposed for autoradiography at -80°C for 24 hours.

STATISTICAL ANALYSIS

Wilcoxon's rank sum test for unpaired samples was used to compare the reactivity scores in *S oedipus* with chronic and acute colitis.

Figure 2: *Saguinus fuscicollis* colon (immunoperoxidase, original magnification ×16). No reactivity with 7E₁₂H₁₂ monoclonal antibody is seen. L=luminal side of the mucosa.

Figure 3: Saguius oedipus colon from an animal with acute colitis (immunoperoxidase, original magnifications $\times 10$ in (A) and $\times 40$ in (B)). Reduction of 7E₁₂H₁₂ immunoreactivity with 'patchy' pattern is evident when compared with Figure 1A.

Figure 4: Saguius oedipus colon from an animal with acute colitis (immunoperoxidase, original magnifications $\times 10$ in (A) and (C) and $\times 40$ in (B) and (D)). Immunoreactivity of 7E₁₂H₁₂ against the colonic epithelium (A&B) is completely blocked by preincubation of the serial sections of the same colon specimen (C&D) with serum from an S oedipus animal with acute colitis, suggesting the presence of circulating antibody against the shared epitope reactive to 7E₁₂H₁₂.

Results

All colonic specimens examined came from animals with chronic or acute colitis except two (one 5 week old *S oedipus* and one 9.1 year old *S fuscicollis*) which showed no findings of colitis. All *S fuscicollis* and *C jacchus* animals had mild chronic colitis. Thirteen of 24 *S oedipus* had acute colitis (Table I).

IMMUNOPEROXIDASE EXPERIMENTS

The anti-Mr 40 000 monoclonal antibody (7E₁₂H₁₂) reacted with each of the 36 colonic specimens from *S oedipus* (Table I and Fig 1A). The only *S oedipus* colon specimen without colitis showed moderately strong reactivity (2+) with 7E₁₂H₁₂ in the colonic epithelial cells, similar to what was observed in *S oedipus* with chronic colitis. However, none of the 10 small bowel specimens from the animals reacted with 7E₁₂H₁₂ (Fig 1B). Neither small bowel nor colonic specimens reacted with the unrelated IgM monoclonal antibody used as negative control. In *S oedipus* the reactivity was localised exclusively to colonic epithelial cells in the crypts and along the lumen (Fig 1A). No appreciable difference in the pattern and intensity of staining was seen between right and left colonic specimens except in a few animals. After treatment with neuraminidase and periodate, the immunoreactivity of 7E₁₂H₁₂ did not change and increased somewhat in several colon tissues, suggesting a better access to the peptide-epitope by the antibody.

In contrast to *S oedipus* colon none of the 10 colonic specimens from *S fuscicollis* reacted with 7E₁₂H₁₂ (Table I, Fig 2). Eight of the 10 colonic mucosal specimens from *C jacchus* did not react with 7E₁₂H₁₂ (Table I). In the remaining two the colonic mucosal epithelium lining the lumen and the glands close to the lumen were totally negative. However, several glands at the deeper level close to the muscularis mucosa were positive (data not shown).

Reduction in the staining intensity with 7E₁₂H₁₂ was observed in colonic specimens from *S oedipus* animals with acute colitis compared with specimens from animals with chronic colitis (p<0.05) (Table I). Age and sex distribution did not significantly differ in the two groups of animals (Table I). The staining pattern in acute colitis specimens seemed to be 'patchy,' since some areas showed a pronounced reduction of reactivity with the monoclonal antibody (Fig 3).

PRESENCE OF ANTIBODIES IN *S oedipus* SERA AGAINST Mr 40 000 PROTEIN-EPITOPE(S) (INHIBITION IMMUNOPEROXIDASE ASSAYS)

Five of six sera from *S oedipus* with acute colitis (Fig 4) and three of four with chronic colitis almost completely inhibited the binding of 7E₁₂H₁₂ to all the seven *S oedipus* colon tissue (Table II). However, none of the four sera from *S oedipus* without colitis changed the immunoreactivity of 7E₁₂H₁₂ against colon tissue. Four of the 10 sera from *S oedipus* with colitis came from the same four animals whose colon tissues were among the seven used for the inhibition immunoperoxidase assay. These four sera also

almost completely inhibited the binding of 7E₁₂H₁₂ to the autologous colonic epithelium (Table II).

IMMUNOTRANSBLOT EXPERIMENTS

The autoradiograph from the transblot experiment performed with extracts from tamarin large and small intestine is shown in Fig 5. When tamarin large intestinal extracts were probed with CCA-IgG the immunoreactivity was observed at Mr 40 000 (lane A2). Tamarin small intestinal extracts did not react with the CCA-IgG (lane A1). Lane A3 shows reactivity of CCA-IgG with a human colon extract.

No reactivity was observed when tamarin small and large intestinal extracts (B₁ and B₂ respectively) and human colon extract (B₃) were probed with IgG extracted from the colon of a patient with Crohn's disease of the colon.

Discussion

The colitis which spontaneously develops in cotton top tamarins shares several features with human ulcerative colitis. The clinical course, the histological findings, the reduction in specific glycoprotein classes, and the pattern of lectin histochemistry reported in tamarin colitis are strikingly similar to those observed in human colitis.^{2-4 6-9}

The results of the present study indicate an additional structural similarity between the colonic mucosa of cotton top tamarins, a model of chronic colitis, and human ulcerative colitis. Furthermore, this study shows that sera from tamarins with colitis contain autoantibodies reactive to the Mr 40 000 protein-epitope as also observed in human ulcerative colitis.^{13 14} The Mr 40 000 colonic protein or cross reactive epitope(s) related to this protein, which are recognised by anti-Mr 40 000 monoclonal antibody as well as ulcerative colitis colon eluted antibody (CCA-IgG), are present in the colonic tissue from tamarins. This is shown both by an immunoperoxidase assay, using 7E₁₂H₁₂ monoclonal antibody and by the immunotransblot study using CCA-IgG. In the immunotransblot experiments the CCA-IgG reacted with an Mr 40 000 protein present in tamarin colon as well as human colon. As in humans, the protein seems to be uniquely associated with colonic tissue, since tamarin small bowel specimens did not react with either the anti-Mr 40 000 monoclonal antibody by immunoperoxidase assay or the CCA-IgG by immunotransblot analysis. The expression of the Mr 40 000 protein in the

TABLE II *S oedipus with colitis have serum antibodies that bind with S oedipus colon blocking the immunoreactivity of 7E₁₂H₁₂*

Sera from <i>S oedipus</i> with:	No of animals	No of sera that blocked the binding of 7E ₁₂ H ₁₂ to <i>S oedipus</i> colon tissue
Acute colitis	6	5* } 80%
Chronic colitis	4	
Normal	4	

*Two sera in each group inhibited the binding of 7E₁₂H₁₂ to autologous colon tissue.

Figure 5: An autoradiograph from transblot experiments after exposure for 24 hours: phosphate buffered saline extracted proteins from tamarin small intestine (lanes A₁B₁), large intestine (lanes A₂B₂), and human colon (lanes A₃B₃) were probed with IgG eluted from the colon of a patient with ulcerative colitis (CCA-IgG) (panel A) and colon eluted IgG from a patient with Crohn's disease involving the colon (panel B). Immunoreactivity is observed at Mr40 000 areas in lanes 2 and 3 containing colon extracts from tamarin and human respectively. Tamarin small intestinal extract did not react. Eluted IgG from the colon specimen with Crohn's colitis also showed no reactivity.

experimental animals such as mice and rats was also observed only in colonic epithelium and not in the small intestinal mucosa.¹⁹ The immunoreactivity, as detected by the immunoperoxidase method, is localised to colonic epithelial cells, both in the crypts and along the luminal surface. This pattern of localisation of the protein in tamarin colon is similar to what is observed in humans.¹⁶ The localisation and the expression of the Mr 40 000 cross reactive epitope(s) were similar in normal colon specimens and in chronic colitis specimens from *S oedipus*. The expression of the Mr 40 000 protein was also similar in specimens obtained from the right and left colon. This differs from what is observed in humans, where an increase of the immunoreactivity in the distal colon and rectum is seen.²⁰

An important difference in the expression of Mr 40 000 cross reactive epitopes was observed between *S oedipus* versus *S fuscicollis* and *C jacchus*, the two species which do not develop colon cancer but may develop colitis.

The nature and similarity of the colitis in these two species compared with *S oedipus* have been debated.⁵⁹ It is intriguing that while each of the 36 colon specimens from all *S oedipus* animals were reactive to 7E₁₂H₁₂, none of the 10 speci-

mens from *S fuscicollis* and eight of the 10 *C jacchus* colon specimens did not react with 7E₁₂H₁₂. This finding shows a major difference in colonic mucosa among these species. Differences among the three species have also been observed by lectin histochemistry of colonic mucosa.¹⁰

Specimens from *S oedipus* animals with acute colitis showed an appreciable reduction of immunoreactivity compared with specimens from normal colon or chronic colitis. This finding could be due to destruction of the normal glandular epithelium associated with acute colitis. In addition, the cross reactive epitopes may also be blocked by the anti-Mr 40 000 autoantibodies.

Eight of 10 (80%) sera from cotton top tamarins with colitis (both acute and chronic) contained circulating antibody against the shared epitope on Mr 40 000 colonic epithelial protein reactive to 7E₁₂H₁₂ monoclonal antibody. These included four sera from autologous animals whose colon tissue was examined with their own sera. None of the sera from normal animals showed such immunoreactivity against tamarin colon tissue.

Future studies are needed to clarify the role of

this epithelial autoantigen, the Mr 40 000 cross reactive protein(s) in the pathogenesis of colitis in tamarins. A better understanding of the pathogenetic mechanism involved in tamarin colitis may provide relevant clues for the study of human ulcerative colitis.

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