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Peer Review File

Contribution of collagen-binding protein Cnm of Streptococcus mutans to induced IgA nephropathy-like nephritis in rats

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

IgAN, as a mucosal immune disease with a complex pathogenesis, has always presented a challenge in the construction of its animal models. In this study, the authors discovered that the Cnm protein produced by Streptococcus mutans can induce IgAN in rats, which is quite intriguing. However, there are several flaws in the methods and results presented by the authors:

1.Why did the authors choose to use rats? In fact, the vast majority of studies attempt to induce IgAN in mice, as rats and mice usually have different characteristics of kidney diseases.

2.What is the basis for the number of bacteria injected intravenously? This should be described fully and clearly in the methods section, and the number of injections also needs to be specified.

3.The authors chose to euthanize the rats 45 days after infection. Why was 45 days chosen? When exactly did the rats develop significant proteinuria and IgA deposition?

4.What were the changes in serum IgA concentration?

5.Pathological images of the renal tubules and interstitium also need to be provided, and the assessment of kidney damage is essential.

6.If only cnm protein is used to treat the rats, will it cause damage to other organs, such as the heart and lungs?

7.This study should have set up a complete time gradient, rather than just presenting data from a 45-day time point, as this does not clarify when the disease characteristics appeared and when they reached a stable state.

8.The article lacks an analysis of the specific pathophysiological mechanisms, and merely presenting the phenotype is insufficient.

Reviewer #2 (Remarks to the Author):

This is an interesting study looking at collagen-binding protein Cnm ability to cause IgA nephritis which has implications for the pathogenesis of IgA nephropathy.

Several suggestions:
1. As it was shown the Cnm protein deposits in the mesangial region of the kidney, is this thought to be the primary mechanism of the pathogenesis that the protein itself is causing injury? If so, could this be expanded on in the discussion?
2. Were the rats confirmed to be bacteremic after infection?
3. Would it be possible to measure IgA or IgG in the sera of the rats to determine with an ELISA that they are specific to Cnm?
4. Were the rats that had the hematuria the same rats that had IgA, C3 and/or IgG deposits in their kidneys and higher mesangial proliferation? Could these experiments be correlated with each other to show potential relationships?
5. Why was only IgG2a tested? Is it possible that there are other IgG subclasses that could be also present? Could you provide some justification for why IgG2a specifically?
6. In Figure 4, the IgG staining for the representative image for rCnm shows diffuse staining but the other representative images only show discrete points of staining. Are there any thoughts for why this is? Was this pattern only present on the one image shown in Figure 4, or was it a specific pattern seen with the rCnm?

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1. Why did the authors choose to use rats? In fact, the vast majority of studies attempt to induce IgAN in mice, as rats and mice usually have different characteristics of kidney diseases.

Thank you for this question and important comment regarding differences between mice and rats. The present study was conducted as a concentration of our two previous studies, in which rats were used as an IgA nephropathy model [Naka S *et al*., 2020 (Reference No. 21); Naka S *et al*., 2021 (Reference No. 22)], thus rats were chosen. The purpose was to examine invasion of the bloodstream by Cnm-positive *S. mutans* through pulp or gingiva, followed by immune activation, leading to IgAN. Therefore, it

was necessary to administer the bacteria intravenously through the jugular vein to allow for bloodstream invasion. In contrast to mice, it is relatively easy to administer bacteria into the jugular vein of rats. In addition, as compared to mice, rat kidneys are considered more suitable for analysis of histopathological findings of tissues and electron microscopy results, since the rat glomerulus is larger.

2. What is the basis for the number of bacteria injected intravenously? This should be described fully and clearly in the methods section, and the number of injections also needs to be specified.

For the present study, it was considered important to determine the number of bacteria to be administered to the IgA model rats. It has been estimated that following invasive dental procedures, approximately 60% of treated patients possess greater than 1×10^4 CFU/mL of bacteria in systemic blood (Seymour RA *et al*., 2000; Lockhart PB *et al*., 2008). When converted to rats, that value is more than 1×10^8 CFU, which was an important factor for determining the amount of bacteria administered in the present study.

In rat jugular vein administration models used in our previous studies, following administration of 1×10^7 CFU of an *S. mutans* MT8148 strain, bacteria were isolated

from blood obtained up to 24 hours later (Nomura R *et al*., 2004), while bacteria were isolated up to 48 hours later after administration of 1×10^9 CFU (Nakano K *et al*., 2005, Nakano K *et al*., 2006). In a rat model of infective endocarditis, 1×10^8 CFU of *S. mutans* TW295 was administered and then the bacteria were isolated from blood obtained up to 7 days later (Otsugu M *et al*., 2017). Nevertheless, there is a possibility that such bacteria could be isolated after longer periods in these models with a heart valve injury induced beforehand as compared to normal rats. Furthermore, the strains used in those studies are different from *S. mutans* SN74 used in this study, a PA- and Cnm-positive strain, while MT8148 is PA-positive and Cnm-negative, and TW295 is PA-negative and Cnm-positive.

In our preliminary experiments conducted for a prior study, IgA model rats died when treated with 1×10^9 CFU of *S. mutans* SN74, whereas no clear findings related to development of IgAN were observed at concentrations below 1×10^7 CFU (Naka S *et al*., 2020). Those results were referred to when deciding the dose of *S. mutans* SN74 at 1×10^8 CFU for the present experiments.

In addition, though only one intravenous administration was used in this study, it has been reported that 1 mg of dental plaque contains more than 1×10^7 bacteria and the organisms can enter the bloodstream at a high frequency when invasive dental procedures are performed (Seymour RA *et al*., 2000; Lockhart PB *et al*., 2008).

Therefore, a single administration was considered adequate to allow the bacteria to enter the bloodstream.

Text related to the above contents has been added to lines 158-185 of the revised Discussion section.

3. The authors chose to euthanize the rats 45 days after infection. Why was 45 days chosen? When exactly did the rats develop significant proteinuria and IgA deposition?

We appreciate this important question. In our previous study, findings showing the progress of bacteria administered intravenously through the jugular vein at 15, 30, 45, and 60 days were obtained [Naka S *et al*., 2020 (Reference 21)]. For the present study, 45 days was chosen, as that is when the greatest levels of mesangial proliferation and IgA deposition were noted in our previous studies.

4. What were the changes in serum IgA concentration?

Although IgA concentration was determined in some of the serum samples, it was impossible to perform the measurements in serum samples from all of the groups as

several rats had already be used for other tests. The results of the measurements performed, which have been added as Supplementary Table 1 in the revised version, showed no significant differences for serum IgA concentrations among the PBS, SN74, and rCnm groups. Related descriptions are now included in the Methods (lines 250-253) and Results (lines 84-87) sections of the revised manuscript.

The appearance of hematuria or proteinuria, as well as increased serum creatinine level and IgA concentration are important clinical markers indicating the pathogenesis of IgAN. In our previous studies, mild hematuria and proteinuria were found in rat models, while serum creatinine levels and serum IgA concentrations were not increased [Naka S et al., 2020 (Reference No. 21); Naka S et al., 2021 (Reference No. 22)]. Based on our previous reports and the present results, we consider that IgAN-like nephritis was developed, but the condition had not yet progressed to the point of renal function decline.

5. Pathological images of the renal tubules and interstitium also need to be provided, and the assessment of kidney damage is essential.

Thank you for these helpful suggestions. We evaluated renal tubules and interstitium using Masson's trichrome (MT) and immunofluorescent (IF) staining with α-SMA antibodies in kidney tissue sections obtained from rats used in this study. There were no obvious abnormalities noted in any of the groups. Furthermore, no obvious atrophy was seen in the tubules, though fibrosis was observed at the inner brush border of the tubules in MT-stained images of rats in the SN74, Comp, and rCnm groups. In addition, α-SMA expression was observed in the IF. Tubular and interstitial lesions associated with IgAN often appear as changes secondary to glomerular damage. Segmental sclerosis, interstitial fibrosis, and tubular atrophy have been reported to be correlated with renal function prognosis, however, the detailed mechanisms have yet to be clarified (Trimarchi H *et al*., 2017; Coppo R *et al*., 2017; D'Amico G 2004). The pathogenesis of individuals affected by these conditions remains largely unknown and additional studies are needed.

Related descriptions have been added to the Methods (lines 264-268, 270-274) and Results (lines 109-113) sections of the revised manuscript. In addition, histological images have been added as Supplementary Fig. 1 and 2.

6. If only cnm protein is used to treat the rats, will it cause damage to other organs,

such as the heart and lungs?

We agree that this is a very important question. The present study targeted the kidneys, and unfortunately hearts and lungs were not observed. During the experimental period, none of the rats died or showed excessive loss of body weight. This issue will be investigated in a future study.

7. This study should have set up a complete time gradient, rather than just presenting data from a 45-day time point, as this does not clarify when the disease characteristics appeared and when they reached a stable state.

As noted above in the reply to comment No. 3, we previously reported findings regarding progression of bacteria administered intravenously through the jugular vein after 15, 30, 45, and 60 days [Naka S *et al*., 2020 (Reference 21)]. The results indicated that it was strongest at 45 days, with recovery shown at 60 days.

8. The article lacks an analysis of the specific pathophysiological mechanisms, and merely presenting the phenotype is insufficient.

The authors agree that understanding of specific pathophysiological mechanisms is important and intend to perform related analyses in a future study. At this point, it is considered possible that of *S. mutans* Cnm proteins may be separated from the bacterium surface, then bind to IgA and other immunoglobulins in blood, leading to induction of glycosylated abnormal IgA (Gd-IgA) expression. On the other hand, the present results revealed that Cnm proteins exist in the dens-deposit region of the kidney. Therefore, those proteins may have an auxiliary role in binding of IgA to the mesangial region, or are possibly related to formation of immune complexes by IgA attached with IgG and C3. In a future study, we intend to examine binding of Cnm protein to other immunoglobulins, including IgA, to clarify the mechanism of IgAN development related to Cnm. A related description has been added to the revised Conclusion (lines 211-219) section.

Reviewer #2 (Remarks to the Author):

This is an interesting study looking at collagen-binding protein Cnm ability to cause

IgA nephritis which has implications for the pathogenesis of IgA nephropathy.

Several suggestions:

1. As it was shown the Cnm protein deposits in the mesangial region of the kidney, is this thought to be the primary mechanism of the pathogenesis that the protein itself is causing injury? If so, could this be expanded on in the discussion?

The authors sincerely appreciate these important comments and questions. In our previous studies, Cnm-positive *S. mutans* organisms were found to be more clearly associated with the pathogenesis of IgAN than Cnm-negative *S. mutans* [Misaki T *et al*., 2015 (Reference 18); Misaki T *et al*., 2016 (Reference 19); Ito S *et al*., 2019 (Reference 20); Naka S *et al*., 2020 (Reference 21); Naka S *et al*., 2021 (Reference 22); Misaki T *at al*., 2023 (Reference 25)]. Results of those studies indicated that the Cnm protein may have auxiliary roles for binding of IgA to the mesangial region, or for attached IgA to form immune complexes with IgG and C3, though no evidence of injury caused by the Cnm protein itself was found.

2. Were the rats confirmed to be bacteremic after infection?

In this study, bacteremic phase was not confirmed immediately after infection. However, blood was obtained at the time of euthanasia and cultured to confirm that the rat was not affected by bacteremia.

In addition, bacteremic phase following infection in a rat jugular vein administration model was confirmed in a previous study conducted by our group (Nomura R *et al*., 2004). Up to 24 hours following administration of 1×10^{7} CFU of an *S. mutans* MT8148 (PA^{+}, Cnm^{-}) strain, bacteria were isolated from blood. In our other studies, following administration of 1×10^{9} CFU, bacteria were isolated up to 48 hours later (Nakano K *et al*., 2005; Nakano K *et al*., 2006), while in a rat model of infective endocarditis given 1×10^{8} CFU of the *S. mutans* TW295 (PA^{-}, Cnm^{+}) strain, bacteria were isolated from blood up to 7 days after administration (Otsugu M *et al*., 2017). Creation of such a model causes injury to the heart valve, thus bacteria are isolated from blood for a longer period of time following administration than seen with normal rats.

Therefore, for the present study, it was expected that administration of 1×10^{8} CFU of *S. mutans* SN74 (PA^{+}, Cnm^{+}) would cause bacteremia for 3-5 days.

3. Would it be possible to measure IgA or IgG in the sera of the rats to determine with an ELISA that they are specific to Cnm?

Findings indicating increased IgA and IgG concentrations are important clinical markers related to the pathogenesis of IgAN. For the present study, serum samples were obtained to measure the serum IgA concentration. There were no significant differences in those concentrations among the PBS, SN74, or rCnm groups. On the other hand, serum IgG levels were significantly higher in the rCnm groups compared to the PBS group. These results are now summarized in Supplementary Table 1, and related descriptions have been added to the Methods (lines 250-253) and Results (lines 84-87) sections of the revised version of the manuscript.

In previous studies using rat models, we have found mild hematuria and proteinuria, while serum creatinine levels and serum IgA concentrations were not increased [Naka S *et al*., 2020 (Reference No. 21); Naka S *et al*., 2021 (Reference No. 22)]. Based on those previous findings along with the present results, we consider that IgAN-like nephritis was developed, whereas progression to renal function decline had not occurred at that point.

Additional studies are needed to determine whether serum IgA or IgG binds specifically to Cnm.

4. Were the rats that had the hematuria the same rats that had IgA, C3 and/or IgG deposits in their kidneys and higher mesangial proliferation? Could these experiments be correlated with each other to show potential relationships?

Thank you for these helpful questions, which led us to perform additional analyses. The rate of correspondence between hematuria-positive status and IgA, C3, and/or IgG deposits was 80% (12/15), higher as compared with hematuria-negative status. Furthermore, mesangial proliferation scoring showed a higher tendency with hematuria-positive as compared with hematuria-negative. Therefore, we considered that these results demonstrated a relationship between hematuria and IgA, C3 and/or IgG deposits, as well as mesangial proliferation.

5. Why was only IgG2a tested? Is it possible that there are other IgG subclasses that could be also present? Could you provide some justification for why IgG2a specifically?

Rats possess four subclasses of IgG; IgG1, IgG2a, IgG2b, and IgG3. The present study used IgG2a, as it is associated with immune responses to IgG2 sugar chains and polysaccharide antigens. Nevertheless, the IgG1 and IgG3 subclasses are also important factors regarding immune response to protein and polypeptide antigens, and should be considered. Based on the reviewer's helpful questions, results of immunostaining using IgG (H+L) antibodies containing all subclasses were examined. The results in Fig. 5 and Fig. 6 have been replaced with those obtained using IgG (H+L) antibodies. A related description has been added to the revised Results (lines 128-130) section.

6. In Figure 4, the IgG staining for the representative image for rCnm shows diffuse staining but the other representative images only show discrete points of staining. Are there any thoughts for why this is? Was this pattern only present on the one image shown in Figure 4, or was it a specific pattern seen with the rCnm?

As noted in our reply to comment 5 above, IgG1, IgG2a, IgG2b, and IgG3 are the four IgG subclasses possessed by rats. rCnm was selected for the present study because it is associated with immune responses to IgG2 sugar chains and polysaccharide antigens. However, the IgG1 and IgG3 subclasses are also important factors regarding immune response to protein and polypeptide antigens, and should be considered. Thus, we reexamined immunostaining using IgG (H+L) antibodies containing all subclasses, and the results in Fig. 5 and Fig. 6 have been replaced with those obtained using IgG (H+L) antibodies. This pattern of IgG deposition may have been improved by changing the antibody.

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

The authors have addressed my concerns.

Reviewer #2 (Remarks to the Author):

The authors addressed many of the initial comments and provided adequate explanations for utilizing certain methodologies.

With regards to the question concerning correlating the hematuria with IgA, C3, and/or IgG deposits, you responded that you did "perform additional analyses [and the] rate of correspondence between hematuria-positive status and IgA, C3, and/or IgG deposits was 80% (12/15), higher as compared with hematuria-negative status. Furthermore, mesangial proliferation scoring showed a higher tendency with hematuria-positive as compared with hematuria-negative. Therefore, we considered that these results demonstrated a relationship between hematuria and IgA, C3 and/or IgG deposits, as well as mesangial proliferation." However, it does not appear this information was added to the results or the discussion. It might be helpful to include these data in the paper as hematuria is a clinical measure/criteria for glomerulonephritis. Furthermore, for future experimental planning, hematuria could be an important time point to indicate when the rat has progressed to tissue disease and used as an experimental endpoint?

Reviewer #1 (Remarks to the Author):

The authors have addressed my concerns.

(Response)
Thank you very much for your peer review. We sincerely appreciate the valuable comments.

Reviewer #2 (Remarks to the Author):

With regards to the question concerning correlating the hematuria with IgA, C3, and/or IgG deposits, you responded that you did "perform additional analyses [and the] rate of correspondence between hematuria-positive status and IgA, C3, and/or IgG deposits was 80% (12/15), higher as compared with hematuria-negative status. Furthermore, mesangial proliferation scoring showed a higher tendency with hematuria-positive as compared with hematuria-negative. Therefore, we considered that these results demonstrated a relationship between hematuria and IgA, C3 and/or IgG deposits, as well as mesangial proliferation." However, it does not appear this information was added to the results or the discussion. It might be helpful to include these data in the paper as hematuria is a clinical measure/criteria for glomerulonephritis. Furthermore, for future experimental planning, hematuria could be an important time point to indicate when the rat has progressed to tissue disease and used as an experimental endpoint?

(Response)
We appreciate the reviewer for this important suggestion. In this study, mesangial proliferation score as well as positive rates of IgA, C3, and/or IgG deposition in the hematuria-positive group were higher than those in the hematuria-negative group. Thus, hematuria was suggested to be associated with mesangial proliferation and deposition of IgA, C3, and/or IgG in glomerular mesangial regions, indicating its importance as an indicator of IgAN in clinical practice. Furthermore, it is possible that hematuria could also be used an important indicator in an IgAN-like nephritis animal model. Sentences regarding the results showing a correlation of hematuria with IgA, C3, and IgG deposition, and also mesangial proliferation scores have been added to the Results and Discussion

sections. Furthermore, a summary of those results is now presented in Supplementary Figure 1.