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ADDENDUM

Collateral damage during antibiotic treatment of *C. difficile* infection in the aged host: Insights into why recurrent disease happens

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ABSTRACT

Clostridium difficile infection (CDI) is one of the most common causes of healthcare-associated infections but an even bigger problem for the aging population. Advanced age leads to higher incidence, higher mortality, and higher recurrences. In our study, recently published in the Journal of Infectious Diseases, we investigated the effect of aging on CDI using a mouse model. We were able to demonstrate that aging leads to worse clinical outcomes, as well as lead to changes in microbiota composition and lower antibody production against *C. difficile* toxin A, but not toxin B. An association between advanced age and lower antibody production against *C. difficile* is a new finding which would explain the effect of aging on CDI outcome. Vancomycin, an anti-*C. difficile* antibiotic, led to similar changes in antibody response, suggesting a connection between microbiome and antibody response in the context of aging, which would require a much more nuanced look at the treatment of CDI.

Clostridium difficile Infection (CDI) is the most common pathogen to cause healthcare-associated infections in the United States and is responsible for an excess cost to the healthcare system of at least 1 billion dollars annually.[1,2] It is an even bigger problem for the aging population. Review of nationwide databases in the US in 2009 shows that the incidence of CDI in people older than 65 is about 10 times higher than in people younger than 65 across various databases.[3] The severity of disease is also higher in the older population, with CDI-related deaths being the 18th most common cause of death in people 65 or older, and 92% of all deaths from CDI occurring in people 65 and older.[4] Not only is aging a risk factor for developing CDI and for severe outcome, but also for recurrent CDI, with odds ratio for recurrence ranging between 1.75 to 6.0 in population older than 65 depending on various studies.[5,6] These statistics suggest that an in-depth investigation into the relationship of advanced age to CDI is of increasing importance.

A unique problem with CDI is the high rate of recurrence. The recurrence rate after an initial episode of CDI is quite high for all patients, ranging from 13.5% to 28.8%.[7,8] In addition to age older than age 65, other risk factors for recurrent disease include severe or fulminant underlying illness, additional antibiotic use after discontinuation of metronidazole or vancomycin, and low serum anti-toxin A IgG concentration.[7,9] These risk factors suggest 2 main mechanisms which may influence CDI recurrence: intestinal microbiota and antibody response. The intestinal microbiota, the population of bacteria which reside in healthy human intestines, provide resistance to C. difficile colonization[10] and therefore pathogenesis of CDI usually involves disruption of this normal microbiota.[11] The diversity of the intestinal microbiota is lower in patients with CDI compared with healthy patients, and is decreased further in recurrent episodes.[12] Antibiotic treatment changes the composition of the microbiota from that of a healthy host and decreases the bacterial diversity.[13] Since treatment of CDI is with antibiotics directed against *C. difficile* bacteria such as metronidazole or vancomycin,[14] these antibiotics themselves can cause more microbiota

changes which may make the host prone to recurrence. Thus, treatment of CDI presents a paradoxical situation where treatment is necessary but the treatment is likely to increase the chance for recurrence. Antibody response, the second potential mechanism for predicting CDI recurrence, has been shown to be an important factor as well, specifically antibody response against *C. difficile* toxins.[5,15,16] Although different antibodies were shown to be important in different studies – IgM anti-toxin A, IgG anti-toxin A, IgA anti-toxin A, IgA anti-toxin B – they all show association between stronger antibody response and lower likelihood of recurrence.[5,15,16] Recent studies on piglet model of CDI[17] and in humans[18] showed that monoclonal antibodies directed against toxin B but not toxin A were effective in preventing recurrence of CDI. These studies confirm the important role anti-toxin B antibody plays in host defense against *C. difficile* and its importance in therapeutics. However, the described previously human studies did show an association of clinical outcome with anti-toxin A antibodies as well. These findings suggest that anti-toxin A antibody along with anti-toxin B antibody levels may be a measure of the robustness of the humoral immune response and still correlates with clinical outcome from CDI. In our model, anti-toxin A antibodies showed the most consistent and reproducible results. IgG anti-toxin B antibodies were measured, but did not show significant difference between young and aged mice or before or after treatment. These inconsistent findings may be secondary to technical challenges encountered with the anti-toxin B assay, including limited amounts of mouse sera for repeat assays at adjusted toxin B and antibody loads and incubation times. However, we found that the anti-toxin A responses we have observed provide insights into what may be occurring in the aged infected host. So far there are no studies looking into factors that affect antibody response to *C. difficile*. Aging has been associated with decreased ability to produce high affinity immunoglobulins[19] and lower antibody response to vaccines[20] but has not been shown to have association with antibody response to *C. difficile* specifically.

In our study, we used a mouse model of CDI to study the effect of aging on CDI, specifically focusing on severity and relapse, and measuring antibody response and intestinal microbiota to explore possible mechanisms of higher recurrence.[21] Aged mice (18 month old) were compared head-to-head with young mice (8 weeks old) during infection with *C. difficile*. For the study of CDI pathogenesis, Syrian hamsters were first used as an animal model and used to demonstrate the role of toxins in pathogenesis.[22,23] Key issues with this model was that the disease was uniformly fatal while diarrhea was not always present, which does not closely replicate the clinical manifestations of human CDI, which is not uniformly fatal, and can often be a mild-to-moderate diarrhea. An additional limitation of the model is that there are relatively few commercially available reagents and assays to study various aspects of immune response to infection and pathogenesis. Genetic techniques to facilitate mechanistic studies are, likewise, limited in the hamster model. The mouse model of CDI using broad spectrum antibiotic exposure was described by Chen et al.[24] which leads to varying severity of disease in accordance with the challenge dose, with diarrhea, more closely mimicking human CDI and could reflect the range of clinical manifestations seen in human CDI. Use of a mouse model offers more tools in the way of readily available mouse specific reagents and genetically modified animals as well. Mouse model also has limitations, one of the limitation being that the susceptibility of the mice to infection varies with microbiota, which is affected by the environment and diet. This may actually more closely reflect human disease than other models, but makes controlling for all the variables difficult. Another limitation is that the immune system of mice is not exactly analogous to humans, which is the limitation for other animal models as well. Furthermore, outcome of infection may vary between mouse strains and *C. difficile* strains. The piglet model has recently come into the spotlight specifically because of overlap in strains infecting humans.[25] CDI infection causes enteritis during the first week of life, and is now the most commonly diagnosed cause of enteritis in neonatal pigs. This is interesting because *C. difficile* in humans had first been isolated in the gut of neonates, but they rarely cause disease. Despite this obvious difference in pathogenesis, a study using gnotobiotic piglet model has shown clinical outcome and histopathologic changes similar to human disease.[26] The piglet model has also recently been used to test the utility of anti-toxin antibody therapy in CDI.[17] This new model presents another good methodology to study effects of therapeutic agents, as it closely resembles human disease in the effect of anti-toxin antibody therapy. As noted prior

however, CDI, although reported in pediatric patients, is more often a disease affecting adults and especially the elderly population, which was the purpose of our study. Therefore in studies looking at the effect of aging or where the age of the host is a factor, another animal model may be more appropriate. For our study, with aging at the end of life, correlating with advanced age such as 65 y or older in humans, being an important factor instead of prematurity in the first

year of life as would be applicable in piglet models, along with the need to measure the microbiota effect, the mouse model is optimal. It should be noted that there is no single animal model that is best reflective of human disease in CDI at present, and while the mouse model is one of the most widely used due to various factors outlined above, it is still an imperfect model, and is a limitation of this study. Aging in the mouse model was associated with higher mortality and prolonged weight loss after CDI, which mirrors the effect of aging observed in the human host.[7,27,28,29] However, the differences were even more striking in the relapse experiment. In this experiment, starting 24 hours after infection, mice were treated with vancomycin which is the treatment of choice for severe CDI.[14,30-32] Treatment with vancomycin prevented the development of symptomatic disease while on treatment but resulted in a relapse of symptomatic disease after stopping vancomycin. During this relapsed disease the difference in clinical outcome was even more dramatic, with 75% mortality in aged mice compared with 0% in young mice (Fig. 1). During relapse, aged mice also experienced more weight loss and higher disease scores.

The striking difference in mortality seen in relapsed disease between aged and young mice raises the question of what is different with initial infection and relapsed disease that makes aged mice so much more susceptible. Changes in the microbiome, with cumulative changes expected from repeated use of antibiotics, would be an obvious explanation.[12] However, conventional wisdom so far would suggest that microbiome mainly affects the susceptibility of the host to becoming colonized with C. difficile bacteria[10] rather than the
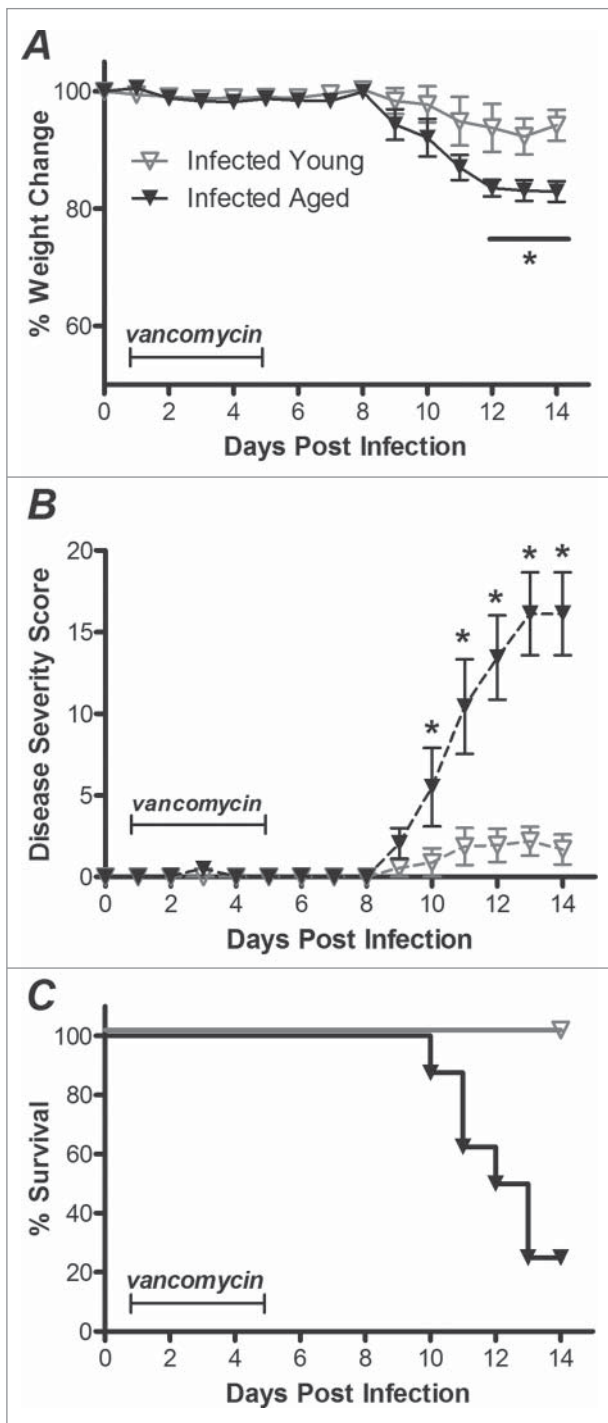

Figure 1. Effect of treatment with vancomycin (50 mg/kg/day) for 5 d (days 1–5 after infection) in Clostridium difficile–infected young mice (aged 2 months) and aged mice (aged 18 months). A. Weight change from baseline, before infection, up to the day of death. *P < .05 by 2-way analysis of variance (ANOVA) with the Bonferroni correction. B. Disease severity scores of surviving mice. *P < .001 by 2-way ANOVA with the Bonferroni correction. C. Survival curve. P < .0001 by the log-rank (Mantel–Cox) test. Figure taken from van Opstal et al., Vancomycin treatment alters humoral immunity and intestinal microbiota in an aged mouse model of Clostridium difficile infection. J Infect Dis 2016. © Edward van Opstal, Glynis L. Kolling, John H. Moorell, Christine M. Coquery, Nekeithia S. Wade, William M. Loo, David T. Bolick, Jae Hyun Shin, Loren D. Erickson, and Cirle A. Warren. Reproduced by permission of the authors. Permission to reuse must be obtained from the rightsholder.
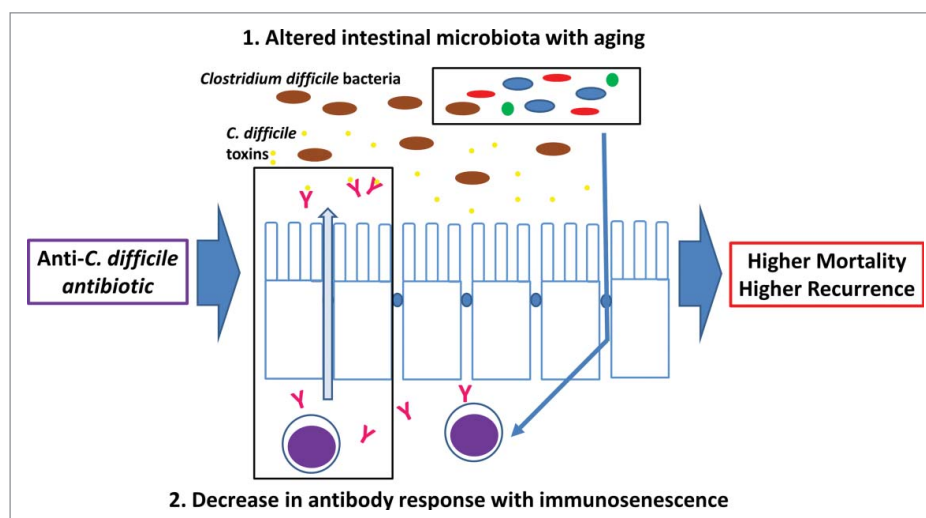
Figure 2. Effect of aging and antibiotic use on *C. difficile* infection outcome. *C. difficile* infection (CDI) experiments using an aged mouse model show significantly worse clinical outcome, including higher mortality. Aged mice also had an alteration in the intestinal microbiota compared with young mice and lower levels of IgG and IgA antibodies against *C. difficile* toxin A (TcdA). Treatment with vancomycin, the treatment of choice against CDI, led to temporary relief from symptoms, but eventually led to even higher mortality in aged mice. Vancomycin also led to lower IgG and IgA response to TcdA. These finding suggest an association with microbiota and antibody response. Modified from figure in Shin et al. Older Is Not Wiser, Immunologically Speaking: Effect of Aging on Host Response to Clostridium difficile Infections. J. Gerontol. A. Biol. Sci. Med. Sci. 2016.

clinical outcome once infection occurs. The finding from this study highlights the critical influence of the microbiota on the outcome of CDI, even after overt disease is established.

Our findings suggest that the 2 important mechanisms that affect rate of recurrence, intestinal microbiota and humoral response, may be linked and explain the difference in outcome between initial versus relapsed disease (Fig. 2). We discovered that vancomycin-treated mice, compared with mice who did not receive any treatment after infection, produced significantly lower levels of IgG and IgA against toxin A, in both aged and young mice (Fig. 3). There is a possibility of lower production of antibodies due to lower pathogen load. Certainly qPCR of *C. difficile* toxin B gene showed lower numbers in the vancomycin-treated group at day 7 in both young and aged mice compared with untreated mice. However, if examined closely, while antibody response to *C. difficile* is definitely lower at day 14 in the aged mice, the number of *C. difficile* bacteria at day 7 is higher by qPCR, demonstrating that pathogen load does not explain fully the differences in antibody response. If an association between microbiota and humoral response can be demonstrated as suggested in this study, this may explain the difference in outcome between young and aged mice and between initial and relapsed infection in our model. In CDI in humans,

antibody response to *C. difficile* has been shown to be the difference between symptomatic infection and asymptomatic colonization[33] as well as between recurrence and resolution of CDI.[5,15,16] These findings suggest an important role for antibody response in CDI pathogenesis.

Regarding the link between microbiota and antibody response, there is a paucity of data in the literature so far. Among the different immunoglobulin classes, IgA is secreted across the intestinal epithelium into the intestinal lumen, where it binds to microbes and other antigens, and can coat and agglutinate its targets to prevent direct interaction with the host, averting a potentially harmful stimulation of the immune system.[34] Consistent with this hypothesis, people deficient in IgA have more bacteria from taxa with potentially inflammatory properties.[35] IgA is generated by gut plasma cells with cooperation of epithelial cells, dendritic cells, and innate lymphoid cells. Therefore, number of IgA- expressing cells in lymphoid tissue are greatly reduced in germ free animals. The effect of microbiota on IgA secretion was demonstrated in a human study, where the number of *Bifidobacterium* and *Lactobacillus* species in the early intestinal microbiota in infants was associated with total levels of secretory IgA measured in the saliva at 6 months.[36] These known findings suggest an interesting possibility that differential IgA binding in different
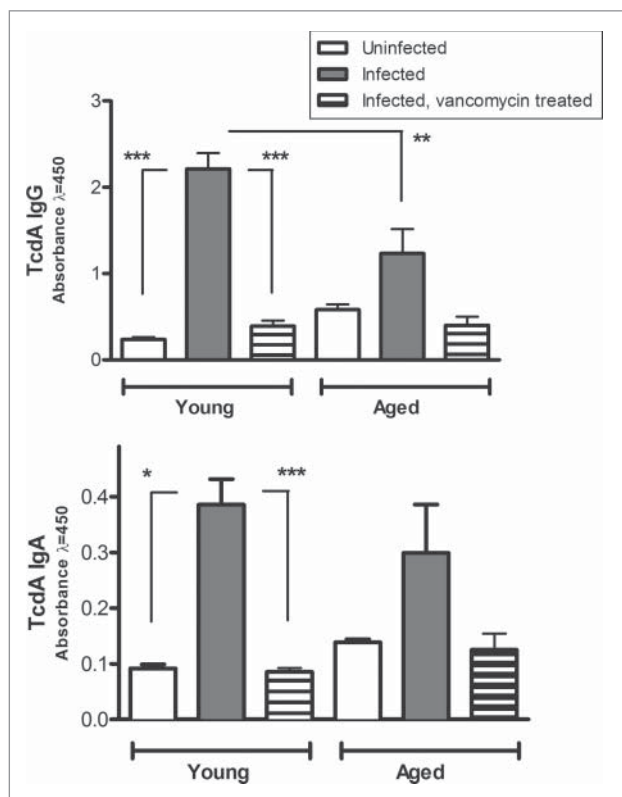
Figure 3. Effect of vancomycin treatment on Clostridium difficile toxin A (TcdA) antibody levels in sera of infected young mice and infected aged mice at day 14 after infection. $^*P < .01$, $^{**}P < .001$, and $^{***}P < .0001$ by 1-way analysis of variance with the Bonferroni correction. Abbreviations: IgA, immunoglobulin A; IgG, immunoglobulin G. Figure taken from van Opstal et al. Vancomycin treatment alters humoral immunity and intestinal microbiota in an aged mouse model of Clostridium difficile infection. J Infect Dis 2016. © Edward van Opstal, Glynis L. Kolling, John H. Moorell, Christine M. Coquery, Nekeithia S. Wade, William M. Loo, David T. Bolick, Jae Hyun Shin, Loren D. Erickson, and Cirle A. Warren. Reproduced by permission of the authors. Permission to reuse must be obtained from the rightsholder.

hosts may lead to a different effective bacterial load or toxin load in CDI leading to differences in outcome despite similar total bacterial or toxin load in the intestine. However, this does not explain the effect of microbiota in the adult host or interaction between the microbiota and other immunoglobulins such as IgG and IgM and its effect on CDI. There are studies demonstrating that probiotic treatment leads to an improvement in IgG and IgA antibody production with influenza vaccines, suggesting that microbiota-humoral immunity interaction in adult host after the initial development of immune system is possible.[37,38]

It is likely that there is an effect of aging on antibody response which is not related to the microbiota. In our study, even without antibiotics, aged mice had lower levels of serum IgG and IgA against toxin A at day 14; which has not been demonstrated previously. In human studies where they were both measured, association between age and antibody production was not observed, although both advanced age and antibody levels were highly correlated with recurrence rates.[5,39] This study is the first to demonstrate an association with advanced age and lower antibody response to CDI. The finding has significant implications on the role of advanced age on CDI. The published findings in the literature suggest a strong relationship between antibody production and development of CDI,[33,40] and recurrence.[5,39] If the statement "aging leads to lower antibody response to *C. difficile* toxins" can be confirmed in subsequent studies, this would explain the reason for why the elderly are more susceptible to CDI and recurrence.

Investigation of aging as a factor in CDI presents a challenging problem. Aging is associated with numerous factors that influences CDI outcome such as antibiotic exposure, healthcare contact, and medical comorbidities, which needs to be controlled when studying the effect of aging.[41] Another challenge is that the changes are detected in various systems simultaneously including humoral immunity and intestinal microbiota as we have seen, along with other factors such as innate immunity and gastrointestinal motility.[41] These factors make study of the effect of aging on CDI crucial, however. The complex interplay of the host factors as detected by differences in aging will shed more light on the pathogenesis of CDI. It may also very well be applied to the investigation of aging as a factor in other infections.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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