A Critical Role for Induced IgM in the Protection against West Nile Virus Infection

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Abstract

In humans, the elderly and immunocompromised are at greatest risk for disseminated West Nile virus (WNV) infection, yet the immunologic basis for this remains unclear. We demonstrated previously that B cells and IgG contributed to the defense against disseminated WNV infection (Diamond, M.S., B. Shrestha, A. Marri, D. Mahan, and M. Engle. 2003. J. Virol. 77: 2578–2586). In this paper, we addressed the function of IgM in controlling WNV infection. C57BL/6j mice (sIgM/H11002/H11002) that were deficient in the production of secreted IgM but capable of expressing surface IgM and secreting other immunoglobulin isotypes were vulnerable to lethal infection, even after inoculation with low doses of WNV. Within 96 h, markedly higher levels of infectious virus were detected in the serum of sIgM/H11002/H11002 mice compared with wild-type mice. The enhanced viremia correlated with higher WNV burdens in the central nervous system, and was also associated with a blunted anti-WNV IgG response. Passive transfer of polyclonal anti-WNV IgM or IgG protected sIgM/H11002/H11002 mice against mortality, although administration of comparable amounts of a nonneutralizing monoclonal anti-WNV IgM provided no protection. In a prospective analysis, a low titer of anti-WNV IgM antibodies at day 4 uniformly predicted mortality in wild-type mice. Thus, the induction of a specific, neutralizing IgM response early in the course of WNV infection limits viremia and dissemination into the central nervous system, and protects against lethal infection.

Key words: flavivirus • antibody • innate immunity • encephalitis • risk factor

Introduction

West Nile virus (WNV) is a single-stranded positive-polarity RNA virus and the etiologic agent of West Nile encephalitis. WNV is maintained in a natural cycle between mosquitoes and birds but also infects humans, horses, and other animals. It is endemic in parts of Africa, Europe, the Middle East, and Asia (1), and outbreaks are now occurring annually in North America (2). Humans develop a febrile illness with a subset of cases progressing to meningitis or encephalitis syndrome (1). Currently, no specific therapy or vaccine has been approved for human use.

Host factors influence the expression of WNV disease in humans (3). Infants, the elderly, and those with impaired immune systems are at greatest risk for severe neurological disease (1, 4, 5). Similarly, in animals, the maturation and integrity of the immune system correlates with resistance to WNV infection (6–8). Through the use of animal models of WNV infection, the immunologic basis for protection is beginning to be understood (9). T and B lymphocytes protect against WNV infection: SCID and RAG1 mice (T and B cell–deficient; references 10, 11) and B cell–deficient mice uniformly succumb to WNV infection (11). Macrophages also have important functions because their depletion increased the neuroinvasiveness of an attenuated strain (12).

Humoral immunity is an essential component of the immune response to WNV and other flaviviruses because neutralizing antibodies limit dissemination of infection. The role of immune IgG in protection has been studied extensively in mouse models of flavivirus infection, includ-
ing WNV. Passive transfer of polyclonal or monoclonal IgG before infection protects mice against lethal flavivirus challenge (11, 13–21). Immune IgG are speculated to protect against WNV infection by direct neutralization of receptor binding, through Fc-receptor–dependent viral clearance, by complement-mediated lysis of virus or infected cells, and by antibody–dependent cytopotoxicity. The importance of antibodies in the protection against WNV infection has been highlighted by recent studies in antibody-deficient mice (11). Mice lacking antibodies developed encephalitis after infection with WNV; high levels of virus and viral RNA were detected both peripherally and in the central nervous system (CNS).

The function of IgM against WNV or other flaviviruses is less well characterized. Low levels of serum IgM antibody against Japanese Encephalitis virus were an independent risk factor for severe neurological deficit in humans (22). Our own studies with B cell–deficient mice demonstrated a ~500-fold increase in viremia 4 d after infection, a time when only immune IgM was detected in the serum from wild-type mice (11). Because passive transfer of immune IgM against WNV, derived from wild-type mice 4 d after infection, prolonged survival of B cell–deficient mice and completely protected wild-type mice, natural or induced IgM could limit WNV infection by controlling viremia and/or by triggering an adaptive B or T cell response (23, 24).

Natural and induced IgM antibodies against WNV may have important protective functions against WNV. Natural IgM is secreted constitutively by CD5+ B-1 cells without specific stimulation, has widely variable binding avidities (10^{-3}–10^{-11} M), and represents an initial defense against pathogens (25–27). IgM mediates direct neutralization of some bacteria and viruses in circulation (26, 28, 29), enhances phagocytosis of pathogens (30), and activates complement (27, 31, 32) to prime the immune response. IgM antibody–antigen complexes are efficiently filtered in the spleen and lymph nodes and may diminish hematogenous spread and infection of critical end organ targets such as the brain or spinal cord (24). In this work, we directly assessed the function of secreted IgM in limiting WNV infection. C57BL/6J mice that did not produce secreted IgM (27, 33, 34) uniformly succumbed to WNV infection. Infection in these mice resulted in higher levels of viremia and CNS viral burdens. Passive transfer of induced, but not natural, IgM protected sIgM mice against lethal WNV infection, but administration of a nonneutralizing IgM monoclonal antibody did not improve outcome. A low titer of anti-WNV IgM antibodies at day 4 after infection in wild-type mice uniformly predicted mortality. Thus, the induction of neutralizing anti-WNV IgM early in the immune response blunts viremia and the spread of infection and is required for survival.

Materials and Methods

Cells, Viruses, and Antibodies. BHK-21 and C6/36 Aedes albopictus cells were cultured as described previously (35, 36). The WNV strain (3000.0259) was isolated in New York in 2000 (37) and obtained from L. Kramer (New York State Department of Health, Albany, NY). All cell culture and in vivo studies used a stock (2 × 10^{8} PFU/ml) of this virus that was propagated (passage 1) once in C6/36 cells. Viruses were diluted in HBSS and 1% heat-inactivated FBS for injection into mice. Hybridoma cells that produce monoclonal antibodies against WNV envelope protein (4G2, IgG2a; reference 38; H5.46, IgM; reference 39) were cultured in DMEM supplemented with 10% FBS. H5.46 is a monoclonal IgM antibody that binds to the E protein of WNV and was used from ascites fluid (39).

Mouse Experiments. All mice used in these experiments were derived from or backcrossed onto the inbred C57BL/6J strain. Mice that lack the ability to secrete IgM due to a targeted mutation that disrupts expression of the secreted but not membrane form of IgM have been described previously (33) and were obtained from M. Carroll (The Center for Blood Research, Harvard Medical School, Boston, MA) and J. Chen (Massachusetts Institute of Technology, Cambridge, MA). The wild type C57BL/6J was purchased from Jackson ImmunoResearch Laboratories. 8–12-wk-old mice were used for all studies and inoculated subcutaneously with WNV by footpad injection after anesthetization with xylazine and ketamine. In some experiments, mice were phlebotomized (0.2 ml of blood) at day 4 after infection. Mouse experiments were approved and performed according to the guidelines of the Washington University School of Medicine Animal Safety Committee. Differences in survival times and outcome were assessed by the Kaplan-Meier analysis and the log-rank test.

Passive Antibody Transfer Experiments. Serum was isolated from naive or infected (day 4 after infection) mice, heat-inactivated for 30 min at 56°C, and stored at −80°C. An aliquot was reserved to confirm levels of specific IgM against WNV. Ascites fluid that contained the H5.46 anti-WNV IgM monoclonal antibody was heat-inactivated, and diluted in HBSS with 1% heat-inactivated FBS. For passive transfer experiments, mice were administered 0.5 ml of serum or H5.46 antibody intraperitoneally 1 d before and after inoculation with 10^{2} PFU of WNV. Purified human γ-globulin with reactivity against WNV was obtained commercially (I. Nur, Omrix Biopharmaceuticals Ltd, Kiryat Ono, Israel) from a WNV endemic region (Israel) that has had repeated outbreaks in recent years. Several batches of pooled human γ globulin (50 mg/ml IgG) were obtained and tested positive by ELISA against WNV antigens and by plaque reduction neutralization titer (PRNT) assay (11, 40). 50 mg/ml IgG negative human γ globulin was obtained from a nonendemic region (Midwestern United States, 2000); purified preparations lacked immune antibodies as judged by ELISA and PRNT assays. In experiments with human γ globulin, mice were administered a single dose of immune or nonimmune purified IgG by intraperitoneal injection at the same time as footpad inoculation of WNV.

Quantitation of Viral Burden in Mice. For analysis of virus in tissues of infected mice, organs were recovered after cardiac perfusion with PBS and dissection, cooled on ice, weighed, homogenized using a Bead-beater apparatus (BioSpec Products, Inc.), and titrated for virus by plaque assay on BHK–21 cells as described previously (11, 36). Serum was obtained from whole blood by phlebotomy of the axillary vein immediately before being killed.

Quantitation of Antibodies. The titer of neutralizing antibodies was determined by a standard plaque-reduction neutralization assay (11, 41). Experiments were performed in duplicate and plaques were scored visually. Results were plotted and the
PRNT for 50% inhibition (PRNT_{50}) was determined. In some experiments, IgM was depleted by immunologic means (11). Serum or diluted ascites were incubated twice with an equal volume of anti-IgM-specific agarose for 1 h at 4°C. After centrifugation, the supernatant (1:4 dilution of original sample) was titrated for PRNT. ELISA confirmed isotype depletion.

The overall titer of antibodies was determined using an ELISA assay against purified WNV antigen. In brief, soluble WNV E protein that was generated from baculovirus-infected SF9 cells (unpublished data) was adsorbed overnight at 4°C to microtiter plates (Maxi-Sorp; Nunc). Nonspecific binding was blocked after incubation with blocking buffer (PBS, 0.05% Tween 20, 3% BSA, and 3% horse serum) for 1 h at 37°C. Plates were incubated with serial dilutions of heat-inactivated serum from infected mice for 1 h at 4°C. After extensive washing, plates were incubated with biotin-conjugated goat anti–mouse IgG (Sigma-Aldrich) and horseradish peroxidase–conjugated streptavidin (Sigma-Aldrich) at 4°C, and developed after the addition of tetramethylbenzidine substrate (Sigma-Aldrich). Optical densities were determined with an automatic ELISA plate reader at 450 nm (Molecular Devices). Optical densities that were twice background (0.06–0.075) binding to BSA were considered positive.

The quantitation of antibodies by isotype was performed similarly with some modifications. Serum was obtained from wild-type or sIgM mice at day 10 after infection and was incubated at a standard 1:40 dilution. Dilutions of isotype-specific antibodies (Southern Biotechnology Associates, Inc.) at a previously optimized concentration (1:2,000–1:4,000) were used. Optical densities were measured at 450 nm, and after subtraction of background binding, a value for each isotype was obtained and directly compared.

Statistical Analyses. For survival analysis, Kaplan–Meier survival curves were plotted using Prism software (GraphPad). Mortality curves were analyzed by the log rank test and average survival times were evaluated using the Mann-Whitney test.

Results

Decreased Survival of sIgM^{−/−} Mice after Infection with WNV

Previous studies in our laboratory with B cell–deficient μMT C57BL/6j mice indicated that B cells and antibody had a critical function in preventing the dissemination of WNV into the CNS. Because a 500-fold difference in viremia between μMT and congenic wild-type mice was observed within 4 d of infection, a time when only specific IgM against WNV was detected in the serum of wild-type mice, we postulated that newly generated anti-WNV IgM had a critical function in preventing the dissemination of WNV infection in wild-type and congenic sIgM mice, we postulated that newly generated anti-WNV IgM was detected in the serum of wild-type mice within 4 d of infection, a time when only specific IgM against WNV was first observed.

To begin to understand the mechanism by which a deficiency in secreted IgM made mice vulnerable to infection by WNV, the levels of infectious virus were measured from serum, spleen, kidney, brain, and spinal cord using plaque assays (11, 42). A time course was performed in wild-type and sIgM^{−/−} mice after inoculation with 10^5 PFU to determine the effect of IgM against WNV on the kinetics and magnitude of infection (Fig. 2).

Viremia. In wild-type mice, viremia was below the level of detection by infectious plaque assay throughout the time course. In contrast, 10^5–10^6 PFU/ml of infectious virus was detectable in sIgM^{−/−} mice between days 4 and 6 after infection (Fig. 2 A, P < 0.001), and subsequently waned to levels below detection. However, when viral RNA in serum was measured by a more sensitive fluorescent RT-PCR assay (11, 42), additional information was obtained (Fig. 2 B). At day 2 after infection, similar levels of WNV RNA were detected (~5,000 copies of WNV RNA/ml) in both wild-type and sIgM^{−/−} mice. At day 4 after infection, a time when anti-WNV IgM was first observed in the serum of wild-type mice (11), sIgM^{−/−} mice had 50-fold higher levels of WNV RNA in serum (P < 0.01). By day 6, WNV RNA was no longer detected in the serum of wild-type mice; in contrast, ~10^6 copies/ml were observed in serum from sIgM^{−/−} mice. By day 8, a time when immune IgG was first measured at significant levels (see Fig. 3); only low levels (~100 copies WNV RNA/ml) were detected in the serum of sIgM^{−/−} mice. Collectively, these studies suggest that a lack of development of specific IgM after WNV infection results in a delayed clearance of virus from serum.

Spleen and Kidney. In spleens from both wild-type and sIgM^{−/−} mice, similar kinetics of WNV accumulation were observed in the serum of wild-type and congenic sIgM mice; in contrast, ~100 copies WNV RNA/ml were observed in the serum of sIgM^{−/−} mice. Collectively, these studies suggest that a lack of development of specific IgM after WNV infection results in a delayed clearance of virus from serum.
observed (Fig. 2 C). Virus levels peaked in the spleen at day 4 after infection, decreased by day 6, and were absent at day 8. Although there was no significant difference in viral burden at day 4 after infection ($\sim 10^4$ PFU/g; $P = 0.3$), there was a small yet significant increase in virus production in the spleens of slgM$^{-/-}$ mice at day 6 after infection ($\sim 10^5$ compared with $10^3$ PFU/g; $P = 0.01$). In contrast, a distinct pattern of WNV infection was observed in a different peripheral organ, the kidney (Fig. 2 D). In wild-type mice, kidney viral burdens were below the level of detection by direct plaque assay at all times measured. However, $>10^3$–$10^4$ PFU of virus was detected in kidneys from slgM$^{-/-}$ mice at days 6 and 9 after initial infection ($P < 0.00001$).

CNS. The kinetics of CNS dissemination between wild-type and slgM$^{-/-}$ mice began similarly. Infectious virus was first detected in the brain at day 6 and in the spinal cord at day 8 after infection in wild-type and slgM$^{-/-}$ mice (Fig. 2, E and F). However, by day 8 after infection, slgM$^{-/-}$ mice had from 10- to 20-fold higher levels of infectious virus in the brain and spinal cord ($P < 0.04$), and by day 10, 100-fold higher levels of WNV were measured in the brains of slgM$^{-/-}$ mice. The difference of CNS viral load between wild-type and slgM$^{-/-}$ mice was not explained by the bias of the survival curves because, by day 10, no individual wild-type mouse that was morbidly infected had viral titers in the brain that approached those in the slgM$^{-/-}$ mice (e.g., maximum wild-type brain titer, $2.1 \times 10^4$ PFU/g; unpublished data).

IgG Response against WNV in slgM$^{-/-}$ Mice

IgM is believed to have an important role in triggering the adaptive immune response (24) as mice that lacked secreted IgM had blunted IgG responses after inoculation with influenza virus (27) or protein antigens (33). To determine whether a deficiency of secreted IgM affected the IgG responses against WNV, an ELISA against purified WNV envelope protein was used to measure serum levels of anti-WNV IgG in wild-type and slgM$^{-/-}$ mice after inoculation.
with WNV. In agreement with our previous work (11), wild-type mice developed a specific anti-WNV IgM response that was detectable within 4 d of infection, and as expected, there was no specific IgM detected in the slgM−/− mice (unpublished data). No specific IgG against WNV was detected in either wild-type or slgM−/− mice until 8 d after infection. The slgM−/− mice responded to WNV infection but the overall levels of virus-specific IgG were reduced compared with wild-type controls at days 8 and 10 after infection (Fig. 3). Isotype-specific analysis revealed markedly lower levels of IgG2b in slgM−/− mice (unpublished data). The results of these experiments suggest that secreted IgM that is generated after WNV infection may trigger an amplified IgG response that facilitates clearance of virus.

Role of Natural and Induced IgM in WNV Protection

Although our studies demonstrated that slgM−/− mice were vulnerable to WNV infection, the nature of the protective IgM response remained unclear. We wished to distinguish the protective function of natural and induced IgM. Natural IgM is constitutively secreted by CD5+ B-1 cells without specific stimulation, has widely variable bind-
The Role of Neutralizing IgM in Protection

Because the absence of secreted IgM resulted in increased viremia at day 4, we speculated that induced IgM might limit WNV infection by neutralizing virus before the development of an adaptive IgG response. As mentioned, pooled serum that was obtained from mice at 4 d after infection contained neutralizing IgM (PRNT_{50} = 1/30) and conferred protection; the neutralization activity could be precleared with anti-IgM but not anti-IgG Sepharose (Table I; reference 11). To assess whether neutralizing activity of IgM was required for protection, we passively transferred H5.46, a monoclonal IgM antibody that recognized the E protein of WNV (39) and lacked neutralizing activity (Table I). An ELISA assay against purified E protein confirmed that similar, if not greater, amounts of the H5.46 antibody were administered. Interestingly, passive transfer of either dilution (1:10 and 1:40 of ascites) of H5.46 anti-WNV IgM to sIgM^-/- mice did not alter the survival time, mortality rate, or viral burden in peripheral or CNS compartments when compared with untreated controls (Table I and unpublished data). Thus, for protection to be transferred, anti-WNV IgM required neutralizing activity.

The IgM Response against WNV as a Predictor of Survival

Because a genetic deficiency of secreted IgM was associated with lethality after WNV infection, we questioned whether variability in anti-WNV IgM levels correlated with survival in wild-type animals. To address this, 7-wk-old wild-type C57BL/6J mice were infected with 10^2 PFU of WNV. At day 4 after infection, animals were phlebotomized and followed clinically for survival. An ELISA was used to evaluate serum for specific IgM titer against purified WNV E protein using a biotin-conjugated goat anti-mouse IgM secondary antibody. 20 animals were included in the study. 8 survived and 12 died after infection. Five of the animals that died had identical titers of 1:5. Solid lines indicate the mean titer.

Discussion

We demonstrated previously that antibody played a critical role in preventing the dissemination of WNV into the CNS (11). Because B cell–deficient mice that lacked antibodies demonstrated a ~500-fold increase in viremia 4 d after infection, a time when only immune IgM was detected in the serum from wild-type mice, we postulated a critical role for IgM in controlling the early phases of WNV infection. In this paper, we directly addressed the function of secreted IgM in limiting the spread of WNV infection. C57BL/6J sIgM^-/- mice that were deficient in the production of secreted IgM but capable of secreting other immu-
noglobulin isotypes were highly vulnerable to lethal infection even after infection with low doses of WNV.

To our knowledge, this is the first paper to show a definitive protective role for secreted IgM against flavivirus infection. Although previous papers have demonstrated that passive administration of IgG or immune serum prevents encephalitis caused by flaviviruses (11, 13, 14, 21, 44–46) and nonflaviviruses (47–54), none demonstrated a definitive role for IgM. Induced IgM does have an important protective role against other pathogens. An absence of virus–induced secreted IgM resulted in 40% and 50% excess mortality in mice after infection with influenza A virus (27) or gastrointestinal bacteria (34), respectively. In comparison, the absence of secreted IgM resulted in 100% lethality in response to WNV infection across a wide (4 log) range of inoculating doses.

The role of natural IgM in protection against viral infection remains controversial. Reconstitution experiments demonstrated that IgM that was induced after virus infection was sufficient to confer protection against WNV, whereas natural IgM provided no protection. These results agree with our previous observations with wild-type and B cell–deficient mice (11): induced IgM prevented mortality in wild-type mice and delayed mortality in B cell–deficient mice, whereas natural IgM had no effect on survival time or rate in B cell–deficient mice. These results contrast with prior reconstitution experiments with other pathogens as follows: transfer of natural IgM conferred protection against influenza A (27) and vesicular stomatitis (26) viruses, and pertussis induced by cecal ligation and puncture (34). Despite our negative data, we speculate that natural IgM may still function to link the innate and adaptive immune responses against WNV (24). However, unlike other pathogens, administration of natural IgM alone does not adequately control WNV infection nor compensate for the production of antigen-triggered induced IgM.

Compared with congenic wild-type mice, no significant difference in WNV infection was observed in slgM−/− mice during the first 2 d after infection. However, by 4 d after infection, markedly higher levels of infectious virus and viral RNA were detected in the serum of slgM−/− mice compared with wild-type mice. Thus, the early anti-WNV IgM response functions, in part, to limit infection by preventing hematogenous spread. Indeed, the enhanced viremia and absence of induced IgM correlated with higher WNV viral burdens in peripheral and CNS tissues at different times after infection. Importantly, significantly higher viral burdens were observed in the kidneys of slgM−/− mice at day 6 after infection, a time that precedes the development of specific anti–WNV IgG antibodies in wild-type mice. The induction of specific IgM against WNV appeared to directly limit spread to peripheral nonlymphoid organs. Although IgM could inhibit WNV dissemination early in the course of infection by preventing virus attachment and entry or by binding C1q and directing complement-mediated lysis of viral particles (24, 43), the experiments with the H5:46 monoclonal IgM antibody suggest that the neutralizing activity of IgM confers protection. The experiments with H5:46 also suggest that IgM binding to WNV in vivo may not result in clinically significant complement-mediated lysis of viral particles, results that agree with in vitro studies that showed that in the presence of complement, nonneutralizing IgM facilitated rather than inhibited viral infection in macrophages, presumably by enhancing viral entry through complement receptor 3 (55, 56). Based on these experiments, we conclude that one critical function of induced IgM is to limit WNV infection by neutralizing virus before the development of an adaptive IgG response.

Nonetheless, the lack of secreted anti-WNV IgM may predispose to lethal infection by additional mechanisms. In addition to neutralizing pathogens in circulation (24, 26, 28), IgM inhibits infection by directly enhancing phagocytosis in lymphoid tissue (30), activating complement (27, 32, 43), and by priming the adaptive immune response (24). Indeed, some of our data suggest that secreted IgM against WNV contributes to the priming of the adaptive immune response. Despite the differences in viremia between wild-type and slgM−/− mice that were observed at day 4 after infection, the levels of virus detected in the brain at day 6 were not significantly different; thus, enhanced viremia was associated with but did not directly correlate with increased CNS viral burden. In addition, by day 8 after infection, a time when ~10–20-fold higher levels of WNV were observed in the CNS of slgM−/− mice, differences in the anti–WNV IgG response were observed: decreased levels of anti-WNV IgG were consistently detected in the slgM−/− mice. These results agree with others that have observed blunted IgG levels in response to viral or protein antigens in mice that lack secreted IgM (27, 33). Because virus eradication in neurons may depend on specific IgG (47, 57), the depressed specific IgG response in slgM−/− mice may contribute to WNV spread in the CNS and injury of neurons.

The importance of induced IgM early in the course of WNV infection was confirmed in wild-type mice by prospective analysis. Mice that had high (≥1:45) anti–WNV IgM titers at day 4 after infection had an improved (70% for IgMhi vs. 40% overall) survival rate. More dramatically, mice that had low (≤1:25) anti–WNV IgM titers at day 4 after infection uniformly died. Thus, the absence of a strong anti–WNV IgM early in the course of infection, by itself, predicted mortality. To our knowledge, this is the first work that definitively demonstrates that a depressed or delayed IgM response against a pathogen in wild-type animals is an independent risk factor for poor outcome. A recent retrospective clinical analysis supports an important role of IgM in flavivirus infections: low levels of anti–Japanese Encephalitis virus IgM antibody were an independent risk factor for neurological disease in humans (22). Although IgM appears to have an important function in limiting WNV infection, it is clear from published works (9–11, 58) that other aspects of the innate (e.g., interferon and...
complement) and adaptive (e.g., T cells) immune system also control WNV infection.

It is intriguing to consider that severe human WNV infection, which is heavily biased toward an elderly and immunocompromised population, occurs, in part, because of a dysfunctional IgM response against WNV early during infection. The elderly frequently have delayed antibody production and shortened durations of protective immunity after viral challenge or immunization (59–61). Although WNV causes neurological disease, it does so in a small subset of cases. As therapies become available, it will be important to target high-risk populations. Natural history studies in humans are currently being designed to identify both clinical and laboratory risk factors for severe WNV disease. Based on the studies presented here, the delayed development of specific, neutralizing anti-WNV IgM is likely to be an independent risk factor for morbidity and mortality.

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