Brief Definitive Report

Unmutated Immunoglobulin M Can Protect Mice from Death by Influenza Virus Infection

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Abstract

To elucidate the role of class switch recombination (CSR) and somatic hypermutation (SHM) in virus infection, we have investigated the influence of the primary and secondary infections of influenza virus on mice deficient of activation-induced cytidine deaminase (AID), which is absolutely required for CSR and SHM. In the primary infection, AID deficiency caused no significant difference in mortality but did cause difference in morbidity. In the secondary infection with a lethal dose of influenza virus, both $AID^{-/-}$ and $AID^{+/-}$ mice survived completely. However, $AID^{-/-}$ mice could not completely block replication of the virus and their body weights decreased severely whereas $AID^{+/-}$ mice showed almost complete prevention from the reinfection. Depletion of $CD8^+$ T cells by administration of an anti-CD8 monoclonal antibody caused slightly severer body weight loss but did not alter the survival rate of $AID^{-/-}$ mice in secondary infection. These results indicate that unmutated immunoglobulin (Ig)M alone is capable of protecting mice from death upon primary and secondary infections. Because the titers of virus-neutralizing antibodies were comparable between $AID^{-/-}$ and $AID^{+/-}$ mice at the time of the secondary infection, a defect of $AID^{-/-}$ mice in protection of morbidity might be due to the absence of either other Ig classes such as IgG, high affinity antibodies with SHM, or both.

Key words: AID • class-switch recombination • somatic hypermutation • antiviral immunity • antibody

Introduction

Prevention of viral infection by antibodies depends on diverse mechanisms such as prevention of viral attachment to the host cell (1, 2), activation of the complement system (3, 4), opsonization (5), antibody-dependent cell-mediated cytotoxicity (6, 7), and inhibition of the release of daughter viruses from infected cells (8–10). Such a wide variety of antibody activities are mediated by a generation of various classes of antibody (IgG, IgA, and IgE) besides IgM and IgD through class switch recombination (CSR; 11). Each class of antibody differs in size, in vivo half-life, ability to bind to Fc receptors, ability to activate complement, sensitivity to digestion by proteolytic enzymes, and the tendency to aggregate (12), and thus CSR determines how captured antigens are eliminated or the locations to which the antibody is delivered.

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Several investigations suggest that CSR may have a vital contribution to the protection against influenza virus infection and/or recovery from the infection (13, 14). For example, different antiviral activities among Ig classes have been reported (13). The passive transfer of virus-specific mAb of IgG class exerted prophylactic and therapeutic effect against influenza virus infection in the SCID mouse model, whereas the transfer of IgM or IgA exerted only prophylactic effect. The protective role of Fc receptor–mediated phagocytosis in influenza virus infection also suggests the importance of CSR (14) because the affinity to the Fc receptor is different among antibody classes, particularly mice IgG subclasses IgG1, IgG2a, and IgG2b, which are able to bind to Fcγ receptors with higher affinity than IgG3 (7). Although these data support the assumption that CSR plays a role in pathology of influenza virus infection, the direct evidence for the involvement of CSR in protection or recovery from viral infection is still missing.

In addition to CSR, another prominent alteration of the Ig gene sequence, somatic hypermutation (SHM), plays a

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critical role in antibody maturation (15). SHM accumulates massive point mutations in the V exon and gives rise to affinity maturation of antibodies in association with selection of B cells expressing high affinity Igs on their surface. The importance of SHM in secondary influenza virus infection is suggested indirectly. Sequence analysis of several antibodies against influenza virus has revealed the accumulation of mutations in secondary, but rarely in primary, antibodies (16).

Recently, activation-induced cytidine deaminase (AID) has been shown to be essential for CSR and SHM (17). The AID gene encodes a protein that has low homology (31%) with apolipoprotein B mRNA-editing enzyme catalytic polypeptide 1, a type of cytidine deaminase (18). AID mRNA was selectively expressed in activated splenic B cells and particularly germinal center B cells (17, 19). AID-deficient ($AID^{-/-}$) mice, generated by gene-targeted mutation, showed complete abrogation of CSR and SHM and also elevated IgM levels in sera as compared with heterozygous mice ($AID^{+/-}$; 17). In human, mutations in the AID gene cause the autosomal recessive hyper IgM syndrome type II that is characterized by higher levels of IgM and the absence of all other Ig classes and SHM (19). Furthermore, ectopic expression of AID alone can induce CSR and SHM in fibroblasts (20, 21).

Availability of $AID^{-/-}$ mice that develop normal hematopoietic cells except for B cells that are deficient in CSR and SHM (17), enables us to investigate the roles of CSR and SHM in viral infections. Here we report that IgM without SHM is capable of supporting complete survival of mice at primary and secondary influenza virus infections with 1 PFU (sublethal dose) and 1,000 PFU (lethal dose), respectively. However, at primary infection $AID^{-/-}$ mice showed delay in elimination of virus and in recovery of body weights and at secondary infection $AID^{-/-}$, but not $AID^{+/-}$, mice showed virus replication and weight loss, indicating that either CSR, SHM, or both play critical roles in the virus infection.

Materials and Methods

Animals and Viruses. $AID^{+/-}$ and $AID^{-/-}$ mice were bred and maintained in the Animal Center of Kyoto University. These mice have (CBA × C57BL/6) × C57BL/6 background and their characters were previously reported (17). All mice used were between 4–6 wk of age. Procedures that involved mice were approved by institutional guidelines for animal care.

Influenza virus, mouse-adapted A/PR/8/34 (mPR8), was grown in the allantoic cavity of 10-d-old embryonated chicken eggs. Virus titers were determined by plaque or 50% tissue culture infectious dose ($TCID_{50}$) assay on MDCK cells. Purified mPR8 used for ELISA was obtained by velocity density gradient centrifugation through a 20–50% linear sucrose gradient. The virion-containing fractions were stored at −80°C until use.

Virus Inoculation. For the primary influenza virus infection, mPR8 was appropriately diluted with PBS containing 0.2% bovine serum albumin (BSA-PBS). Mice were anesthetized with ether and then inoculated intranasally with 50 μl mPR8. For the secondary influenza virus infection, mice were inoculated with 1 PFU of mPR8 as described above and 6 wk later they were challenged intranasally with a lethal dose (100 LD_{50} or 1,000 PFU) of mPR8 in 50 μl BSA-PBS. These mice were monitored daily for their survival and weight for 4 wk.

Titration of Virus in the Lungs. Lungs were homogenized in 2.5 ml ice cold RPMI 1640. The homogenates were centrifuged at 500 *g* for 5 min to remove cell debris and the supernatants were stored at −80°C until assay. To determine $TCID_{50}$ of virus in the lungs, confluent monolayers of MDCK cells on 96-well microtiter plates were infected with 10-fold serial dilutions of lung homogenates. After 6–7 d of incubation at 34°C, MDCK cells were fixed and stained with crystal violet to detect the cytopathic effect (CPE) caused by influenza virus infection. The wells with CPE were counted and $TCID_{50}$ was calculated according to the Reed and Muench method.

Detection of Influenza Virus–specific Antibodies in Sera. Influenza virus–specific antibodies in sera were detected by ELISA as previously described (22). In brief, the wells of 96-well microtiter plates were coated with purified PR8 virus that had been solubilized with disruption buffer (0.05 M Tris-HCl, pH 7.8, containing 0.5% Triton X-100, and 0.6 M KCl) at room temperature. Diluted sera were transferred onto the viral protein–coated plates. After incubation for 60 min at room temperature, the plates were washed and horseradish peroxidase–conjugated secondary antibody was added to the wells. The secondary antibodies used in this study were sheep anti–mouse Igs (Amersham Biosciences) for total antibody detection, goat anti–mouse IgM specific for μ heavy chain (Zymed Laboratories), and rat anti–mouse IgG specific for γ heavy chains (Zymed Laboratories). Endpoint antibody titers were expressed as the reciprocal dilution of the last dilution that gave optical densities at 405 nm of ≥0.1 U above the optical density of negative controls.

Virus-neutralizing titers of sera were determined according to Benton et al. (23), and the reciprocal dilution of the last dilution that reduced the CPE by 50% was taken as the neutralizing titer.

In Vivo Depletion of $CD8^+$ T Cells. $AID^{-/-}$ mice were depleted of $CD8^+$ T cells by intraperitoneal administration of diluted mouse ascites fluid containing the rat anti–mouse CD8 mAb 53-6.7. Each mouse received 0.5 ml ascites fluid 3 d before influenza virus challenge, on the day of the challenge, on day 3 after the challenge, and then at 2-d intervals until the completion of the experiment. To verify depletion of $CD8^+$ T cells, flow cytometric analysis was performed using a FACScan™ (Becton Dickinson). Splenocytes (5×10^5 cells) were prepared from mice and stained with FITC-conjugated anti-CD8 mAb (YTS169.4; Cedarlane) and PE-conjugated anti-CD4 mAb (GK1.5; Leinco Technologies, Inc.). By the analysis, it was confirmed that 93–99% of $CD8^+$ T cells were depleted by this procedure.

Results and Discussion

AID Is Not Essential to Survival of Mice from Primary Influenza Infection. To examine whether CSR and SHM are required to protect from primary influenza virus infection, $AID^{-/-}$ and $AID^{+/-}$ mice were inoculated intranasally with various doses of mPR8 and their survival and morbidity, which was monitored by weight loss, was measured (Fig. 1). The mice of both genotypes showed no significant difference in the survival curve with the identical LD_{50} value (4.68 PFU) of mPR8. Regardless of genotypes, the mice inoculated with 100 PFU mPR8 were completely killed by day 10 and the inoculation of 10 PFU mPR8 caused 80%

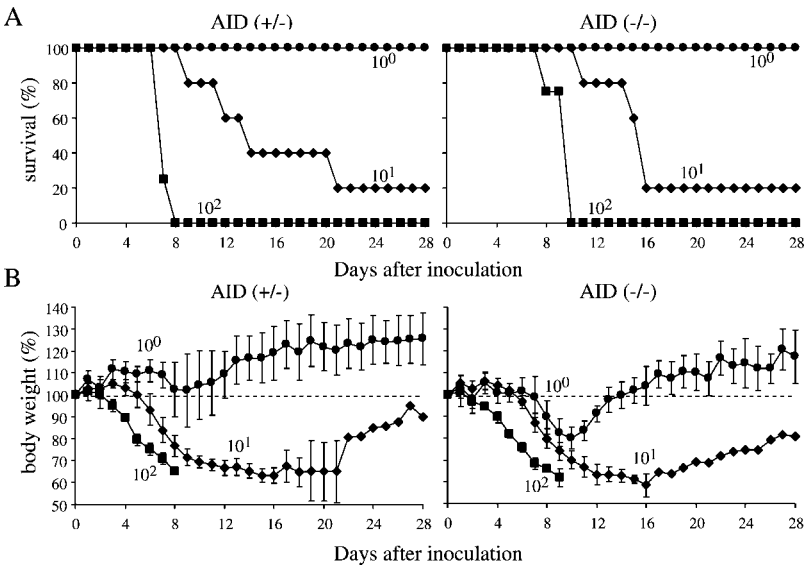

Figure 1. Susceptibility of AID$^{-/-}$ mice to influenza virus infection. Five mice of each genotype, AID$^{-/-}$ and AID$^{+/-}$, were inoculated intranasally with various doses of mPR8 (●, 10^0 PFU; ◆, 10^1 PFU; ■, 10^2 PFU) and monitored for 4 wk for their survival (A) and body weight (B) daily. The weight is expressed by percent of the weight on day 0 and datum points represent the mean of survived mice. Dashed line shows basal level of body weight and error bars represent standard deviations.

death by day 21 (Fig. 1 A). All of the mice of both genotypes survived when inoculated with 1 PFU mPR8.

The rate of weight loss by virus infection was dependent on inoculated doses of the virus in both genotypes and the mice inoculated with >10 PFU reduced their body weights much more rapidly until death than 1 PFU–infected mice (Fig. 1 B). The average weight loss by 1 PFU mPR8 infection was slightly greater in AID$^{-/-}$ mice than that in AID$^{+/-}$ mice. The lowest weight of mice and the day when mice showed the lowest weight were statistically different between AID$^{-/-}$ mice ($n = 20$) and AID$^{+/-}$ mice ($n = 27$). Student's t test for the lowest weight and Mann-Whitney's U-test for the lowest day revealed $P < 0.005$ and $P < 0.01$, respectively. In AID$^{-/-}$ mice the recovery from the weight loss was also delayed. When the day on which mice regained to the initial body weight was compared, the difference between 2 genotypes was statistically significant (Mann-Whitney's U test, $P < 0.05$).

In spite of similar survival rate of AID$^{-/-}$ and AID$^{+/-}$ mice at primary influenza virus infection, there was difference in the replication of mPR8 in the lungs between AID$^{-/-}$ and AID$^{+/-}$ mice (Fig. 2). The virus titers in the lungs of mice infected with 1 PFU mPR8 increased similarly in both genotypes until day 6. However, considerable titers of virus were detected on day 8 in AID$^{-/-}$ mice, but not in AID$^{+/-}$ mice, indicating a defect in virus elimination in AID$^{-/-}$ mice. By day 10, virus was eliminated in both AID$^{-/-}$ and AID$^{+/-}$ mice. The slightly slower elimination of virus in AID$^{-/-}$ mice is consistent with their delayed recovery of the body weight.

Induction of Similar Levels of Virus Neutralizing Antibodies in AID-proficient and -deficient Mice. Antibody response specific to mPR8 was clearly altered by AID deficiency (Fig. 3). The amounts of total antibodies specific to mPR8 in sera began to increase on day 8 in AID$^{+/-}$ mice inoculated with 1 PFU mPR8, reaching a plateau around day 28. The plateau level was maintained until secondary virus infection (day 42). In AID$^{-/-}$ mice, the response of virus-specific total antibodies was basically similar to that in AID$^{+/-}$ mice. An abrupt increment of antibodies was detected on day 8 and then their amounts gradually increased to a plateau level in AID$^{-/-}$ mice, which was maintained until secondary virus infection like in AID$^{+/-}$ mice. Although the average plateau level in AID$^{-/-}$ mice was lower than that in AID$^{+/-}$ mice, the time period to reach the plateau level was shorter in AID$^{-/-}$ mice; around day 10 as compared with around day 28 in AID$^{+/-}$ mice.

When IgM and IgG classes in virus-specific antibodies were quantified separately, the differences between AID$^{-/-}$ and AID$^{+/-}$ mice were much more obvious. The time course of IgM levels in sera of AID$^{-/-}$ mice was essentially similar to that of total antibodies as expected, whereas the

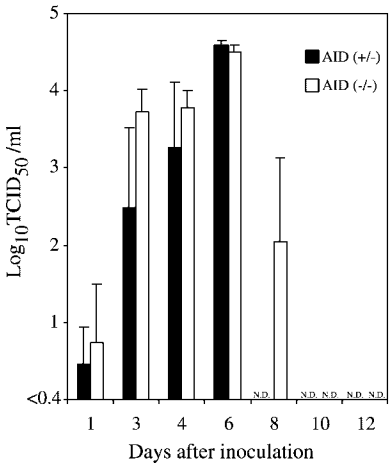

Figure 2. Virus replication in the lungs of AID$^{-/-}$ mice. Three to five mice of each genotype, AID$^{-/-}$ or AID$^{+/-}$, were inoculated intranasally with 1 PFU mPR8. At indicated days after inoculation, lungs were collected and homogenized. Virus titers in the homogenates were determined on MDCK cells as described in Materials and Methods. Results are expressed as the mean titer ± standard deviation. N.D., not detectable.

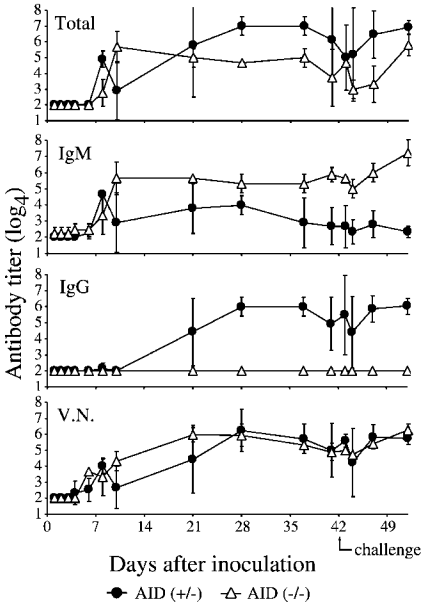

Figure 3. Influenza virus–specific antibody response in $AID^{-/-}$ mice. Mice of each genotype, $AID^{-/-}$ or $AID^{+/-}$, were intranasally inoculated with 1 PFU mPR8 and 42 d later they were challenged intranasally with a lethal dose (1,000 PFU) of mPR8 virus (arrows). Blood was taken at the indicated time points and virus-specific titers of total antibodies, IgM and IgG, and virus-neutralizing antibody titers in the sera were determined as described in Materials and Methods. Symbols and error bars represent means and standard deviations for three to five mice per group. The limit of detection in this assay was $4^{2.6}$. Titers $<4^{2.6}$ were set to 4^2 for calculation of means and standard deviations.

level of IgM in $AID^{+/-}$ mice decreased after the peak on day 8 and in most mice (three of the five mice in Fig. 3 and in other experiments 16 of 22 mice), virus-specific IgM was below the detectable level on day 41. The long-maintained high level of IgM in $AID^{-/-}$ mice is probably due to the absence of CSR and suggests that the CSR may contribute to the reduction of antigen-specific IgM response in wild-type mice.

As expected, the IgG response in $AID^{+/-}$ mice was totally different from that in $AID^{-/-}$ mice. In the sera of mPR8-infected $AID^{+/-}$ mice, virus-specific IgG was detectable on day 8, only 2 d later than the detection of IgM, and then gradually increased until day 28 and thereafter maintained a plateau level like the time course of total antibodies. In $AID^{-/-}$ mice, virus-specific IgG was not detected at all.

The time course of neutralizing antibody response was similar between the two genotypes in spite of the difference in the total antibody titers. Less amounts of virus-specific total antibodies might be compensated by higher levels of IgM in sera of $AID^{-/-}$ mice.

AID Is Required for Inhibition of Viral Replication but Not for Host Survival at the Secondary Lethal Challenge. Next, we examined the role of CSR and SHM in the secondary influenza virus infection. AID-deficient and -proficient mice were inoculated with 1 PFU mPR8 and 6 wk later the mice were challenged intranasally with a lethal dose (1,000 PFU or 100 LD_{50}) of mPR8. Regardless of the genotypes, all of the challenged mice survived completely (unpublished data).

The morbidity assessed by the body weight change, however, was distinct between $AID^{-/-}$ and $AID^{+/-}$ mice. Whereas $AID^{+/-}$ mice showed no sign of the decrease in the body weight, $AID^{-/-}$ mice lost their weight severely for the first 6 d after the challenge (Fig. 4 A). Weight loss was recovered to their initial body weights by 17 d after the challenge in all of $AID^{-/-}$ mice. The replication of secondarily challenged virus was completely prevented in $AID^{+/-}$ mice with no detection of virus during a 5-d period after infection. In contrast, vigorous viral replication was observed in preimmunized $AID^{-/-}$ mice challenged with the lethal dose of mPR8 (Table I). The titers of virus recovered in preimmunized $AID^{-/-}$ mice were almost equivalent to those in mock-immunized $AID^{+/-}$ mice (unpublished data). These data indicate that the primary virus immunization of $AID^{-/-}$ mice was incompetent for prevention of viral replication as well as of morbidity upon secondary virus infection.

Virus-specific IgM titers in sera were not significantly increased by the secondary influenza virus infection in $AID^{+/-}$ mice (Fig. 3). On the other hand, in $AID^{-/-}$ mice, virus-specific IgM as well as total antibodies began to increase on day 5 after challenge (47 d after primary infection) and kept increasing until day 10. The total virus-specific antibodies on day 10 were comparable between $AID^{-/-}$ and $AID^{+/-}$ mice.

Although $AID^{-/-}$ mice survived from secondary influenza virus infection, severe weight loss and vigorous viral replication were observed. By contrast, immunocompetent $AID^{+/-}$ mice completely protected themselves from the lethal virus challenge in the secondary infection. It is remarkable that virus was not detected even on day 1 after challenge without significant increase in the antibody titer in $AID^{+/-}$ mice (Figs. 3 and 4 A, and Table I). We assume that inoculated virus was completely neutralized with the antibody induced by primary infection in $AID^{+/-}$ mice but not in $AID^{-/-}$ mice.

Because the memory CTL response against influenza virus in $AID^{-/-}$ mice was comparable to that in $AID^{+/-}$ mice (unpublished data) and virus-specific memory CTL response was first observed in the lymph nodes attached to lungs on day 2 after secondary influenza virus infection (24), it is unlikely that the $CD8^+$ CTL response played a major role in the protection against secondary virus infection observed in $AID^{+/-}$ mice. Given the fact that the protection against secondary virus infection of the lethal dose is not complete in $AID^{-/-}$ mice, it is likely that virus-specific IgG or IgM antibodies with SHM play critical roles in protecting mice from the secondary influenza virus challenge. In fact, Palladino et al. (13) have demonstrated that adoptive transfer of monoclonal IgM provides mice with complete protection and blockade of viral replication, although therapeutically ineffective, upon lethal influenza virus infection. The low avidity of IgM to virus antigen because of the lack of SHM could be the cause of less effi-

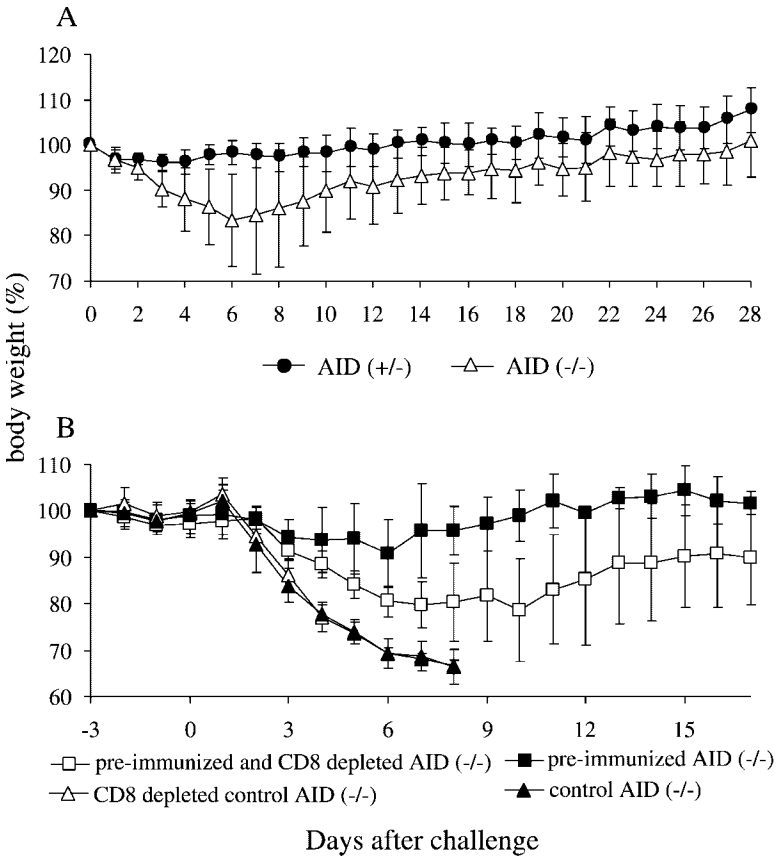

Figure 4. Effect of secondary virus challenge on the body weight of $AID^{-/-}$ mice. (A) Mice of each genotype, $AID^{-/-}$ (△, $n = 7$) or $AID^{+/-}$ (●, $n = 8$), were intranasally inoculated with 1 PFU mPR8. (B) $AID^{-/-}$ mice were intranasally inoculated with 1 PFU mPR8 (□, $n = 8$; ■, $n = 6$) or mock inoculated (△, $n = 7$; ▲, $n = 6$). Part of the mice were depleted of $CD8^+$ CTL cell as described in Materials and Methods by the administration of anti-CD8 mAb before challenge (△ and □). 42 d after inoculation, mice were challenged intranasally with a lethal dose (1,000 PFU) of mPR8. The weights of the mice were monitored daily for 4 wk after the challenge (A) or after the day of the first antibody administration (B). Datum points represent the mean of infected mice. Error bars represent standard deviation.

cient protection against the secondary virus challenge in $AID^{-/-}$ mice.

$CD8^+$ CTL Is Not Required for Survival of $AID^{-/-}$ Mice at the Secondary Lethal Challenge. Although the critical role of CTL in secondary virus infection of $AID^{+/-}$ mice is unlikely, contribution of $CD8^+$ CTL to the protection of $AID^{-/-}$ mice is not excluded. To clarify this point, preimmunized $AID^{-/-}$ mice were depleted of $CD8^+$ T cells by the administration of an anti-CD8 mAb and challenged with a lethal dose of influenza virus. $CD8^+$ CTL-depleted $AID^{-/-}$ mice showed slightly severer body weight loss than challenged control $AID^{-/-}$ mice that had been mock treated and maintained intact $CD8^+$ CTL (Fig. 4 B). The depletion of $CD8^+$ CTL, however, did not alter the survival rate of $AID^{-/-}$ mice and all of the immunized $AID^{-/-}$ mice survived regardless of depletion of $CD8^+$ CTL. These data indicate that the survival of $AID^{-/-}$ mice at secondary lethal virus challenge should be mediated mainly by virus-specific antibody and not by CTL.

Table I. *Virus Titers in the Lungs after Secondary Virus Challenge*

	Genotype of mice	
Days after challenge	$AID^{+/-}$[a]	$AID^{-/-}$[a]
1	N.D.[b]	—[c]
2	N.D.	5.44 ± 0.96
5	N.D.	4.78 ± 0.51

[a]Three to five mice were intranasally inoculated with 1 PFU mPR8 and 42 d later they were challenged intranasally with a lethal dose (100 LD_{50}) of mPR8. At the indicated days after the challenge, lungs were collected and homogenized. Virus titers in the homogenates were determined on MDCK cells as described in Materials and Methods. Results are expressed as the mean titer ± standard deviation of $\log_{10}$ $TCID_{50}$/ml.
[b]Not detectable.
[c]Not done.

Unmutated IgM Is Sufficient for Survival of Mice from Influenza Virus Infection. Survival rate in $AID^{-/-}$ mice was similar to those in $AID^{+/-}$ mice, whereas the clearance of virus and recovery of body weight in $AID^{-/-}$ mice was delayed. These data indicate that in the early phase of influenza virus infection AID does not play a critical role in the survival battle against the virus. It is noteworthy that low levels of virus-specific IgM were already reproducibly detected 1 d after infection in $AID^{-/-}$ mice, which is in general agreement with increased levels of IgM in these animals (17). IgM in $AID^{-/-}$ mice should not have SHM and are unlikely to be induced by virus infection because the same level of virus-specific IgM was detected in the PBS-inoculated $AID^{-/-}$ mice (unpublished data). Because the sharp increase of virus-specific antibodies roughly coincided with the decrease of virus titers in the lungs of $AID^{+/-}$ and $AID^{-/-}$ mice (Figs. 2 and 3), we assume that virus-specific

unmutated antibodies should play some role even in the later phase of the primary infection.

A recent study has demonstrated the essential role of IgM for protection from infection with influenza virus because secreted sIgM-deficient ($sIgM^{-/-}$) mice that can produce other isotypes show much higher susceptibility to influenza virus (25). In the infection of $sIgM^{-/-}$ mice with influenza virus, survival rate is lower and lung virus load is higher than those in wild-type mice. Because the presence of protective IgM in unimmunized wild-type mice was also reported (25, 26), the increased level of IgM in $AID^{-/-}$ mice may help their survival against influenza virus infection.

Concluding Remark. In the primary influenza virus infection, survival rates of $AID^{-/-}$ and $AID^{+/-}$ mice were identical but the morbidity of $AID^{-/-}$ mice was somewhat severer than that of $AID^{+/-}$ mice. In the secondary infection with the lethal dose, complete protection from death was observed in both mice but only $AID^{-/-}$ mice showed severe morbidity. Depletion of $CD8^+$ CTL did not affect the survival of $AID^{-/-}$ mice in the secondary infection. Taken together, unmutated IgM appears to be able to control virus replication to the extent that prevents animals from death but unable to eliminate virus quickly and completely, thus resulting in severer morbidity. It is worth noting that antigen-induced high titers of unmutated IgM protected $AID^{-/-}$ mice from the lethal dose of secondary influenza virus challenge. CSR and SHM appear to play a vital role for the efficient protection in the primary as well as secondary infection, although their involvement is not absolute in survival of the animals.

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