Brief Definitive Report

Resistance to and Recovery from Lethal Influenza Virus Infection in B Lymphocyte–deficient Mice

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Summary

In the adaptive immune response to most viruses, both the cellular and humoral arms of the immune system play complementary roles in eliminating virus and virus-infected cells and in promoting recovery. To evaluate the relative contribution of $CD4^+$ and $CD8^+$ effector T lymphocytes in virus clearance and recovery, we have examined the host response to lethal type A influenza virus infection in B lymphocyte–deficient mice with a targeted disruption in the immunoglobulin mu heavy chain. Our results indicate that naive B cell–deficient mice have a 50–100-fold greater susceptibility to lethal type A influenza virus infection than do wild type mice. However, after priming with sublethal doses of influenza, immune B cell–deficient animals show an enhanced resistance to lethal virus infection. This finding indicates that an antibody-independent immune-mediated antiviral mechanism accounts for the increased resistance to lethal virus challenge. To assess the contribution of influenza-specific $CD4^+$ and $CD8^+$ effector T cells in this process, defined clonal populations of influenza-specific $CD4^+$ and $CD8^+$ effector T cells were adoptively transferred into lethally infected B cell–deficient mice. Cloned $CD8^+$ effectors efficiently promoted recovery from lethal infection, whereas cloned $CD4^+$ T cells conferred only partial protection. These results suggest that memory T lymphocytes can act independently of a humoral immune response in order to confer resistance to influenza infection in immune individuals. The potential implications of these results for vaccination against human influenza infection are discussed.

For many viral infections, it has been suggested that the cell-mediated immune response plays a dominant role in recovery from disease, whereas the humoral, or B cell, response is important for protection against free virus and in preventing reinfection upon secondary exposure to an identical or cross-reactive virus (1, 2). In the case of influenza virus pneumonia, the disease can be cured either by a $CD8^+$ T cell response alone (3, 4), or, in the absence of $CD8^+$ T cells, by a $CD4^+$ T cell and concomitant B cell response (5, 6). In addition, studies examining the T cell–B cell interactions occurring in response to influenza virus infection have found that (*a*) adoptively transferred $CD4^+$ T cells can provide help for the production of neutralizing anti-influenza virus antibody (7); (*b*) this antibody response leads to virus clearance from the lungs of infected T cell–deficient (nude) mice (7); and (*c*) transfer of neutralizing anti-hemagglutinin (HA) antibody alone into infected SCID mice (deficient in T and B cells) leads to influenza virus clearance from the lungs of these immunodeficient mice (2, 8).

Since both activated $CD4^+$ and, to a lesser extent, activated $CD8^+$ T lymphocytes produce cytokines that can augment B lymphocyte differentiation and the production of neutralizing antibodies, the precise role of T cells in orchestrating recovery from experimental influenza infection is not well defined. To assess the contribution of T lymphocyte effector activity, we have examined the response of mice with a targeted mutation in the membrane exon of the immunoglobulin μ chain gene (B cell–deficient or μ knockout [KO] mice) to lethal primary and challenge type A influenza virus infection and the effectiveness of transferred $CD4^+$ and $CD8^+$ T cells in virus clearance. We find that while B cell–deficient mice are more susceptible to lethal influenza virus infection than are wild-type mice, earlier vaccination of μKO mice leads to enhanced resistance to lethal influenza challenge. Also, $CD8^+$ T cells are more efficient than $CD4^+$ T cells in promoting virus clearance and recovery in the absence of B lymphocytes. Our results indicate that the presence of influenza-specific memory T lymphocytes in an immune individual results in increased resistance to subsequent infection in the absence of neutralizing antibody.

Materials and Methods

Animals. Pathogen-free male and female C57Bl/6 ($H\text{-}2^b$) mice were purchased from Taconic Farms, Inc. (Germantown, NY)

J. Exp. Med. © The Rockefeller University Press • 0022-1007/97/12/2063/06 $2.00
Volume 186, Number 12, December 15, 1997 2063–2068
http://www.jem.org

and used at 6–12 wk of age. Breeding pairs of B cell–deficient (H-2^b) mice with a targeted mutation in the membrane exon of the immunoglobulin μ chain gene, or μKO mice, provided by K. Rajewsky (University of Cologne, Germany) (9), were bred and maintained in a colony at the University of Virginia, and were used at 6–12 wk of age.

Viruses. Influenza virus strain A/JAPAN/57 (A/JAPAN/305/57 [H2N2]) was grown in the allantoic cavity of 10-d-old embryonated hens' eggs and stored as infectious allantoic fluid as previously described (10). Determination of virus titer, expressed as hemagglutinating units, was done as previously described (10). Influenza virus strain A/JAPAN/57 used for intranasal inoculation and adoptive transfer procedures was mouse adapted by serial passage in mouse lung and is lethal at concentrations as low as one hemagglutinating units (equivalent to 10^4 EID_{50}, or egg infectious dose, units). For procedures requiring a sublethal challenge of influenza, an egg-adapted, antigenically identical strain of A/JAPAN/57 was used for intranasal inoculation. This preparation is not lethal for the mice used in these studies at concentrations equivalent to 10^8 EID_{50} units.

Intranasal Influenza Virus Inoculation. Intranasal inoculation of mice and determination of LD_{50} values was performed as previously described (11). To evaluate a dose response to intranasal virus, groups of 5–10 age-matched animals received serial 10-fold dilutions of allantoic fluid in cold PBS ranging from 10^{-2} to 10^{-6} and animals were watched daily for morbidity and/or mortality. Each group consisted of both male and female mice as no differences have been observed when using age-matched mice.

Bulk and Clonal T Lymphocyte Lines. Spleen cells from μKO and C57Bl/6 mice which had received a sublethal intranasal dose of A/JAPAN/57 12 d before were harvested and processed as previously described (11). Subsequently, these bulk cultures were stimulated in vitro with influenza A/JAPAN/57 virus-infected, gamma-irradiated (2,000 rad) C57Bl/6 spleen cells every 7–10 d in complete medium (IMDM [GIBCO BRL, Gaithersburg, MD]), 10% HIFCS, 1% glutamine, 5×10^{-5} M 2-ME, and antibiotics) with 15 U/ml human recombinant IL-2 (huRIL-2; Biosource International, Inc., Camarillo, CA). The procedures developed to establish and maintain the T cell clones used in these studies have been described in detail elsewhere (11).

Assays for Cell-mediated Cytotoxicity. The ^{51}Cr-release cytotoxicity assay and lysis calculations were carried out as previously described in detail (10–12). Effector/target ratios ranged from 5:1 to 50:1 depending on assay. Spontaneous release was <15%.

Adoptive Transfer Procedure. Adoptive transfer of day 6 viable cloned cells was performed as previously described (11, 12). 8–12-wk-old C57Bl/6 and B cell–deficient mice were intranasally inoculated with 10 LD_{50} influenza A/JAPAN/57 virus, and within 30 min 10^7 clone cells in 0.5 ml Iscove's medium were injected intravenously. Control mice were injected intravenously with 0.5 ml Iscove's medium alone. Mice were watched daily for 21 d for morbidity and/or mortality.

Results

To assess the impact of the presence or absence of B cells on susceptibility to infection with influenza virus, cohorts of age-matched C57Bl/6 and μKO mice were inoculated intranasally with varying doses of infectious mouse-adapted A/JAPAN/57 influenza virus (10^{-3}–10^{-7} dilutions of a stock preparation with an infectious titer of 10^7 EID_{50}/ml in embryonated eggs). As shown in Fig. 1, μKO mice were

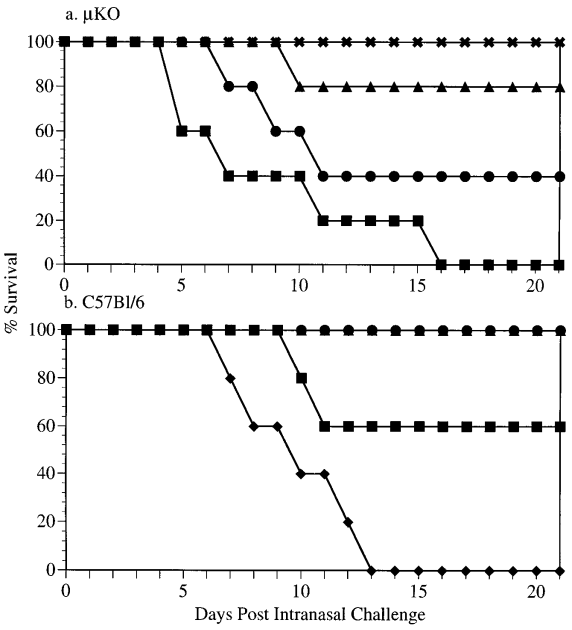

Figure 1. B cell–deficient mice are more susceptible to influenza viral challenge. μKO (*a*) and C57Bl/6 (*b*) mice were intranasally inoculated with varying doses (◆, 10^{-3} dilution; ■, 10^{-4} dilution; ●, 10^{-5} dilution; ▲, 10^{-6} dilution; and ✕, 10^{-7} dilution) of mouse adapted influenza virus and watched for 21 d for morbidity and mortality.

~100-fold more susceptible to lethal virus infection than were wild-type C57Bl/6 mice. The LD_{50} of this mouse-adapted A/JAPAN/57 influenza virus was $10^{3.8}$ EID_{50} units in wild-type C57Bl/6 mice and $10^{1.7}$ EID_{50} units in μKO mice, respectively. The titer of mouse-adapted virus used for these studies was ~10^7 EID_{50} units, which corresponds to an LD_{50} of 10^3 EID_{50} units in C57Bl/6 mice and an LD_{50} of 10^1–10^2 EID_{50} units in μKO mice.

It is well established that the host immune response to primary influenza virus infection results both in a cell-mediated immune response with the induction of $CD8^+$ CTLs and activated $CD4^+$ T cells, and in a humoral response with the production of neutralizing antibody (13–15). Therefore, one likely explanation for increased susceptibility of μKO mice to influenza infection is the inability of μKO mice to mount a neutralizing antibody response. Indeed, μKO mice are unable to mount a serum influenza–specific antibody response after influenza infection (data not shown).

To determine if recovery from primary intranasal influenza infection in μKO mice correlated with the presence of cellular immune effectors, e.g., CTLs, at the site of infection, groups of μKO and age-matched C57Bl/6 mice were sublethally infected with the mouse-adapted A/JAPAN/57 virus. On day 12 after infection, the mice were killed, lungs were excised, and mononuclear cells infiltrating the lungs were collected and tested for in vitro cytolytic activity. As Fig. 2 *a* shows, mononuclear cells from the lungs of infected μKO mice and from conventional mice exhibited a comparable degree of specific cytolytic activity on virus-infected target cells in vitro. Similar results were obtained when T cells were obtained at d 12 from lungs of wild-type

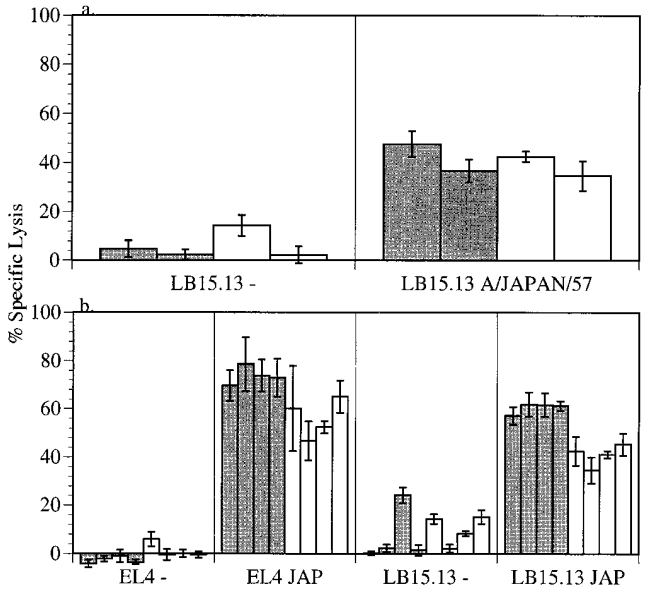

Figure 2. μKO mice can initiate and maintain an influenza-specific CTL response after challenge with influenza virus. (*a*) Lungs were removed from μKO (*shaded bars*) and C57BL/6 (*open bars*) mice on day 12 after intranasal viral challenge with a sublethal dose of A/JAPAN/57 (attenuated strain). Cell suspensions from the lungs were obtained by processing through a sieve, and were Ficoll purified and plated for a final effector/targer (E/T) ratio of 50:1. Assay time was 6 h and spontaneous release for all targets was <20%. Results of two separate experiments with two mice per experimental group are shown. (*b*) Influenza specific bulks from four individual μKO (*shaded bars*) and from C57BL/6 (*open bars*) mice were tested for their ability to lyse uninfected and A/JAPAN/57 infected class II negative (EL4) and class I and II positive (LB15.13) target cells in a standard chromium release assay. Assay time = 6 h; E/T = 10:1. Spontaneous release for all targets was <15%. Experiment is representative of three separate experiments.

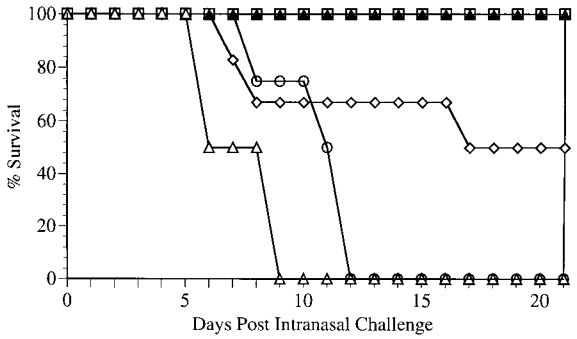

Figure 3. B cell–deficient mice are more susceptible to rechallenge with influenza. Groups of 7–12 age-matched μKO (*open symbols*) and C57Bl/6 (*closed symbols*) mice were intranasally infected with attenuated A/JAPAN/57. 28 d later the animals were rechallenged intranasally with the dilutions of mouse adapted A/JAPAN/57: △, 10^{-1} dilution; ○, 10^{-2} dilution; ◇, 10^{-3} dilution; □, 10^{-4} dilution. Animals were followed for 21 d for mortality. Data is representative of two experiments.

and B cell–deficient mice recovering from nonlethal intranasal infection with an attenuated A/JAPAN/57 virus preparation (data not shown).

The finding of virus-specific cytolytic activity in the lungs of μKO mice recovering from sublethal primary infection raised the possibility that primary infection would also result in the development of virus-specific memory $CD8^+$ (and presumably $CD4^+$) T lymphocytes capable of responding to challenge infection with virus. To further examine this hypothesis, two experimental approaches were employed. First, immune splenocytes from μKO and C57Bl/6 mice were taken 28 d after priming by sublethal infection with attenuated virus. These splenocytes were restimulated once in vitro and subsequently tested for CTL activity. As shown in Fig. 2 *b*, the in vitro secondary CTL response to A/JAPAN/57-infected stimulators between vaccinated μKO and C57Bl/6 mice was comparable. Second, groups of μKO and wild-type mice were first vaccinated by intranasal infection with a live attenuated (egg-adapted) A/JAPAN/57 virus preparation. This attenuated virus stock has an LD_{50} for μKO mice ~1,000-fold higher than the challenge mouse-adapted stock and is uniformly nonlethal for conventional mice. It clears from the lungs of both conventional and B cell–deficient mice by day 14 after infection (data not shown). 28 d after vaccination, when CTL activity was no longer detectable in the lungs of infected animals, the vaccinated mice were challenged by intranasal infection with the aggressive mouse-adapted A/JAPAN/57 virus.

Fig. 3 shows the results of this priming/challenge study. As expected, conventional mice, which have neutralizing antiviral antibody both in the circulation and locally in the respiratory tract, were uniformly resistant to lethal infection with mouse-adapted virus at virus doses up to 10^6 EID_{50} units (i.e., the equivalent of an innoculum of 10^3 LD_{50} doses for a naive conventional mouse). By contrast, challenge infection of B lymphocyte–deficient μKO mice resulted in death (Fig. 3). However, the immune μKO mice demonstrated a 100-fold greater resistance to challenge infection with mouse-adapted virus than did naive μKO mice (LD_{50} values of $10^{1.7}$ EID_{50} units and 10^4 EID_{50} units for mouse-adapted virus in naive and vaccinated μKO mice, respectively). These results suggest that the enhanced resistance to lethal virus challenge observed in the primed B cell–deficient mice was due to the activation of virus-specific memory T lymphocytes in response to challenge infection.

In view of the evidence for both antibody independent clearance of virus during primary infection of μKO mice and enhanced resistance of these mice to lethal infection after priming, it was of interest to assess the contribution of virus-specific $CD4^+$ and $CD8^+$ effector T lymphocytes to recovery from infection in B cell–deficient mice. To examine this, we adoptively transferred clonal populations of A/JAPAN/57 virus-specific $CD4^+$ and $CD8^+$ T lymphocytes into lethally infected conventional and μKO mice. The clones used for these studies were H-2^b haplotype-restricted $CD4^+$ T cells (i.e., 4D7) and $CD8^+$ T cells (i.e., 11E4 and B1.11), which have been previously characterized by us and have been shown to promote recovery when adoptively transferred into lethally infected C57Bl/6 mice (11). Fig. 4 shows the survival data for one adoptive transfer (representative of five independent experiments)

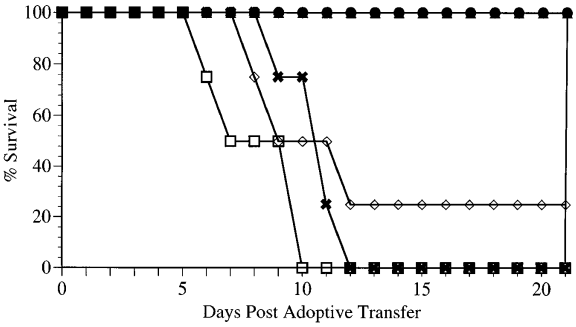

Figure 4. CD8$^+$, but not CD4$^+$, T cell clones promote full recovery in B cell–deficient mice. B cell–deficient (μKO) mice were infected intranasally with a 10 LD_{50} dose of mouse-adapted A/JAPAN/57 virus followed by an intravenous injection of 10^7 cells. The cells transferred included 4D7 (◇, a CD4$^+$ clone), 11E4 (▲, a CD8$^+$ clone), and B1.11 (●, a CD8$^+$ clone). Mice receiving intranasal influenza, but no cell transfer, are denoted as □. C57Bl/6 mice receiving intranasal influenza without cell transfer are also shown (✕). Each group represents 5–7 animals. Adoptive transfer of these clones and media control into lethally challenged C57Bl/6 mice was done simultaneously as a control.

carried out with these influenza-specific CD4$^+$ and CD8$^+$ T lymphocyte clonal effectors. As a control for each experiment, the same number of influenza-specific CD4$^+$ or CD8$^+$ T cell clones were adoptively transferred into lethally challenged C57Bl/6 mice, and all three clones consistently promoted 100% survival as previously published by this laboratory (11). The results of the adoptive transfers into C57Bl/6 mice are not shown as they are identical to those in our previous publication (11), but results of the viral challenge without concomitant clone transfer are shown in Fig. 4. In addition to promoting recovery in C57Bl/6 mice, both CD8$^+$ T cell clones efficiently promoted recovery in lethally infected μKO mice (Fig. 4). On the other hand, the adoptively transferred clone of CD4$^+$ T cells conferred partial protection in infected μKO mice with only 20% of the clone recipients surviving lethal infection (Fig. 4). In four other independent transfer experiments using these clonal populations of CD4$^+$ and CD8$^+$ T cells, the overall survival for lethally infected μKO recipients of the virus-specific CD4$^+$ T cell clone ranged from 20 to 60%, whereas the CD8$^+$ T cell clones reproducibly promoted recovery of 100% of infected μKO recipients.

This difference in the efficiency of virus clearance by CD4$^+$ and CD8$^+$ immune effectors was not a unique property of these three cloned T cell populations. In companion experiments, bulk cultures of A/JAPAN/57 immune splenocytes produced outcomes similar to their clonal counterparts upon adoptive transfer into lethally infected conventional and B cell–deficient recipients. Fractionated bulk populations of activated CD4$^+$ T cells promoted recovery in 100% of conventional recipients and in only 40%–60% of μKO recipients (data not shown). Like the virus-specific CD8$^+$ clones (Fig. 4), bulk populations of CD8$^+$ CTL effectors were uniformly protective after adoptive transfer into either conventional or B cell–deficient recipients.

Discussion

In this report we have examined recovery from lethal pulmonary influenza infection in μKO mice. We found that B lymphocyte–deficient mice show greater susceptibility to lethal primary influenza infection than do conventional mice with an intact B cell compartment. However, after vaccination with attenuated virus, the μKO mice demonstrate enhanced resistance to secondary challenge infection with virulent virus. This finding is consistent with the presence of virus-specific memory T lymphocytes, which can respond to and promote recovery from lethal challenge infection, in the vaccinated animals. Finally, we have observed that virus-specific CD4$^+$ and CD8$^+$ effector T lymphocytes differ in their capacity to clear virus and to promote recovery in infected B cell–deficient recipients.

The essential role of neutralizing antiviral antibody in protection against reinfection has been well established for influenza and most other viruses (2, 14, 16). The finding that B lymphocyte–deficient mice demonstrate increased sensitivity to lethal primary infection with type A influenza suggests an important role for antiviral antibody in recovery from primary influenza virus infection. Palladino et al. have previously provided compelling evidence for a critical role of neutralizing antibody in virus clearance during primary influenza infection in the mouse (2). The findings reported here support this concept. However, although no gross abnormalities in immune responsiveness have been reported to date in μKO mice (9, 17, 18), more subtle alterations in the host response to influenza in animals lacking mature B220$^+$ B cells (e.g., delayed T cell response kinetics) may contribute to their increased susceptibility to lethal influenza infection. It is noteworthy that Topham et al. recently reported efficient clearing of influenza HKX-31 influenza strain in μKO mice after sublethal infection (19). The findings reported here are in agreement with those results; however, our results further suggest that although virus clearance can be achieved in the absence of mature B cells, a humoral immune response to the virus also makes an important contribution to the control of virus replication and recovery from primary infection with a virulent virus strain.

A characteristic feature of influenza infection in the human is the susceptibility of immune individuals (primed by earlier vaccination or natural infection) to infection with serologically distinct variant virus strains of the same type A influenza subtype as that which arises in the human population through antigenic drift (20). Since these circulating viruses also share conserved antigenic epitopes recognizable by human and murine CD4$^+$ and particularly CD8$^+$ T lymphocytes (13, 21) the contribution in immune individuals of influenza-specific memory T lymphocytes directed to these conserved antigenic epitopes in resistance to and recovery from subsequent infection with drift variants has generally been considered minimal (1). The results reported here suggest otherwise. Earlier priming of B lymphocyte–deficient μKO mice with a live attenuated virus resulted in a >100-fold increase in the resistance of vaccinated mice to lethal challenge infection. This finding implies that, like preexist-

ing neutralizing anti-influenza antibody, memory T lymphocytes present in influenza-immune individuals can act to modify the course and severity of subsequent influenza infections. In a recent report on the outcome of lymphocytic choriomeningitis virus (LCMV) infection in B lymphocyte–deficient mice, the presence of LCMV-specific memory T cells induced by earlier vaccination likewise resulted in enhanced resistance to challenge infection (22).

Our finding that the secondary CTL response elicited in primed B cell–deficient mice is comparable to the CTL response in immune animals with an intact B lymphocyte compartment is in keeping with other recent reports in viral and nonviral models that demonstrated normal development and maintenance of $CD8^+$ T cell memory in mice lacking mature B lymphocytes (22, 23). However, as noted above, T lymphocytes which differentiate in the absence of B cells may develop compensatory mechanisms not normally expressed by T lymphocytes developing and responding in the presence of an intact B cell compartment. To assess the relative contribution of $CD4^+$ and $CD8^+$ T cells to virus clearance and recovery in μKO mice, we chose to adoptively transfer defined clonal and bulk populations of virus-specific effector $CD4^+$ and $CD8^+$ T cells derived from B cell–positive donors into lethally infected μKO recipients.

The results of this adoptive transfer analysis were clear cut. Virus-specific $CD8^+$ T lymphocyte effectors were equally effective at protecting from lethal infection and at promoting recovery in both conventional and B lymphocyte–deficient recipients. On the other hand $CD4^+$ T lymphocyte effectors were as effective as $CD8^+$ T lymphocytes in protecting mice with an intact B cell compartment, but promoted recovery in only a fraction of lethally infected μKO recipients. This result is in keeping with earlier observations of Scherle and co-workers that influenza-specific $CD4^+$ T lymphocytes function in vivo to clear influenza infection primarily by collaborating with B lymphocytes in the production of antiviral antibody (7, 24).

However, it should be emphasized that we reproducibly observed virus clearance and recovery in 20–60% of lethally infected B lymphocyte–deficient mice after transfer of activated virus-specific $CD4^+$ T cells. Thus, one or more antibody-independent effector mechanisms appear to be used by $CD4^+$ effector T cells to confer resistance to lethal challenge in some μKO recipients. Since the $CD4^+$ T cell clone used in this study, 4D7, has been previously shown to express virus-specific MHC class II–restricted cytolytic activity in vitro (11) direct cytolysis of influenza-infected respiratory epithelial cells would be the probable mechanism to account for the antiviral effect of transferred $CD4^+$ T cell effectors in μKO recipients. The recent studies of Tripp et al. on virus clearance by $CD4^+$ T cells in influenza-infected MHC class II–deficient mice provide compelling evidence against direct cytolysis as a critical in vivo effector mechanism of virus-specific effector $CD4^+$ T lymphocytes (25). A more likely explanation for the antiviral effect of transferred $CD4^+$ T cells in μKO mice is that upon contact with infected MHC class II–positive cells the $CD4^+$ effectors release proinflammatory cytokines that could accelerate the generation of specific $CD8^+$ T cell effectors in the infected recipient and/or amplify nonadaptive innate immune effectors mechanism with antiviral activity.

The ultimate goal of vaccination against viruses is to prevent the establishment of infection and this can be most effectively achieved by the presence of preexisting neutralizing antibody at the initial site of infection. In the case of type A influenza virus and other viruses that can undergo extensive antigenic variation, vaccination to prevent infection is difficult to achieve. Therefore, it may be more realistic to also consider vaccination strategies that lessen the severity of influenza infection. The results of the murine influenza model reported here strongly suggest that, in an immune individual, primed populations of influenza-specific memory T lymphocytes can act to modify the course and severity of subsequent influenza infection. These findings further imply that to be most effective a vaccine against human influenza should not only elicit a neutralizing antibody response but should also efficiently prime virus-specific memory $CD8^+$ and $CD4^+$ T lymphocytes.

This work was supported by Physician Scientist Award K11-AI01086 (M.B. Graham) from the National Institute of Allergy and Infectious Diseases and National Institutes of Health grants HL-33391, AI-15608, and AI-28317 (T.J. Braciale).

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Received for publication 11 August 1997 and in revised form 14 October 1997.

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