Brief Definitive Report

Transgenic Mice Lacking Class I Major Histocompatibility Complex-restricted T Cells Have Delayed Viral Clearance and Increased Mortality after Influenza Virus Challenge

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Summary

To investigate the role of $CD8^+$ T lymphocytes in recovery from influenza pneumonia, we used transgenic mice either homozygous (−/−) or heterozygous (+/−) for β_2-microglobulin (β_2-M) gene disruption. These mice lack major histocompatibility complex-restricted class I ($CD8^+$) T cells. We found that after challenge with a nonlethal influenza virus, the β_2-M (−/−) mice had significantly delayed pulmonary viral clearance. Furthermore, after challenge with a more virulent influenza virus, the β_2-M (−/−) mice had a significantly higher mortality rate than did control mice. Thus, $CD8^+$ T cells are important in recovery from virulent influenza infections, but other host defense mechanisms can clear the respiratory tract of more benign infections.

Mice infected with influenza virus generate class I MHC-restricted $CD8^+$ cytotoxic T lymphocytes that in vitro lyse influenza-sensitized targets and that in vivo function to clear the respiratory tract infection and promote clinical recovery (1). Experimental data supporting this paradigm come from studies in mice that have been adoptively immunized by antiinfluenza $CD8^+$ splenocytes or clones (2–5). These studies showed that mice receiving such cells had reduced mortality and more efficient viral clearance after infection with influenza virus (2–5).

Whether class II MHC-restricted T cells can also function to clear virus is currently under investigation. After adoptive transfer of class II cytolytic clones, mice have been shown to clear virus (6). Eichelberger et al. (7) addressed the role of $CD4^+$ T cells by using mice that either had been depleted of $CD8^+$ T cells by in vivo treatment with anti-CD8 mAb, or had disrupted β_2-microglobulin (β_2-M) genes. Their data suggested that these mice cleared virus similarly to normal mice (7), and cast some doubt on the relative importance of $CD8^+$ T cells in host defense against viral infections.

In this report, we also used transgenic homozygous for the β_2-M defect and found that although these animals can eventually eliminate influenza virus from their lungs, clearance is significantly delayed. Further, these animals are more susceptible to death from a more virulent strain of influenza virus.

Materials and Methods

Mice. Mice were housed under barrier conditions at the transgenic animal facility of the University of Florida, Gainesville. Mice transgenic for a homozygous (−/−) or heterozygous (+/−) gene disruption for β_2-M were derived from a breeding pair obtained from Dr. O. Smithies, University of North Carolina, Chapel Hill, NC (8). PCR was used to screen for the mutation and confirmed by Southern blot hybridization (8). Control mice were C57BL/6. All mice were females and 4–16 wk old when used.

Viruses. Two viruses were used: influenza A/Port Chalmers/1/73 (H3N2) and influenza A/Puerto Rico/8/34 (H1N1). Both viruses were grown in fertilized hen's eggs. The mice were infected by the intranasal route with $\sim 10^6 - 10^7$ 50% tissue culture infective dose ($TCID_{50}$) after pentobarbital (0.1 mg i.p.) anesthesia. Virus was isolated from the mice by grinding the lungs and adding fresh triplicate 10-fold dilutions of 0.1-ml aliquots in MEM with 10% FCS into Madin Darby canine kidney cells (3×10^4/well) in a 96-well plate. The plates were incubated at 34°C for 24 h, the media removed and replaced with MEM with 0.2% trypsin (Sigma Chemical Co., St. Louis, MO), incubated for an additional 4 d at 34°C, and 0.04 ml of 5% chicken red blood cell suspension added to each well. The plates were refrigerated for 2 h, and wells exhibiting hemadsorption were considered positive. The $TCID_{50}$ was calculated using the method of Reed and Muench (9). We found that $TCID_{50}$ results are about 0.5 $\log_{10}$ lower than injecting virus into the allantoic cavity of fertilized hen's eggs (unpublished observations). $\log_{10}$ $TCID_{50}$ of undetectable virus was defined as < -1.

Statistical Analysis. All data were analyzed by Fisher's exact test.

Mortality data were further analyzed using the SURVIVAL procedure of SPSS (SPSS, Inc., Chicago, IL) on a PDP 11/74 computer (Digital Electronics Corporation, Maynard, MA).

Results and Discussion

Clearance of Influenza Virus. After a total respiratory tract infection of normal young mice, virus reaches a peak titer of about 10^5 $TCID_{50}$, and is cleared from the lungs of most mice by days 6–8 (10, 11). This coincides with the peak pulmonary cellular immune response (10, 12, and our unpublished data). To determine whether β_2-M (+/−) mice could eliminate influenza from their lungs similar to normal mice, we killed mice 8 d postchallenge with H3N2 virus. Similar to normal mice, the β_2-M (+/−) mice, which express class I MHC glycoproteins and have functional $CD8^+$ CTLs, had all cleared virus by this day (Table 1). In contrast, 4/5 β_2-M (−/−) mice ($p < 0.05$, Fisher's exact test) were still infected on this day (with a mean titer of $10^{3.3}$).

Table 1 also shows that by day 15, all three β_2-M (−/−) mice had cleared virus from their lungs. These data are consistent with Eichelberger et al. (7) who showed that on day 10, only one of four β_2-M (−/−) mice was shedding virus (in undiluted lung homogenate), and on day 13, all mice had cleared virus. The mechanism for this clearance remains obscure. Although it is most likely mediated by $CD4^+$ cells, Eichelberger et al. reported extremely low T cell–mediated cytotoxicity in pulmonary lavage cells from influenza-infected β_2-M (−/−) mice (7). Similarly, $CD4^-$ $CD8^-$, γ/δ T cells are believed to be nonphagocytic (7, 13), and the β_2-M (−/−) mice lack NK cell activity (7). Antiinfluenza IgG antibody alone is not believed to be sufficient for recovery from influenza infection (14), though a recent report suggests that a mixture of antiinfluenza IgA, IgG, and IgM antibodies may be (15). Because $CD4^+$ and B lymphocytes are seemingly intact in the β_2-M (−/−) mice (7, 16), their antibody response should be normal. Hence, antibody differences do not seem to be able to explain the differences in viral clearance at day 8, but could account for the eventual clearance.

Table 1. *Clearance of H3N2 influenza Virus after a Respiratory Tract Challenge*

	Pulmonary Virus Titers (Log_{10} $TCID_{50}$)	
Group	Day 8	Day 15
β_2-M (−/−)	<−1, 1.45, 1.45, 4.2, 5.3	<−1*
β_2-M (+/−)	<−1*	ND

Anesthetized β_2-M (−/−) and β_2-M (+/−) were infected intranasally with ~10^6 $TCID_{50}$ H3N2 virus, killed on days 8 and 15 post infection, and virus quantitated by tissue culture. Three of the β_2-M (−/−) mice died (on days 4, 6, and 10) vs two of the β_2-M (+/−) mice (on days 6 and 7).

* All mice in these groups (five β_2-M [+/−] and three β_2-M [−/−]) had titers <−1.

Survival after Challenge with a Nonvirulent Influenza Virus. When anesthetized mice are inoculated intranasally with influenza, there is an initial infection of the total respiratory tract (nose, trachea, and lungs) (17). We therefore evaluated the effect of the β_2-M disruption on the ability of mice to survive by challenging the mice with a more virulent H1N1 virus. Our results are shown in Fig. 1 and demonstrate that significantly more β_2-M (−/−) mice died than either β_2-M (+/−) or control C57BL/6 mice. This difference is statistically significant by either Fisher's exact test (β_2-M [−/−] vs. β_2-M [+/−], $p < 0.05$; β_2-M [−/−] vs. C57BL/6, $p < 0.005$), or the survival analysis (Lee-Desu statistic = 8.74, d.f., = 2, $p = 0.013$; β_2-M [−/−] vs. β_2-M [+/−], $p = 0.084$; β_2-M [−/−] vs. C57BL/6, $p = 0.0054$).

Eichelberger et al. (7) evaluated viral clearance viral clearance from β_2-M (−/−) mice and found one of four and zero of four mice shedding virus at days 10 and 13, respectively. They also reported that mice depleted of $CD8^+$ T cells by anti-$CD8^+$ antibody were similar to controls in that they shed virus on days 5 and 7, but not on days 10 or 13. Others, not reasonably, have interpreted this as normal recovery (15). Our data on viral shedding are consistent with Eichelberger et al. (7). However, having shown that on day 8 four of five β_2-M (+/−) mice and zero of five β_2-M (−/−) mice shed virus, our data demonstrate a definite delay in viral clearance in the absence of $CD8^+$ T cells. The pathologic significance of this defect is clearly illustrated by the challenge experiment using a more virulent strain of influenza virus (Fig. 1). Functional $CD8^+$ T cells are necessary for survival when the influenza virus challenge is more virulent.

We agree with the conclusion of Eichelberger et al. (7) that there is "a redundancy in cell-mediated effector mechanisms, at least in young, healthy adult mice infected with a virus that grows mainly in surface epithelium." However, our data show that $CD8^+$ T cells are essential for recovery when the challenge is sufficiently virulent. Furthermore, deple-

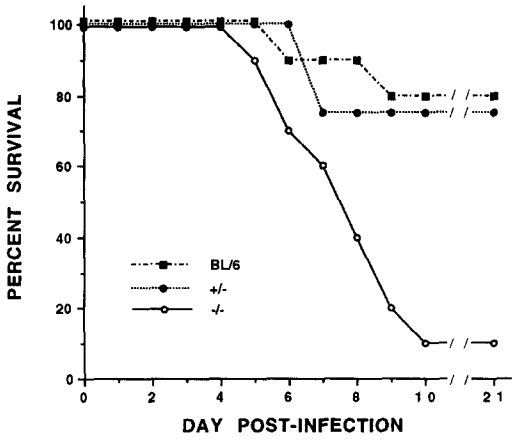

Figure 1. Survival of mice after respiratory challenge with influenza A H1N1. Homozygous β_2-M (−/−) (n = 10), heterozygous β_2-M (+/−) (n = 4), and C57BL/6 (BL/6) (n = 10) mice were given ~10^7 $TCID_{50}$ of H1N1 while under anesthesia and observed for 21 d.

tion of CD4$^+$ cells causes little delay in viral clearance (12), and adoptive transfer of class II MHC-restricted clones enhances influenza clearance, but has no significant effect on survival when a lethal dose of influenza is used (6). We conclude that CD8$^+$ class I MHC-restricted T cells are the primary mechanism responsible for recovery, as first proposed by Zinkernagel and Doherty (18).

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References

1. Yewdell, J.W., and C.J. Hackett. 1989. Specificity and function of T lymphocytes induced by influenza A viruses. *In* The Influenza Viruses. R.M. Krug, editor. Plenum Press, New York. 361–429.
2. Yap, K.L., G.L. Ada, and I.F.C. McKenzie. 1978. Transfer of specific cytotoxic T lymphocytes protects mice inoculated with influenza virus. *Nature (Lond.).* 273:238.
3. Yap, K.L., and G.L. Ada. 1978. The recovery of mice from influenza A virus infection: adoptive transfer of immunity with influenza virus-specific cytotoxic T lymphocytes recognizing a common virion antigen. *Scand. J. Immunol.* 8:413.
4. Wells, M.A., F.A. Ennis, and P. Albrecht. 1981. Recovery from a viral respiratory infection. II. Passive transfer of immune spleen cells to mice with influenza pneumonia. *J. Immunol.* 126:1042.
5. Taylor, P.M., and B.A. Askonas. 1986. Influenza nucleoprotein-specific cytotoxic T-cell clones are protective *in vivo*. *Immunology.* 58:417.
6. Hioe, C.E., and V.S. Hinshaw. 1989. Induction and activity of class II-restricted, Lyt-2$^+$ cytolytic T lymphocytes specific for the influenza H5 hemagglutinin. *J. Immunol.* 142:2482.
7. Eichelberger, M., W. Allan, M. Zijlstra, R. Jaenisch, and P.C. Doherty. 1991. Clearance of influenza virus respiratory infection in mice lacking class I major histocompatibility complex-restricted CD8$^+$ T cells. *J. Exp. Med.* 174:875.
8. Koller, B.H., and O. Smithies. 1989. Inactivating the β_2-microglobulin locus in mouse embryonic stem cells by homologous recombination. *Proc. Natl. Acad. Sci. USA.* 86:8932.
9. Reed, L.J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* 27:493.
10. Yap, K.L., and G.L. Ada. 1978. Cytotoxic T cells in the lungs of mice infected with an influenza A virus. *Scand. J. Immunol.* 7:73.
11. Bender, B.S., M.P. Johnson, and P.A. Small. 1991. Influenza in senescent mice: impaired cytotoxic T-lymphocyte activity is correlated with prolonged infection. *Immunology.* 72:514.
12. Allan, W., Z. Tabi, A. Cleary, and P.C. Doherty. 1990. Cellular events in the lymph node and lung of mice with influenza. Consequences of depleting CD4$^+$ T cells. *J. Immunol.* 144: 3980.
13. Doherty, P.C., W. Allan, M. Eichelberger, S. Hou, K. Bottomly, and S. Carding. 1991. Involvement of $\gamma\delta$ T cells in respiratory virus infections. *Curr. Top. Microbiol. Immunol.* 173:291.
14. Kris, R.M., R.A. Yetter, R. Cogliano, R. Ramphal, and P.A. Small. 1988. Passive serum antibody causes temporary recovery from influenza virus infection of the nose, trachea and lung of nude mice. *Immunology.* 63:349.
15. Scherle, P.A., G. Palladino, and W. Gerhard. 1992. Mice can recover from pulmonary influenza virus infection in the absence of class I-restricted cytotoxic T cells. *J. Immunol.* 148:212.
16. Koller, B.H., P. Marrack, J.W. Kappler, and O. Smithies. 1990. Normal development of mice deficient in β_2M, MHC class I proteins, and CD8$^+$ T cells. *Science (Wash. DC).* 248:1227.
17. Yetter, R.A., S. Lehrer, R. Ramphal, and P.A. Small, Jr. 1980. Outcome of influenza infection: effect of site of initial infection and heterotypic immunity. *Infect. Immun.* 29:654.
18. Zinkernagel, R.M., and P.C. Doherty. 1979. MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity, function, and responsiveness. *Adv. Immunol.* 27:51.