

## Comment

## Cytotoxic T lymphocyte memory: role in cross-protective immunity against influenza?

G.F. Rimmelzwaan and A.D.M.E. Osterhaus

MHC class I restricted CD8<sup>+</sup> cytotoxic T lymphocytes (CTL) have been shown to play a role in the clearance of several virus infections and thus in the prevention of disease symptoms they may cause<sup>1-9</sup>.

Although the induction of protective CTL responses directed to influenza virus has been the subject of several studies (see *Table 1* for review), a role of these cells in

the protection from subsequent influenza virus infections is controversial. Despite the existence of conserved CTL epitopes present in e.g. the nucleoprotein (NP)<sup>10, 11</sup> of influenza virus strains that infect humans in subsequent seasons, there is apparently little or no protective effect mediated by CTLs directed against these epitopes<sup>12</sup>. For this reason it is argued that predominantly the presence of virus neutralizing (VN) antibodies with the relevant specificities for the hemagglutinin (HA) and the neuraminidase (NA) membrane glycoproteins of the actually circulating influenza virus strains is protective: protection against

infection proved to be correlated with VN serum antibody titers, induced by natural infection, vaccination or passive transfer<sup>13</sup>. In animal models it was demonstrated that CTL responses are not a requirement for the recovery from influenza infections (Eichelberger *et al.*, 1991<sup>33</sup>; Scherle *et al.*, 1992<sup>34</sup>) and that vaccination with recombinant vaccinia viruses (rVV) expressing the NP or other internal proteins, resulted in virus-specific MHC class I restricted CTL responses but did not protect against a lethal challenge with influenza virus A/PR/8/34 (H1N1)<sup>14-16</sup>. However, in some cases a protective effect could be observed after adoptive transfer of CTLs of this specificity<sup>8, 16, 17</sup>. Immunization with rVVs expressing the HA or NA proteins led to the induction of virus-neutralizing antibodies and complete protection from challenge infection. Furthermore it was shown that in  $\beta$ 2m<sup>-</sup> knockout mice, which lack

Department of Virology, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. (Received 14 October 1994; accepted 19 October 1994)

**Table 1** Induction of protection through CTL responses by influenza subunit preparations

Protein	Virus	Presentation form	Species	Immune response demonstrated	Protection	Reference
NP	X/31 H3N2	Adjuvant: none	Balb/c mice (H-2 <sup>d</sup> )	CTL	Yes	Wraith <i>et al.</i> <sup>25</sup>
rNP	A/PR/8/34 H1N1	Adjuvant: Al(OH <sub>3</sub> )	Balb/c, B10.A (5R) mice	Th	Yes	Tite <i>et al.</i> <sup>18</sup>
rNP	A/PR/8/34 H1N1	<i>Salmonella typhimurium</i>	Balb/c mice	Th	Yes	Tite <i>et al.</i> <sup>19</sup>
rNP	A/PR/8/34 H1N1	DNA	Balb/c mice	CTL	Yes	Brett <i>et al.</i> <sup>26</sup>
rNP	A/HK/68 H3N2					Ulmer <i>et al.</i> <sup>24</sup>
HA	A/PR/8/34 H1N1	Vaccinia virus	B6 and $\beta$ 2m <sup>-</sup> mice (H-2 <sup>b</sup> )		No	Epstein <i>et al.</i> <sup>15</sup>
NA		Vaccinia virus	B6 and $\beta$ 2m <sup>-</sup> mice	VN	Yes	
PA, NS1, NS2, PB1, PB2, M1, M2, NP, alone or as a mixture		Vaccinia virus	B6 and $\beta$ 2m <sup>-</sup> mice	VN	Yes	
HA		Vaccinia virus	B6 and $\beta$ 2m <sup>-</sup> mice		No	
HA		Vaccinia virus-IL-2	(old) mice	VN/CTL	Yes	Ben-Yehuda <i>et al.</i> <sup>27</sup>
HA		NS1-HA2 fusion protein	nude mice	VN/ $\gamma$ IFN	Yes	Karupiah <i>et al.</i> <sup>22</sup>
HA	H1/H2		Balb/c mice	CTL	Yes	Kuwano <i>et al.</i> <sup>28</sup>
HA	H1H1	Vaccinia virus	CBA/h mice	VN	Yes	Andrew <i>et al.</i> <sup>16</sup>
NP	H1N1	Vaccinia virus	CBA/h mice	ND	No	Andrew <i>et al.</i> <sup>16</sup>
NP 147-155	H1N1	Vaccinia virus	Balb/c mice	Pulmonary CTL	No	Lawson <i>et al.</i> <sup>14</sup>
NP	H1N1	Vaccinia virus	Balb/c mice	Pulmonary CTL	No	Lawson <i>et al.</i> <sup>14</sup>
NP	H7N1	Vaccinia virus	C57 BL/6 (H-2b) CBA/J (H-2k) mice	CTL	No	Stitz <i>et al.</i> <sup>29</sup>
NP	H7N1	Cell associated	idem	CTL	No	Stitz <i>et al.</i> <sup>29</sup>
NP	H2N2	ISCOM	Balb/c and C3H (H-2k) mice	No CTL	Partially	Weiss <i>et al.</i> <sup>30</sup>
NP	H5N2/H5N8	Fowl pox	chicken		No	Webster <i>et al.</i> <sup>31</sup>
NP	H1N1	Vaccinia virus	outbred ddY mice	CTL in Balb/c	No, reduced pulmonary virus titers d7 p.i.	Endo <i>et al.</i> <sup>22</sup>

CD8<sup>+</sup> CTL, HA and NA protein expressing rVVs induced protection that was fully mediated by VN serum antibodies. However, it should be realized that in most of the studies which failed to induce protective immunity by mechanisms other than VN antibodies, rVVs were used as expression system for viral proteins like NP. In other experiments using purified NP or recombinant NP as immunogens, either presented as soluble protein or by *Salmonella typhimurium* as a vector, protection could be induced. With *Salmonella* as an antigen delivery system for NP or with recombinant NP in Al(OH)<sub>3</sub>, strong T helper (Th) cell responses were induced. No direct association of protection with virus-specific MHC class I restricted CTLs was demonstrated<sup>18, 19</sup>, probably since antigens presented in this way are highly inefficient in entering the endogenous pathway of antigen processing<sup>20, 21</sup>. A mechanism underlying this protection may be the accelerated induction of VN antibodies and CTLs as a result of strong T helper cell responses after infection with the challenge virus. However, since the immunized mice in these experiments were also protected from challenge with an influenza B virus, the action of non-specific antiviral mechanisms such as the production of IFN $\gamma$  could not be excluded<sup>19</sup>. Studies in nude mice showed that a rVV coexpressing IL-2 along with HA may confer protection in mice in the absence of T cells and suggested a role for specific IgM antibodies, natural killer cell activity and IFN $\gamma$ <sup>22</sup>.

Recently, alternative systems have been explored for the presentation of NP to the immune system including nucleic acid vaccination: plasmid DNA, which encodes a foreign protein under control of a eukaryotic promoter sequence, was directly injected into muscle tissue of recipient mice. Inoculation of mice with influenza NP encoding DNA, resulted in decreased virus titers in the lung, inhibited mass loss and increased survival rates not only after a homologous challenge with influenza A/PR/8/34 (H1N1), but also after a heterologous challenge with influenza virus A/HK/68 (H3N2). In parallel experiments neither the passive transfer of NP-specific antibodies, nor the immunization with purified NP

could confer protection. In the NP-DNA inoculated mice, NP-specific CTL were demonstrated which were absent in mice immunized with the purified protein.

The observation that humans may develop influenza upon infection with newly emerging virus strains and that mice are not protected against a virus challenge after vaccination with rVV expressing the NP protein, in spite of the induction of CTL responses, suggests an inadequate induction of CTL memory.

Recently it was shown that for the induction of protective cytotoxic T cell memory towards lymphocytic choriomeningitis virus (LCMV) infection, the persistence of viral antigen was required<sup>23</sup>. LCMV-specific long-term CTL memory was only maintained in persistent LCMV infection. Vaccination with rVVs expressing LCMV proteins resulted in a poor CTL memory, which correlated with the rVV dose and depended on the LCMV protein expressed. Adoptive transfer experiments with spleen cells from immunized mice confirmed that for the maintenance of CTL memory the presence of viral antigen was essential. These findings are in contrast to results recently obtained in adoptive transfer experiments, which were performed with irradiative recipient mice (Mullbacher, 1994<sup>25</sup>; Hou *et al.*, 1994<sup>26</sup>), suggesting that thus acquired influenza virus specific CTL memory can be maintained in the absence of antigen. The protective effect of these CTLs was, however, not studied by challenge infection.

Therefore we speculate that antiviral protection mediated by memory CTL is short-lived when induced by non-persistent infection with, for example, influenza virus. Similarly, vaccination with rVV expressing the relevant influenza proteins or live attenuated influenza vaccines may only induce short-lived CTL responses in the absence of persisting antigen. In contrast the persistent low level *in vivo* expression of NP after inoculation with NP-DNA<sup>24</sup>, may lead to protective immunity mediated by specific CTL.

Therefore it may be expected that if indeed the development of future influenza vaccines should take advantage of the existence of

conserved CTL epitopes<sup>10, 11</sup>, resulting in the induction of inter- and intratypic cross-protection, they should be based upon strategies leading to the persistent exposure of the immune system to the relevant viral antigens.

## REFERENCES

- 1 Doherty, P.C., Allan, W. and Eichelberg, M. Roles of  $\alpha\beta$  and  $\gamma$  T cell subsets in viral immunity. *Annu. Rev. Immunol.* 1992, **10**, 123–151
- 2 Cannon, J., Scott, E.J., Taylor, G. and Askonas, B.A. Clearance of persistent respiratory syncytial virus infection in immunodeficient mice following transfer of primed T cells. *Virology* 1987, **62**, 133–183
- 3 Reddehase, M.J., Mutter, W., Munch, K., Buhning, H.J. and Koszinowski, U.H. CD8-positive T lymphocytes specific for murine cytomegalovirus immediate-early antigens mediate protective immunity. *J. Virol.* 1987, **61**, 3102–3108
- 4 Kast, W.M., Bronkhorst, A.M., De Waal, L.P. and Melief, C.J.M. Cooperation between cytotoxic and helper T lymphocytes in protection against a lethal sendai virus infection. *J. Exp. Med.* 1986, **164**, 723–738
- 5 Klavinskis, L.S., Whitton, J.L., Joly, E. and Oldstone, M.B.A. Vaccination and protection from a lethal virus infection: identification, incorporation and use of a cytotoxic T lymphocyte glycoprotein epitope. *Virology* 1990, **178**, 393–400
- 6 Lehmann-Grube, F.D., Moskopidhis, D. and Lohrer, J. Recovery from acute virus infection. *Ann. NY Acad. Sci.* 1988, **532**, 238–255
- 7 Askonas, B.A., Taylor, P.M. and Esquivel, F. Cytotoxic T cell in influenza infection. *Ann. NY Acad. Sci.* 1988, **532**, 230–237
- 8 Yap, K.L., Ada, G.L. and McKenzie, I.S.C. Transfer of specific cytotoxic T cells protects mice inoculated with influenza viruses. *Nature* 1978, **273**, 238–239
- 9 Van Binnendijk, R.S., Poelen, M.C.M., Kuipers, K.C., Osterhaus, A.D.M.E. and Uytendaele, F.G.C.M. The predominance of CD8<sup>+</sup> cells after infection with measles virus suggests a role for CD8<sup>+</sup> class I MHC-restricted cytotoxic T lymphocytes (CTL) in recovery from measles. Clonal analyses of human CD8<sup>+</sup> class I MHC restricted CTL. *J. Immunol.* 1990, **144**, 2394–2399
- 10 Townsend, A.R.M. and Skehel, J.J. The influenza A virus nucleoprotein gene controls the induction of both subtype specific and cross-reactive cytotoxic T cells. *J. Exp. Med.* 1984, **160**, 552–563
- 11 Yewdell, J.W., Bennink, J.R., Smith, G.L. and Moss, B. Influenza A virus nucleoprotein is a major target antigen for crossreactive antiinfluenza A virus cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA* 1985, **82**, 1785–1789
- 12 Gotch, F., McMichael, Smith, G. and Moss, B. Identification of viral molecules recognized by influenza-specific human cytotoxic T lymphocytes. *J. Exp. Med.* 1987, **165**, 408–416
- 13 Murphy, B.R. and Webster, R.G. Orthomyxoviruses. In: *Virology*, 2nd ed. (Eds Fields, B.N., Knipe, D.M., Chanock, R.M., Hirsch, M.S., Melnick, J.L., Monath, T.P. and Roizman, B.). Raven Press, New York, 1990, p. 1091
- 14 Lawson, C.M., Bennink, J.R., Restifo, N.P., Yewdell, J.W. and Murphy, B.R. Primary pulmonary cytotoxic T lymphocytes induced by immunization with a vaccinia virus recombinant expressing influenza A virus nucleoprotein peptide do not protect mice against challenge. *J. Virol.* 1994, **68**, 3505–3511

- 15 Epstein, S.L., Misplon, J.A., Lawson, C.M., Subbarao, E.K., Connors, M. and Murphy, B.R.  $\beta$ 2-Microglobulin-deficient mice can be protected against influenza A infection by vaccination with vaccinia-influenza recombinants expressing hemagglutinin and neuraminidase. *J. Immunol.* 1993, **150**, 5484–5493
- 16 Andrew, M.E., Coupar, B.E.H., Boyle, D.B. and Ada, G.L. The roles of influenza virus hemagglutinin and nucleoprotein in protection: analysis using vaccinia virus recombinants. *Scand. J. Immunol.* 1987, **25**, 21–28
- 17 Taylor, P.M. and Askonas, B.A. Influenza nucleoprotein-specific cytotoxic T-cell clones are protective *in vivo*. *Immunology* 1986, **58**, 417–420
- 18 Tite, J.P., Hughes-Jenkins, C., O'Callaghan, D., Dougan, G., Russel, S.M., Gao, K.M. and Liew, F.Y. Antiviral immunity induced by recombinant nucleoprotein of influenza virus. II Protection from influenza infection and mechanism of protection. *Immunology* 1990a, **71**, 202
- 19 Tite, J.P., Gao, X.-M., Hughes-Jenkins, C., Lipscombe, M., O'Callaghan, D., Dougan, G. and Liew, F.Y. Antiviral immunity induced by recombinant nucleoprotein of influenza virus. III Delivery of recombinant nucleoprotein to the immune system using attenuated *Salmonella typhimurium* as a live carrier. *Immunology* 1990b, **70**, 540
- 20 Yewdell, J.W. and Bennink, J.R. Cell biology of antigen processing and presentation to major histocompatibility complex class I molecule-restricted T lymphocytes. *Adv. Immunol.* 1992, **62**, 1–123
- 21 Monaco, J.J. Pathways of antigen processing. A molecular model of MHC class I restricted antigen processing. *Immunol. Today* 1992, **13**, 173–178
- 22 Karupiah, G., Ramsay, A.J., Ramshaw, I.A. and Blanden, R.V. Recombinant vaccine vector-induced protection of athymic, nude mice from influenza A virus infection. Analysis of protective mechanisms. *Scand. J. Immunol.* 1992, **36**, 99–105
- 23 Oehen, S., Waldner, H., Kündig, T.M., Hengartner, H. and Zinkernagel, R.M. Antivirally protective cytotoxic T cell memory to lymphocytic choriomeningitis virus is governed by persisting antigen. *J. Exp. Med.* 1992, **176**, 1273–1281
- 24 Ulmer, J.B., Donnelly, J.J., Parker, S.E., Rhodes, G.H., Felgner, P.L., Dworki, V.J. *et al.* Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* 1993, **259**, 1745–1749
- 25 Wraith, D.C., Vessey, A.E. and Askonas, B.A. Purified influenza virus nucleoprotein protects mice from lethal infection. *J. Gen. Virol.* 1987, **68**, 433
- 26 Brett, S.J., Rhodes, J., Liew, F.Y. and Tite, J.P. Comparison of antigen presentation of influenza A nucleoprotein expressed in attenuated AroA-*Salmonella typhimurium* with that of live virus. *J. Immunol.* 1993, **150**, 2869–2884
- 27 Ben-Yehuda, A., Ehleiter, D., Hu, A. and Wexler, M.E. Recombinant vaccinia virus expressing the PR/8 influenza hemagglutinin gene overcomes the impaired immune response and increased susceptibility of old mice to influenza infection. *J. Infect. Dis.* 1993, **168**, 352–357
- 28 Kuwano, K., Scott, M., Young, J.F. and Ennis, F.A. Active immunization against virus infections due to antigenic drift by induction of crossreactive cytotoxic T lymphocytes. *J. Exp. Med.* 1989, **169**, 1361–1371
- 29 Stitz, L., Schmitz, C., Binder, D., Zinkernagel, R., Paoletti, E. and Becht, H. Characterization and immunological properties of influenza A virus nucleoprotein (NP): cell-associated NP isolated from infected cells or viral NP expressed by vaccinia recombinant virus do not confer protection. *J. Gen. Virol.* 1990, **71**, 1169–1179
- 30 Weiss, H.P., Stitz, L. and Becht, H. Immunogenic properties of ISCOM prepared with influenza virus nucleoprotein. *Arch. Virol.* 1990, **114**, 109–120
- 31 Webster, R.G., Kawaoka, Y., Taylor, J., Weinberg, R. and Paoletti, E. Efficacy of nucleoprotein and haemagglutinin antigens expressed in fowlpox virus as vaccine for influenza in chickens. *Vaccine* 1991, **9**, 303–308
- 32 Endo, A., Itamura, S., Iinuma, H., Funahashi, S., Shida, H., Koide, F., Nerome, K. and Oya, A. Homotypic and heterotypic protection against influenza virus infection in mice by recombinant vaccinia virus expressing the haemagglutinin or nucleoprotein gene of influenza virus. *J. Gen. Virol.* 1991, **72**, 699–703.
- 33 Eichelberger, M., Allan, W., Zijlstra, M., Jaenisch, R. and Doherty, P.C. Clearance of influenza virus respiratory infection in mice lacking class I major histocompatibility complex-restricted CD8<sup>+</sup> T cells. *J. Exp. Med.* 1991, **174**, 875–880
- 34 Scherle, P.A., Pailadino, G. and Gerhard, W. Mice can recover from pulmonary influenza virus infection in the absence of class I-restricted cytotoxic T cells. *J. Immunol.* 1992, **148**, 212–217
- 35 Mullbacher, A. The long-term maintenance of cytotoxic T cell memory does not require persistence of antigen. *J. Exp. Med.* 1994, **179**, 317–321
- 36 Hou, S., Hyland, L., Ryan, K.W., Portner, A. and Doherty, P.C. Virus specific CD8<sup>+</sup> T-cell memory determined by clonal burst size. *Nature* 1994, **369**, 652–654