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Anti-viral immunity induced by recombinant nucleoprotein of influenza A virus

II. PROTECTION FROM INFLUENZA INFECTION AND MECHANISM OF PROTECTION

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SUMMARY

Protection against influenza A virus infection in mice immunized with recombinant nucleoprotein (rNP) was studied. Nucleoprotein-immune mice were protected against a lethal challenge with A/Puerto Rico/8/34 (A/PR8) virus but showed considerable morbidity before recovery. Local boosting of the immune system with rNP by intranasal immunization improved the protection in NP-immune mice, and the decrease in morbidity after infection was reflected in accelerated clearance of virus from lungs. However, immune, boosted mice also rapidly cleared an antigenically unrelated influenza B virus from their lungs. Mice immunized with rNP precipitated with alhydrogel, that subsequently developed significant resistance to virus infection, failed to generate detectable levels of class I major histocompatability complex (MHC)-restricted killer cells. Furthermore, B10.A(5R) mice that are non-responders for NP-specific class I killer cells could also be protected by immunization with rNP. In contrast, rNP-immunized mice developed strong proliferative T-cell responses to rNP. These data argue for an important role for helper T cells rather than virus-specific class I cytotoxic T cells in protection against influenza virus infection induced by rNP.

INTRODUCTION

Influenza is an important disease both in terms of mortality in vulnerable individuals and of morbidity in healthy adults. However, the changing antigenicity of the viral glycoproteins limits the effectiveness of current vaccination programmes. These are further confounded by a shortfall in vaccine production compared to the numbers in the vulnerable high-risk groups and a low level of public acceptance of the available vaccine. Thus an ideal influenza vaccine should overcome the antigenic variability of the virus, be amenable to high level production and be acceptable to the general public.

It has recently been demonstrated that much of the cell-mediate response to influenza is directed against internal proteins such as the nucleoprotein and matrix protein (Townsend & Skehel, 1984; Yewdell *et al.*, 1985). These proteins are much less subject to antigenic variation than are the surface glycoproteins and neuraminadase (Sleigh & Both, 1981). Such observations have led to the hypotheseis that immunization with nucleoprotein may generate cross-reactive protective immunity to influenza virus. Wraith, Vessey & Askonas (1986) have reported that immunization with nucleoprotein purified from A/X-31 (H3N2) influenza virus protects mice from a subsequent lethal infection with A/Puerto Rico/8/34 (A/PR8; H1N1).

In a previous report (Tite *et al.*, 1988), we have described the expression and purification of nucleoprotein (NP) from the A/NT/60/68 influenza virus in *Salmonella typhimurium* and demonstrated the ability of this recombinant protein to generate cross-reactive anti-viral immunity. In this study we demonstrate that this immunity endows protection against infection with A/PR8 and hence confirm the earlier finding of Wraith *et al.* (1986) using a recombinant (r) derived NP. However, in contrast to the purified NP, the rNP does not induce class I major histocompatibility complex (MHC)-restricted cytotoxic T cells, and the protection appears to be mediated by class II MHC-restricted helper T cells.

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Abbreviations: A/PR8, A/Puerto Rico/8/34; ASC, antibody-secreting cell; Clicks' EHAA medium, Clicks' extra high amino acid medium; LSM, lymphocyte separation medium; NP, nucleoprotein; OVA, ovalbumin; rNP, recombinant nucleoprotein

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MATERIALS AND METHODS

Animals

BALB/c mice were bred in the animal facility at Wellcome Biotech. B10.A(5R) and C57BL/10 mice were purchased from Olac Ltd (Bicester, Oxon, U.K.).

Immunization

Mice were immunized with alhydrogel-precipitated antigen intraperitoneally or subcutaneously in the base of the tail, as indicated in the text. Alhydrogel was obtained from Wellcome Biotech.

Antigens and viruses

Recombinant-derived nucleoprotein (rNP) from *S. typhimurium* expressing the cloned NP gene from A/NT/60/68 (Jones & Brownlee, 1985) was purified as described previously (Tite *et al.*, 1988). Viruses were grown in embryonated chicken eggs and either used as allantoic fluid, for target cell sensitization and preparation of infected stimulator cells, or purified by ultracentrifugation (Tite *et al.*, 1988), for use as antigen in lymph node cell proliferation assays. The following viruses were used: A/Puerto Rico/8/34 (A/PR8), A/Okuda/57, B/Lee/40.

Virus challenge and assay of lung virus titres

Mice were challenged with A/R8 virus of B/Lee/40 either by aerosol inhalation in a chamber or by intranasal application of solution applied to the nose-pad of mice anaesthetized with ether. In the case of lethal challenge, deaths were recorded over a 21-day period. In some experiments a sublethal dose of virus was given and immunity assessed by weight loss measurements or by measurement of virus titres in the lungs. Lung virus titres were measured using a plaque assay on Madin–Darby canine kidney (MDCK) monolayers. 9×10^6 MDCK cells in Medium 199 (Flow, Herts, U.K.) plus 10% foetal calf serum (FCS) were plated into 6-well plates (Nunc, Roskilde, Denmark) and left overnight at 37° and 5% CO_2. After thorough washing, dilutions of virus in phosphate-buffered saline (PBS) were added in 0·5 ml and adsorbed on to the monolayers for 1 hr, after which time the virus was removed by aspiration and an overlay containing 0·5% agarose, 100 μg/ml DEAE-dextran and 1 μg/ml trypsin in serum-free Medium 199 was added. The plates were incubated for 48 hr at 37° before formalin fixation and staining with crystal violet.

^{51}Cr-release assays

Target cells were P815(H-2^d) and EL4(H-2^b) tumour cell lines. Virus-infected targets were prepared by incubating 2×10^6 tumour cells with allantoic fluid containing 400 haemagglutinating units (HAU) of virus and 100 μl of $Na^{51}CrO_4$ in a total volume of 1 ml serum-free Clicks' EHAA medium for 2 hr at 37°. Peptide-pulsed targets were prepared similarly except that peptide at a final concentration of 100–200 μg/ml was included instead of virus. Effector cells were titrated in V-bottomed microtitre plates before addition of 10^4 ^{51}Cr-labelled target cells per well. The plates were then spun for 5 min at 1500 r.p.m. and incubated for 6 hr at 37° before supernatants were removed for the calculation of specific ^{51}Cr release (Tite & Janeway, 1984).

In vitro *generation of secondary cytotoxic T lymphocytes*

Spleen cells (2×10^7) from immunized mice were cultured with 5×10^6 virus-infected (400 HAU/2×10^7 spleen cells) syngeneic spleen cells in 10 ml of Clicks' EHAA medium containing 10% FCS for 5 days at 37°, 5% CO^2. At the end of this period the cells were harvested and titrated in ^{51}Cr-release assays as described in the previous section. Target cells used were P815 and EL4.

Table 1. Protection from lethal infection of A/PR8 virus

Exp.	Primary immunization	Secondary immunization	Route	$Al(OH)_3$	Survivor/ total
1	250 μg	250 μg	i.p.	+	5/5
	50 μg	50 μg	i.p.	+	6/6
	10 μg	10 μg	i.p.	+	5/5
	—	—	i.p.	+	0/5
2	250 μg	10 μg	i.p.	+	4/5
	50 μg	10 μg	i.p.	+	4/4
	10 μg	10 μg	i.p.	+	4/5
	—	—	i.p.	+	0/5
3	10 μg	10 μg	i.p.	+	4/6
	—	—	i.p.	+	0/6
4	10 μg	10 μg	i.p.	+	3/6
	10 μg	10 μg	i.m.	+	3/6
	10 μg	10 μg	s.c.	+	4/6
	—	—	—	+	0/6
5	10 μg	10 μg	i.p.	+	3/6
	—	—	i.p.	+	0/6

Mice were immunized with various doses of rNP and alhydrogel as indicated and challenged with approximately $5 \times LD_{50}$ of A/PR8 virus as an aerosol in an enclosed chamber. Deaths were recorded over a 21-day period.

Lymph node proliferation assay

The inguinal and periaortic lymph nodes were removed from mice immunized subcutaneously at the base of the tail and single cell suspensions prepared by gently grinding between two frosted glass slides. The resulting single cell suspension was washed twice in PBS and the cells resuspended at a concentration of 4×10^6 cells/ml in Clicks' EHAA medium containing 0·5% normal mouse serum. This suspension was added to microtitre wells (96-well; Costar, Cambridge, MA) and antigen added at the indicated concentration. The cultures were then incubated for 3 days before pulsing for 18 hr with tritiated thymidine. Cultures were harvested on an automatic cell harvester (Skatron, Sterling, VA) and counted in a β-counter (β-plate; LKB, Uppsala Sweden).

RESULTS

Protection from infection with A/PR8 influenza virus by immunization with rNP

BALB/c mice were immunized and boosted intraperitoneally with 10 μg of rNP precipitated with alhydrogel and subsequently challenged with 5 LD_{50} of mouse-adapted A/PR8 virus. The results of several experiments are presented in Table 1, which shows that such an immunization schedule resulted in approximately 75% survival compared to control mice in which mortality was 100%. The protected mice did, however, show considerable morbidity, which could be monitored by measure-

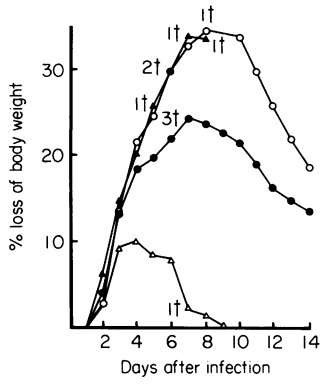

Figure 1. Groups of six BALB/c mice were immunized and boosted intraperitoneally with 10 μg rNP plus 100 μg alhydrogel. Four weeks after secondary immunization, some groups were immunized intranasally with 5 μg rNP in PBS. All groups were challenged intranasally with 2 HAU of A/PR8 virus 7 days later. Mice were weighed daily and percentage loss of body weight calculated. (▲) Alhydrogel alone (0/6 survivors); (○) alhydrogel plus intranasal rNP (2/6 survivors); (●) rNP plus alhydrogel (6/6 survivors); (△) rNP plus alhydrogel plus intranasal rNP (5/6 survivors).

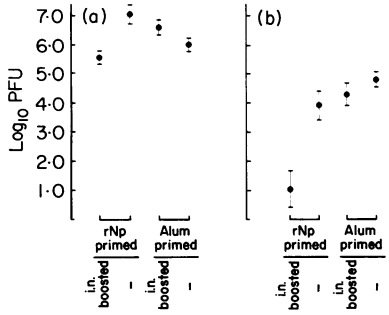

Figure 2. BALB/c mice were immunized as is detailed in the legend to Fig. 1. Mice were challenged with 0·02 HAU of A/PR8 virus and killed either 2 (a) or 6 days (b) post-challenge to assay levels of virus in the lungs. Alumalhydrogel.

ment of loss in body weight. The data in Fig. 1 illustrate this point; immunization with rNP protected mice from a lethal infection with A/PR8 virus, but only after undergoing severe weight loss. However, rNP immune mice boosted intranasally with rNP 7 days before lethal challenge performed much better than mice receiving no intranasal boost, undergoing only marginal weight loss. Control mice receiving just the intranasal immunization showed exactly the same morbidity as the unimmunized controls (Fig. 1). These data suggest that the recruitment of immune cells to the lung by intranasal immunization with rNP is beneficial to anti-viral immunity.

Viral replication in the lungs of rNP immunized mice

The high level of morbidity seen in rNP immune mice that subsequently resolved their infection suggests that the virus underwent replication in the lungs of these mice, with the observed pathological consequences. Figure 2 indicates that this was indeed the case; 48 hr after viral challenge, all groups had

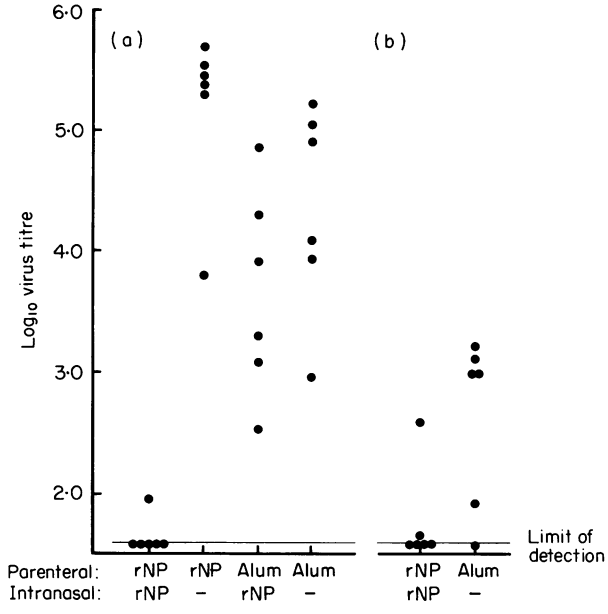

Figure 3. BALB/c mice were immunized as in Fig. 1. Groups of six mice were challenged with either A/PR8 virus (0·02 HAU) (a) or B/Lee/40 virus (0·2 HAU) (b) as indicated. Lungs were removed 6 days after infection and lung virus titres measured.

similar levels of virus in the lungs. However, by Day 6 after infection the group that had been primed parenterally and boosted intranasally with rNP had essentially cleared virus from the lungs, whereas mice in the other groups each had 10^4–10^5 virions in their lungs.

Specificity of protection after rNP immunization

In order to test the specificity of protection endowed by rNP priming and subsequent intranasal boost with rNP, mice were immunized and subsequently challenged with either A/PR8 virus or B/Lee/40 virus. Figure 3 shows that not only was A/PR8 virus cleared from lungs of immunized mice by Day 6, but immunized mice receiving B/Lee/40 also had dramatically reduced titres compared to the control groups. Since the nucleoproteins of A and B virus do not cross-react at the T-cell level (data not shown; Tite *et al.*, 1988), this strongly suggests that the effector mechanism operating in protected mice has a non-specific element.

Role of cytotoxic T cells in protection

The non-specificity of the protection seen in Fig. 3 suggests that it is not attributable to a highly specific effector mechanism such as virus-specific cytotoxic T lymphocytes (CTL). However, Wraith & Askonas (1985) have demonstrated that immunization with nucleoprotein purified from X-31 virus primes virus-specific CTL, which could be detected after *in vitro* secondary stimulation. Figure 4a demonstrates that immunization of BALB/c mice with rNP precipitated with alhydrogel, which led to protection from viral challenge, induced T cells capable of proliferating specifically to virus in lymph nodes draining the site of subcutaneous immunization. However, these protected mice did not develop detectable levels of virus-specific CTL (Fig. 4b). Further evidence suggesting that NP-specific cytotoxic T

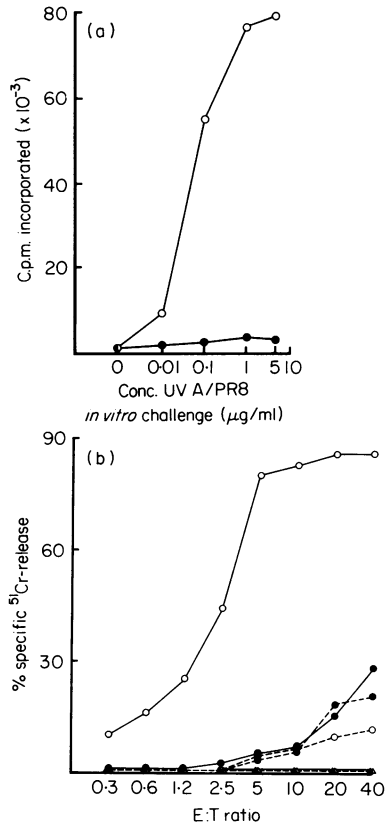

Figure 4. BALB/c mice were immunized and boosted subcutaneously with 10 μg rNP plus alhydrogel. Draining lymph nodes were taken 10 days after the second immunization. In (a), lymph node cells (LNC) were restimulated *in vitro* with purified UV inactivated A/PR8 and DNA synthesis measured by the incorporation of [^{3}H]thymidine for 18 hr at the end of a 4-day culture. (○) rNP plus alhydrogel; (●) alhydrogel alone. In (b) LNC from rNP immune mice, control mice or spleen cells from mice infected several months previously with infectious A/PR8 virus were restimulated *in vitro* with A/PR8-infected and irradiated BALB/c spleen cells. After 5 days, the cultures were assayed for cytotoxic T cells by ^{51}Cr-release assay against A/PR8-infected P815 cells or uninfected P815 cells. (○——○) A/PR8-immune spleen cells, A/PR8-infected target cells; (○----○) A/PR8-immune spleen cells, uninfected target cells; (●——●) rNP-immune LNC, A/PR8-infected target cells; (●----●) rNP-immune LNC, uninfected target cells; (▲----▲) alhydrogel-immune LNC, A/PR8-infected target cells; (△----△) alhydrogel-immune LNC, uninfected target cells.

Table 2. Protection of B10.A(5R) mice from lethal infection with A/PR8 by pre-immunization with rNP

Immunization schedule*	Survivors/total	%	HAI titre†
2 × rNP + alhydrogel i.p.	8/8	100	5·96 ± 0·74
2 × alhydrogel i.p.	0/8	0	NT

* B10.A5R mice were immunized as indicated above with 10 μg rNP and 100 μg aldhydrogel or alhydrogel alone before challenge with A/PR8. Survival was monitored over a 21-day period, at which time survivors were bled and the sera tested for HAI titre against A/PR8 virus.

† Log_2 titre; geometric mean ± SD.

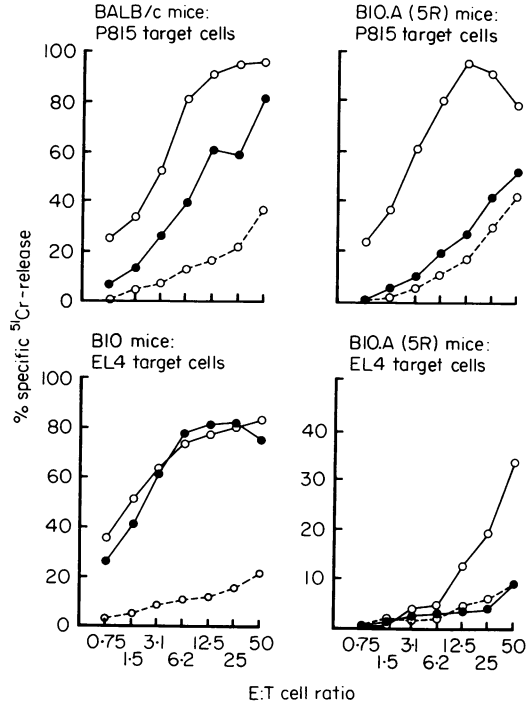

Figure 5. Spleen cells from rNP-immune B10A(5R) mice that had survived a lethal challenge with A/PR8 virus were stimulated *in vitro* with A/PR8-infected syngeneic spleen cells and assayed for CTL function 5 days later. As positive controls, spleen cells from sublethally infected BALB/c and C57BL/10 mice were also stimulated in a similar manner. Target cells were either A/PR8 virus infected (○——○), CTL peptide pulsed (●——●) or untreated (○----○). For P815 target cells, a synthetic peptide corresponding to residues 147–161 of NP was used and for EL4 targets, a peptide corresponding amino acids to 365–380 of NP, was used. Supernatants were removed after 6 hr.

cells may not play an important role in the protection from infection in the present system was provided by experiments using B10.A (5R) mice. B10.A(5R) mice are low or non-responders for the generation of NP-specific, class I MHC gene product-restricted CTL. This is due to the fact that both K^b and D^d are low responder alleles for NP-specific class I-restricted CTL. Immunization of B10.A(5R) mice with alhydrogel-precipitated rNP generated cells which proliferated *in vitro* in response to purified virus (data not shown), indicating that helper cell responsiveness was not compromised in these mice. B10.A(5R) mice immunized with rNP were highly resistant to a lethal challenge of A/PR8 virus (Table 2). The mortality in the control group was 100%, whereas all of the rNP immune mice survived. Sera from the survivors were tested for haemagglutination inhibition (HAI) titres and all were found to possess high levels of HAI antibody. Spleen cells from the surviving group were also tested for the ability to generate NP-specific CTL (Figure 5). As positive controls, spleen cells from immune BALB/c (K^d D^d) and B6(K^b D^b) mice were also stimulated *in vitro* with A/PR8 virus-infected syngeneic spleen cells. Target cells were either P815 (K^d D^d) or EL4 (K^b D^b) cells that had been infected with A/PR8 virus or had been pulsed with synthetic

peptides corresponding to the dominant nucleoprotein CTL epitope in the respective strain. Figure 5 shows that whereas the BALB/c and B6 mice generated strong anti-viral and anti-peptide CTL responses, the 5R mice developed essentially no NP peptide-specific CTL activity. These mice did, however, produce a strong CTL response against virus-infected P815 cells, indicating that virus infection had occurred, and also a weak but significant anti-viral CTL response against A/PR8-infected EL4. The latter result was expected as it has been demonstrated (Pala & Askonas, 1986) that K^b is a low responder allele for CTL responses to whole influenza virus. Therefore, mice immunized with rNP and resistant to lethal challenge did not generate NP-specific CTL, arguing for an alternative mechanism other than killer cells in protection.

DISCUSSION

Immunization of mice with recombinant DNA-derived influenza NP was found to protect mice from a subsequent lethal infection of the mouse-adapted influenza virus A/PR8. These data are consistent with those of Wraith *et al.* (1986), who reported a similar efficacy with NP prepared from A/X-31 virus. Our data, in confirming these observations, also allay any concerns about other viral proteins contributing to the protection seen with biochemically purified virus-derived NP. As with the protection observed with purified viral NP and vaccinia virus constructs expressing the NP gene (Andrews *et al.*, 1986), the protection observed after immunization with recombinant NP was not complete and was associated with a considerable degree of morbidity. Analysis of virus replication in the lungs of mice protected from lethal infection in this manner indicated that virus replication occurred at a level indistinguishable from control mice up to 6 days after infection. Protection in these mice presumably reflects a very late event which eventually leads to clearance of virus from the lung before irreversible damage occurs. We therefore attempted to improve the quality of protection by deliberately boosting the respiratory system with NP before viral challenge. In mice immunized systemically and boosted intranasally with rNP, the morbidity observed after infection was minimal and virus was cleared from the lungs by Day 6. However, in these optimally protected mice, there was still clear evidence of early viral replication indeed, 48 hr after infection there was no significant difference in lung virus titres between the protected mice and the control mice. This observation argues strongly against the possibility that inflammation of lung epithelial cells prevents initial viral infection and subsequent replication. These data suggest that recruitment of immune lymphocytes to the site of infection dramatically improves the protection endowed by rNP immunization. The analysis of virus-specific antibody-forming cells in the lung confirms that this does indeed occur (J. P. Tite, unpublished observations).

The increased efficiency of protection when antigen is used to boost the local immune response could conceivably be achieved in two ways. One possibility is that virus-specific T cells are recruited to the lung and these T cells are specifically restimulated by viral NP produced during the early stages of the infection, and this leads to the curtailment of virus replication. Alternatively, boosting intranasally with rNP specifically recruits T cells to the lung, which remain non-specifically activated to mediate viral clearance. In an attempt to decide between these two alternatives, mice which had been parenterally immunized and boosted intranasally with rNP were challenged with B/Lee/40, a B-type virus with an antigenically unrelated NP molecule. The immune mice had markedly reduced levels of virus in the lungs 6 days after challenge compared to control mice. This suggests that there is at least an element of non-specificity in the observed protection. However, since B/Lee does not grow as well in lungs as A/PR8 virus, it is difficult to discount the possibility that there may also be a specific component to the protection against A/PR8, but the non-specific element is sufficient to reduce the titre of the type B virus. Indeed, the fact that clearance of virus is only observed at late stages after the initial infection is puzzling if the protection is completely non-specific, as one would expect such a mechanism to act at the early stages of infection. In rNP immune mice that have not received intranasal immunization it is likely that a similar protective mechanism prevails, but in this case the production of NP during the early phases of viral infection is required before the recruitment and activation of rNP-primed lymphocytes can occur. This explains the higher level of morbidity in these mice and the delay in the onset of recovery compared to rNP-immune and intranasally boosted mice.

In our attempts to analyse the mechanism of NP-induced protection, several other lines of evidence argue against class I MHC-restricted CTL playing a major role in protection in this experimental system. We failed to induce class I MHC-restricted CTL but could readily activate helper T cells with rNP. Wraith & Askonas (1985) have reported that immunization with viral NP generated cross-reactive class I MHC-restricted CTL. There are possible differences in experimental procedures which may explain this discrepancy. Firstly, our preparation was adjuvanted with alhydrogel (we found this to be necessary in order to obtain protection) whereas the viral NP was used in soluble form. Secondly, the viral NP was contaminated with haemagglutinin (HA) which may have had a co-operative effect for NP-specific CTL generation (e.g. by forming micelles), which could not happen with the recombinant preparation. There is no doubt that $CD8^+$ CTL are important in anti-viral immunity, but using this immunization schedule we could find no evidence for priming of such cells.

Further evidence supporting mechanisms other than NP-specific CTL playing a major role in protection is provided by the ability to protect B10.A(5R) mice from lethal infection with rNP. B10.A(5R) mice are low or non-responders for CTL responses to NP. This finding would not preclude the possibility that CTL to other antigens are important in protection, the generation of these CTL being enhanced by immune NP-specific helper T cells. Our unpublished data measuring CTL responses in the lungs of protected mice undergoing infection did not, however, provide support for this hypothesis. The non-specificity of protection after intranasal boosting with rNP also argues against the accelerated production of neutralizing antibodies as the protective mechanism and would tend to favour a non-specific effector mechanism such as the production of interferon-gamma. We are currently attempting to analyse the protective mechanism with neutralizing antibodies to cytokines such as interferon-gamma and tumour necrosis factor. Furthermore, we are investigating the duration of the protective effect; it is conceivable that the short-term non-specific protection observed may be superceded by a long-term specific protection

as the recruited immune lymphocytes become quiescent and resident in the lung.

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