Purified Influenza Virus Nucleoprotein Protects Mice from Lethal Infection

By DAVID C. WRAITH,* ANGELA E. VESSEY AND BRIGITTE A. ASKONAS

Division of Immunology, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K.

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SUMMARY

Local administration of nucleoprotein purified from X31 (H3N2) influenza A virus primed for A virus cross-reactive cytotoxic T cells and resulted in substantial protection (75%) of mice from a lethal challenge with the heterologous mouse-adapted A/PR/8/34 (H1N1) virus. By following the course of a lethal virus challenge we found that nucleoprotein priming did not prevent virus infection but rather aided recovery. Nucleoprotein-primed mice suffered initial symptoms of infection, i.e. weight loss and surface temperature changes, but started to recover after approximately 7 days. We suggest that such heterotypic protection can be attributed to priming of A virus cross-reactive cytotoxic T cells.

INTRODUCTION

Influenza A virus infection of mouse and man induces antibodies to variable regions of the haemagglutinin (HA) and neuraminidase (NA) surface glycoproteins. Virus-neutralizing antibodies to HA constitute the primary defence against homologous virus infection and provide immunological pressure leading to antigenic drift within and shift between subtypes (Potter & Oxford, 1979; Virelizier et al., 1979). By contrast, a major proportion of anti-influenza cytotoxic T (T\textsubscript{C}) cells in mouse and man are A virus cross-reactive in that they do not discriminate between serologically distinct type A influenza viruses (Effros et al., 1977; McMichael & Askonas, 1978; Zweerink et al., 1977a). Such influenza virus-specific T\textsubscript{C} cells reduce virus replication in the lungs of mice infected with a heterologous virus (Lin & Askonas, 1981; Yap & Ada, 1978) and furthermore can protect against lethal infection (Lukacher et al., 1984; Taylor & Askonas, 1986).

Current influenza vaccines, whether purified HA/NA or inactivated virus, effectively stimulate subtype- or variant-specific antibody and helper T (T\textsubscript{H}) cells (Ada et al., 1981) but induce insignificant A virus cross-reactive T\textsubscript{C} cell responses in mice (Reiss & Schulman, 1980; Webster & Askonas, 1980). An improved vaccination strategy would clearly aim to induce both neutralizing antibodies and A virus cross-reactive T cells.

Recent reports have defined the internal viral nucleoprotein (NP) as an important target antigen for A virus cross-reactive T\textsubscript{C} cells (Townsend et al., 1984; Yewdell et al., 1985) and this led us to test the immunogenecity of purified NP for these cells in mice. We have previously shown that NP administered intraperitoneally induces high levels of such cross-reactive T\textsubscript{C} memory cells and may therefore represent a good vaccine candidate (Wriath & Askonas, 1985). We have extended this work and here show that mice primed subcutaneously with NP are substantially protected from a lethal dose of mouse-adapted A/PR/8/34 virus. By following weight loss resulting from viral anorexia, it is clear that NP vaccination enhances recovery from disease as opposed to preventing infection per se.
METHODS

Chemicals. Sodium deoxycholate (product no. D 6750), bromelain (product no. B 2252 and batch no. 113F 0585) and lubrol (product no. L 3753) were purchased from Sigma. Ammonium deoxycholate (Am.DOC) was prepared from deoxycholic acid as previously described (Laver & Webster, 1976).

Animals. BALB/c (H-2b) mice were bred and maintained under specific pathogen-free conditions at this Institute.

Viruses. Influenza virus strains A/PR/8/34 (H1N1), A/USSR/90/77 (H1N1), A/X31 (H3N2) recombinant expressing genes for the internal proteins from A/PR/8/34 and glycoproteins from A/Hong Kong/8/73 were grown in the allantoic cavity of 10-day embryonated eggs and stored frozen at -70 °C.

Purification of influenza virus HA. A/X31 virus was purified (Brand & Skehel, 1972) and resuspended at 2 mg/ml in 15 mM-Tris–HCl pH 8.1 containing 0.5% lubrol. After 30 min on ice, virus cores were pelleted at 100000 g for 1 h. The supernatant was layered over a gradient of 5 to 40% sucrose in 15 mM-Tris–HCl pH 8.1 containing 0.5% sodium deoxycholate, and centrifuged for 16 h at 150000 g. The protein peak, which comigrated with haemagglutinating activity at 15% sucrose, was dialysed to remove detergent and analysed by SDS–PAGE (Laemmli, 1970) under non-reducing conditions. Silver staining detected only a band comigrating with HA (data not shown).

NP preparation. A/X31 (H3N2) virus was purified and treated with bromelain as previously described (Brand & Skehel, 1972), in order to remove the majority of surface glycoproteins (>95% of HA activity). Virus cores were resuspended to 4 mg/ml and treated with 0.2% Am.DOC (Wraith & Askonas, 1985). After 5 h the insoluble matrix (M) protein was removed by centrifugation, leaving purified NP in the supernatant (Laver & Webster, 1976). This preparation was collected, extensively dialysed and stored in aliquots at -70 °C.

SDS–PAGE. Protein fractions were analysed by SDS–PAGE on 5 to 20% gel under non-reducing conditions (Laemmli, 1970) and stained with Coomassie Brilliant Blue.

Radioimmunoassay for influenza virus proteins. To estimate possible HA contamination of the NP preparation a direct antibody binding immunoassay was set up using lubrol-purified HA as a standard and Am.DOC-purified NP-coated plastic microtitre wells as previously described (Hackett et al., 1980). The monoclonal antibody HC-19 (IgG2a), specific for the HA (H3 subtype) of influenza A virus, was kindly provided by A. Douglas of this Institute. Protein A affinity-purified antibody was radioiodinated to a specific activity of 5 gCi/gg using the chloramine-T method 2 described by Mason & Williams (1980) and used at 30000 c.p.m./well. After 3 h, plates were washed eight times and the amount of antibody bound to each well was measured in a gamma counter.

Immunization protocols. Three-month-old BALB/c mice were infected intranasally (i.n.) with influenza A/X31 (H3N2) virus as described previously (Zweerink et al., 1977b). Alternatively, purified NP was diluted to 0.1 mg/ml in phosphate-buffered saline pH 7.2 (PBS) and injected either intraperitoneally (i.p.) (0.5 ml), intramuscularly (i.m.) (0.1 ml) or subcutaneously (s.c.) (0.1 ml). Some mice received an identical boost dose after 3 to 4 weeks. Control mice received the same volumes of PBS alone.

In vivo secondary stimulation of Tc cells. At least 1 month after immunization of BALB/c mice, either i.n. with infectious virus or by injection of purified NP, spleen cells were restimulated in vitro at 1 x 10⁶ cells/ml in RPMI 1640 medium containing 10% (v/v) foetal calf serum and supplemented with penicillin, streptomycin and L-glutamine (RPMI/10) by the addition of 2 x 10⁵ syngeneic spleen cells per ml infected with A/X31 virus (Zweerink et al., 1977b). After 5 days, viable cells were collected and tested for cytotoxicity.

Cytotoxicity assay. P815 (DBA/2, H-2b, mastocytoma) cells were labelled with ⁵¹Cr, infected with influenza virus for 1.5 h and cytotoxicity assays were performed for 6 h as previously described (Zweerink et al., 1977b).

A/PR/8/34 lethal challenge. At least 1 month after the last injection of NP, immunized and control mice were challenged with a lethal dose of the mouse-adapted A/PR/8/34 (H1N1) virus. Each mouse was anaesthetized and received 50 μl of a 5 x 10⁶ dilution in PBS of freshly thawed allantoic fluid i.n. (0.1 HAU per mouse). This dose corresponds to 8 to 16 LD₅₀ for BALB/c mice.

Body temperature and weight. Body temperature was measured using a Heimann KT 15 infrared thermometer (lens aperture 2.5 mm) held approx. 0.5 cm from the left eye. In trial experiments this technique was repeatable to within 0.5 °C. Mice were weighed on a Torbal balance.

RESULTS

NP preparation

To avoid variability in effectiveness of the NP material, we ensured that more than 95% of HA activity was removed from X31 virus by bromelain treatment and that core concentration for Am.DOC NP preparation was optimized at 4 mg/ml. Under these conditions 20 to 25% of total protein was recovered in the supernatant and on SDS–PAGE this fraction proved to be highly enriched for NP. Fig. 1 shows an SDS–polyacrylamide gel of purified X31 virus before
Influenza NP induces heterotypic immunity

Fig. 1. SDS–PAGE of Am.DOC NP preparation. The 5 to 20% acrylamide gradient gel was run under non-reducing conditions and influenza virus proteins were identified by mobility relative to standards as previously described (Wraith & Askonas, 1985). (a) Mol. wt. standards with relative mol. wt. (× 10^-3) indicated, (b) A/X31 virus prior to bromelain treatment (1.5 mg/ml), (c) A/X31 virus after bromelain treatment (1 mg/ml) and (d) NP supernatant from Am.DOC-extracted virus.

Fig. 2. Radioimmunoassay for influenza virus proteins. Wells were coated with a serial dilution of lubrol-purified HA (Δ), Am.DOC-purified NP (●) or bovine serum albumin (○), incubated with 125I-labelled HC-19 antibody and the amount of radioactivity bound was measured at each dilution. The particular Am.DOC preparation tested in this experiment contained approximately 20 ng HA/μg NP.

We wished to examine whether A virus cross-reactive Tc cells could be induced by local injection of NP (s.c. or i.m.) in addition to the i.p. immunization previously described (Wraith & Askonas, 1985). A single dose of NP (10 μg), administered by either route, effectively primed for

NP priming of anti-influenza A virus Tc cells

A direct monoclonal antibody binding assay has allowed us to show that the Am.DOC NP preparations had only low levels of contaminating HA (1 to 3% w/w) (Fig. 2). HA contamination was reduced in comparison to previously reported preparations (Wraith & Askonas, 1985) by obtaining a more effective batch of bromelain.
Fig. 3. Cross-reactive Tc response *in vitro* after vaccination with Am.DOC NP. Tc cells were generated from BALB/c mice either injected with NP (a) 10 μg i.m., (b) two i.m. doses of 10 μg at 1 month interval, (c) 10 μg s.c. or (d) primed i.n. with A/X31 virus (Zweerink *et al.*, 1977b). After at least 1 month spleen cells were co-cultured with A/X31-infected syngeneic spleen cells for 5 days prior to testing for cytotoxicity. Targets were P815 (H-2k) cells infected with A/X31 (○), P815 infected with A/USSR (△) and P815 infected with B/Hong Kong (●). K/T, killer to target cell ratio at 2 × 104 targets per well. Assays were set up and percentage lysis was calculated as previously described (Zweerink *et al.*, 1977b). Background release of 51Cr was less than 10%.

Table 1. *Recovery from a lethal dose of influenza A virus after Am.DOC NP vaccination*  

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* BALB/c mice were injected with two 10 μg doses of NP s.c. at 4 week intervals or with one 50 μg dose i.p. After 1 month mice were challenged with 0.1 HAU of A/PR/8/34 virus i.n. In each experiment this dose was lethal for an identical group of age- and batch-matched mice previously injected with isotonic PBS alone (control primed). Mice primed 1 month previously by intranasal infection with X31 virus (4.3 HAU) were protected from this dose of virus.

influenza A virus cross-reactive Tc memory cells which could be restimulated *in vitro* to give high levels of cytotoxicity directed to targets infected with either X31 (H3N2) or USSR (H1N1) viruses (Fig. 3a and c). The level of Tc priming was considerably enhanced with a similar NP dose after 1 month (Fig. 3b) but, even so, was not as high as that achieved by i.n. infection (Fig. 3d). No activity was detected in spleen cells from control mice injected with PBS alone (Wraith & Askonas, 1985).

**NP-primed mice are protected from lethal influenza virus infection**

Since A virus cross-reactive Tc have been shown to be protective, it was important to see whether NP priming could protect hosts against lethal influenza infection. In Table 1 we list a series of experiments in which BALB/c mice [control or primed with NP prepared from X31 (H3N2) virus] were infected i.n. with a lethal dose of A/PR/8/34 (H1N1) virus. All control infected mice died (43/45 by day 14, 45/45 by day 20), while of 45 NP-primed mice 34 had survived by day 60. Priming with NP prepared from X31 virus thus gave an overall protection of...
Influenza NP induces heterotypic immunity

75% against a lethal infection with the heterologous influenza A/PR/8/34 virus. In parallel experiments, all mice primed by i.n. infection with A/X31 virus survived a similar challenge with A/PR/8/34 (Table 1).

**NP vaccine aids recovery from but does not inhibit infection with A/PR/8/34 virus**

In initial experiments mice primed with NP were visibly affected by virus challenge. We therefore chose to follow the course of infection by monitoring body temperature and weight. On challenge with A/PR/8/34 virus normal mice rapidly lost weight, presumably as a result of viral anorexia. In the experiment illustrated in Fig. 4, NP-primed mice initially lost weight in parallel with controls, but by day 8 to 10 primed mice were recovering while control mice became progressively more sick. Body temperature was also measured in order to observe fever resulting from infection. Surprisingly, challenged mice showed a gradual decrease in temperature, as measured at the eye surface, in parallel with body weight (Fig. 4). Again, in the group of mice primed with NP, this decline was reversed by day 8 to 10 and temperatures returned to normal by day 20. Mice primed by infection with A/X31 showed neither weight nor temperature loss on challenge with the heterologous A/PR/8/34 virus (data not shown).

**DISCUSSION**

The present study shows that local injection of mice with purified NP, prepared from X31 (H3N2) virus, primes for A virus cross-reactive Tc memory cells and leads to protection of hosts against i.n. infection with a lethal dose of A/PR/8/34 (H1N1) influenza virus. Temperature and weight measurements during the course of infection indicated that infection was not prevented but that recovery started after a week of infection and was complete by day 20. Over a series of experiments 75% of NP-primed mice were protected for at least 60 days. Complete protection
against the heterologous challenge was only observed in mice primed by i.n. infection with X31 virus. Such increased protection correlated well with the level of Tc memory cells which was higher in i.n. infected than in NP-primed mice.

We had previously shown that an NP/HA fraction, from X31 virus, induced A virus cross-reactive Tc memory cells while a matrix (M) protein fraction was ineffective (Wraith & Askonas, 1985). The NP preparation described in this paper has been modified from our previous report to contain less than 3% (w/w) of HA. This low level of HA (H3) contamination cannot account for the protection observed since neither neutralizing antibodies to H3 nor H3-specific murine Tn or Tc cells recognize H1 of A/PR8/34, the challenge virus. Although one human HA subtype cross-reactive Th clone has been described (Lamb & Green, 1983) evidence against cross-reactivity of murine HA-specific Th cells has come from an extensive analysis of H3-specific Th clones none of which reacted with H2 or H1 (Mills et al., 1986). Through the use of cloned HA genes it has been established that there is no recognition of H1 by H3-specific murine Tc cells (Bennink et al., 1986; Townsend et al., 1984).

The mechanism of protection in our NP-primed mice is unlikely to be due to NP-specific antibodies since these are not neutralizing and no antiviral function has been assigned to them (Virelizier et al., 1979). The most likely basis for the protective effect of NP priming is through induction of NP-specific A virus cross-reactive Tc and Th cells.

We favour an important role of Tc cells for the following reasons. Influenza A virus cross-reactive Tc cell clones can confer protection against infection by any type A virus (Lin & Askonas, 1981; Lukacher et al., 1984). In earlier experiments the clonal specificity for individual influenza proteins was not known. Recently, however, Taylor & Askonas (1986) have found that NP-specific and A virus cross-reactive Tc cell clones can protect mice from lethal infection with A/PR/8/34 or reduce lung virus titres in mice challenged with a sublethal dose of A/JAP/305/57 virus. These latter findings are particularly encouraging because although NP-specific A virus cross-reactive Tc cells are detected at relatively high levels in vitro (Townsend et al., 1984; Yewdell et al., 1985), an in vivo effector function had not been described. Also, in the present study, there is a correlation between levels of cross-reactive Tc priming and the ability of mice to recover from lethal challenge. Mice primed by i.n. infection with X31 showed high levels of memory Tc cell priming on secondary challenge in vitro and were totally protected from a lethal infection displaying none of the symptoms of virus infection on challenge with the heterologous A/PR/8/34 virus. NP priming of mice induced significant but lower levels of memory Tc and 75% of such mice survived but showed transient symptoms of viral infection.

The arguments in favour of Tc cell induction as a means of producing heterotypic immunity to influenza A virus does not and should not exclude Th cells. NP-specific Th cell clones have been described in mouse and man (Hurwitz et al., 1985; Lamb et al., 1982a) and we find anti-NP Th cells in NP-primed mice (F. R. Esquivel, D. C. Wraith & B. A. Askonas, unpublished results). At present there is no evidence for direct antiviral effects of NP-specific Th cells, but clearly such cells may contribute to protection by amplifying antibody and/or Tc responses. Certainly M protein-specific Th cells can collaborate with B cells in the production of anti-HA antibodies (Lamb et al., 1982b) and mice primed with purified M protein mount an enhanced primary antibody response to HA on subsequent challenge with infectious virus (Russell & Liew, 1979).

The question arises whether NP might be usefully applied as a vaccine in humans. Studies in mice have shown that there are low responder major histocompatibility complex alleles for Tc recognition of NP which could exclude some individuals, but that on a population basis NP remains an important target antigen (Pala & Askonas, 1986). Although it is premature to extrapolate from mouse to man, our studies indicate that, following immunization with NP, symptoms of disease could be less severe and recovery more rapid even though influenza infection would not be prevented. As NP-reactive Tc cells have been described in humans (Fleischer et al., 1985; McMichael et al., 1986) this proposal warrants further investigation.

REFERENCES


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