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Influenza nucleoprotein-specific cytotoxic T-cell clones are protective *in vivo*

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SUMMARY

Influenza nucleoprotein (NP) serves as a target antigen on abortively infected cells for cytotoxic T cells (T_c) cross-reactive for all type A influenza viruses, and it can also prime mice for such T_c. It is important to test the protective ability of NP-specific T_c clones *in vivo* in a productive influenza infection. In this report, we show that T_c clones of this antigenic specificity protect mice against a lethal influenza infection on transfer to syngeneic recipients, and also that they reduce virus titres in the lungs and trachea of mice challenged with homologous or heterologous type A influenza viruses. Simultaneous injection of IL-2 to maintain the viability of the T_c clones is not essential, but has made the clonal transfer experiments highly reproducible.

INTRODUCTION

An important function of T_c in influenza infections was demonstrated with the finding that murine T_c clones could limit virus replication *in vivo*. This has been demonstrated for subtype-specific and influenza A virus cross-reactive T_c clones (Lin & Askonas, 1981; Lukacher, Braciale & Braciale, 1984), but the viral target antigens for these clones had not been mapped. In recent studies, the non-glycosylated internal NP was shown to serve as an important target antigen for influenza A virus cross-reactive T_c (Townsend *et al.*, 1984a; Yewdell *et al.*, 1985). This antigen, however, does not account for the specificity of all influenza A virus cross-reactive T_c (Pala, Townsend & Askonas, 1986). To date, the definition of the NP as a target antigen has relied on studies using recombinant influenza viruses to abortively infect target cells (Townsend *et al.*, 1984b), the recognition of a cloned NP gene product expressed in mouse L cells (Townsend *et al.*, 1984a), and also vaccination of mice or infection of target cells with a vaccinia virus recombinant into which the NP has been inserted (Yewdell *et al.*, 1985).

In view of the possibility that purified NP may have potential as a vaccine (Wraith & Askonas, 1985), its relevance as a target antigen in a situation involving a productive influenza infection in the lungs or trachea of infected mice was investigated. We have transferred several influenza NP-specific T_c clones into syngeneic mice infected with lethal or sublethal doses of different A type viruses. Our study demonstrates that NP-specific T_c clones can limit influenza virus replication *in vivo* and also protect against a lethal infection. We find that the presence of IL-2 makes these clonal transfers highly reproducible, while not always being essential for protective effects of T_c.

Abbreviations: HA, influenza haemagglutinin; HAU, haemagglutination units; IL-2, interleukin-2; m.o.i., multiplicity of infection; NP, influenza nucleoprotein; p.f.u., plaque-forming units; T_c, cytotoxic T-cells.

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MATERIALS AND METHODS

Influenza viruses

Strains A/X31 (H3N2), A/JAP/305/57 (H2N2) and A/PR/8/34 (H1N1) were grown in the allantoic cavity of 10-day-old embryonated chicken eggs and stored at $-70°$. The highest dilution of virus infected allantoic fluid that agglutinates a 0·5% solution of fowl red blood cells is considered to contain 1 HAU (Hirst, 1941).

Vaccinia recombinant viruses

Two vaccinia recombinant viruses were used: V69 (NP-Vac) contains the A/PR/8 NP gene as previously described (Yewdell *et al.*, 1985) and V64 (H1-Vac) contains the A/PR/8 HA gene (Smith, Murphy & Moss, 1983). These viruses were grown in Hep-2 cells at 37° for 2–3 days. Infected cells were pelleted, disrupted by freeze-thawing and sonication, and aliquots of stock virus (2×10^7 p.f.u./100 μl) were then stored at $-70°$. Virus titres were determined by standard p.f.u. assays on Hep-2 cells.

Mice

BALB/c and C57BL/6 mice aged 3–5 months were bred under specific pathogen-free conditions at the National Institute for Medical Research, London.

Influenza-specific T_c-cell clones

The BALB/c T_c clones T9/13 and BA4 have been previously described (Taylor, Wraith & Askonas, 1985). T_c clones B4 and

B8 were derived by D. C. Wraith from C57BL mice primed with A/X31 (260 HAU i.p.). For optimal growth, all the T_c clones require IL-2 in the form of Con A-stimulated rat spleen cell supernatant or PMA-stimulated EL4 cell supernatant. They also require regular stimulation in the form of A/X31-infected syngeneic thioglycollate-induced macrophages (Taylor *et al.*, 1985). T_c clones were transferred 7–10 days after antigenic stimulation.

Cytotoxicity assays

P815 (H-2^d) target cells were washed, infected for 1 hr with NP-Vac or HA-Vac (m.o.i. 0·5 p.f.u./cell), then washed and incubated at 37° for 15 hr. Influenza-infected target cells were infected with A/X31 virus (1000 HAU/10^7 cells) for 1·5 hr at 37° (Zweerink *et al.*, 1977). All targets were labelled with ^{51}Cr and plated out at 1×10^4/microwell. T_c clones were added at an initial K/T of 4:1. Specific target lysis was calculated following a 5-hr incubation period at 37° (Zweerink *et al.*, 1977).

T_c protection experiments

T_c clones were resuspended in BSS with 5% haemaccel (Hoechst, Hounslow, Middlesex) for i.v. injection into mice that had been intranasally infected with influenza virus 1–2 hr previously. Where indicated, human recombinant IL-2 (a kind gift of Sandoz, Vienna, Austria) was added to the cells to be transferred (100 ng/mouse). A further inoculation of IL-2 was administered at 24 hr i.p. (0·25–0·5 μg). On specified days post-infection, lungs and intrathoracic trachea (about 0·5 cm in 1 ml PBS, Yetter *et al.*, 1980) were removed and homogenized. Influenza virus was titrated in the allantoic cavities of 10-day-old chick embryos and infectivity titres are expressed as egg infectious doses (EID_{50}) in $\log_{10}$ terms (Lin & Askonas, 1981).

RESULTS

NP specificity of selected anti-influenza T_c clones

We have previously described BALB/c clones T9/13 and BA4 and shown them to be K^d-restricted and cross-reactive for influenza A viruses (Taylor *et al.*, 1985). However, it had not been possible to map their antigen specificity until NP-Vac became available. Figure 1 shows that the NP-Vac-infected target cells were lysed nearly as efficiently as X31-infected targets by both the T_c clones, while the HA-Vac-infected cells were not lysed. This established that these clones recognize NP in conjunction with K^d. The NP specificity of the two D^b-restricted and PR8 variant-specific C57BL T_c clones (B4 and B8) was established by lysis of target cells infected with a series of type A recombinant influenza viruses (D. C. Wraith and A. Vessey, unpublished observations), essentially as described for clone A3.1 (Townsend *et al.*, 1984b).

NP-specific T_c clones inhibit virus replication *in vivo*

We wished to investigate whether NP-specific T_c clones were capable of limiting virus replication in infected syngeneic hosts. Table 1 shows a three to four log reduction in lung virus titres following transfer of two influenza A virus cross-reactive NP-specific T_c clones into A/Jap- or A/X31-infected BALB/c mice. We also wished to test whether these clones were active against

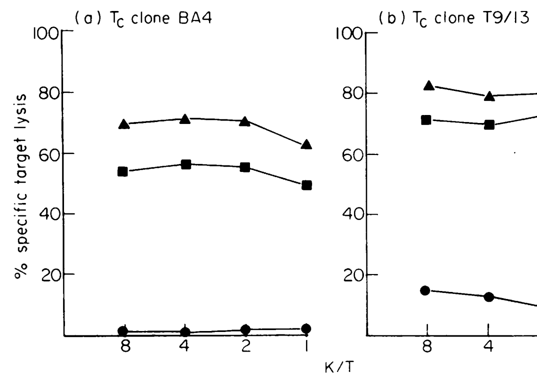

Figure 1. Influenza A virus cross-reactive T_c clones BA4 and T9/13 are influenza NP-specific. BALB/c T_c clones were selected from donor mice primed by infection with A/X31 (BA4) or A/JAP (T9/13). Target cells (see Materials and Methods) were P815-infected with: (▲) A/X31, (■) V69 (A/PR/8 NP-Vac) and (●) V64 (A/PR/8 H1-Vac). Target lysis was calculated following a 5-hr cytotoxicity assay. Spontaneous ^{51}Cr release in the absence of T_c = 11–12%.

virus replication in the trachea. Experiment 3 (Table 1) shows that T_c clone T9/13 led to a reduction of virus in the trachea of infected mice. A lower but significant reduction in virus titre in the lungs was observed following transfer of C57BL clone B8 into syngeneic infected hosts.

Our use of IL-2 in attempts to keep T_c cells more viable on transfer, and possibly to improve their migration properties *in vivo*, was prompted by variability in protective effects of our earlier T_c clones (Taylor & Askonas, 1983). Table 1 shows that the injection of IL-2 alone into infected mice has no significant effect; however, IL-2 in conjunction with cloned T_c can result in lower virus titres in the lungs than transfer of T_c in the absence of IL-2 (Table 1, Experiment 2). Additional experiments (not illustrated) have shown that IL-2 is not always essential, but does make the protective effects highly reproducible.

NP-specific cloned T_c can protect against a lethal influenza infection

Adoptive transfer of NP-specific T_c clones into mice conferred protection against a lethal infection of the mouse-adapted A/PR/8 virus. An i.n. infection with as little as 0·1 HAU of this particular virus normally leads to death of the mice within 1–2 weeks of infection. Table 2 and Fig. 2 present typical experiments and show that the transfer of NP-specific T_c clones enables the mice to survive this type of virulent infection.

DISCUSSION

The process of antigenic variation by influenza viruses leads to the production of new variants which escape neutralization of subtype-specific antibodies. Thus, in considering new types of effective vaccines against this disease, it would be of great value to produce one that could elicit A or B virus cross-reactive T_c as well as neutralizing antibodies.

Recent experiments (Wraith & Askonas, 1985) reported that an ammonium deoxycholate preparation of this antigen was

Table 1. NP-specific T_c clones reduce replication of homologous and heterologous virus in the lungs of syngeneic mice

Exp.	T_c clone transfer	Cell no.	Host infection (i.n.)	IL-2		Virus titres (EID_{50}) Lung	Trachea
1	—	—	A/Jap (12 HAU)	−	Day 4	4·5	
	BA4	9×10^6	A/Jap (12 HAU)	+		1·6	ND
	—	—	A/Jap (12 HAU)	+		3·8	
2	—	—	A/X31 (4·3 HAU)	−	Day 6	5·0	
	T9/13	1×10^7	A/X31 (4·3 HAU)	+		1·0	ND
	T9/13	1×10^7	A/X31 (4·3 HAU)	−		2·8	
	—	—	A/X31 (4·3 HAU)	+		4·5	
3	—	—	A/X31 (4·3 HAU)	+	Day 6	5·5	5·0
	T9/13	9×10^6	A/X31 (4·3 HAU)	+		2·8	3·5
4	—	—	A/X31 (4·3 HAU)	+	Day 6	5·8	ND
	B8	1×10^7	A/X31 (4·3 HAU)	+		4·2	

BA4 and T9/13 (BALB/c influenza A virus cross-reactive NP-specific clones) were transferred into BALB/c mice. B8 (a C57BL A/PR/8 NP-specific T_c clone) was transferred into C57BL infected mice. One to two hours post i.n. infection, T_c clones were injected i.v. + 100 ng IL-2. Twenty-four hours later, 0·5 μg IL-2 was injected i.p. into groups of mice as indicated. Infected control mice receive same regime of IL-2 inoculation. Three mice/group.

Table 2. NP-specific T_c clones protect mice against lethal influenza infection

T_c clone	Mouse strain	A/PR/8 virus	Survival Day 16	Day 30
—	C57BL.6	+	1/6	1/6
B4	C57BL.6	+	4/4	4/4
—	BALB/c	+	0/6	—
T9/13.BA4*	BALB/c	+	3/3	3/3

T_c clones injected i.v. 2 hr post i.n. infection with a lethal dose of A/PR/8 virus (0·1 HAU). IL-2 as in Table 1 legend.

* T9/13 and BA4 cloned T_c (equal numbers mixed).

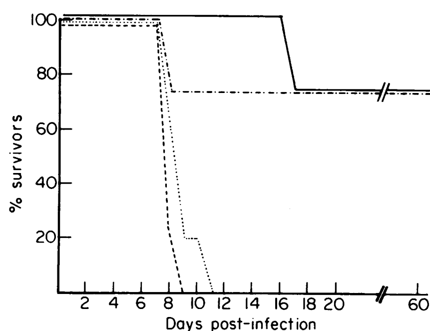

Figure 2. NP-specific T_c clone T9/13 confers protection in mice infected with a lethal dose of A/PR8. T_c clone injected i.v. into BALB/c mice 1–2 hr post-infection with 0·1 HAU of A/PR8. IL-2 as in Table 1 legend. (——) T9/13 (four mice); (·—·—) T9/13 + IL-2 (four mice); (----) IL-2 (no T_c cells) (three mice); (····) control infection (five mice).

successful in priming mice for significant levels of influenza-specific cross-reactive T_c. The definition of NP as an important target antigen for influenza A virus cross-reactive T_c emerged from experiments using target cells transfected with a cloned NP gene (Townsend *et al.*, 1984a) and also cells infected with the NP-Vac virus recombinant (Yewdell *et al.*, 1985). However, it was not clear whether NP-specific T_c had a protective role in a *productive* influenza virus infection *in vivo*. Since we mapped the recognition of several of our selected influenza-specific T_c clones to NP using typed recombinant viruses or NP-Vac-infected target cells, we examined their role, if any, in protection against influenza infection by transferring them to infected hosts. Although the protective ability of influenza-specific T_c clones has been demonstrated before (Lin & Askonas, 1981; Lukacher *et al.*, 1984), the clones used were of unknown viral antigen specificity. In this study, we show that a series of NP-specific T_c are capable of limiting virus replication in the lungs and trachea of influenza-infected hosts and can protect against lethal infection. Our experiments show that these NP-specific T_c clones are recognizing NP in a productive viral infection in the lung tissues or trachea, and not only with non-permissive target cells, *in vitro*. This has not been demonstrated before, and is important in view of our poor understanding of the mechanisms by which internal viral antigens are expressed on the infected cell surface, and lack of NP detection at the cell surface of NP-transfected targets recognized by T_c (Townsend *et al.*, 1984a). The results provide evidence of the relevance of T_c clones of this specificity *in vivo*. This lends support for the potential use of NP as a vaccine candidate. Experiments in which mice primed with purified influenza NP were challenged with lethal doses of A/PR/8 (D. C. Wraith and B. A. Askonas, manuscript submitted) resulted in greatly reduced severity of infection and in recovery.

We have previously reported a lack of protection by some of our antigen-dependent T_c clones (Taylor & Askonas, 1983). Following reports of the efficacy of simultaneous injection of IL-2 in aiding survival of long-term tissue cultured cells *in vivo* (for review, see Cheever & Greenberg, 1985), we used this to supplement our clonal cell transfers. The reliability this measure has produced in our adoptive transfer systems has been valuable, whilst not always essential. A similar effect has been described by Rouse *et al.* (1985), where HSV-specific T-cell lines showed greater efficiency of virus clearance *in vivo* if injected with IL-2.

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