# BIOLOGICAL PROPERTIES OF AN INFLUENZA A VIRUS-SPECIFIC KILLER T CELL CLONE

## Inhibition of Virus Replication In Vivo and Induction of Delayed-Type Hypersensitivity Reactions

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We have described the selection and cloning of a killer T cell line, L4, of BALB/c origin, which grows continuously in the presence of T cell growth factor(s), carries the Lyt-2 marker, and retains H-2 restriction (1). Similar to cytotoxic T cells ($T_c$)[1] induced in vivo in spleen and lung after influenza virus infection of mice (2–4), L4 killer cells are cross-reactive for the different type A influenza viruses, but do not recognize type B influenza virus-infected cells (1).

The role of the cross-reactive $T_c$ in protection against and recovery from influenza infection or the limitation of spread of the infective virus is still uncertain. We have been able to establish a good correlation between levels of memory $T_c$ and cross-protection against different type A influenza viruses in vivo (5). In addition, T cells from primed mice lead to a reduction of replication of homologous virus in the recipient mice on adoptive transfer, and this effect is abolished by treatment with anti-Lyt-2 serum and complement (6). Protection only occurs when donor cells and recipient mice share the K and D end of H-2. Because $T_c$ are restricted at the K and D end of H-2, this implied that $T_c$ might be the effector cells. However, both types of evidence are merely circumstantial in view of the complex interactions of T cell subsets in lymphoid organs; in the second type of experiment, allogeneic effects or host response to allogeneic K and D regions of H-2 cannot be excluded.

In this study we therefore wished to test the effect of clone L4 on virus replication in vivo after influenza virus infection, and found significant reduction in lung virus replication and enhanced survival time of syngeneic mice after lethal influenza infection. We also studied whether the cloned L4 cells, known to actively lyse infected target cells, can also effect skin sensitization reactions. In general, it is thought that delayed-type hypersensitivity (DTH) reactions are the responsibility of effector T cells ($T_d$) restricted to the I region and bearing Lyt-$1^+2^-$ surface markers (7, 8). However, in lymphocytic choriomeningitis infection of mice, Zinkernagel (9) described that K- and D-region compatibility was required for effective transfer of DTH. Some K and D restriction of DTH was also found in a hapten system (10). Recently, two groups

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[1] *Abbreviations used in this paper:* DTH, delayed-type hypersensitivity; $EID_{50}$, egg infectious dose; FCS, fetal calf serum; HAU, virus hemagglutinin units; RS, rat cell supernate containing T cell growth factor(s); $T_c$, cytotoxic T cells; $T_d$, effector T cells for DTH.

of workers looked at DTH after influenza infection and found $T_d$ cells cross-reactive for all type A viruses (11, 12). Transfer of DTH reactivity from mice sensitized with infective virus was I-region dependent (12), when mice were challenged with inactivated virus in the footpad. However, an H-2 K- and D-end restriction was also demonstrated after challenge with infective virus (13), when the cells transferred were Lyt-$1^-2^+$. This raised the question as to whether Lyt-$2^+$ $T_c$, known to kill virus-infected target cells with a K- and D-region restriction, were identical to the K- and D-restricted T cells effeting DTH. We could demonstrate that the influenza virus-specific killer T cell clone was able to induce a DTH reaction in the footpad of syngeneic mice.

During our work, the L4 killer cells had to be recloned repeatedly to maintain their killer activity. Occasionally we obtained clones of cells that lost their ability to kill influenza virus-infected target cells and appeared to lose their receptors for target cells. Cells from such a clone (L4/N) did not induce DTH reactions.

## Materials and Methods

*Mice.* BALB/c mice bred under specific-pathogen-free conditions at the National Institute for Medical Research, London (NIMR) were used at 4–6 mo of age.

*Influenza Virus Strains.* The following influenza viruses were used: A/X-31 ($H_3N_2$), A/USSR/90/77 ($H_1N_1$), A/Jap/Bel ($H_2N_1$), and A/Jap/305 ($H_2N_2$). Virus was grown in the allantoic membrane of 10-d-old embryonated chicken eggs and stored frozen at −70°C. Purified virus for skin sensitization assays was a kind gift from Dr. J. Skehel of the NIMR. Inactivation of virus was by ultraviolet irradiation from a 20-W light source at a distance of 12 cm for 7 min.

*Mouse Influenza Infection and Titration of Virus in the Lung.* BALB/c mice were infected with 5 hemagglutin units (HAU) of A/X-31 or 100 HAU of A/USSR/90/77 intranasally. The lungs of groups of three to five mice were removed 3 d after infection. The lungs were ground to give a 10% extract (5). 10-fold serial dilutions were inoculated into the allantoic sac of 11-d-old embryonated chicken eggs. The eggs were incubated at 35°C and tested 48 h later for hemagglutinating activity for 0.5% chicken erythrocytes. Infectivity titers were estimated by a simplified Reed and Muench procedure and are expressed as egg infectious doses ($EID_{50}$) in $\log_{10}$ terms (14).

*Continuous Culture and Cloning of L4 Killer Cells.* The selection of the BALB/c killer clone L4 was described previously (1). The methods we used followed instructions given by others (15–17) for the selection of T cell lines with other specificities growing in the presence of T cell growth factor. Briefly, we restimulated cultures of primed spleen cells every 11–14 d with syngeneic A/X-31-infected lymphoblasts (2) over a 2-month period, and then grew the $T_c$ in rat cell supernates containing T cell growth factor(s).

For each stimulation, $5 \times 10^5$–$7 \times 10^5$ viable cells/ml were cultured with $10^5$ infected stimulator cells in 3 ml of RPMI-1640-10% fetal calf serum (FCS)-$5 \times 10^{-5}$ M mercaptoethanol in 7-ml Bijou vials (Sterilin Ltd, Richmond, Surrey, England). When viability remained >80%, $10^5$ cells/ml were grown in the above medium containing 30% rat cell supernate containing T cell growth factor(s) (RS), and split every 3–4 d. RS was prepared by stimulating $3 \times 10^6$ rat spleen cells/ml with 4 μg/ml Concanavalin A for 48 h in RPMI-1640 medium containing 10% FCS. After 2–3 wk, the cytotoxic cell population was cloned in plastic 96-well flat bottomed microtiter plates (Linbro Chemical Co., Hamden, Conn. or Nunc, Roskilde, Denmark). Peritoneal cells ($10^5$/well in 0.1 ml) were permitted to settle overnight, irradiated at 1,600 rad, washed free of loose cells, and the cultured $T_c$ were added at a density of 2 cells/well in 0.2 ml medium as above, but also containing 10% RS. Cells growing in single foci were grown up in Costar plates and their cytotoxicity was tested. Cells in well L4, which killed influenza A virus-infected cells, were recloned at 1 cell/well in the same way. All the subclones were very highly cytotoxic; clones L4/2 and L4/11 provided our stock and were also frozen in 10% dimethyl sulfoxide. The L4 cells were maintained in RPMI-1640 medium containing 10% FCS, 10% RS, 100 μg/ml ampicillin, and 100 μg/ml kanamycin. They did not require the presence of influenza

virus-infected stimulator cells, which did not seem to improve growth. To maintain the killer activity of L4 cells, they needed regular recloning on irradiated macrophages (1 cell/well) every 2 wk. The cell line has therefore been recloned regularly over the last year, and any clones we propagated came from a single growing focus. All the L4 killer cells stain brilliantly with fluorescent monoclonal anti-Lyt-2 (1).

*Cytotoxicity Assay.* T cell-mediated lysis was assayed on influenza A virus-infected, $^{51}$Cr-labeled P815 mastocytoma target cells (H-2$^d$) as described previously (3, 18). P815 cells were passaged weekly in DBA/2 or (CBA/H × DBA/2) $F_1$ mice. They were infected for 1.5 h with influenza virus (3, 18), washed free of excess virus, and plated into 96-well round-bottomed plastic Linbro plates at $10^4$ target cells/well. $T_c$ were added at various ratios, and the assay was for 6 h at 37°C.

*DTH Assay.* Normal spleen cells, L4 $T_c$, L4/N nonkillers, or spleen cells from mice sensitized with 1,000 HAU of purified A/X-31 virus subcutaneously 48 h after cyclophosphamide treatment (100 mg/kg) were tested for DTH activity. The cells and purified A influenza virus (6,000 HAU or 6 μg) were injected into the right hindfootpad in a vol of 40 μl, and the left footpad received the same volume of phosphate-buffered saline (PBS). There were four to five mice per group. All experiments were controlled with cells alone or virus alone in the right footpad. At various time intervals, the footpad thickness was measured with a pair of dial calipers (Carobronze Ltd., London) (calibrated in divisions of 0.05 mm). Increase in footpad thickness was calculated as the difference of readings between the right vs. left side divided by the mean thickness of the feet, as measured before challenge (12). The peak of the delayed reaction occurred at ~24 h. Results are expressed as the arithmetic mean ± SE.

*Killer Cell Receptors.* A proportion of $T_c$ form pairs and clusters when mixed with influenza A-virus infected target cells, which can be marked by fluorescence after the uptake of fluorescein diacetate. We used a modification by W. A. Taylor (personal communication) of the method described by Berke et al. (19) for allogeneic cells. P815 cells were infected overnight with A/X-31 virus (18) at 33°C; $10^7$ infected cells were then incubated with 10 μl of fluorescein diacetate (5 mg/ml acetone) in 1 ml medium for 15 min at 20°C. They were washed with RPMI-1640 medium, and $10^6$ targets were incubated with $10^6$ $T_c$ in 1 ml for 3 min at 37°C, and 10 min at 20°C. The cells were centrifuged at 250 *g* for 10 min and resuspended with a Pasteur pipette 20 times. The proportion of $T_c$ forming pairs or clusters with infected fluorescent targets was counted (at least 500 cells counted/slide) in a Leitz fluorescence microscope (E. Leitz, Wetzlar, Federal Republic of Germany).

*Immunofluorescent Staining of L4 Cell Clones.* L4 cells were pelleted by centrifugation, washed with RPMI-1640 medium, and resuspended in RPMI-1640 medium containing 10% FCS, 10 mM $NaN_3$, and 0.03 M Hepes buffer at pH 7.2 ($10^6$ cells/0.1 ml per group). The cells were then exposed (0°C for 30 min) to rat monoclonal antisera directed against (*a*) Lyt-1 (clone 53-7) and (*b*) Lyt-2 (clone 53-6.7), a kind gift of Dr. L. Herzenberg, Stanford University School of Medicine, Stanford, Calif. Cells were subsequently washed three times in the cold, and then stained with fluorescein-labeled antibodies to rat Ig previousy absorbed with mouse Ig preparations. The cells were washed again three times in the cold, and cell smears were prepared and fixed for 5 min in ethanol at 20°C.

## Results

*Phenotype of L4 Killer Cell Clones.* Two subclones of L4 killer cells have been cultured in our laboratory for >1 yr. To maintain their cytotoxic properties for influenza A virus-infected H-2$^d$ target cells, we have had to reclone the cytotoxic L4 cells at regular intervals on irradiated macrophages. Active $T_c$ maintained their surface properties and phenotypes. We could not detect any Ig$^+$ cells, nor Lyt-1$^+$ cells in the L4 clonal populations by fluorescent staining. 98–100% of the cells stained with monoclonal antisera to Lyt-2 and Thy-1.2; all the L4 cloned cells stain brilliantly with fluorescent *Vicia vallosa* lectin (experiments carried out by P. Banga, Middlesex Hospital, London). Clone L4 killing is restricted to the D or L end of H-2 (Y.-L. Lin and B. A. Askonas, unpublished observation).

*L4 $T_c$ Inhibit Influenza Virus Replication.* We wished to test some biological properties of cloned L4 killer cells in vivo. The L4 cells decline and die in culture rapidly (within 1 d) if RS is withheld from the medium. In view of this rapid decline in the health of the cells, we had to use experimental systems that permitted rapid homing of the cells to the appropriate area. In the mouse, influenza virus will replicate in epithelial cells of the lung after intranasal infection, and infective virus can be titred in lung extracts, peaking on day 3 of infection (5). When slightly larger doses of virus are used for intranasal infection and the animals are sublethally irradiated, death ensues.

To clarify whether $T_c$ are effective in limiting virus replication in vivo, we transferred cloned L4 $T_c$ cells intravenously into syngeneic BALB/c mice after infection with either the homologous A/X-31 ($H_3N_2$) virus, which was used originally during the selection of L4, or a heterologous influenza virus (A/USSR/90/77 $H_1N_1$). Table I illustrates a typical experiment in we show that clone L4 killer cells protect against lethal influenza infection with the homologous A influenza virus.

Intranasal infection of sublethally irradiated BALB/c with 20–30 HAU of A/X-31 virus leads to death within 19 d. After transfer of $3 \times 10^6$ L4 cells, three of four mice survived for >4 mo. Transfer of normal spleen cells delayed death somewhat (three of four mice died by 30 d), but survival was far shorter than after L4 cell transfer.

We also wished to test whether cloned L4 killer cells are active in limiting virus replication in the lungs of nonirradiated infected mice in vivo. We were particularly interested to see whether they were effective against a heterologous type A influenza virus. BALB/c mice were therefore infected intranasally with A/USSR/90/77 influenza virus, and 24 h later, L4 cells were transferred intravenously into the infected mice. 3 d later, the lungs were removed, extracted, lung extract inoculated into embryonated eggs, and the virus titers (HA) assayed (Materials and Methods). Table II shows the results. The lung virus titer in the mice receiving L4 cells was three orders of magnitude lower than in control mice that received virus alone. Similarly, L4 cells reduced the lung virus replication after intranasal infection of A/X-31 virus, which was used initially to select the L4 clone (Table II). A/USSR/90/77 replicates less efficiently than A/X-31, and therefore higher doses of A/USSR/90/77 are required to infect mice reproducibly. A clone of $T_c$ cells thus led to the reduction of virus replication in the lungs of infected mice and also offered protection against death from a lethal infection.

*Selection of a Nonkiller Clone Derived from L4 Cells.* L4 cytotoxic cells tended to become less efficient killers of infected target cells after some weeks in tissue culture. On recloning such cells, we derived a clone (L4/N) that did not have the ability of

TABLE I

*Cloned L4 $T_c$ Protect against Influenza Infection*

| Cells transferred | Virus infection | Dead/total | Survival |
|---|---|---|---|
| | | | *d* |
| None | A/X-31 | 4/4 | <19 |
| $3 \times 10^6$ normal spleen cells | A/X-31 | 3/4 | <30 |
| $3 \times 10^6$ clone L4 $T_c$ | A/X-31 | 3/4 | >80 |

BALB/c host mice, irradiated with 450 rad, were challenged with 20 HAU A/X-31 intranasally on the day before cell transfer. Cells were transferred intravenously.

TABLE II

*Cloned L4 $T_c$ Inhibit In Vivo Replication of Heterologous Influenza Virus in the Lung*

| Exp. | Treatment of host | | Lung virus titers* at day 3 |
|---|---|---|---|
| | Cells | Influenza virus | |
| | | | *$EID_{50}$* |
| 1 | — | A/USSR/90/77 | 7.8 |
| | L4 | A/USSR/90/77 | 4.8 |
| 2 | — | A/X-31 | >7 |
| | L4 | A/X-31 | 4.5 |

$3 \times 10^6$ L4 cells were transferred intravenously 1 d after intranasal infection of BALB/c mice with 100 HAU A/USSR/90/77 ($H_1N_1$) or 5 HAU A/X-31. L4 cells were originally selected by stimulation with A/X-31 ($H_3N_2$) influenza virus. Three mice/group titered individually.

* $Log_{10}$ $EID_{50}$ (Materials and Methods).

TABLE III

*L4 $T_c$ Bind Influenza A Virus-infected Target Cells*

| Cell source | Percentage of L4 $T_c$ binding targets* |
|---|---|
| L4 killer clone | 18.6 |
| L4/N nonkiller clone | 4.0 |
| Normal spleen cells | 4.0 |

* P815 target cells infected with A/X-31 infuenza virus, after uptake of fluorescein diacetate, were mixed with $T_c$ or nonkiller cells (Materials and Methods). 500 cells per slide were counted in the fluorescence microscope.

killing type A influenza virus-infected cells, even at higher killer:target cell ratios, and seemed to have lost receptors for recognition of infected target cells. L4/N cells did not form clusters with influenza virus-infected P815 target cells, in contrast to the L4 cells which actively lysed A influenza virus-infected cells; 18–25% of cytotoxic L4 cells formed pairs or clusters with infected targets (Table III). The nonkiller clone L4/N was used as control in the DTH experiments described below.

*Cloned L4 $T_c$ Effect a DTH Reaction.* Leung and Ada found recently (13) that influenza virus infection of mice can sensitize two types of T cell subpopulations. Provided that infective virus is also used for the elicitation of the DTH reaction, Lyt-$1^+2^-$ T cells restricted to the I region and Lyt-$1^-2^+$ cells restricted to the D or K end of H-2 can both induce a DTH reaction in the footpad of mice. Because $T_c$ are restricted to the K and/or D region of H-2 and bear the Lyt-$2^+$ phenotype, it was of interest to test whether $T_c$ active in killing infected target cells were responsible for DTH reactions. We therefore injected clone L4 $T_c$ into the footpads of mice, challenged them with purified virus, and measured the footpad at various time intervals. Virus alone induces an immediate footpad enlargement of similar magnitude to that observed after injection of virus plus L4 $T_c$. However, this immediate reaction caused by virus declines rapidly, and by 24 h, the footpad of mice receiving virus alone is only very slightly enlarged. However, the group of mice receiving $T_c$ clone L4 plus virus had a significantly enlarged footpad 24 h after injection, as in a DTH

reaction (Fig. 1). The same pattern of reactivity occurs when the reaction is elicited with A influenza virus inactivated by ultraviolet irradiation (data not shown).

Every experiment was controlled with groups of mice receiving influenza A virus alone or L4 cells alone. The mean increase in footpad swelling (~25%) at 24 h is of very similar magnitude in comparison to the transfer of spleen cells from mice sensitized with infective virus after cyclophosphamide treatment of the mice (Table IV). We also controlled the experiment by transferring the nonkiller clone L4/N into the footpad plus A/X-31 virus. This did not induce significant footpad swelling (Table IV). The induction of a delayed skin reaction by L4 killer cells was equally strong upon challenge with a heterologous influenza A virus (A/Jap/Bel [$H_2N_1$]) (Table V). L4 killer cells are therefore cross-reactive for the different type A influenza viruses for DTH reactions and for the lysis of virus-infected targets. L4 killer cells administered intravenously did not induce a significant delayed reaction on virus challenge in the footpad. The L4 cells die very quickly in the absence of T cell growth factor, and presumably, do not migrate to the footpad in sufficient numbers before death. The time-course of footpad swelling would exclude the possibility that the increase in footpad thickness is a result of growth of the L4 cells. The nonkiller clone (L4/N) grows far more vigorously than the cytotoxic L4 clones, and yet does not induce footpad swelling. Once more, the skin reaction by L4 cells and viruses does not last, and the footpad is back to normal by 48 h. Our results therefore indicate that a clone of Lyt-$1^-2^+$ T-cells (1), which actively kills influenza cells of the same H-2

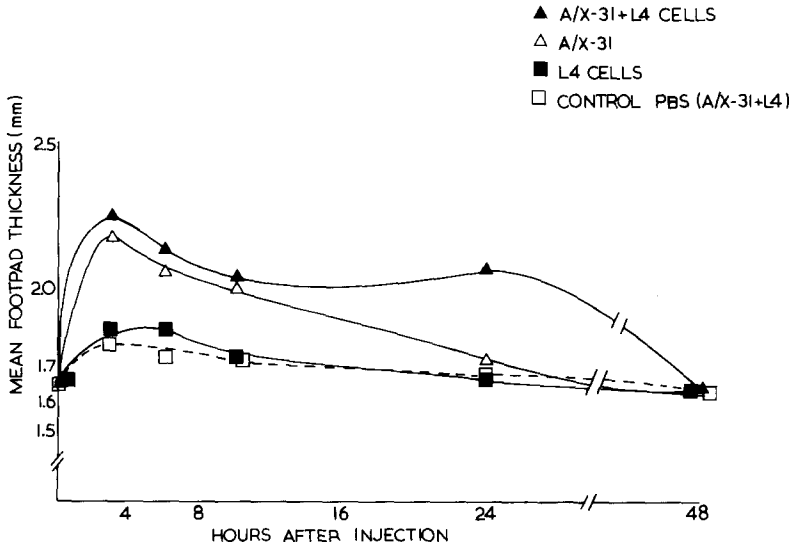


FIG. 1. Induction of DTH reaction by $T_c$ clone L4. $5 \times 10^5$ L4 killer cells and/or 6 μg of purified A/X-31 virus (6,000 HAU) were injected into the right footpad in a vol of 40 μl, and the left footpad received an equal volume of PBS (Materials and Methods). Measurements were taken with a dial caliper at various times after injection. There were five mice per group. SE, ±0.018–0.022 in the different groups. PBS injections and measurements were carried out on each mouse. Footpad thickness after PBS injection is only illustrated for one group of mice, because the results were the same in each case. Injection into right footpad: group 1: ▲, Clone L4 and A/X-31 virus; group 2: △, A/X-31 virus; group 3: ■, L4 cells. Injection into left footpad: □, PBS (control footpads for group 1).

TABLE IV
*Cloned L4 $T_c$ Effect DTH Reactions*

| Cells transferred | No. of cells | A/X-31 virus | Mean increase in footpad thickness* |
|---|---|---|---|
| | | | *percentage ± SE* |
| L4 | $6 \times 10^5$ | + | 25.1 ± 1.4 |
| — | — | + | 3.0 ± 1.2 |
| L4 | $6 \times 10^5$ | − | 2.3 ± 0.8 |
| Nonkiller clone L4/N | $5 \times 10^5$ | + | 2.3 ± 0.8 |
| Sensitized cells | $3 \times 10^6$ | − | 3.1 ± 0 |
| Sensitized cells | $3 \times 10^6$ | + | 26.1 ± 2.9 |

Groups of four BALB/c mice were injected into the right footpad with L4 cells, spleen cells sensitized with infective virus, or a nonkiller clone (L4/N) derived from L4, and/or 6 μg purified, infective A/X-31 virus in a total vol of 40 μl. The left footpad was injected with 40 μl medium only. Footpad thickness was measured 24 h after injection.

* $\frac{\text{Right (experimental)} - \text{left foot (control [PBS])}}{\text{normal footpad thickness}}$.

TABLE V
*Cloned L4 $T_c$ Effect DTH Reaction to A/Jap/Bel Influenza Virus*

| Cells transferred ($6 \times 10^5$) | Influenza virus | 24-h mean increase in footpad thickness |
|---|---|---|
| | | *percentage ± SE* |
| Cloned L4 cells | — | 3.0 ± 1.2 |
| Cloned L4 cells | A/Jap/Bel | 31.8 ± 1.8 |
| — | A/Jap/Bel | 3.1 ± 1.4 |

Five BALB/c mice per group. L4 $T_c$ cells and/or purified A/Jap/Bel influenza virus (5 μg = 5,000 HAU) were injected into the right footpad, and the left footpad received an equal volume of PBS. Footpad thickness and its increase over control footpad measured as in Table IV.

haplotype, can also effect a DTH skin reaction in the presence of infective or inactivated influenza virus.

## Discussion

Much of the literature on our defense against influenza has focused on circulating antibody responses, which have been shown to protect against infection with homologous virus, but not against infection with variants or different subtypes of influenza virus. On the other hand, cross-protection between different subtypes of influenza A virus has been reported in the absence of antibodies of appropriate specificity (20). The finding that the major proportion of $T_c$ induced by infection does not distinguish between the different type A influenza viruses in mouse and man (2–4, 21, 22) has raised the question of whether $T_c$ play a major role in our immune control of influenza. Studies in nude mice have indicated previously that antibodies alone were not sufficient for recovery from influenza (23). It is important to assess which T cell subsets play an important role in influenza. Recent studies by Yap et al. (6) indicated that T cells restricted to the K and D regions of H-2 limited virus replication in the lungs of infected mice. Lyt-$1^-2^+$ spleen T cells, primed by infecting mice, reduced the

replication of virus in the lungs of mice infected with the homologous influenza virus on transfer, provided donor cells and hosts shared the K and D region of H-2. Differences in the I region between donor and host did not result in reduction of lung virus titers. Because $T_c$ are K- and D-region-restricted $T_d$ effector cells in virus infections (24) this suggested that $T_c$ is the cell type active in inhibiting virus replication. As $T_c$ memory cells in influenza are cross-reactive for the type A influenza viruses, one might expect that their protective role would extend to all type A influenza viruses, not merely the homologous influenza virus. There is a correlation between the level of $T_c$ memory in primed mice and cross-protection against infection with different type A influenza viruses (5). However, in the whole animal, the immune network interactions are highly complex and it is difficult to pinpoint a single cell type.

In this study, we could test directly whether active $T_c$ act to limit replication of type A influenza virus, be it homologous or heterologous. Cloned L4 killer cells were transferred either into sublethally irradiated mice after challenge with a lethal infection, or into normal mice infected intranasally with a heterologous influenza virus. Clone L4 protected against a lethal infection with A/X-31 virus, and reduced the replication of homologous or heterologous influenza virus in the lungs of infected mice. The experiment thus showed that clone L4 $T_c$ are cross-reactive for the type A influenza viruses not only in vitro, but also in vivo, and can limit virus replication.

Yap et al. (6), on the basis of their cell transfer experiments, excluded Lyt-$1^+2^-$ $T_d$ cells from playing a major role in limiting virus replication (6). However, recently they reported that $T_d$ cells are not only I-region restricted, but can also be K- and D-region restricted when mice are challenged locally with infective influenza virus (13). This raises the question whether the K- and D-restricted Ly-$2^+$ killer cells could be responsible for the DTH reaction, or whether a nonkiller T cell subset would effect K- or D-region-restricted DTH reactions.

We find that clone L4 cells that are Lyt-$1^-2^+$ and actively lyse virus-infected target cells also induce footpad swelling in the presence of type A influenza virus. There is a significant increase in footpad swelling 24 h after injection of L4 cells and A/X-31 or A/Jap/Bel virus, compared with the control groups that received injections of the virus or L4 cells alone. The experiment was controlled also by injection of A/X-31 virus and a clone of L4 cells (L4/N), which had lost its ability to recognize or to lyse virus-infected target cells. This combination did not result in a 24-h reaction. In our experiments, L4 killer cells induced DTH skin reactions to challenge with both inactivated as well as infective virus. Our failure to transfer DTH by intravenous injection of $3 \times 10^6$ L4 killer cells presumably can be attributed to poor homing to the footpad areas of the cells derived from tissue culture, and their rapid death in the absence of T cell growth factor(s). On the other hand, L4 cells effectively reach the lung after intravenous transfer to an infected mouse, and this should explain their capacity to reduce the replication of influenza virus in the lung. A pilot experiment had shown that $^{51}$Cr-labeled L4 cells were trapped in the liver, spleen, and lung on transfer to virus-infected mice (data not shown).

We therefore conclude that Lyt-$1^-2^+$ $T_c$ can also induce DTH skin reactions in addition to their ability to kill infected target cells. Whether the two functions are a property of the cells during different phases of the cell cycle or whether the same cell at any given time can act either as a killer cell or act to induce DTH reactions as

required is not clear. It is also possible that the two functions are combined, and that killer T cells secrete lymphokines causing the cell infiltration observed in DTH on interaction with the infected target cell.

Therefore, although $T_c$ may not protect us entirely against infection, it seems clear that a $T_c$ response can limit virus replication in infected hosts and aid recovery. The cloned $T_c$ should enable us to evaluate further the role of $T_c$ as part of our defence mechanisms in influenza.

## Summary

We tested two biological properties of a continuously growing mouse cytotoxic T cell line, L4, which is specific for influenza A virus and has been cloned and recloned many times. We previously reported that L4 cells are H-2 restricted and cross-reactive for all type A influenza viruses, whereas they do not recognize type B influenza viruses. They bear Thy-1 and Lyt-2 markers (1).

In the present study, we show that L4 cytotoxic T cells protect mice against a lethal influenza infection on transfer to syngeneic recipients, and reduce virus titers in the lungs of mice challenged with a heterologous type A influenza virus. This provides further support for the active role of cytotoxic T cells in limiting virus replication in influenza infection. We could also demonstrate that the cloned cytotoxic T cells induce a delayed-type hypersensitivity skin reaction in the footpads of mice challenged with live or inactivated influenza virus. This reaction can be observed at 24 h, but has declined by 48 h. A clone of cells derived from L4 that has lost its cytotoxic potential and its ability to recognize infected cells did not induce a delayed-type hypersensitivity reaction in the presence of virus. Thus, cytotoxic T cells actively killing influenza virus-infected cells are able to induce a delayed-type hypersensitivity skin reaction to homologous and heterologous type A influenza viruses.

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