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Murine CD4+ T cell clones vary in function *in vitro* and in influenza infection *in vivo*

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Abstract

Several CD4+ T_h1 clones specific for influenza haemagglutinin or nucleoprotein were transferred into syngeneic mice after intranasal influenza infection to examine whether they accelerate viral clearance *in vivo* similarly to CD8+ cytotoxic T cells. We observed changes in functional properties of the CD4+ clones *in vitro* and variable effects on the course of infection *in vivo*. While some clones resulted in more rapid virus clearance, others had no protective effect, but rather exacerbated illness symptoms. Our results reflect problems in the *in vivo* use of CD4+ T cell clones maintained in long-term culture. Their IL-2 and IL-5 release and cytolytic activity varied, while IL-3 and γ-IFN production as well as DTH induction were more stable. CD4+ T cells primed by infection became cytolytic only after prolonged culture. The data point to the fine balance between exacerbation of disease and protection by CD4+ T cells.

Introduction

Cloned influenza specific cytotoxic T cells (CTL) which are CD8+ and class I MHC restricted can protect against lethal influenza infection of mice and accelerate clearance of lung virus by day 6 (1 – 3). This effect is reflected by a more rapid recovery from lung pathology (4). Since CD4+ T cell responses are far more easily induced than CD8+ CTL by subunit vaccines, we wished to know whether CD4+ T cells played a role in heterotypic immunity following influenza infection by accelerating viral clearance similarly to CD8+ CTL (1 – 3) Antigen stimulation induces the secretion of similar levels of γ-IFN by both T cell subpopulations (T_h1 cells and CD8+ T cells) (e.g. 5), but the two T cell subsets differ in other functions, such as the levels of interleukin release, DTH induction, and cytolytic activity.

Previous experiments with CD4+ cells have been very limited A single HA-specific clone transferred into nu/nu mice amplified antibody responses and resulted in viral clearance by 2 weeks of infection (6). A neuraminidase-specific clone (7) or primed spleen cells selected for expression of Lyt-1 led to survival of mice given a lethal influenza challenge (8). However, the short-term effector function of CD4+ cells had not been examined. In earlier experiments we found that *in vivo* depletion of CD8+ T cells by monoclonal antibodies (9) was not sufficient to abolish the generation of significant CD8+ CTL responses to influenza (10). Therefore in the present study we transferred several CD4+ and influenza-specific T cell clones into infected hosts to observe their short-term effect on virus replication in the lung of influenza infected syngeneic mice. Our results emphasize the difficulties arising in the use of CD4+ T cell clones in long-term culture to study *in vivo* function The effector function *in vivo* varied between individual T_h clones and within the life of a single clone with time in culture and changes in interleukin secretion or cytolytic activity could be observed

Methods

Mice

BALB/c a mice, aged 3 – 5 months, were bred under specific pathogen-free conditions at NIMR, Mill Hill, London.

Influenza virus and proteins

Influenza viruses A/X31 (H3N2 recombinant with H1N1 internal proteins) and A/PR/8/34 (H1N1) were grown in the allantoic cavity of 10-day-old embryonated chicken eggs, and stored at – 70°C as infectious allantoic fluid. Virus was inactivated by irradiation at a distance of 10 cm from a 15 W UV lamp for 10 min.

Purified HA from bromelain digested A/X31 (11) was kindly provided by D. Stevens (NIMR). The remaining viral cores were treated wtih ammonium deoxycholate (12) to precipitate the matrix protein and to allow purification of nucleoprotein (NP) (13).

Selection and maintenance of CD4+ T cell clones

The general method has been described by Mills *et al.* (14). In brief, BALB/c mice were infected i.n. with A/X31 virus (2-5 HAU). After 2 – 4 months spleen cells were cultured at 1 – 2 × 10^6/ml,

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with UV inactivated A/X31 unless otherwise indicated. Four days later cells were cloned by limiting dilution in the presence of virus and APC (normal spleen cells, 2000 rad, at 5×10^5/well) with a source of IL-2 (supernatant from 48 h stimulation of rat spleen cells with Con A at 5 μg/ml). Clones were maintained in 25 cm^2 flasks by the following regime: every 10 – 12 days 2×10^5 cells/ml cells were stimulated with antigen and APC at 2×10^6 cells/ml). After 3 – 5 days, 5% IL-2 was added to the cultures.

This method of selection proved ineffective in obtaining NP-specific CD4+ clones. Clone 2F3 was selected by using purified NP instead of A/X31 for the cloning procedure. For clone 13.3, donor spleen cells were depleted *in vitro* with C and anti-Lyt-2 mAb HO2 2 (15) and J11d (anti-Ig; 16), and cells were cloned directly using UV-inactivated A/PR/8 virus as the antigen. Both NP-specific clones grew optimally with NP or less pure virus core (1 μg/ml). The time of culture of our clones was calculated starting at 2 weeks after cloning, i.e. once reliable clonal growth had occurred, it does not necessarily reflect continuous growth, as clones at times were frozen and thawed.

Antigen recognition assays

Clones were tested for antigen specificity 10 – 12 days after antigen stimulation (14). T_h cells (10^4) were cultured with 4×10^5 APC and purified antigen (0.1 – 10 μg/ml) in 200 μl medium in 96-well microplates. After 48 – 72 h cells were pulsed with 0 5 μCi [^{3}H]thymidine ([^{3}H]TdR) for 4 – 6 h before harvesting.

T cell transfer and lung virus titration

Mice were infected i.n. with 4 – 5 HAU A/X31 virus (3 – 4 mice group). After 1 – 2 h, CD4+ T cells (in BSS with 5% haemaccel, Hoechst, Hounslow, Middlesex) were mixed with recombinant human IL-2, generously provided by Hoffman La Roche (Basel), and transferred i.v. into the infected hosts (5000 units/mouse). On days 6 – 7 post-infection, lungs within a group were pooled and homogenized before dilution and virus titration in the allantoic cavities of 10-day-old chick embryos. Infectivity titres are expressed as EID_{50} in $\log_{10}$ terms (1).

Class II MHC restricted target cell lysis

Target cells were the A20.2J B cell lymphoma line (17) (kindly donated by J. Tite, Wellcome Labs, Beckenham, Kent), which expresses both class I and class II MHC molecules on its surface. Following a 1 h incubation with infectious A/X31 virus (1000 HAU/10^7 cells) and ^{51}Cr (100 μCi/10^7 cells), cells were washed and a 6 h ^{51}Cr release assay performed as described previously (18).

Interleukin assays

The cloned CD4+ cells ($2 - 5 \times 10^5$/ml) were washed and, 10 – 12 days after antigen stimulation, stimulated with UV-inactivated A/X31 virus at 100 HAU/ml, HA, NP, or influenza core protein (1 μg/ml), and syngeneic, normal spleen cells (irradiated at 2000 rad) as APC. Following incubation at 37°C, cell-free supernatants were stored at −20°C and interleukins assayed (see below). The stimulation index (SI) was calculated as follows:

$$SI = \frac{[^3H]TdR \text{ (cpm) incorporation; antigen stimulated supernatants}}{\text{cpm cell supernatants; no antigen}}$$

Interleukin assays relating to a single clone at various times of culture were carried out on the same day.

IL-2

Two-fold dilutions of cell supernatants (50 μl/well) were tested in triplicate in RPMI/5 in round-bottom 96-well microtitre plates. CTLL cells, maintained in RPMI/10 with a source of IL-2, were washed three times before plating out at 10^4 cells in 50 μl in RPMI/5. Each assay included control supernatants (no antigen) and a known IL-2 supernatant. Following a 20 h incubation period at 37°C, cells were pulsed with [^{3}H]TdR (0.5 μCi/well) for 4 h Cells were then harvested and processed for scintillation counting.

IL-3

32D cells (19) were maintained in RPMI/10 and 5% WEHI3 supernatant [obtained from dense cultures of WEHI3 cells (20)]. The assay conditions for IL-3 are essentially identical to the method described above for IL-2, but using 32D cells for the read out.

IL-5

Frozen stocks of T cell-depleted BCL_1 lymphoma cells (21) were generously provided by Dr Arturo Gonzalez (NIMR). Two-fold dilutions of supernatants were tested in triplicate in RPMI/5, in flat-bottom microtitre plates Thawed, washed BCL_1 cells were plated out at 2.5×10^4 cells/well (total volume 200 μl/well). Plates were incubated at 37°C for 44 h, and cells then pulsed with [^{3}H]TdR (0.5 μCi/well) for 4 h before harvest and scintillation counting. BCL_1 cells can also respond to IL-2, thus IL-5 could only be assessed in the absence of IL-2.

γ-IFN

The γ-IFN content in supernatants from antigen-stimulated T_h cells was kindly assayed by Dr A. Meager (NIBSC, South Mimms) using a highly specific radioimmunoassay which utilizes two mAbs specific for γ-IFN (5). All results were standardized with the murine γ-IFN standard NIH Gg02-901-533.

Delayed type hypersensitivity assay

T_h clones in varying cell numbers were injected with 5 μg of purified A/X31 virus in a volume of 30 μl into the right hind footpads of four mice per group. An equal volume of PBS was injected into the left hind footpad. Footpad thickness was measured at 24 h with dial calipers (Pocotest, FRG).

$$\% \text{ footpad increase} = \frac{\text{right (experimental)} - \text{left (PBS)}}{\text{normal footpad}} \times 100$$

The low increases in footpad size seen at control sites (virus or cells alone) were subtracted.

Results

Characterization of CD4+ T cell clones

The phenotype of the clones used in this study was ascertained by fluorescent antibody staining [anti-L3T4, mAb YTS 191.1; anti-Lyt-2, mAb YTS 169.4 (9)]. No Lyt-2+ cells were detected and 90% of the cells were L3T4+. This phenotype was stable

during the entire culture time. We refer to these cells as CD4+ T cells or T_h. Antigen specificity was established by stimulating the cloned cells with different concentrations (0.1 – 10 μg/ml) of purified H3 (from X31 virus) or NP (derived from A/PR/8/34 virus) as described by Mills *et al.* (14). On antigen stimulation, all the clones when first selected secreted IL-2 and IFN (not illustrated) and belonged to the T_h1 subset (22). Anti-IL-2 (mAb S4B5, 1/500 ascites) was shown to totally block CTLL proliferation to confirm that the lymphokine released was IL-2 (Fig. 1). Two BALB/c NP-specific and A virus cross-reactive clones 2F3 and 13.3 and H3-specific clone 2A12 were derived by F.E., and three other BALB/c H3-specific CD4+ clones (BAE5, BA 5.2, BA 5.6) were kindly given by D. B. Thomas and C. Graham, NIMR (23). All the clones used were class II MHC restricted. Stimulation was tested with APC from congenic mice (not illustrated). When clones became cytolytic they lysed A/X31-infected A20.2J targets (expressing I-A^d and I-E^d) but not infected P815 cells (H-2^d), which are good targets for class I MHC-restricted CD8+ CTL.

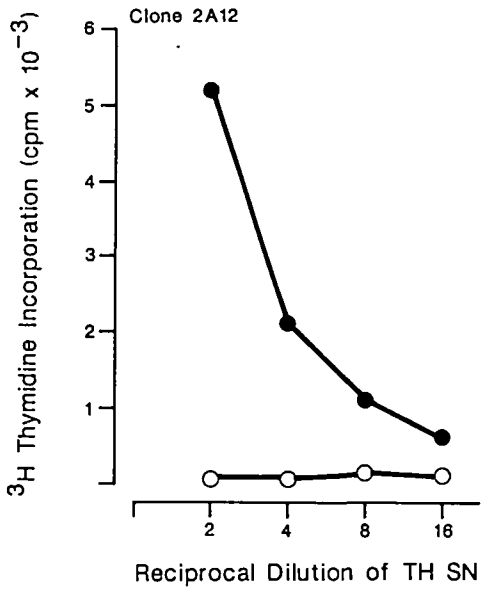

Fig. 1. Antigen-induced IL-2 production by CD4+ T cell clone 2A12 6 weeks after cloning. Proliferation of CTLL in the presence of supernatant of A/X31 stimulated 2A12 cells (●—●); this is totally inhibited by 1/500 dilution of mAb S4B6 (anti-IL-2) ascites (○—○).

Functional variation of HA-specific CD4+ T cell clones

In pilot experiments we transferred a CD4+ influenza-specific T cell line into sublethally irradiated mice infected with X31 virus. Three to four million cells were lethal to the hosts within a few days, while the infected mice without T cells survived. This prompted us to transfer newly available HA-specific clones into infected but not irradiated syngeneic mice. To obtain sufficient numbers of cells long-term culture was necessary. Table 1 illustrates that individual clones specific for H3, the haemagglutinin of the X31 virus, differed in their effects *in vivo*. Transfer of two of the clones (6 – 9 × 10^6 cells) exacerbated illness symptoms and had no significant effect on lung virus titres. One of them (clone 5.6) appeared to slightly enhance lung virus titres. In contrast, transfer of the two other H3-specific clones at similar cell numbers led to a reduction in virus recoverable from the lung on day 6 At the time of cell transfer, these clones were able to lyse A20.2J targets infected with X31 virus but at much lower efficiency than our CD8+ CTL Clones 2A12 and BAE 5 were also tested for DTH induction (20 – 30% increase in footpad thickness by 2.5 × 10^5 cloned cells). The other two clones had not been tested for cytolytic ability at the time of the cell transfers.

These experiments showed that CD4+ T cell clones recognizing the same viral protein vary in their effector function *in vivo* and we wondered whether this might be attributable to changes in interleukin production or other properties between clones during their long-term maintenance *in vitro*. The next clones selected were analysed accordingly at the time of the cell transfers.

Transfer of CD4+ NP-specific T cell clones

We were particularly interested to examine whether influenza A virus cross-reactive CD4+ T cells were able to accelerate viral

Table 1. HA-specific CD4+ T cell clones differ in their *in vivo* effect

Clone	Weeks in culture	Cell transfer (no. × 10^{-6})	Lung virus titre (d6), $\log_{10}$ EID_{50}	% A/X31-specific A20-2J lysis[a]	DTH[b] % A/X31-specific footpad increase
BA5 2	18	–	5 2	ND	ND
		9	4.8[c]		
BA5 6	24	–	5.2	ND	ND
		6	6.2[c]		
2A12	9	–	4.2	15	ND
		9	2 8		
	14	–	4.5	24	33
		4	2.0		
BAE5	15	–	5.5	31	26
		8	2.8		

The H3-specific CD4+ T cell clones were BALB/c derived. Cells were transferred i.v into syngeneic mice 2 h after i.n. A/X31 infection and lung virus titred on day 6 of infection.

[a] K/T = 10.

[b] 2.5 × 10^5 cloned cells/footpad.

[c] Increased sickness symptoms.

Table 2. NP-specific CD4+ T cell clones can enhance disease or recovery

Clone	Weeks in culture	Cell transfer (no. × 10^{-6})	Lung virus titre (d6), $\log_{10}$ EID_{50}	% A/X31-specific A20-2J lysis[a]	DTH[b] % A/X31-specific footpad increase
13 3	8	–	4 5	ND	ND
		5	4.5[c]		
	10	–	4.5	ND	ND
		8	4 8		
	13	–	5 5	27	11
		2	4.5		
		8	3.5		
2F3	10	–	4 5 (0/3 died)	ND	ND
		5	5 7[c] (1/3 died)		
	18	–	5 0	0	20
		5	6.0[c]		
	21	–	4 5	22	ND
		5	4 5		
		8	4.5		

BALB/c CD4+ T cell clones were transferred i v 2 h after A/X31 i n infection of hosts
[a] K/T = 10.
[b] 2 5 × 10^5 cloned cells
[c] Mice very ill.

clearance similarly to CD8+ T cell clones and possibly to play a part in partial heterotypic protection. We were able to select two NP-specific and A virus cross-reactive CD4+ clones which enabled us to study several parameters over several weeks of culture antigen-induced interleukin production *in vitro*, cytolytic capacity, and *in vivo* function (DTH induction and viral clearance).

Clone 13.3 grew well and was first tested after 8 weeks of culture. Transfer of only 5 million cells enhanced sickness symptoms in comparison to control-infected mice; within several days of infection and cell transfer the host mice were severely ill (sweating, ruffled fur, huddling, and tachypnoic signs), though the lung virus titres were the same as in the control-infected mice. After another 2 weeks of culture even higher numbers of cells (8 × 10^6) did not affect viral replication in the lung, while after 13 weeks of culture the same number of cloned cells transferred resulted in a two-log reduction of lung virus titre by day 6. Interleukin production had changed during the interval between transfers. IL-2 release was not detectable any more by 13 weeks of culture, while IL-3 production had increased. At the time of protective activity clone 13.3 was able to lyse X31-infected A20 2J target cells (Table 2 and Fig. 2).

The second NP-specific clone 2F3 was transferred first after 10 weeks in culture; again 5 × 10^6 cells made the host mice very ill and the lung virus titre was slightly higher than in the control mice 6 days post-infection. One-third of the mice died by day 5. After a few more weeks of culture, a similar effect could be observed. Another test after 21 weeks of culture did not exacerbate illness symptoms and no effect was noted on the lung virus titre when 8 × 10^6 cloned cells were transferred (Table 2). With this number of 13.3 cells a two-log reduction in lung virus resulted.

Lymphokine assays at the time of cell transfer are illustrated in Fig. 2. IL-2 production was down-regulated by 18 weeks in culture, at which time IL-5 induction was detectable. The only difference between the last two transfers of clone 2F3 was an increase in cytolytic activity. This clone could not lyse the influenza-infected targets at the time of the first two cell transfers.

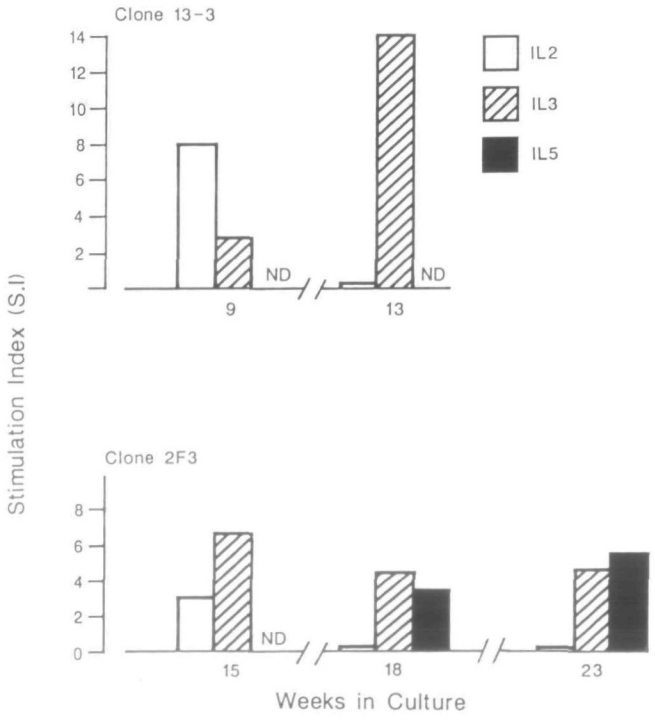

Fig. 2. Antigen-induced IL release by CD4+ clones. Supernatants collected 24 h after antigen stimulation of NP-specific clones 13 3 and 2F3 were tested for interleukin activity by proliferation of CTLL (IL-2), 32D (IL-3), or BCL_1 cells (IL-5)

$$SI = \frac{\text{cpm in antigen stimulated SN}}{\text{cpm SN (no antigen)}}$$

DTH responses induced by the protective and non-protective clones were of similar magnitude, and both clones continued to produce high levels of γ-IFN (1000 – 2600 units/ml/5 × 10^5 cells) in the presence of antigen (not illustrated).

Our data show very clearly that CD4+ T cells can vary in their *in vitro* and *in vivo* functions during long-term culture but at present it is not yet possible to correlate individual functional changes with the observed effects.

Discussion

Influenza-specific CD4+ T cell clones, described above, early after selection produced IL-2, IL-3, and γ-IFN in response to antigen and thus belonged to the T_h1 subset (22). This subset is also considered to be inflammatory and to induce strong DTH reactions (24,25).

Our present results emphasize the difficulties that arise in the use of CD4+ T cell clones for functional studies *in vivo* since their properties *in vitro* and *in vivo* can change with time in culture The variable effects of several influenza-specific CD4+ T cell clones on lung virus replication and exacerbation of illness symptoms that we observed does not correlate with viral specificity (HA or NP) of the T cells. Not only do different clones vary in their effects, but a given clone can change with time in culture in regard to antigen-induced interleukin production, cytolytic activity and *in vivo* effector function. Our clones continued to secrete γ-IFN (not illustrated) and IL-3 on antigen contact during many weeks of culture, but IL-2 production by all clones was down-regulated *in vitro* while IL-5 production became evident with time IL-5 production by T_h1 cells has been previously reported (25). CD4+ memory T cells primed by i.n. influenza infection of mice are not cytolytic when stimulated with antigen for several days (F. Esquivel, unpublished results); however, our CD4+ T cell clones acquired cytotoxic capacity to a variable degree in tissue culture, as also reported for human T cells by Fleischer (26). In contrast, Tite *et al.* (27) reported that rNP in adjuvant induces CD4+ memory cells that become cytolytic within a few days of culture.

Since HA-specific T_h cells are virus subtype specific, our main interest concerned the A virus cross-reactive NP-specific CD4+ T cells Clone 13.3 started to accelerate viral clearance after >10 weeks *in vitro* when cytotoxicity was found but IL-2 production was not detectable. Clone 2F3 never protected—up to 18 weeks of culture it exacerbated illness symptoms and, if anything, enhanced virus replication rather than reducing it At that stage it was not cytotoxic and IL-2 production had stopped Later, 2F3 cells were able to lyse infected target cells and did not affect lung virus titres.

Preliminary analyses of lung pathology in influenza-infected mice after transfer of CD4+ clones (with Dr C. D. Mackenzie) suggest that in contrast to the rapid recovery of the epithelium seen with our CD8+ CTL clones (4), early recovery of epithelium is not evident after transfer of CD4+ clone 2F3. This clone induced a mixed cellular infiltrate, including macrophages and polymorphonuclear cells, in contrast to CD8+ clones, which induce a predominantly lymphocytic infiltrate (4).

Detailed analysis of the lung histology will be required to understand how some CD4+ T cell clones might exacerbate illness, strong inflammatory responses may well lead to enhanced lung pathology. CD4+ T cells secrete numerous mediators, new ones are stil being discovered, and with further knowledge one hopes to understand which CD4+ T cell-derived mediators would be responsible for the variation in effector function of CD4+ T cell clones after prolonged culture. Differences in the role of T_h1 and T_h2 cells have been observed, for example, in Leishmaniasis (28,29). As the role of T cell subpopulations is being analysed in more infections, it becomes clear that a given T cell subpopulation (be it CD8+ or CD4+) can exert either beneficial effects or exacerbate immunopathology and illness in different virus infections or the same infection depending on the site of infection, i e. LCMV (30,31). Quantitative differences were observed in respiratory syncytial virus infection of mice. Low numbers of transferred CD8+ T cells were helpful, while high numbers augmented pathology (32).

Our present observations indicate that CD4+ T cells are less effective in early influenza virus clearance than CD8+ T cells. Coronavirus-specific CD4+ T cell clones also were poor in viral clearance (33). In spite of migration problems, results with our CD8+ CTL clones have been far more consistent than with the CD4+ clones, presumably due to the many more mediators produced by CD4+ cells. Continuous detailed functional analyses will be required at the time of clonal transfers to define protective or deleterious effects *in vivo* by CD4+ T cells. Our results point to the fine balance that can exist between exacerbation of illness or enhanced viral clearance by virus-specific T cells.

Abbreviations

CTL	cytotoxic T cells
T_h	T-helper cells
HA, NP	haemagglutinin and nucleoprotein of influenza virus
HAU	haemagglutination units
DTH	delayed type hypersensitivity
γ-IFN	γ-interferon
RPMI/10	RPMI 1640 + 10% fetal calf serum
TdR	thymidine

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