

## Establishment and characterization of canine parvovirus-specific murine CD4<sup>+</sup> T cell clones and their use for the delineation of T cell epitopes

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Canine parvovirus (CPV)-specific T cell clones were generated by culturing lymph node cells from CPV-immunized BALB/c mice at limiting dilutions in the presence of CPV antigen and interleukin-2 (IL-2). All isolated T cell clones exhibited the cell surface phenotype Thy1<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>-</sup> and proliferated specifically in response to CPV antigen. After stimulation with CPV antigen in culture the T cell clones produced IL-2 and proliferated in the absence of exogenous IL-2. Naive mice to which CPV-specific T

cell clones had been adoptively transferred developed a CPV-specific delayed type hypersensitivity reaction upon simultaneous intracutaneous injection of CPV in their ears. The ability of recombinant viral fusion proteins, representing the VP<sub>2</sub> capsid protein of the antigenically closely related feline panleukopenia virus and of synthetic peptides derived from the amino acid sequence of the VP<sub>2</sub> of CPV, to stimulate these T cell clones enabled the identification of T cell epitopes.

### Introduction

Canine parvovirus (CPV) is a member of the autonomous replicating parvoviruses and is aetiologically associated with enteritis and myocarditis in dogs. Since the virus was discovered in 1978 (Appel *et al.*, 1979; Burtonboy *et al.*, 1979; Carmichael *et al.*, 1983; Gagnon & Povey, 1979; Osterhaus *et al.*, 1980) live attenuated vaccines and, with limited success, inactivated vaccines have been used to prevent CPV infection and disease in dogs (Pollock & Carmichael, 1982*a, b*). In pups, especially susceptible to the disease, maternally derived antibodies interfere with the replication of live attenuated vaccines.

Synthetic peptides of CPV proteins, if presented in a proper immunogenic form, might induce immunity to CPV under circumstances where conventional live attenuated vaccines or inactivated vaccines cannot (Pollock & Carmichael, 1982*a*). Knowledge about the location and structures representing B cell and T cell epitopes on the proteins of the virus will be necessary for a better understanding of immunity to CPV in the dog and possibly for the development of a new generation of CPV vaccines, like synthetic peptide, recombinant DNA and idio-type vaccines. Identification of immunodominant helper T cell antigenic determinants should facilitate the development of a highly immunogenic

carrier-free vaccine that induces T cell and B cell immunity. Although many reports have provided evidence that the specific antibody response is of major importance in the protection against infection with CPV, very little is known about the epitopes responsible for the induction of neutralizing antibodies. Studies using monoclonal antibodies have provided information about the natural variation of CPV (Parrish *et al.*, 1985) and about B cell epitopes present on the viral proteins of CPV (Surleraux *et al.*, 1987).

Although for the generation of an antibody response the activation of T helper (Th) cells is essential, the immune response to CPV mediated by T cells has so far not been studied. Since it has been demonstrated that immunodominant T cell epitopes exist and are recognized by T cell clones in association with major histocompatibility complex (MHC) molecules of different animal species (Sinigaglia *et al.*, 1988; Hurwitz *et al.*, 1984; Berzofsky *et al.*, 1988), the generation of CPV-specific mouse T cell clones may provide tools for the delineation of epitopes of CPV recognized by canine T cells.

In the present study we describe the establishment and characterization of CPV-specific mouse T cell clones and their use for the delineation of T cell epitopes using *Escherichia coli* fusion products of VP<sub>2</sub> of the antigenically closely related feline panleukopenia virus (FPV)



and of synthetic peptides resembling CPV amino acid sequences.

## Methods

**Viruses and antigens.** CPV (strain 780916) (Carmichael *et al.*, 1981) was purified by immunoaffinity chromatography (IAC) from culture supernatant of infected A-72 cells, as previously described (Rimmelzwaan *et al.*, 1987), and after dialysis against phosphate-buffered saline (PBS) was not further treated. Rabies virus (Pitman-Moore strain) and measles virus (Edmonston strain), propagated in dog kidney cells and Vero cells, respectively, were used as control antigens. Rabies virus antigen was prepared from a concentrated, purified virus suspension, inactivated with  $\beta$ -propiolactone according to standard methods (van Wezel *et al.*, 1978). Measles virus antigen was purified by density centrifugation on sucrose and inactivated by u.v. irradiation (De Vries *et al.*, 1988).

**FPV fusion proteins.** Expression of FPV capsid proteins in *E. coli* was accomplished in the plasmid ptrpLE', which contains the *trp* promoter and the  $\Delta$ 1413 deletion, which fuses the *trp* leader peptide to the *trpE* gene (Miozzari & Yanofsky, 1978). The plasmid ptrpLEFPV has the *HpaII*-*EcoRI* fragment (nucleotides 1652 to 3942; Carlson *et al.*, 1985) containing the FPV capsid protein gene inserted in frame into the *HpaII* site in the LE' gene 40 codons downstream from the ATG of the LE' gene. Deletions of various lengths of the LEFPV fusion gene were constructed by digesting ptrpLEFPV with appropriate restriction enzymes and religating the products. The FPV sequences contained in the various deletion plasmids are: 1652 to 2741 (*HaeEco* $\Delta$ ), 1652 to 2287 (*NcoEco* $\Delta$ 6) and 1652 to 1976 (*NcoEco* $\Delta$ 3). All plasmids were maintained in *E. coli* strain HB101. Synthesis of the fusion proteins was induced in the bacteria by tryptophan starvation. The culture was collected by centrifugation (2500 g for 5 min) and the pellet was resuspended in 0.01 M-Tris-HCl buffer pH 8.0 containing 0.001 M-EDTA and was disrupted by sonication for a total of 3 min in 30 s bursts. The lysate was centrifuged for 15 min at 10000 g and the pellet containing the insoluble fusion proteins was dissolved in either SDS (0.5%) or urea (8 M) in 0.01 M-Tris-HCl pH 8.0 containing 0.001 M-EDTA. The extent of induction and the purity of the fusion proteins were analysed by SDS-PAGE according to Laemmli (1970).

**Synthetic peptides.** Peptides were synthesized by the solid-phase method (Erickson & Merrifield, 1976) using polystyrene resin and T-Boc-protected amino acids. All peptides were synthesized with a cysteine residue at the carboxy terminus to facilitate coupling to a carrier protein. The following side-chain-protecting groups were used: *o*-bromobenzyloxycarbonyl for tyrosine and lysine; *o*-benzyl for threonine, serine, aspartic acid and glutamic acid; tosyl for arginine; *n*-formyl for tryptophan; and *n*-dinitrophenyl for histidine. The couplings were carried out with a 2.5- to 10-fold molar excess of the amino acid derivative and *N,N'*-dicyclohexylcarbodiimide. For asparagine and glutamine an equimolar amount of *n*-hydroxybenzotriazole was added. The peptides were cleaved from the resin with anhydrous hydrofluoric acid, extracted with ether and lyophilized.

**Immunization.** Female BALB/c mice (8 to 16 weeks of age), free from known pathogenic mouse viruses, including mouse parvoviruses, raised in the barrier maintained facilities of the Bilthoven laboratory, were immunized according to a method recently described by Ziola *et al.* (1987). Briefly, mice were injected intraperitoneally with 200 mg of cyclophosphamide (Asta-Werke) per kg mouse 2 days before immunization with antigen. Eight  $\mu$ g of IAC-purified CPV (25  $\mu$ g/ml) was mixed with 100  $\mu$ g dimethyldioctadecylammonium bromide (DDA)

(Kodak) and injected into the hind leg muscles and footpads of each mouse.

**T cell growth factor (TCGF).** Interleukin-2 (IL-2)-containing medium was prepared according to the method of Gillis *et al.* (1978). Briefly, rat splenocytes were activated with phorbol myristate acetate (Sigma) for 24 h at 37 °C and stimulated with 5  $\mu$ g/ml concanavalin A (Sigma) for 2 h. The cells were washed three times and cultured in RPMI medium supplemented with 10% foetal calf serum (FCS), antibiotics and glutamine for another 24 h. After centrifugation at 1200 g for 10 min the supernatant was collected and tested for IL-2 content using the IL-2-dependent CTLL line (Gillis *et al.*, 1978).

**Cloning of CPV-specific T cells.** Seven days after immunization mice were sacrificed and the draining lymph nodes (LN) were removed. LN cell suspensions were prepared by forcing the lymph nodes through a nylon mesh. The cells were washed in Iscove's medium containing penicillin (100 units/ml), streptomycin (100  $\mu$ g/ml) and 2 mM-glutamine. Lymphocytes were cloned directly from the suspension of the LN cell population by the limiting dilution technique. Different concentrations of cells from the LN cell suspension were incubated with  $3 \times 10^5$   $\gamma$ -irradiated (1500 rad) syngeneic spleen cells as antigen-presenting cells (APC) in Iscove's medium containing antibiotics, 2 mM-glutamine, 10% FCS (Boehringer) and 0.25  $\mu$ g IAC-purified CPV per well in 96-well round-bottomed microtitre plates (Greiner Labortechnik). Growing clones were restimulated every 7 days with IAC-purified CPV in the presence of irradiated syngeneic spleen cells. Cloned cells were cultured in the presence of 5 to 10% TCGF as a source of IL-2.

**Proliferation assay.** Four to 7 days before testing, clones were grown in the presence of  $3 \times 10^5$  irradiated spleen cells and IL-2, but in the absence of CPV. The proliferation assay was performed in 96-well round-bottomed plates (Greiner Labortechnik). Ten-thousand to 30000 cloned T cells were cultured together with  $3 \times 10^5$  irradiated syngeneic spleen cells and antigen or control antigens in Iscove's medium containing antibiotics, glutamine and 10% FCS, with or without 2% IL-2-containing culture supernatant, in a total volume of 150  $\mu$ l. As a control T cells were cultured with irradiated spleen cells without antigen. After 2 days the cells were labelled with [<sup>3</sup>H]thymidine (1  $\mu$ Ci/well) (Amersham) for 16 h and harvested on glass fibre paper with a cell harvester (Skatron). Incorporation was measured in a scintillation counter. For bulk cultures  $3 \times 10^5$  LN cells were cultured in the same medium, but without IL-2-containing culture supernatant and 8% autologous serum instead of FCS.

**Phenotypic analysis of T cell clones.** Cells were collected from culture plates by centrifugation through Lympholyte M (Cedarlane). After washing, cells were incubated with a monoclonal antibody (1  $\mu$ g/10<sup>6</sup> cells) for 60 min at 4 °C in suspension. The monoclonal antibodies anti-Thy1, 2, anti-Lyt1, anti-Lyt2 and anti-L3T4 were used (Becton Dickinson). The cells were washed in PBS containing 2% FCS and incubated with monoclonal anti-rat antibody ( $\kappa$ ) conjugated to fluorescein isothiocyanate (1  $\mu$ g/10<sup>6</sup> cells) at 4 °C. The cells were washed again in PBS supplemented with 2% FCS and resuspended in PBS with 2% FCS and 2% BSA (Boseral, Organon Teknika). Fluorescence was measured using a fluorescence-activated cell sorter (Becton Dickinson FACS systems).

**IL-2 assay.** T cells were treated as described for the proliferation assay and washed three times with medium without IL-2 before testing. Ten thousand T cells were co-cultured with  $3 \times 10^5$  irradiated spleen cells as APCs in the presence of CPV and control antigen, or without antigen for 24 h at 37 °C. Culture supernatants were tested for their ability to induce proliferation of the IL-2-dependent CTLL line. Culture supernatant of MLA 144 cells was used as a positive control.



After incubation for 16 h [ $^3\text{H}$ ]thymidine uptake was measured (Gillis *et al.*, 1978).

*Adoptive transfer of T cells and measurement of delayed type hypersensitivity (DTH).* Two days after cyclophosphamide treatment CPV-specific or control mouse T cell clones (measles virus-specific T cell clones; De Vries *et al.*, 1988) were transferred to naive recipients by intracutaneous injection of  $2 \times 10^5$  T cells together with 0.6  $\mu\text{g}$  CPV or 0.8  $\mu\text{g}$  measles virus into the right ear, or T cells alone in the left ear. Ear swelling was measured at 24 h intervals after transfer with a Mitutoyo micrometer. Swelling was expressed in  $\mu\text{m}$  as the thickness of the right ear minus the thickness of the left ear.

## Results

### Isolation and specificity of T cell clones

Lymph node cells of CPV-immunized mice were seeded at a density of  $10^4$  cells per well and stimulated with IAC-purified CPV. After 6 to 8 days 14% of the wells showed cell growth. Numbers of wells showing cell growth did not increase with prolonged incubation up to 21 days. In a second cloning procedure 16% of the wells seeded at  $3 \times 10^4$  cells per well contained proliferating cells after 8 days of culture. According to the Poisson distribution the growing T cells have a high probability ( $\geq 98\%$ ) of being monoclonal.

Eleven clones were isolated that showed proliferative responses to IAC-purified CPV, indicating their specificity for CPV antigens. These responses were antigen dose-dependent, as is shown for five clones in Fig. 1. The specificity of the T cell clones for CPV was further established using different antigen preparations in the proliferation assay. As shown in Table 1 for six of the clones, the T cell clones could not be stimulated by measles virus or rabies virus antigens in concentrations that have been shown to be optimal for the stimulation of measles virus- and rabies virus-specific T cell clones, respectively (H. Bunschoten *et al.*, unpublished; De Vries *et al.*, 1988). Furthermore, the culture supernatant of non-infected A-72 cells, which were used to propagate CPV, was not able to induce proliferation, whereas culture supernatant of CPV-infected A-72 cells did show stimulation of T cell clones. Stimulation of rabies virus- or measles virus-specific T cell clones using IAC-purified CPV as an antigen did not result in proliferation of the clones (data not shown).

All T cell clones were found to exhibit the phenotype  $\text{Thy1}^+$ ,  $\text{Lyt1}^+$ ,  $\text{CD4}^+$ ,  $\text{CD8}^-$ , as demonstrated by immunofluorescence (data not shown).

### Determination of fine specificity of the CPV-specific T cell clones

In order to study whether the T cell clones specifically recognized sequences of  $\text{VP}_2$  of CPV, the capacity of *E.*

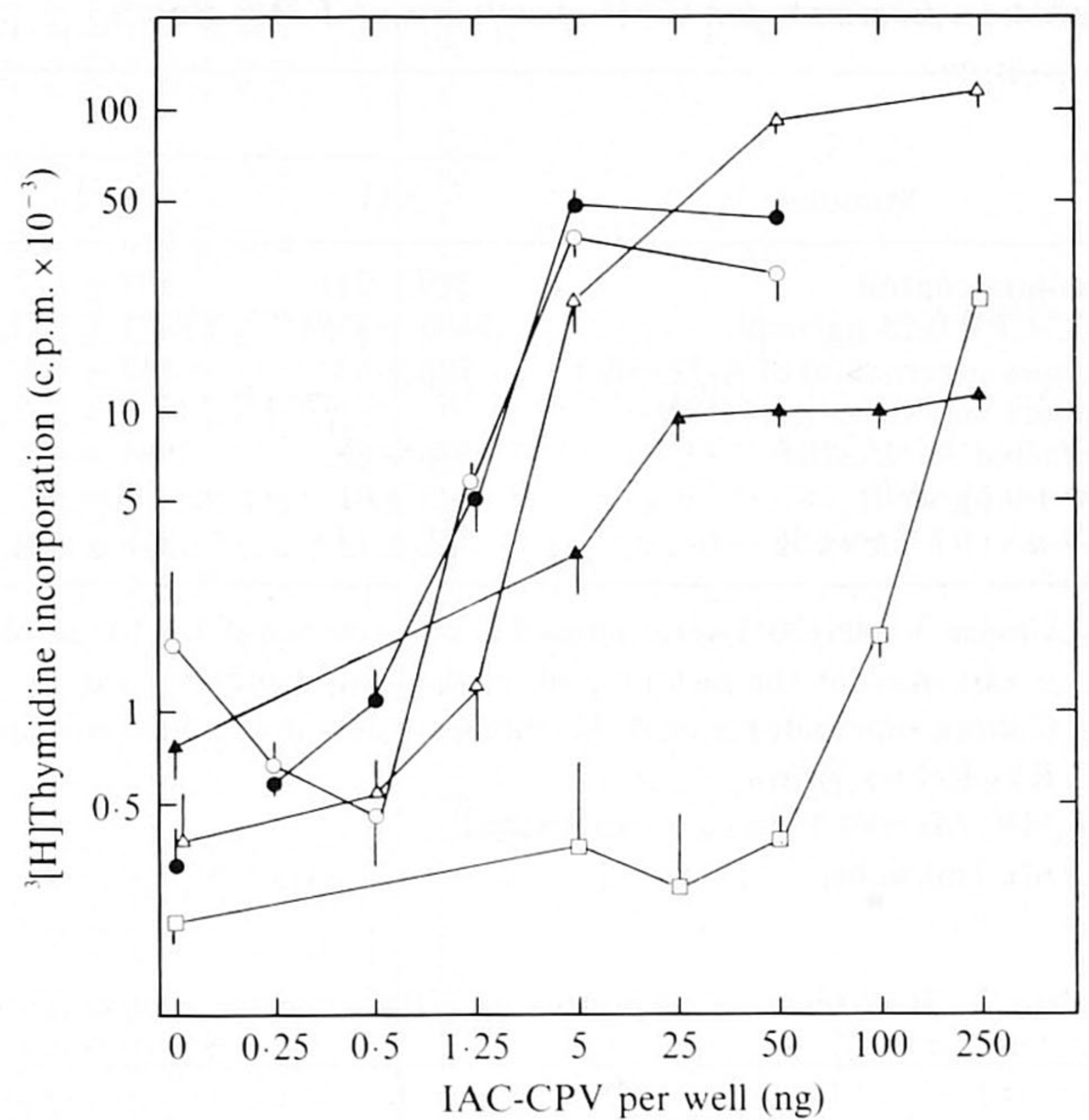


Fig. 1. Dose-dependent proliferation of CPV-reactive T cell clones P2 (●), P8 (○), P29 (▲), P45 (△) and 29G (□). Cloned T cells ( $10^4$ ) were cultured with IAC-purified CPV in the presence of  $3 \times 10^5$  autologous irradiated spleen cells as APCs. Results are expressed as the mean c.p.m. of triplicate cultures  $\pm$  S.D.

*coli* fusion proteins representing different fragments of  $\text{VP}_2$  of FPV to stimulate CPV-reactive T cells was determined. As shown in Table 2, all fusion proteins tested were able to stimulate LN cells from mice immunized with CPV, indicating that T cell epitopes are present on each of these fusion products. The LN cells could not be stimulated by the product of the *E. coli* strain, which did not contain FPV sequences. Five out of six clones tested (P2, P8, P29, P35 and P45) could be stimulated by fusion protein LEFPV, representing the complete amino acid sequence of  $\text{VP}_2$  of FPV (amino acids 1 to 584). T cell clone P29 could also be stimulated by fusion protein *HaeEco* $\Delta$  (1 to 351), which did not stimulate clones P2, P8 and P45. Stimulation with *NcoEco* $\Delta$ 6 (1 to 200) and *NcoEco* $\Delta$ 3 (1 to 95) did not result in a proliferative response of any of the T cell clones.

The fine specificity of the CPV-specific T cell clones was further determined with synthetic peptides derived from the CPV sequence (Reed *et al.*, 1988). The results in Table 3 show that T cell clone P2 could be stimulated by peptide 6 (532 to 546), but not by the partially overlapping peptides 8 (522 to 536) and 9 (542 to 556), or any of the other peptides tested. T cell clones P8 and P45 could be stimulated by peptide 8 (522 to 536), but not by any of the other peptides tested, including partially overlapping peptides 10 (512 to 526) and 6 (532 to 546). T cell clone P35 was only stimulated by peptide 9 (542 to 556) and not by any of the other peptides, including



Table 1. Evidence for CPV specificity of T cell clones

Stimulus	T cell clone*					
	29G	P2	P8	P35	P29	P45
Medium control	319 ± 211	807 ± 372	298 ± 157	557 ± 154	787 ± 193	473 ± 169
IAC-CPV 0.25 µg/well	9490 ± 3586	43475 ± 12316	59807 ± 13159	7561 ± 235	11105 ± 347	81556 ± 10607
Culture supernatant of A-72 cells†	196 ± 53	262 ± 111	152 ± 23	ND	1229 ± 211	116 ± 45
Culture supernatant of CPV-infected A-72 cells†	4386 ± 85	2588 ± 431	4206 ± 157	ND	3906 ± 313	3725 ± 172
RV 1.0 µg/well‡	277 ± 91	706 ± 397	272 ± 14	ND	751 ± 276	350 ± 137
MV-u.v. 0.8 µg/well§	369 ± 173	919 ± 500	308 ± 93	ND	426 ± 136	460 ± 167

\* Cloned T cells ( $10^4$ ) were cultured in the presence of  $3 \times 10^5$  autologous irradiated spleen cells as APCs and stimulated as indicated. Proliferation is expressed as the mean c.p.m. of triplicate cultures ± s.d.

† Culture supernatants of A-72 cells were added at a final dilution of 1:15.

‡ RV, Rabies virus.

§ MV, Measles virus, u.v.-inactivated.

|| ND, Not done.

Table 2. Proliferative responses of CPV-reactive mouse T cell clones and LN cells to ptrpLEFPV fusion proteins

Proteins expressed by	Amino acid residue of VP <sub>2</sub> expressed	T cell clone*						LN cells†
		29G	P2	P8	P35	P45	P29	
ptrpLE	—	1591 ± 243‡	2124 ± 350	1524 ± 69	1934 ± 485	1926 ± 342	857 ± 160	2306 ± 62
ptrpLEFPV	1-584	1814 ± 386	23649 ± 2817	72108 ± 7334	3844 ± 294	28666 ± 1182	7254 ± 428	22462 ± 2826
HaeEco	1-351	1815 ± 313	2245 ± 569	1487 ± 360	1764 ± 281	2403 ± 338	3407 ± 484	16386 ± 1815
NcoEcoΔ6	1-200	1549 ± 151	2283 ± 26	1536 ± 23	1976 ± 224	2186 ± 43	1395 ± 366	13326 ± 651
NcoEcoΔ3	1-95	1788 ± 262	1980 ± 66	1336 ± 25	1941 ± 385	2000 ± 231	962 ± 136	13969 ± 2432

\* Cloned T cells ( $10^4$ ) were cultured with fusion proteins in the presence of  $3 \times 10^5$  autologous irradiated spleen cells as APCs.

† LN cells ( $10^5$ ) from CPV/DDA-immunized mice were cultured per well.

‡ Fusion protein (2 µg) was added per well. Proliferation is expressed as the mean c.p.m. of triplicate cultures ± s.d.

peptides 6 (532 to 546) and 12 (522 to 561), which partially overlapped with peptide 9 (542 to 556). The proliferative responses of the clones to the respective peptides were similar to the responses observed using CPV as a stimulating antigen. These results were obtained in two independent experiments.

#### Functional analysis of T cell clones

After stimulation with IAC-purified CPV all T cell clones produced IL-2, as shown in Table 4. When T cells were grown in the absence of CPV, or with control antigen, no IL-2 production could be detected. We also tested these clones for their ability to proliferate in the absence of IL-2-containing culture supernatant. In all cases the cells showed CPV-induced proliferation and addition of exogenous IL-2 did not enhance their proliferative responses (not shown).

Simultaneous subcutaneous injection of purified CPV with T cell clones P2 and 29G into the ears of syngeneic mice induced a strong DTH response, histologically characterized by infiltration of mononuclear cells at the site of inflammation (not shown). This response was

significantly more pronounced than the response obtained when the T cell clones were inoculated in the presence of measles virus control antigen (Fig. 2). In the same experiment the measles virus-specific T cell clone 20Fl was shown to induce ear swelling in the presence of measles virus antigen, but not in the presence of CPV.

#### Discussion

In the present study CPV-specific CD4<sup>+</sup> mouse T cell clones were isolated from the lymph nodes of mice inoculated with CPV and DDA after pretreatment with cyclophosphamide, according to a method previously described (Smith & Ziola, 1986; Ziola *et al.*, 1987). This method was chosen since it requires only small amounts of viral antigen for the induction of antigen-specific T cell responses (Smith & Ziola, 1986; Ziola *et al.*, 1987). It should be realized that this protocol introduces a bias towards the generation of predominantly CD4<sup>+</sup> T cells. The antigen-induced IL-2 production and the induction of a DTH response upon transfer of CPV-specific T cell clones P2 and 29G on challenge with CPV, indicate that



Table 3. Proliferative responses of CPV-reactive mouse T cell clones to synthetic peptides of CPV VP<sub>2</sub>\*

Peptide Positions	Sequence	Clone P2			Clone P8			Clone P35			Clone P45		
		10 µg/well	5 µg/well	2 µg/well	10 µg/well	5 µg/well	2 µg/well	10 µg/well	2.5 µg/well	0.6 µg/well	10 µg/well	5 µg/well	2 µg/well
17	1-11 MSDGAVQPDGG	1.1 ± 0.7	1.4 ± 1.4	0.6 ± 0.7	0.2 ± 0.3	0.4 ± 0.4	0.2 ± 0.1	NT†	NT	NT	0.6 ± 0.6	0.4 ± 0.1	0.5 ± 0.2
18	8-20 PDGGQPAVRNERA	1.1 ± 0.5	1.0 ± 0.4	0.6 ± 0.3	0.3 ± 0.3	0.2 ± 0.0	0.2 ± 0.1	NT	NT	NT	0.6 ± 0.3	0.2 ± 0.1	0.8 ± 0.5
19	41-55 STGTFNNQTEFKFLE	1.2 ± 1.6	0.7 ± 0.2	0.8 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.2	NT	NT	NT	0.3 ± 0.4	0.2 ± 0.2	0.4 ± 0.2
20	60-72 EITANSSRLVHLN	0.9 ± 0.8	2.6 ± 2.3	1.1 ± 0.4	0.1 ± 0.1	1.1 ± 1.1	0.4 ± 0.3	NT	NT	NT	0.1 ± 0.1	0.7 ± 0.2	0.5 ± 0.2
131	147-163 NVVLKTVSESATQPPTK	1.0 ± 0.3	0.5 ± 0.4	NT	0.2 ± 0.1	0.2 ± 0.2	NT	NT	NT	NT	0.2 ± 0.0	0.4 ± 0.2	NT
135	189-202 AMRSETLGFYPWKP	1.0 ± 0.8	0.4 ± 0.1	NT	0.3 ± 0.1	0.2 ± 0.1	NT	NT	NT	NT	0.1 ± 0.0	0.2 ± 0.0	NT
136	297-317 SEGATNFGDIGVQQDKRRGVT	0.8 ± 0.2	0.6 ± 0.4	NT	0.3 ± 0.1	0.2 ± 0.0	NT	NT	NT	NT	0.2 ± 0.1	0.4 ± 0.3	NT
3	368-382 ENQAADGDPYAFGR	1.5 ± 1.0	0.8 ± 0.0	0.9 ± 0.6	0.3 ± 0.2	0.4 ± 0.3	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.1 ± 0.0	0.5 ± 0.5	0.8 ± 0.5	0.1 ± 0.0
2	385-398 GQKTTTGETPERF	0.8 ± 1.1	1.3 ± 0.9	0.5 ± 0.6	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.4 ± 0.0	0.5 ± 0.5	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.2
4	426-441 NDNVLLPTDPIGKKTG	0.5 ± 0.4	0.7 ± 0.3	1.4 ± 1.2	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	0.1 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.3 ± 0.2	0.3 ± 0.2
11	441-457 GINYTNIFNTYGPPLTAL	0.4 ± 0.3	0.2 ± 0.1	0.7 ± 0.3	0.5 ± 0.2	0.2 ± 0.1	0.3 ± 0.1	0.6 ± 0.4	0.7 ± 0.6	0.5 ± 0.1	0.6 ± 0.1	0.2 ± 0.1	0.5 ± 0.2
129	441-469 GINYTNIFNTYGPPLTALNNVPPVYPNGQI	0.6 ± 0.2	0.4 ± 0.0	NT	0.3 ± 0.2	0.4 ± 0.2	NT	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	NT
7	486-515 APFVCQNNCPGQLFVKVAPNLTNEYDPDAS	0.8 ± 0.3	0.9 ± 0.4	0.5 ± 0.3	0.3 ± 0.2	0.5 ± 0.2	0.2 ± 0.1	NT	NT	NT	0.8 ± 0.9	0.1 ± 0.1	1.1 ± 1.4
13	501-516 KVAPNLTNEYDPDASA	0.6 ± 0.7	1.5 ± 1.3	0.4 ± 0.4	0.5 ± 0.3	0.3 ± 0.2	0.2 ± 0.2	0.2 ± 0.1	0.8 ± 0.2	0.2 ± 0.1	0.6 ± 0.4	0.3 ± 0.1	0.3 ± 0.1
10	512-526 PDASANMSRIVTYS	1.6 ± 1.3	2.0 ± 1.6	1.5 ± 1.5	0.6 ± 0.4	0.3 ± 0.2	0.5 ± 0.1	0.5 ± 0.5	0.3 ± 0.2	0.1 ± 0.0	0.5 ± 0.4	0.4 ± 0.1	0.9 ± 0.7
8	522-536 VTYSDFWVKGLVFK	1.0 ± 0.5	0.9 ± 0.8	1.0 ± 0.2	7.1 ± 1.1	15.9 ± 3.0	24.5 ± 4.4	0.8 ± 0.2	0.5 ± 0.3	0.8 ± 0.2	9.0 ± 1.1	23.2 ± 2.2	35.4 ± 3.5
6	532-546 KLVFKAKLRASHTWN	111.4 ± 12.2	85.9 ± 4.4	34.4 ± 2.9	0.3 ± 0.1	0.5 ± 0.5	0.5 ± 0.5	0.5 ± 0.5	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.2	0.4 ± 0.3	0.8 ± 0.6
9	542-556 SHTWNPIQQMSINVD	0.9 ± 0.7	0.7 ± 0.3	1.5 ± 0.9	0.3 ± 0.0	0.3 ± 0.4	0.3 ± 0.1	7.7 ± 0.4	6.5 ± 0.2	3.6 ± 0.8	0.6 ± 0.3	0.4 ± 0.1	0.3 ± 0.1
12	552-561 SINVDNQFN	1.2 ± 0.8	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.2 ± 0.0	0.2 ± 0.1	0.4 ± 0.2	0.6 ± 0.7	0.3 ± 0.1	0.2 ± 0.0	0.1 ± 0.1	0.1 ± 0.0
117	570-584 KIVYEKSQLAPRKLY	0.7 ± 0.3	0.8 ± 0.9	0.2 ± 0.1	1.1 ± 1.4	0.5 ± 0.6	0.3 ± 0.3	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.7 ± 0.1	0.1 ± 0.0
CPV 1-584		80.4 ± 2.7			38.7 ± 0.9			7.6 ± 0.3			12.7 ± 1.7		
Medium control		0.8 ± 0.4			0.3 ± 0.2			0.5 ± 0.3			0.3 ± 0.1		

\* Cloned T cells (10<sup>4</sup>) were cultured in the presence of 3 × 10<sup>5</sup> autologous irradiated spleen cells as APCs and stimulated with synthetic peptides as indicated. Proliferation is expressed as the mean c.p.m. × 10<sup>-3</sup> of triplicate cultures ± s.d.

† NT, Not tested.



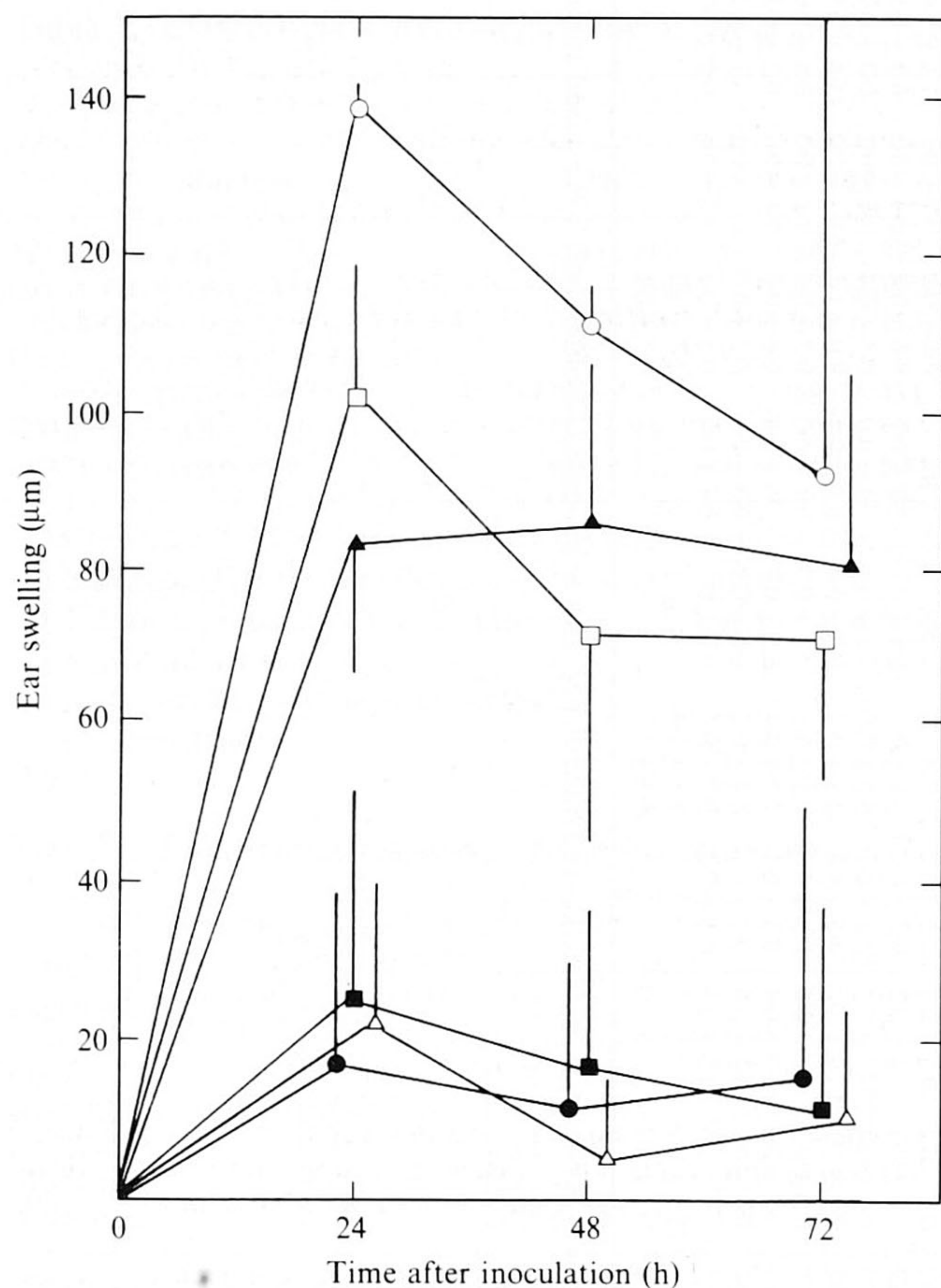


Fig. 2. Transfer of DTH by antigen-specific T cell clones. A mouse ear swelling test was used to assay DTH reaction induced by CPV-specific T cell clones P2 (□) and 29G (○) and measles virus-specific T cell clone 20F1 (△), when inoculated into the right ear with 0.6 µg IAC-purified CPV (open symbols) or 0.8 µg measles virus (closed symbols). Results are expressed as the thickness of the right ear minus thickness of the left ear, which only received T cells, and represent the mean of three mice  $\pm$  s.d.

Table 4. *IL-2 production by CPV-specific mouse T cell clones in response to stimulation with antigen\**

Clone	Stimulus	
	Medium control	IAC-CPV 0.25 µg/well
29G	174 $\pm$ 53	5861 $\pm$ 100
P2	116 $\pm$ 8	89607 $\pm$ 9964
P8	1751 $\pm$ 318	95062 $\pm$ 8412
P29	744 $\pm$ 21	75721 $\pm$ 4345
P35	442 $\pm$ 57	38817 $\pm$ 1168
P45	1560 $\pm$ 632	67385 $\pm$ 2499
Medium	280 $\pm$ 122	
Supernatant of MLA 144 cells	93125 $\pm$ 10221	

\* CPV-specific T cells ( $10^4$ ) were cultured in the presence of  $3 \times 10^5$  autologous irradiated spleen cells and 0.25 µg IAC-purified CPV per well. Supernatants were harvested after 24 h and subsequently tested at a 1:1 dilution for induction of proliferation of an IL-2-sensitive cell line (CTTL). Results are expressed as the mean c.p.m.  $\pm$  s.d. of triplicate cultures of the CTTL cells.

the T cell clones belong to the Th1 subset of Th cells (Cher & Mosmann, 1987; Mosmann *et al.*, 1986).

Although it has been suggested that T cells belonging to this subset of inflammatory Th1 cells are not able to provide help for a specific antibody response (Bottomly, 1988), recent experiments have demonstrated that B cells can conjugate to either type of Th cell (Sanders *et al.*, 1988). Furthermore, it has been demonstrated that Th1 clones may also function as classical Th cells able to provide help for a specific antibody response (De Kruyff *et al.*, 1989) (for review see Mosmann & Coffman, 1989). For the initial determination of CPV specificity of the T cell clones, IAC-purified CPV preparations were used. This procedure yields highly purified CPV preparations, as demonstrated by electron microscopy and analysis by SDS gel electrophoresis (Rimmelzwaan *et al.*, 1987). Induction of a dose-dependent proliferative T cell response by IAC-purified CPV was a strong indication that the clones were CPV-specific. This specificity was further confirmed by showing that the T cell clones could be stimulated by culture supernatant of CPV-infected A-72 cells and not by culture supernatant of non-infected A-72 cells, measles virus or rabies virus antigens, which were used as control antigens.

In a first attempt to locate T cell epitopes on VP<sub>2</sub> of CPV we used bacterial fusion products of VP<sub>2</sub> of FPV, a virus antigenically closely related to CPV, with an amino acid homology of 99% for the capsid genes (Reed *et al.*, 1988). VP<sub>2</sub> was chosen since the viral capsid consists predominantly of VP<sub>2</sub> (85%), which contains the whole amino acid sequence of VP<sub>3</sub> and most of the amino acid sequence of VP<sub>1</sub>. Based on the reactivity of the T cell clones and LN cells from CPV-immunized mice, at least three different regions containing Th cell epitopes could be identified. The C-terminal region within amino acid sequence 352 to 584 was recognized by T cell clones P2, P8, P35 and P45. These clones only proliferated upon stimulation with the fusion protein LEFPV, representing the whole amino acid sequence of FPV, but not with fusion proteins representing smaller parts of VP<sub>2</sub>. T cell clone P29 responded to stimulation with fusion proteins LEFPV and *HaeEco*Δ (amino acids 1 to 351), but not to stimulation with *NcoEco*Δ6 (1 to 200) or *NcoEco*Δ3 (1 to 95), indicating that an epitope within region 200 to 351 is recognized by clone P29. T cell clone 29G did not respond to any of the fusion proteins tested, which may indicate that clone 29G recognized an epitope present on VP<sub>1</sub> and not on VP<sub>2</sub>, or that the epitope recognized is present only on CPV and not on FPV. Alternatively, it cannot be excluded that higher concentrations of antigen are required to stimulate this clone and, due to toxicity, these could not be met by adding bacterial fusion protein. LN cells from CPV-immunized mice responded to stimulation with all fusion proteins tested, suggesting



that a T cell epitope is present in the amino-terminal amino acid sequence 1 to 95. From this study it is not clear whether sequence 96 to 200 contains T cell epitopes. The level of the response of the LN cells to LEFPV compared with the response to other fusion proteins may confirm that T cell epitopes are present in the amino acid sequence 352 to 584. In order to map T cell epitopes of CPV more precisely, synthetic peptides were used to stimulate CPV-specific T cell clones. Within the sequence 522 to 556 of VP<sub>2</sub> three distinct epitopes could be identified, using three partially overlapping synthetic peptides, peptide 8 (522 to 536), peptide 6 (532 to 546) and peptide 9 (542 to 556), each recognized by distinct T cell clones. It has been shown that T cell epitopes in proteins are likely to be located in sequences of high amphipathicity (Berzofsky *et al.*, 1986; Margalit *et al.*, 1987). Plotting of the amphipathicity values of VP<sub>2</sub> of CPV indeed provided evidence that regions of high amphipathicity are present in the sequence of peptides 8, 6 and 9 (data not shown). Although we realize that different species express different T cell repertoires and that T cell epitopes that have been found to be immunogenic in mice may not be immunogenic in dogs, several examples exist of inter-species cross-reactive epitopes for Th cells. Such epitopes have been identified in the gp120 envelope proteins of human immunodeficiency virus (Berzofsky *et al.*, 1988), influenza virus haemagglutinin (Hurwitz *et al.*, 1984) and the circumsporozoite protein of the malaria parasite *Plasmodium falciparum* (Sinigaglia *et al.*, 1988). Preliminary experiments indicate that peptides 8, 6 and 9 may represent immunodominant T cell epitopes in dogs. These peptides were used to stimulate peripheral blood mononuclear cells from nine CPV-immune dogs *in vitro*. Five of these dogs showed a strong proliferative response *in vitro* after stimulation with peptides 8, 6 and 9 (G. F. Rimmelzwaan *et al.*, unpublished observations). Experiments are now being conducted to examine these responses at the clonal level. The identification of immunodominant T cell epitopes in dogs may contribute to the construction of a carrier-free synthetic peptide vaccine.

The authors wish to thank Ms C. Kruyssen and Ms M. Eskens for preparing the manuscript. This work was supported by 'De Bond tot Bescherming van Honden', The Hague, The Netherlands.

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(Received 27 September 1989; Accepted 10 January 1990)