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Delineation of canine parvovirus T cell epitopes with peripheral blood mononuclear cells and T cell clones from immunized dogs

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Three synthetic peptides derived from the amino acid sequence of VP_2 of canine parvovirus (CPV) which were recently shown to represent three distinct T cell epitopes for BALB/c mice could prime BALB/c mice for a CPV-specific proliferative T cell response upon immunization. Proliferative responses of peripheral blood mononuclear cells (PBMC) from CPV-immunized dogs upon stimulation with these and other peptides, covering the major part of the sequence of VP_2', identified the presence of T cell epitopes for this species. Most of these epitopes were recognized by PBMC from only a minority of the dogs tested. With three newly generated canine $Thyl^+$ T cell clones, which recognized CPV antigen in association with major histocompatibility complex class II molecules, two distinct T cell epitopes were identified within the unique sequence of VP_1.

Introduction

Canine parvovirus (CPV) is a member of the autonomously replicating viruses of the Parvoviridae family and may be associated with infectious diarrhoea and myocarditis in dogs (Appel *et al.*, 1979; Burtonboy *et al.*, 1979; Gagnon & Povey, 1979; Osterhaus *et al.*, 1980). The CPV capsid consists of three related proteins, which differ in size and are the product of one single gene. The three viral proteins that can be distinguished are designated VP_1, VP_2' and VP_2. VP_2' is a protein consisting of 584 amino acids (Rhode, 1985; Reed *et al.*, 1988; Parrish *et al.*, 1988). VP_1 is a protein with 143 additional amino acids at the N terminus of VP_2' and VP_2 is the result of a proteolytic cleavage of VP_2' (Paradiso *et al.*, 1982). Since the virus was discovered in 1978, live attenuated and inactivated vaccines, based on CPV or the closely related feline panleukopenia virus (FPV), have been used to prevent CPV infection and disease in dogs (for a review see Appel & Parrish, 1987). Although live vaccines based on CPV have been especially successful, problems with vaccine failure in puppies have been attributed to interference by maternally derived antibodies.

For a better understanding of the induction and the regulation of the canine immune response to CPV, the identification of B and T cell epitopes of the virus is of major importance. It is generally accepted that virus neutralizing (VN) antibodies play a major role in the protection against CPV infection (Pollock & Carmichael, 1982; Meunier *et al.*, 1985). Several studies to define the number of VN-inducing B cell epitopes of CPV have been conducted with monoclonal antibodies but limited information is available at present on their structural features and location (Parrish & Carmichael, 1983; Parrish *et al.*, 1985; Rimmelzwaan *et al.*, 1987; Surleraux *et al.*, 1987).

Since virus-specific T helper (Th) cells should be considered to play an essential role in the regulation of the antibody response to CPV, we have focused not only on the delineation of B cell epitopes, but also on the identification and localization of Th cell epitopes in our studies on the immune response to CPV. We have recently identified three T cell epitopes within the amino acid sequence of VP_2 of CPV, using synthetic peptides and CPV-specific murine Th cell clones (Rimmelzwaan *et al.*, 1990).

In the present paper we have extended these studies by showing that a CPV-specific T cell response can be induced in mice with peptides representing these epitopes. In addition we have tested these and other peptides derived from the VP_2' and/or VP_1 sequence *in vitro*, for their ability to stimulate peripheral blood mononuclear cells (PBMC) or CPV-specific T cell clones derived from CPV-immunized dogs. Part of these studies was carried out with the newly developed pepscan

method, using series of partially overlapping synthetic peptides. It was shown that, among others, the three epitopes recognized by murine Th cells on VP_2 also stimulated canine PBMC to proliferative responses. Finally, two canine T cell epitopes were identified within the unique sequence of VP_1, by showing that canine CPV-specific T cell clones could be stimulated by peptides representing these epitopes.

Methods

Virus preparations. CPV (strain 780916) (Carmichael *et al.*, 1981) was propagated in canine A-72 cells (Binn *et al.*, 1980) as described previously (Rimmelzwaan *et al.*, 1987). Culture supernatant of CPV-infected (haemagglutination titre 1024) or non-infected cultures were used as antigen for stimulation (Rimmelzwaan *et al.*, 1990). CPV was purified by immunoaffinity chromatography (IAC) from culture supernatant of infected A-72 cells and inactivated with β-propiolactone as described previously (Rimmelzwaan *et al.*, 1987; Van Wezel *et al.*, 1978).

Fusion proteins and synthetic peptides from the VP_1 amino acid sequences. The preparation of fusion proteins of VP_2' of FPV has been described previously (Carlson *et al.*, 1985; Rimmelzwaan *et al.*, 1990). The synthesis of peptides from the amino acid sequences of CPV (Rhode *et al.*, 1985; Parrish *et al.*, 1988; Reed *et al.*, 1988) and FPV (Carlson *et al.*, 1985) according to the solid-phase method (Erickson & Merrifield, 1976) has been described (Rimmelzwaan *et al.*, 1990). All available products from the sequence of VP_1 and VP_2' are listed in Table 1.

Table 1. *Peptides derived from the amino acid sequence of the structural proteins of CPV and/or FPV used for the stimulation of canine or murine lymphoid cells*

Peptide	VP_2' positions	Sequence
17	1–11	MSDGAVQPDGG
18	8–20	PDGGQPAVRNERA
19	41–55	STGTFNNQTEFKFLE
20	60–72	EITANSSRLVHLN
21*	71–83	LNMPESENYKRVV
22*	85–94	NNMDKTAVKG
133*	96–116	MALDDTHVQIVTPWSLVDANA
131	147–163	NVVLKTVSESATQPPTK
135	189–202	AMRSETLGFYPWKP
136	297–317	SEGATNFGDIGVQQDKRRGVT
161*	316–338	VTQMGNTDYITEATIMRPAEVGY
3	368–382	ENQAADGDPRYAFGR
2	385–389	GQKTTTTGETPERF
15*	411–425	AGDWIQNINFNLPVT
4	426–441	NDNVLLPTDPIGGKTG
11	441–457	GINYTNIFNTYGPLTAL
129	441–469	GINYTNIFNTYGPLTALNNVPPVYPNGQI
7	486–515	APFVCQNNCPGQLFVKVAPNLTNEYDPDAS
13	501–516	KVAPNLTNEYDPDASA
10	512–526	PDASANMSRIVTYSD
8†	522–536	VTYSDFWWKGKLVFK
6†	532–546	KLVFKAKLRASHTWN
9†	542–556	SHTWNPIQQMSINVD
12	552–561	SINVDNQFNY
117	570–584	KIVYEKSQLAPRKLY
ptrpLEFPV‡	1–584	
HaeEco‡	1–351	
*NcoEco*Δ6‡	1–200	
*NcoEco*Δ3‡	1–95	

Peptide	VP_1 positions	Sequence
207	35–52	SDAAAKEHDEAYAAYLRS
208	79–94	GKIGHYFFRAKKAIAP
212	116–133	KPPPHIFINLAKKKKAGA

* Peptides were synthesized on the basis of the amino acid sequence of VP_2' of FPV (Carlson *et al.*, 1985), resulting in five peptides which differed in one or two amino acid residues as compared to the amino acid sequence of CPV (Reed *et al.*, 1988; Rhode, 1985; Parrish *et al.*, 1988).

† Synthetic peptides that recently have been shown to represent T cell epitopes of CPV in BALB/c mice (Rimmelzwaan *et al.*, 1990).

‡ Fusion proteins of FPV (Carlson *et al.*, 1985) representing the complete or incomplete amino acid sequence of VP_2'.

Pepscan of VP_1-specific amino acid sequence. A set of 45 partially overlapping peptides was synthesized with a peptide length of 12 residues. Synthesis started at the N terminus of VP_1 of CPV, the first peptide containing amino acids (aa) 1 to 12, the second aa 4 to 15, etc. In this way the complete sequence of 143 amino acids unique to VP_1 of CPV was covered essentially according to the method described previously (Geysen *et al.*, 1984). The peptides were detached from the solid phase (van der Zee *et al.*, 1989) and tested for their ability to stimulate CPV-specific dog T cell clones in a proliferative assay (see below).

Induction of CPV-specific T cell immunity in mice. 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 methods recently described for the generation of CPV-specific murine T cell clones (Ziola *et al.*, 1987; Rimmelzwaan *et al.*, 1990). Briefly, were injected intraperitoneally with 200 mg per kg mouse of cyclophosphamide (Astawerke) 2 days before immunization with antigen. Eight μg of IAC-purified CPV or 5 μg of synthetic peptide were mixed with 100 μg dimethyl dioctadecylammonium bromide (DDA; Eastman Kodak) and injected into the hind leg muscles and footpads of each mouse. Seven days after immunization the draining lymph nodes were removed. Lymph node cell suspensions were prepared and assayed for proliferative responses to CPV and synthetic peptides as described (Rimmelzwaan *et al.*, 1990).

Results were expressed as stimulation indices (SI) which represent the ratio of the mean proliferation of triplicate cultures after stimulation to medium controls.

Proliferative assay for dog PBMC. Heparinized blood was collected from conventionally maintained, apparently healthy beagle dogs (age 2 to 8 years) that were revaccinated annually against CPV. The last vaccination had taken place between 3 and 12 months before sampling. Samples were also taken from CPV-seronegative dogs that had been kept under specific pathogen-free conditions in the animal facilities of Harlan/Olac CPB Zeist, The Netherlands.

PBMC were obtained by sedimentation of heparinized blood on Ficoll-Isopaque. PBMC were cultured in round-bottomed 96-well microtitre plates (Greiner Labor Technik) at a density of 10^5 cells per well in 150 μl Iscove's medium supplemented with 10% (v/v) pooled canine serum, 2 mM-L-glutamine, penicillin [100 units (U)/ml] streptomycin (100 μg/ml) and 10^{-5} M-2-mercaptoethanol, referred to below as culture medium. IAC-purified CPV was added in doses ranging from 60 to 300 ng/well. Synthetic peptides were added in doses ranging from 0·5 to 10 μg/well. PBMC were incubated for seven days at 37 °C and then pulse-labelled with 1 μCi of [^{3}H]thymidine for the last 16 h of culture. Cells were harvested and the incorporated [^{3}H]thymidine was measured in a scintillation counter (1205 Beta plate, LKB). Results were expressed as SI.

Cloning of canine T cells. PBMC were cultured in round-bottomed wells at a density of 10^5 cells/well in 150 μl culture medium supplemented with 0·3 μg β-propiolactone-inactivated IAC-purified CPV. After 12 days of culture, proliferating T cell blasts were cloned by limiting dilution (0·5 cell/well) in round-bottomed wells. To each well 5×10^4 irradiated (3000 rad) autologous PBMC, 0·3 μg IAC-purified CPV and 2 U recombinant interleukin 2 (IL-2) (Boehringer Mannheim) were added. Ten to 12 days later growing clones were expanded, kept at a density of 2×10^4 cells per well and restimulated with IAC-purified CPV every 10 to 12 days of culture. They were identified as T cell clones by immunofluorescence (see below).

Proliferation assays for T cell clones. Growing T cell clones were removed from culture 7 to 12 days after the last stimulation with IAC-purified CPV and washed three times. Cloned T cells (10^4) were cultured in culture medium in round-bottomed microtitre wells in the presence of 10^5 autologous irradiated (3000 rad) PBMC as antigen-presenting cells (APC), which had been incubated with the respective antigens (CPV or peptides) for 2 h at 37 °C. The T cell clones were then incubated for 4 days at 37 °C and labelled with 1 μCi of [^{3}H]thymidine during the last 16 h of culture. Cells were harvested and the radioactivity incorporated was determined in a scintillation counter (1205 Beta plate, LKB). Results were expressed as the mean c.p.m. ± S.D. of triplicate cultures.

Major histocompatibility complex (MHC) restriction of antigen recognition. Two monoclonal antibodies (MAbs) directed to human MHC class I (B1.1.G.6) and class II (7.5.10.1) antigens (both kindly provided by F. Koning, Academic Hospital, Leiden, The Netherlands) which were shown to cross-react with canine MHC molecules (Doveren *et al.*, 1985), as shown in Fig. 2(*a*), were added in appropriate dilutions to 10^5 antigen-pulsed irradiated autologous APC 2 h before co-cultivation with 10^4 cloned T cells as described above.

Proliferative response of T cell clones to antigen-bearing particles. The polypeptide specificity of T cell clones was determined according to a method described by Abou-Zeid *et al.* (1987). Cloned T cells (3×10^4) were cultured in culture medium in flat-bottomed microtitre wells in the presence of 3×10^5 autologous irradiated (3000 rad) PBMC as APC, which had been pulsed with antigen-bearing particles for 2 h at 37 °C. Antigen-bearing particles were obtained by dissolving protein bands, which had been cut from nitrocellulose membranes used in a Western blotting assay, in DMSO (20 mm²/250 μl DMSO). After 1 h incubation at room temperature an equal volume of 0·1 M-carbonate buffer pH 9·6 was added dropwise, while the suspension was shaken vigorously in a vortex mixer. The particle suspension was centrifuged at 10000 *g* for 10 min and resuspended in phosphate-buffered saline (PBS). The T cell clones were incubated for 4 days at 37 °C and then assayed for proliferative responses as described above.

Immunofluorescence. After washing with PBS supplemented with 2% foetal calf serum (FCS), cells were incubated for 60 min at 4 °C with appropriate dilutions of ascitic fluids containing MAbs directed to the Thy-1 antigen on canine T cells (MAb F3–20–7; McKenzie & Fabre 1981) (kindly provided by Dr W. A. Buurman, Biomedical Centre, Hospital St Annadal, University of Limburg, Maastricht, The Netherlands with the permission of Dr J. Fabre, Blond McIndoe Centre, East Grinstead, U.K.) or to the human MHC class II (MAb 7.5.10.1) or MHC class I (MAb B1.1.G.6) described above. The cells were subsequently washed with PBS containing 2% FCS and incubated for 1 h at 4 °C with anti-mouse IgG antibody preparation conjugated to fluorescein isothiocyanate (Becton Dickinson). The T cells were washed again and resuspended in PBS with 2% FCS and 2% bovine serum albumin (Boseral, Organon Teknika). Fluorescence was measured in a fluorescence-activated cell sorter (FACS; Becton Dickinson FACS systems).

Results

Immunogenicity of peptides representing CPV T cell epitopes for BALB/c mice

In order to investigate the immunogenicity of synthetic peptides which have recently been shown to define three T cell epitopes of CPV recognized by BALB/c mouse T cell clones (Rimmelzwaan *et al.*, 1990), cyclophosphamide-treated BALB/c mice were immunized with 5 μg of peptide mixed with DDA. After 7 days their draining lymph nodes were collected and lymph node cell proliferation assays were performed with CPV or peptides as stimulus. Peptide 8 (aa 522 to 536), peptide 6

Table 2. *Induction of T cell immunity to CPV in mice by immunization with synthetic peptides*

Stimulus	Amount (μg/well)	Proliferative response of lymph node cells from mice immunized with* Peptide 6	Peptide 8	Peptide 9
IAC-purified PVC	0·3	4.3†	6·7	2·6
	0·12	5·1	6·5	2·3
	0·06	3·6	5·5	2·2
Peptide 6	5	12·0	1·9	0·4
	2·5	15·0	2·0	0·5
	0·5	10·8	1·9	0·9
Peptide 8	5	1·1	15·3	0·5
	2·5	0·8	16·2	0·6
	0·5	1·2	13·9	0·7
Peptide 9	5	0·9	1·5	2·6
	2·5	0·8	2·4	1·9
	0·5	1·1	1·5	1·3

* Proliferation of lymph node cells *in vitro* after immunization with 5 μg of peptide mixed with DDA. Lymph node cells were cultured (10^5 per well) in the presence of different quantities of peptides or CPV as indicated.

† Results are expressed as SI (cells + peptide or CPV/cells + medium). Background proliferation of the lymph node cells from mice immunized with peptides 6, 8 or 9 was 1200 ± 486, 2405 ± 677 or 3799 ± 1053, respectively.

(aa 532 to 546) and peptide 9 (aa 542 to 556) induced not only a proliferative response to the immunizing peptide, but also a proliferative response to CPV (Table 2). Immunization with an irrelevant 15 amino acid control peptide did not result in a specific response upon stimulation with IAC-purified CPV or the peptides (not shown). This indicates that BALB/c mice can be primed with these peptides for an antiviral response.

Proliferative responses of PBMC of CPV-immune dogs to synthetic peptides derived from the VP_2' sequence

Fig. 1 summarizes the proliferative responses of PBMC from eight CPV-immune dogs to synthetic peptides derived from the sequence of VP_2'. Cells were cultured with peptide for 7 days and an SI of 2·5 or more was considered positive. The SI of the dogs tested in response to IAC-purified CPV ranged from 3·3 to 35·4. There was considerable variability in the response patterns to individual peptides. Three peptides (peptides 18, 136 and 129) did not stimulate PBMC from any of the CPV-immune dogs tested. Several other peptides had relatively low stimulating effects (peptides 17, 19, 20, 22, 131, 7 and 117) on PBMC from one or two CPV-immune dogs. No peptide could activate cells obtained from all CPV-immune dogs. However, peptide 133 activated PBMC from seven out of eight dogs tested. The highest SI were found after stimulation of PBMC of CPV-immune dogs

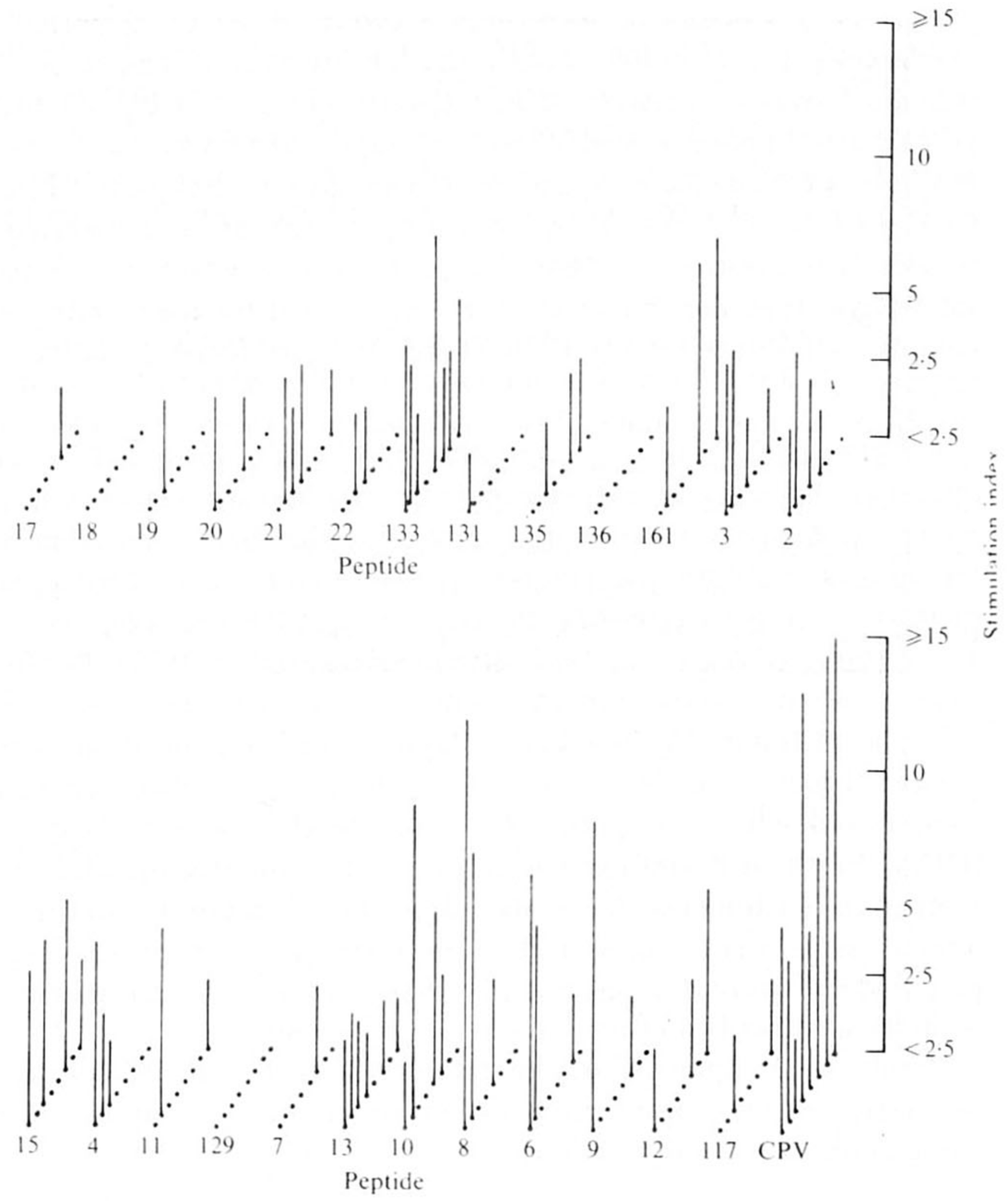

Fig. 1. Proliferative responses of PBMC from eight CPV-immunized dogs to synthetic peptides derived from the amino acid sequence of VP_2' (listed in Table 1). Each dot represents the response of one dog. PBMC (10^5) were cultured in the presence of 10 μg peptide per well or 0·3 μg IAC-purified CPV as a positive control.

with peptides 8, 6 or 9 which have recently been found to represent T cell epitopes for BALB/c mice (Rimmelzwaan *et al.*, 1990). PBMC from five out of eight dogs tested could be activated by at least one of these three peptides. PBMC from five dogs not immunized against CPV did not respond to these peptides (data not shown).

Establishment of CPV-specific canine T cell clones

In order to analyse T cell epitopes at the clonal level, a procedure to clone CPV-specific canine T lymphocytes was established. After stimulation of PBMC from two CPV-immunized dogs in bulk cultures with IAC-purified CPV to expand antigen-specific T cells, cells were seeded at a density of 0·5 cell/well and stimulated with IAC-CPV. After 12 days 6 to 20% of the wells showed cell proliferation. Three stable clones were isolated from two dogs, DM 1.1 and DM 1.2 from the first and DM 2.1 from the second dog. They were found to be CPV antigen-specific as they showed a proliferative response to culture supernatant of CPV-infected A-72 cells and IAC-purified CPV, but not to culture supernatant of non-infected A-72 cells (Table 3).

Phenotypic analysis of the T cell clones by immuno-

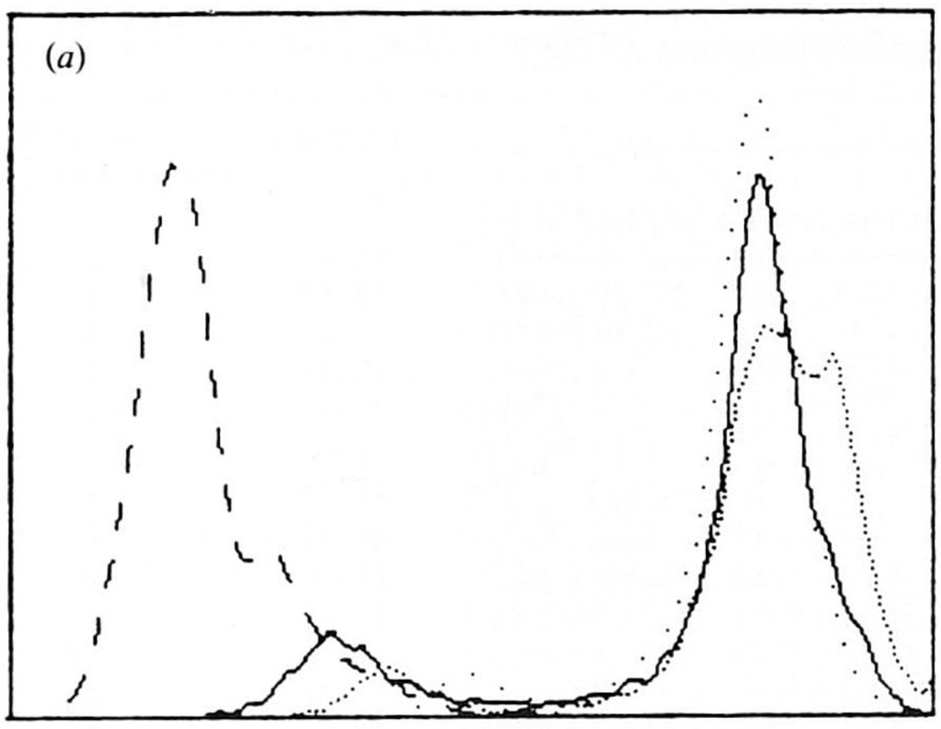

Fig. 2. FACS analysis of canine PBMC and CPV-specific T cell clones. The fluorescence patterns of PBMC of one representative dog (*a*) or of one representative T cell (*b*) clone are given. Cells were incubated with buffer (---), MHC or MAb directed to canine Thyl (——), MHC class I (. . .) or MHC class II (...) as indicated in Methods.

Table 3. *Proliferative response of canine T cell clones to CPV*

Stimulus	Clone DM 1.1*	Clone DM 1.2	Clone DM 2.1
Medium control	567 ± 150	633 ± 115	150 ± 43
Culture supernatant of control A-72 cells†	527 ± 282	595 ± 40	120 ± 49
Culture supernatant of infected A-72 cells†	2421 ± 263	2791 ± 267	1074 ± 246
IAC-purified CPV	34444 ± 3911	30385 ± 3643	15857 ± 1928

* Cloned T cells (10^4) were cultured in the presence of 10^5 autologous irradiated PBMC 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.

fluorescence showed that they were Thyl$^+$ and expressed MHC class II molecules (Fig. 2*b*), as has been described previously for resting and activated canine T cells (Doveren *et al.*, 1985). The reactivities of the monoclonal antibodies with canine PBMC were confirmed in a parallel experiment with canine PBMC (Fig. 2*a*). Proliferation of all three clones upon stimulation with IAC-purified CPV could be inhibited to more than 60% (64, 61 and 63% for clones DM 1.1, DM 1.2 and DM 2.1, respectively) by addition of the appropriate dilution of the class II-specific MAb, whereas no significant reduction (more than 5%) was observed upon the addition of the class I-specific MAb.

Determination of fine specificity of canine T cell clones

In a first attempt to localize the T cell epitopes recognized by T cell clones DM 1.1, DM 1.2 and DM 2.1, we tested the panel of synthetic peptides and FPV fusion proteins derived from the amino acid sequence of VP_2' (Table 1) for their ability to activate these clones. None of these products could stimulate any of the T cell clones to proliferate (data not shown). This could suggest either that these T cell clones recognize epitopes on CPV not present on FPV or that they recognize an epitope within the unique sequence of VP_1. The former posibility seems to be unlikely, since an amino acid sequence similarity of 99% between CPV and FPV has been described (Reed *et al.*, 1988).

Stimulation of clone DM 2.1 with VP_1 antigen-bearing particles from a nitrocellulose membrane, but not with VP_2 antigen-bearing particles, resulted in a proliferative response. This strongly indicated that indeed at least one T cell epitope was localized in the unique sequence of VP_1 (Table 4). In order to further delineate potential canine T cell epitopes within this sequence of 143 amino acids, three peptides were synthesized based on high amphipathicity values found in this sequence (data not shown), because it has been

Table 4. *Determination of polypeptide specificity of clone DM 2.1. Proliferative responses of clone DM 2.1 to CPV structural proteins**

Stimulus	Radioactivity (c.p.m.)
Particle-bound VP_1†	13669 ± 565
Particle-bound VP_2	4952 ± 347
Control particle‡	5929 ± 653
Medium	5264 ± 1244
IAC-purified CPV	56911 ± 5604

* Cloned T cells (3×10^4) were cultured in the presence of 5×10^5 irradiated autologous PBMC in flat-bottomed plates and stimulated as indicated. Proliferation is expressed as the mean c.p.m. of triplicate cultures ± S.D.

† Antigen-bearing particles were prepared as described in Methods. The estimated quantity of viral protein added to the wells was 0·1 μg.

‡ As control, nitrocellulose membrane particles devoid of any protein were used.

Table 5. *Proliferative responses of CPV-reactive dog T cell clones to synthetic peptides of CPV VP_1**

Peptide	Positions	Sequence	Amount (μg/well)	Clone DM 1.1	Clone DM 1.2	Clone DM 2.1
207	35–52	SDAAAKEHDEAYAAYLRS	5	NT†	3269 ± 934	3189 ± 1305
			2	NT	2298 ± 1362	3564 ± 1392
			1	7296 ± 4751	1097 ± 701	3677 ± 794
			0·5	7296 ± 4751	1097 ± 701	3677 ± 794
208	79–94	GKIGHYFFRAKKAIAP	5	7704 ± 1230	1774 ± 1926	2914 ± 750
			2	6772 ± 800	859 ± 332	5995 ± 3030
			1	6246 ± 3309	986 ± 515	3862 ± 477
			0·5	4511 ± 3138	480 ± 154	4036 ± 2118
212	116–133	KPPPHIFINLAKKKKAGA	5	16575 ± 367	75605 ± 11640	4180 ± 310
			2	19638 ± 1964	77062 ± 7394	5364 ± 1451
			1	17620 ± 1428	79819 ± 5408	3780 ± 1978
			0·5	21224 ± 350	92592 ± 3244	3622 ± 284
IAC-purified CPV			0·3	29959 ± 7311	29943 ± 4600	57659 ± 6311
Medium control				6972 ± 6109	2298 ± 1419	5256 ± 3258

* Cloned T cells (10^4) were cultured in the presence of 10^5 autologous irradiated PBMC as APC and stimulated with synthetic peptides as indicated.

† NT, Not tested.

shown that T cell epitopes in proteins are likely to be located within sequences of high amphipathicity (Berzofsky *et al.*, 1986; Margalit *et al.*, 1987). Clones DM 1.1 and DM 1.2 could be stimulated to a proliferative response by one of the three synthetic peptides, peptide 212, KPPPHIFINLAKKKKAGA (aa 116 to 133). T cell clone DM 2.1 did not proliferate in response to any of these three synthetic peptides (Table 5). The specificity of the clones was further analysed with the pepscan method. The sequence of 143 amino acids was covered by using 45 partially overlapping peptides. The proliferative responses of T cell clones DM 1.1, DM 1.2 and DM 2.1 induced by stimulation with these peptides are shown in Table 6. T cell clone DM 2.1 proliferated in response to peptide 20, LYFSPADQRFID (aa 58 to 69), but not to the adjacent partially overlapping peptides 19 (aa 55 to 66) or 21 (aa 61 to 72). T cell clones DM 1.1 and DM 1.2 could be stimulated to a proliferative response by peptide 40, PPHIFINLAKKK (aa 118 to 129) and the adjacent partially overlapping peptide 41, IFINLAKKKKAG (aa 121 to 132). This was in agreement with the observation that these two T cell clones also proliferated in response to stimulation with peptide 212 (aa 116 to 133).

Discussion

In the present paper we have extended previous studies in inbred BALB/c mice, for which three Th cell epitopes on VP_2 of CPV have been delineated. First we showed that with synthetic peptides representing each of these three epitopes, priming for proliferative T cell responses could be achieved. In bulk stimulations of PBMC from eight CPV-immunized dogs with these and other synthetic peptides, it was shown that T cells of a significant proportion of these dogs also recognized these three epitopes. In addition it was shown that various other peptides representing sequences of VP_2′ could induce proliferative responses *in vitro* in PBMC of certain CPV-immunized dogs. The majority of the synthetic peptides stimulated cells from only a minority of the dogs, suggesting that these responses are restricted to certain allelic forms of MHC molecules as has also been shown in various other systems (for a review see Arnon, 1987). This is probably an important problem that would arise in a peptide vaccine approach. Not all individual dogs in an outbred population will respond to any single antigenic determinant, since the allelic form of the MHC molecules strongly influences which of the peptides are recognized by the immune system. Thus in order to develop a synthetic peptide CPV vaccine, applicable to the entire dog population, individual dogs of various MHC haplotypes would have to be studied to ascertain which peptides are predominantly used as T cell epitopes. On the other hand it has been shown that certain peptides can function as T cell epitopes with several allelic forms of class II molecules (Brett *et al.*, 1989; Panina-Bordignon *et al.*, 1989). Such peptides would be of particular interest for peptide vaccine composition. Examples of such peptides may be peptides 13 or 133 which were shown to stimulate PBMC from six and seven of the eight dogs tested, respectively.

Although the stimulation of PBMC in bulk cultures with peptides is the method of choice for the assessment of the presence and immunodominance of certain T cell epitopes, for more detailed functional and mechanistic studies the use of specific T cell clones seems more

Table 6. *VP_1 pepscan peptide recognition by CPV-specific dog T cell clones**

Peptide	Positions	Sequence	Clone DM 1.1	Clone DM 1.2	Clone DM 2.1
1	1–12	MAPPAKRARRGL	0·5 ± 0·2	0·1 ± 0·1	1·7 ± 1·1
2	4–15	PAKRARRGLVPP	1·7 ± 1·9	0·4 ± 0·3	3·0 ± 0·1
3	7–18	RARRGLVPPGYK	0·5 ± 0·5	0·1 ± 0·1	0·6 ± 0·2
4	10–21	RGLVPPGYKYLG	0·6 ± 0·2	0·2 ± 0·1	1·2 ± 0·3
5	13–24	VPPGYKYLGPGN	0·4 ± 0·1	0·3 ± 0·2	2·8 ± 1·4
6	16–27	GYKYLGPGNSLD	0·4 ± 0·2	0·1 ± 0·0	2·1 ± 1·4
7	19–30	YLGPGNSLDQGE	0·2 ± 0·0	0·5 ± 0·5	0·2 ± 0·2
8	22–33	PGNSLDQGEPTN	0·1 ± 0·0	0·1 ± 0·1	2·8 ± 2·8
9	25–36	SLDQGEPTNPSD	0·3 ± 0·1	0·1 ± 0·0	1·2 ± 1·0
10	28–39	QGEPTNPSDAAA	0·4 ± 0·3	0·2 ± 0·1	1·0 ± 0·1
11	31–42	PTNPSDAAAKEH	0·3 ± 0·1	0·1 ± 0·1	1·5 ± 1·0
12	34–45	PSDAAAKEHDEA	0·6 ± 0·3	0·1 ± 0·0	1·0 ± 0·0
13	37–48	AAAKEHDEAYAA	0·2 ± 0·1	0·2 ± 0·1	1·1 ± 0·6
14	40–51	KEHDEAYAAYLR	0·1 ± 0·1	0·1 ± 0·0	0·3 ± 0·1
15	43–54	DEAYAAYLRSGK	0·1 ± 0·0	0·1 ± 0·0	1·4 ± 0·4
16	46–57	YAAYLRSGKNPY	0·2 ± 0·0	0·1 ± 0·0	0·5 ± 0·0
17	49–60	YLRSGKNPYLYF	0·2 ± 0·0	0·0 ± 0·0	0·3 ± 0·1
18	52–63	SGKNPYLYFSPA	0·1 ± 0·0	0·1 ± 0·0	0·3 ± 0·1
19	55–66	NPYLYFSPADQR	0·1 ± 0·0	0·1 ± 0·1	1·0 ± 0·5
20	58–69	LYFSPADQRFID	0·3 ± 0·0	0·1 ± 0·0	22·0 ± 6·2
21	61–72	SPADQRFIDQTK	0·2 ± 0·1	0·1 ± 0·0	1·6 ± 0·8
22	64–75	DQRFIDQTKDAK	0·3 ± 0·3	0·1 ± 0·0	1·8 ± 1·5
23	67–78	FIDQTKDAKDWG	0·3 ± 0·2	0·1 ± 0·0	1·3 ± 0·3
24	70–81	QTKDAKDWGGKI	0·1 ± 0·0	0·1 ± 0·0	1·3 ± 0·7
25	73–84	DAKDWGGKIGHY	0·5 ± 0·3	0·1 ± 0·0	1·1 ± 0·2
26	76–87	DWGGKIGHYFFR	0·1 ± 0·0	0·1 ± 0·0	0·5 ± 0·2
27	79–90	GKIGHYFFRAKK	0·1 ± 0·0	0·1 ± 0·0	1·3 ± 0·2
28	82–93	GHYFFRAKKAIA	0·1 ± 0·0	0·1 ± 0·0	0·5 ± 0·1
29	85–96	FFRAKKAIAPYL	0·5 ± 0·3	0·1 ± 0·0	1·3 ± 0·8
30	88–99	AKKAIAPYLTDT	0·1 ± 0·0	0·1 ± 0·0	1·1 ± 1·0
31	91–102	AIAPYLTDTPDH	0·1 ± 0·0	0·1 ± 0·0	0·2 ± 0·1
32	94–105	PYLTDTPDHPST	0·1 ± 0·0	0·1 ± 0·0	0·2 ± 0·0
33	97–108	TDTPDHPSTSRP	0·4 ± 0·1	0·1 ± 0·0	0·6 ± 0·2
34	100–111	PDHPSTSRPTKP	0·1 ± 0·0	0·0 ± 0·0	0·9 ± 0·5
35	103–114	PSTSRPTKPTKR	0·3 ± 0·1	0·1 ± 0·1	2·2 ± 1·3
36	106–117	SRPTKPTKRSKP	0·1 ± 0·0	0·1 ± 0·0	1·5 ± 0·1
37	109–120	TKPTKRSKPPPH	0·1 ± 0·0	0·1 ± 0·0	0·8 ± 0·3
38	112–123	TKRSKPPPHIFI	0·1 ± 0·0	0·1 ± 0·0	1·0 ± 0·2
39	115–126	SKPPPHIFINLA	0·1 ± 0·0	0·1 ± 0·0	0·8 ± 0·2
40	118–129	PPHIFINLAKKK	6·1 ± 0·5	31·0 ± 0·4	1·2 ± 0·1
41	121–132	IFINLAKKKKAG	7·8 ± 1·6	17·6 ± 2·1	2·1 ± 0·8
42	124–135	NLAKKKKAGAGQ	0·1 ± 0·1	0·1 ± 0·0	0·8 ± 0·5
43	127–138	KKKKAGAGQVKR	0·2 ± 0·1	0·1 ± 0·0	0·6 ± 0·2
44	130–141	KAGAGQVKRDNL	0·3 ± 0·1	0·1 ± 0·0	0·5 ± 0·1
45	132–143	GAGQVKRDNQAP	0·1 ± 0·1	0·1 ± 0·0	0·4 ± 0·0
IAC-purified CPV			19·3 ± 7·2	22·4 ± 0·6	53·7 ± 9·4
Medium control			1·2 ± 0·7	0·2 ± 0·0	2·1 ± 0·2

* Cloned T cells (10^4) were cultured in the presence of 10^5 autologous irradiated PBMC as APC and stimulated with synthetic peptides as indicated. Proliferation is expressed as the mean c.p.m. $\times 10^{-3}$ of triplicate cultures ± S.D.

appropriate. The generation of virus-specific canine T cell clones has not been described previously. Using IAC-purified CPV for the stimulation of PBMC of CPV-immunized dogs, we succeeded in generating three CPV-specific T cell clones from two dogs, which expressed the canine Thyl marker. Since a MAb to human MHC class II antigen, cross-reactive with canine MHC class II antigen, could inhibit the proliferative responses of these clones, we have reason to speculate that these clones belong to a $CD4^+$ subset of T cells in this species. It is generally accepted that proliferative responses of T cells bearing either CD4 or CD8 markers on their surface are restricted by MHC class II or class I molecules, respectively (Swain, 1983).

Specificity of these three clones for CPV was confirmed by showing a proliferative response of these cells to the supernatant of CPV-infected canine A-72 cells and not to the supernatant of non-infected control cells. As it could not be excluded that infectious CPV present in the supernatant of the infected A-72 cells

could have interfered with the outcome of the test results in this assay, it was demonstrated that IAC-purified CPV, which had been inactivated with β-propiolactone, also could stimulate the T cell clones to proliferation. All the synthetic peptides and recombinant fusion proteins representing sequences of VP_2' of CPV and FPV, respectively, failed to stimulate these three clones to proliferation. Because one of the fusion proteins used (ptrpLEFPV) covers the whole sequence of VP_2' of FPV and the sequence similarity between CPV and FPV is 99% (Reed *et al.*, 1988), we speculated that the most likely explanation for this failure would be that these three clones would recognize one or more epitopes within the unique 143 amino acid sequence of VP_1. The first indication of such an epitope came from experiments in which one of the clones (DM 2.1) could be stimulated with VP_1-bearing particles obtained from a nitrocellulose membrane. For further identification of T cell epitopes, three peptides were synthesized on the basis of high amphipathicity values. One of these, peptide 212, could stimulate two T cell clones (DM 1.1 and DM 1.2) to proliferation. The specificity of these three clones was further analysed in a modification of the pepscan method using a series of partially overlapping synthetic peptides (Geysen *et al.*, 1984). By this method we showed that T cell clones DM 1.1 and DM 1.2 reacted with an epitope defined by peptides 40 and 41 (aa sequence 118 to 132; PPHIFINLAKKKKAG), which is in agreement with the finding that these clones are also stimulated by peptide 212 (aa sequence 116 to 133; KPPPHIFINLAKKKKAGA). T cell clone DM 2.1 was shown to recognize an epitope defined by peptide 20 (aa sequence 58 to 69; LYFSPADQRFID) and not by the adjacent partially overlapping peptides 19 and 21. Also this is in agreement with the data obtained by stimulation with the peptides synthesized on the basis of high amphipathicity values, as the sequence of peptide 20 does not overlap any of these three peptides. Since no data about stimulation of PBMC from different dogs with these two T cell epitopes are available, their level of immunodominance in the outbred dog population is not clear at present.

Further studies identifying canine B and T cell epitopes of CPV and their immunodominance are in progress. The results may broaden our understanding of the protective immune response to CPV and eventually enable the construction of novel generations of recombinant or synthetic CPV vaccines.

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