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A synthetic peptide derived from the amino acid sequence of canine parvovirus structural proteins which defines a B cell epitope and elicits antiviral antibody in BALB c mice

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Synthetic peptides, recombinant fusion proteins and mouse monoclonal antibodies were used to delineate a B cell epitope of the VP'_2 structural protein of canine parvovirus (CPV). Although this epitope is not preferentially recognized in the normal antibody response to CPV, virus-specific antibodies could be induced in BALB/c mice with a synthetic peptide representing the epitope. The potential of this non-dominant B cell epitope to induce antiviral immunity in the presence of maternal CPV-specific antibodies, is discussed.

The capsid of canine parvovirus (CPV) is composed of three related proteins which differ in size and are the product of a single gene. These three structural viral proteins (VPs) are designated VP_1, VP'_2 and VP_2 and have M_rs of 82·5K, 67K and 63·5K, respectively. VP_1 is a protein of 727 amino acids; VP'_2 overlaps the sequence of VP_1, but lacks 143 amino acids at the N terminus of VP_1 (Fig. 1*a*); and VP_2 is the result of a proteolytic cleavage of VP'_2 (Paradiso *et al.*, 1982; Rhode, 1985; Reed *et al.*, 1988; Parrish *et al.*, 1988).

For a better understanding of the humoral and cell-mediated immune responses to CPV, and for the construction of novel generations of vaccines based on recombinant proteins or synthetic peptides, the delineation of B and T cell epitopes should be considered of major importance. Recently we described the first delineation of T cell epitopes of CPV using synthetic peptides and CPV-specific murine and canine T cell clones (Rimmelzwaan *et al.*, 1990*b*, *c*). Using anti-CPV mouse monoclonal antibodies (MAbs) it has been shown that at least two distinct antigenic sites for B cell epitopes should exist on CPV (Rimmelzwaan *et al.*, 1987; Parrish *et al.*, 1982; Parrish & Carmichael, 1983). Although it has been shown that CPV-specific MAbs can recognize conformation-dependent or conformation-independent epitopes (Surleraux *et al.*, 1987), the exact location of these B cell epitopes within the CPV structural proteins has not been identified. Of four CPV haemagglutinin-specific MAbs that also recognize feline panleukopenia virus (FPV), three were shown to react with one epitope and one MAb (MAb H-2) was shown to react with another epitope of CPV (Rimmelzwaan *et al.*, 1987). Testing of these four MAbs in Western blot analysis showed that only MAb H-2 recognized all the denatured VPs of CPV in this assay (Fig. 2). This suggested that MAb H-2 recognized a conformation-independent epitope (H-2 epitope) present on all three VPs.

To determine the location of this H-2 epitope, the reactivity of MAb H-2 was determined with a panel of previously described recombinant fusion proteins (Fig. 1*a*; Carlson *et al.*, 1985; Rimmelzwaan *et al.*, 1990*b*) in a double-antibody sandwich ELISA, essentially as previously described (Rimmelzwaan *et al.*, 1987, 1990*a*). The nomenclature of the fusion proteins used is in accordance with their original description. Microtitre plates (Titertek type III; Flow Laboratories) were coated with 250 ng of Protein A-purified MAb H-2 per well (100 µl) in 0·1 M-carbonate buffer pH 9·6 (coating buffer), and subsequently blocked with phosphate-buffered saline (PBS) containing 0·05% Tween-80 (Merck) and 1% bovine serum albumin (BSA) (Sigma) (PBS/TB). Serial twofold dilutions (100 µl) of the recombinant fusion proteins in PBS/TB were added and incubated for 1 h at 37 °C. Subsequently, the binding of the protein to the plates was detected with horseradish peroxidase (HRP)-conjugated MAb H-2. Between each step the plates were washed with demineralized water containing 0·05% Tween-80 (DWT) and finally the plates were read by determining absorbance values at 450 nm (A_{450}), after developing with tetramethyl benzidine and H_2O_2 as substrate. Immunoaffinity chromatography-purified CPV (IAC-CPV) (Rimmelzwaan *et al.*, 1987) was included as a positive control. MAb H-2 reacted with recombinant fusion proteins ptrpLEFPV [amino acids (aa) 1 to 584], *HaeEco*Δ (aa 1 to 351) and *NcoEco*Δ6 (aa 1 to 200) in a dose-dependent way, whereas no reactivity

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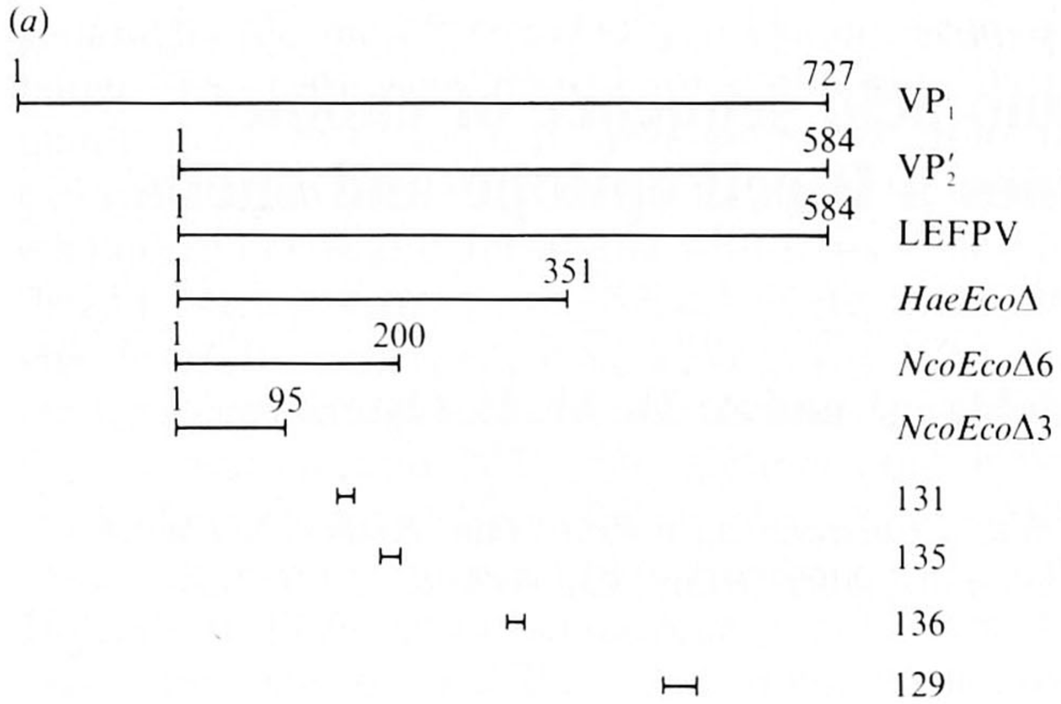

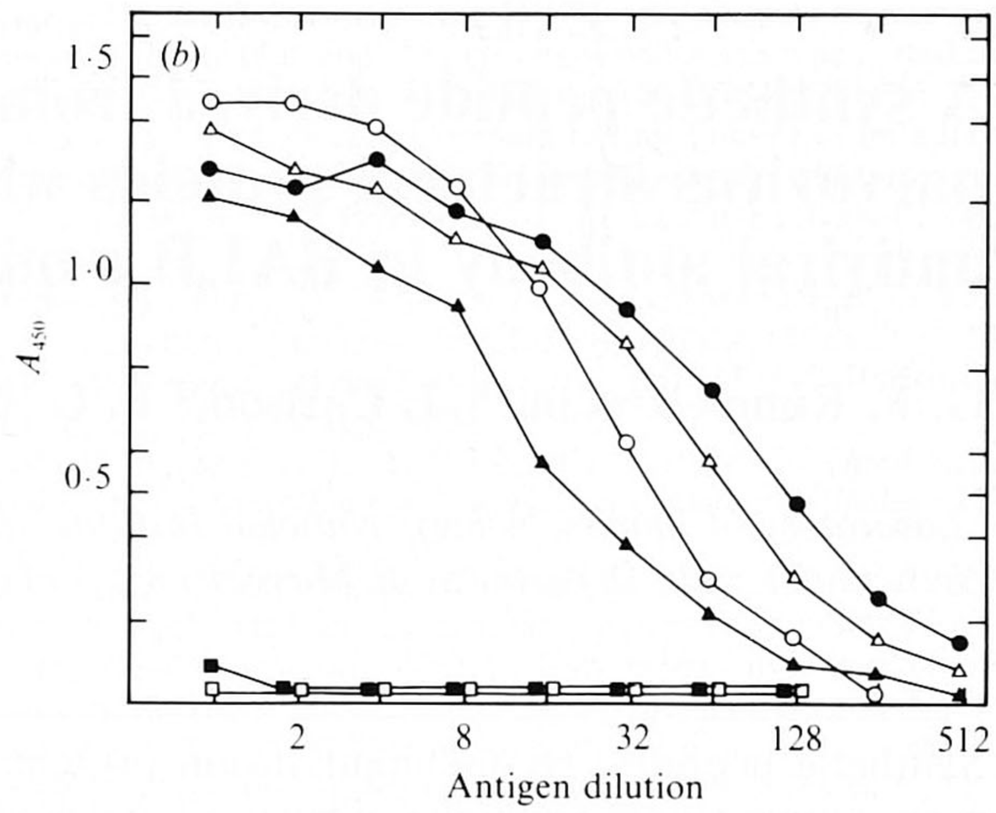

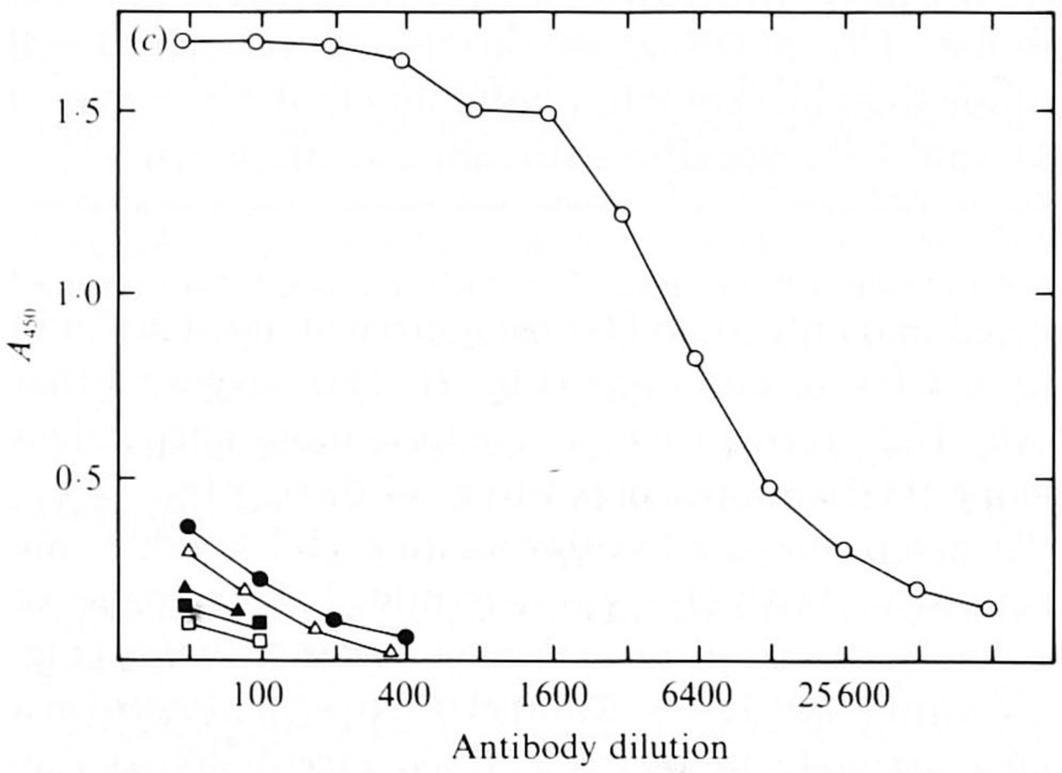

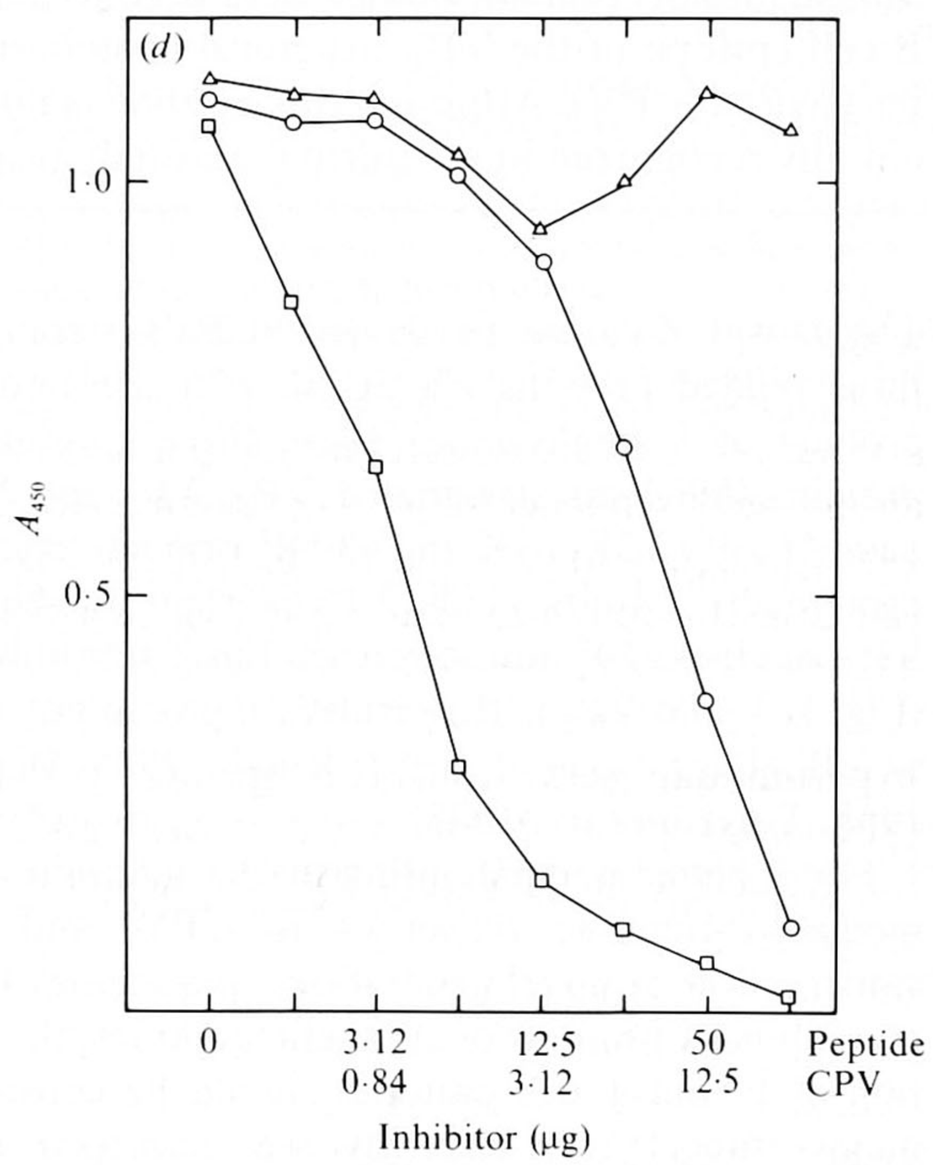

Fig. 1. (*a*) Recombinant fusion proteins and synthetic peptides used in this study. The fusion proteins ptrpLEFPV (aa 1 to 584), *HaeEco*Δ (aa 1 to 351), *NcoEco*Δ6 (aa 1 to 200) and *NcoEco*Δ3 (aa 1 to 95) represent the complete amino acid sequence of VP'_2 of FPV, or smaller fragments of it. Peptides 131 (aa 147 to 163), 135 (aa 189 to 202), 136 (aa 297 to 317), 129 (aa 441 to 469) and 117 (570 to 584) were derived from the amino acid sequence of VP'_2 of CPV. (*b*) Binding of anti-CPV MAb H-2 to fusion proteins ptrpLEFPV (●), *HaeEco*Δ (Δ), *NcoEco*Δ6 (▲), *NcoEco*Δ3 (□) and controls, bacterial protein LE (■) and IAC-CPV (○), as determined by reactivity in the DAS-ELISA. (*c*) Binding of anti-CPV MAbs or control MAb to different peptides as determined in the indirect ELISA with peptides bound to the solid phase. The reactivities of anti-CPV MAbs H-2 (○) and H-1 (□) or control MAb (△) with peptide 131 are shown. The reactivities of these MAbs to peptide 129 are indicated with closed symbols. The binding of MAb H-1 to peptides 131 and 129 was representative of the reactivities of the other anti-CPV MAbs (not shown). Binding to peptide 129 was representative of the binding to other peptides tested (not shown). (*d*) Inhibition of binding of anti-CPV MAb H-2 to solid phase-bound CPV by preincubation of MAb H-2 with different amounts of peptide 131 (○), peptide 129 (△) or IAC-CPV (□), as determined in competition ELISA.

was observed with recombinant fusion protein *NcoEco*Δ3 (aa 1 to 95) and control bacterial protein LE, which has no FPV amino acid sequences (Fig. 1*b*). The observed reactivity with the recombinant proteins is probably dependent on their aggregation because, in monomeric form, no reactivity would be expected when the same antibody is used for capturing and detecting the antigen. This suggested that the H-2 epitope was located within the amino acid sequence 95 to 200 of VP'_2.

Table 1. *Antibody titres induced in BALB/c mice by immunization with peptides*

Mouse no.	Immunized with	Detection of antibodies directed to*																						
		Peptide 131					Peptide 129					IAC-CPV							Rabies virus						
		d0†	d7	d14	d28	d35	d0	d7	d14	d28	d35	d0	d7	d14	d28	d42	d63	d70	d0	d7	d14	d28	d42	d63	d70
533	500 µg peptide	–‡	80	160	320	> 640	–	–	–	–	–	–	–	–	160	640	> 640	640	–	–	–	–	–	–	–
534	131–KLH	–	80	160	320	> 640	–	–	–	–	–	–	–	–	160	640	> 640	> 640	–	–	–	–	–	–	–
535	400 µg peptide	–	–	40	80	320	–	–	–	–	–	–	–	–	–	640	> 640	640	–	–	–	–	–	–	–
536	131–KLH	–	–	20	40	320	–	–	–	–	–	–	–	–	–	320	640	640	–	–	–	–	–	–	–
537	200 µg peptide	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	80	640	–	–	–	–	–	–	–
538	131–KLH	–	–	80	320	> 640	–	–	–	–	–	–	–	–	160	640	640	640	–	–	–	–	–	–	–
539	500 µg	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
540	control peptide–KLH	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Anti-CPV mouse serum				–					–						> 640							–			
MAb H-2				5×10^4					–						$> 10^4$							–			

* Peptide- or virus-specific antibodies were detected in an indirect ELISA using solid phase-bound peptides or virus by endpoint titration.
† Days post-immunization.
‡ –, < 20.

To delineate further the location of the H-2 epitope within this amino acid sequence, the reactivity of the four anti-CPV MAbs with a selected panel of previously synthesized peptides representing parts of the CPV amino acid sequence (Fig. 1*a*; Rimmelzwaan *et al.*, 1990*b*), i.e. peptide 131 (aa 147 to 163), peptide 135 (aa 189 to 202), peptide 136 (aa 297 to 317), peptide 129 (aa 441 to 469) and peptide 117 (aa 570 to 584), was determined in an indirect ELISA. In addition, pre- and hyperimmune sera from two specific pathogen-free (SPF) dogs, three SPF cats and 10 SPF BALB/c mice immunized with CPV or FPV [serum antibody titres ranging from 320 to 2048 in the haemagglutination inhibition (HI) assay] were tested in the indirect ELISA. In this assay, peptides were coated onto microtitre strips (Costar EIA/RIA strip plate-8) by incubating 1 µg peptide in coating buffer (100 µl) for 18 h at 20 °C. After washing the strips twice with DWT, they were blocked with PBS/TB. This buffer, supplemented with 5% NaCl, was also used as the diluent in subsequent incubation steps. After washing with DWT, twofold serial dilutions (100 µl) of MAb ascitic fluids (anti-CPV titre $> 10^4$ in HI) or the sera were incubated for 1 h at 37 °C. After washing again, HRP-conjugated goat anti-species specific IgG (Cappel, Cooper Biomedical) was incubated at the appropriate dilutions for 1 h at 37 °C. It was shown that only MAb H-2 reacted with peptide 131. This reactivity proved to be dose-dependent. None of these MAbs, including MAb H-2, reacted with any of the other peptides in this assay (Fig. 1*c*). Also, the sera from the dogs, cats and BALB/c mice failed to react with any of these peptides in these assays (not shown).

The specificity of the reactivity between MAb H-2 and peptide 131 was confirmed in a competition ELISA, which determined the inhibition of the reactivity of MAb H-2 with plate-bound IAC-CPV by preincubation with the different synthetic peptides. Microtitre plates (Titerek type III; Flow Laboratories) were coated with 50 ng IAC-CPV per well (50 µl) by incubation for 16 h at 20 °C in coating buffer. After washing with DWT, HRP-conjugated MAb H-2 was added (50 µl) at a concentration giving 75% of the maximal A_{450}, after preincubation with different amounts of the respective peptides or IAC-CPV. After incubation for 1 h at 37 °C, plates were washed and read as described above. Only preincubation with peptide 131 or IAC-CPV resulted in a significant inhibition (> 15% of the maximal A_{450} of the binding of MAb H-2. However, IAC-CPV inhibited the binding of MAb H-2 to CPV more efficiently than peptide 131, which suggests that peptide 131 does not fully mimic the H-2 epitope. This inhibition proved to be dose-dependent (Fig. 1*d*). None of these peptides inhibited the binding of the other three anti-CPV MAbs to IAC-CPV (not shown). These inhibition data were in agreement with the findings obtained with the recombinant fusion proteins of FPV and indicated that the H-2 epitope is located within the amino acid sequence between aa 147 and aa 163 of VP_2', which is identical for CPV and FPV. They also indicated that the antibody response to the H-2 epitope is not a major part of the antibody response to CPV in the three species tested and therefore the H-2 epitope may be considered a non-dominant epitope.

The observed failure of conventional vaccines to induce CPV immunity in puppies in certain cases can usually be attributed to interference by maternally derived antibodies (Pollock & Carmichael, 1982). Therefore, the use of a peptide vaccine which is based in part

on non-dominant epitopes, that would not be recognized by maternal antibodies, might overcome this problem in the immunization of young animals.

To test the validity of this concept we conducted immunization experiments with peptide 131 in BALB/c mice, in which the H-2 epitope is also not a major part of the CPV antibody response. However, because we succeeded in raising a BALB/c mouse MAb to this epitope, it can be considered immunogenic in this mouse strain under certain conditions. Six SPF BALB/c mice were immunized once with different amounts (200 to 500 μg) of peptide 131 and two were immunized with a 15 amino acid control peptide in Freund's complete adjuvant. Both peptides were conjugated to keyhole limpet haemocyanin (KLH) with glutaraldehyde before immunization. As shown in Table 1, within 14 days five out of six mice immunized with peptide 131 developed an anti-peptide 131 response as demonstrated in the indirect ELISA described above. None of these mice developed an anti-peptide 129 response. Within 63 days, all six mice immunized with peptide 131 also developed CPV-specific antibodies as demonstrated by indirect ELISA, whereas none of these developed rabies virus-specific antibodies, as measured in a similar ELISA but using rabies virus as antigen (UytdeHaag *et al.*, 1983).

The specificity of this response to CPV antigen was confirmed by showing that sera from mice with ELISA titres > 160 were also positive in the HI assay (titre $\geqslant 32$) and in the immunofluorescence assay on CPV-infected A-72 cells (titre $\geqslant 40$) (data not shown). These sera were also shown to react with the VPs of CPV in Western blot analysis (Fig. 2). Although MAb H-2, which recognizes peptide 131, does neutralize CPV, no CPV-neutralizing serum antibodies were found in any of the mice immunized with peptide 131. Also, 14 days after booster immunization of these mice with the same materials in Freund's incomplete adjuvant on day 200, no CPV-neutralizing serum antibodies could be demonstrated. This may indicate that the affinity of the antibodies elicited by peptide immunization for CPV is too low for virus neutralization, which could be because the peptide chosen does not represent the complete epitope recognized by MAb H-2.

Although lack of virus-neutralizing activity *in vitro* does not necessarily mean lack of protection against infection, it does appear that the H-2 epitope must be located more precisely, e.g. by a pepscan procedure (Geysen *et al.*, 1984) covering this region, before embarking on further immunization studies in mice and subsequently in dogs. If it does prove possible to identify a peptide structure within this region which does induce CPV-neutralizing antibodies, it would be interesting to copolymerize this peptide with other peptides representing immunodominant T cell epitopes for mice and/or dogs, as we described recently (Rimmelzwaan *et al.*, 1990*b*, *c*). This may provide an immunogenic peptide that not only induces B cell immunity but that will also allow the induction of CPV-specific T cell memory, which would facilitate a proper secondary immune response upon challenge with virulent CPV. After combining the non-dominant B cell epitope with such T cell epitopes, the next step towards a composite synthetic CPV vaccine should be the incorporation of more

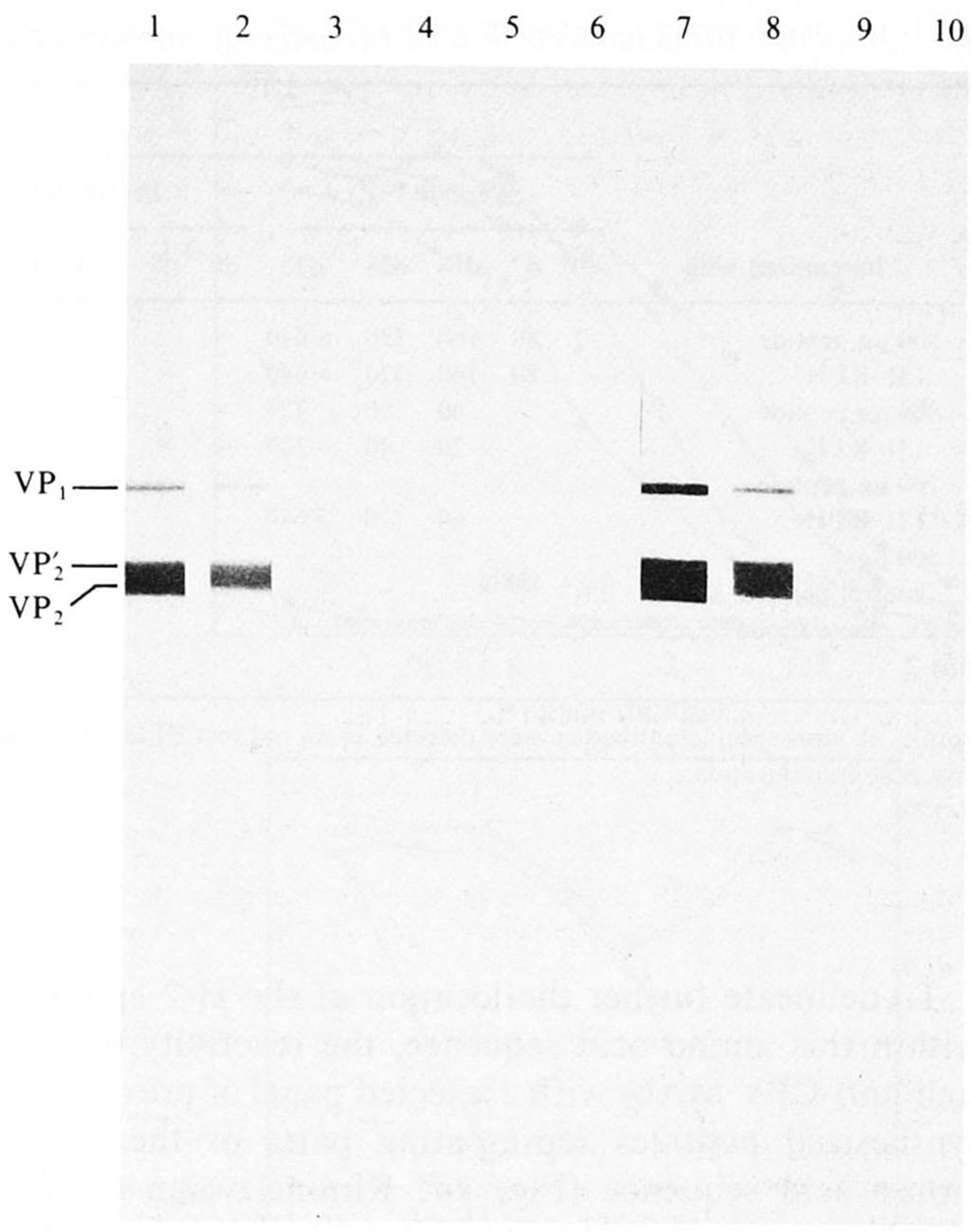

Fig. 2. Western blot analysis of anti-CPV MAbs and anti-peptide sera. IAC-CPV was denatured in the presence of SDS and 2-mercaptoethanol and the individual viral proteins were separated on a 12% polyacrylamide gel using the discontinuous buffer system (Laemmli, 1970). Polypeptides were then electrically transferred to nitrocellulose sheets (Towbin *et al.*, 1979). For immunostaining, the nitrocellulose was blocked with 1% BSA and individual lanes were incubated for 1 h at 20 °C with ascitic fluids of anti-CPV MAb H-2 diluted 1:100 (lane 1) or 1:500 (lane 2) or of anti-CPV MAb H-1 diluted 1:100 (lane 3) or 1:500 (lane 4); BALB/c mouse preimmune serum diluted 1:50 (lane 5) or 1:100 (lane 6); pool of anti-peptide 131 sera from mice 533 and 534 obtained 42 days after immunization, diluted 1:50 (lane 7) or 1:100 (lane 8); and a pool of control peptide sera from mice 539 and 540 obtained 42 days after immunization, diluted 1:50 (lane 9) or 1:100 (lane 10). After washing with PBS containing 0·1% BSA the lanes were incubated with a gold-labelled anti-mouse IgG antibody preparation and the gold signal was subsequently silver-enhanced according to the manufacturers instructions (Janssen Biotech). The reactivities obtained with MAb H-1 were representative of the reactivities of all three MAbs reactive with this site (not shown).

peptides representing other B cell epitopes to minimize the risk of the emergence of escape variants of the virus upon vaccination. Whether this approach will eventually lead to the construction of a peptide-based vaccine against CPV infection remains to be shown.

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