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ORIGINAL ARTICLE

Characterization of T helper (Th)1- and Th2-type immune responses caused by baculovirus-expressed protein derived from the S2 domain of feline infectious peritonitis virus, and exploration of the Th1 and Th2 epitopes in a mouse model

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ABSTRACT

Feline infectious peritonitis virus (FIPV) may cause a lethal infection in cats. Antibody-dependent enhancement (ADE) of FIPV infection has been recognized, and cellular immunity is considered to play an important role in preventing the onset of feline infectious peritonitis. In the present study, whether or not the T helper (Th)1 epitope was present in the spike (S)2 domain was investigated, the ADE epitope being thought to be absent from this domain. Three kinds of protein derived from the C-terminal S2 domain of S protein of the FIPV KU-2 strain were developed using a baculovirus expression system. These expressed proteins were the pre-coil region which is the N-terminal side of the putative fusion protein (FP), the region from FP to the heptad repeat (HR)2 (FP-HR2) region, and the inter-helical region which is sandwiched between HR1 and HR2. The ability of three baculovirus-expressed proteins to induce Th1- and Th2-type immune responses was investigated in a mouse model. It was shown that FP-HR2 protein induced marked Th1- and Th2-type immune responses. Furthermore, 30 peptides derived from the FP-HR2 region were synthesized. Five and 16 peptides which included the Th1 and Th2 epitopes, respectively, were identified. Of these, four peptides which included both Th1 and Th2 epitopes were identified. These findings suggest that the identification of Th1 epitopes in the S2 domain of FIPV has important implications in the cat.

Key words coronavirus, feline infectious peritonitis virus, Th1-type immune response.

FIP is a virus-induced, chronically progressive, immunologically-mediated, and usually fatal disease in domestic and wild *Felidae*. The causative agent of this disease is FIPV, which belongs to the family *Coronaviridae*, genus *Coronavirus*. In an attempt to prevent FIP, various vaccines, such as virulence-attenuated live or inactivated FIPV vaccines, have been investigated. However none have shown sufficient efficacy, rather these vaccines have enhanced the onset of FIP (1–7). Compared to antibody-negative kittens, intraperitoneal inoculation

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List of Abbreviations: ADE, antibody-dependent enhancement; CTL, cytotoxic T lymphocyte; FIP, feline infectious peritonitis; FIPV, feline infectious peritonitis virus; FP, fusion protein; HLA, human leukocyte antigen; HR, heptad repeat; IFN, interferon; IH, inter-helical; IL, interleukin; M, membrane; MHC, major histocompatibility complex; N, nucleocapsid; PC, pre-coil; RT, reverse transcription; S, spike; SARS-CoV, severe acute respiratory syndrome-associated coronavirus; SPF, specific-pathogen-free; Tc, T cytotoxic; $TCID_{50}$, median tissue culture infective dose; Th, T helper.

with FIPV induces more severe clinical signs in anti-FIPV antibody-positive kittens, and in kittens which have undergone passive immunization with serum or purified IgG from antibody-positive cats (8, 9). This phenomenon is referred to as ADE of viral infection. The ADE of FIPV infection is a serious obstacle to its vaccine-based prevention. Cellular immunity is considered to play an important role in preventing the onset of FIP (2). Thus, it is essential that vaccines against FIPV infection induce a cellular immune response.

FIPV consists of three major proteins, N, M, and S, which are classified as class I virus fusion proteins (10, 11). S protein exists as radially protruding trimers on the viral envelope, and can be structurally or functionally divided into two domains, namely the S1 and S2 domains, representing the N-terminal globular head and C-terminal membrane-bound stalk, respectively. The C-terminal S2 domain sequentially contains the PC region, putative FP, 4,3 hydrophobic HR1, IH domain, HR2, and the cluster aromatic amino acid domain from the N-terminal, and is responsible for driving viral and target cell membrane fusion (10, 12). The N-terminal S1 domain contains receptor-binding, neutralizing antibody-binding, and ADE epitopes (13–19). Recently, it was reported that Th1/Tc1 epitopes are present in the S1 and S2 domains of SARS-CoV, which belongs to the *Coronaviridae*, as does FIPV (20–25). However, it is not yet known whether the Th1/Tc1 epitope is present in the S1 and/or S2 domain of FIPV.

In the present study, we examined whether the Th1 epitope was present in the S2 domain, a domain in which it was thought that the ADE epitope was absent (13, 14, 16, 18, 19). We performed two experiments using a mouse model. Firstly, we investigated the immunogenicity of three baculovirus-expressed proteins, PC, IH, and FP-HR2 proteins, derived from the S2 domain of the type I FIPV KU-2 strain (Fig. 1). It has been suggested that FP-HR2 protein has marked immunogenicity. Secondly, we searched for epitopes inducing a Th1- and/or Th2-type immune response using 30 synthetic peptides derived from the FP-HR2 region. As a result, we identified 5 and 16 peptides including Th1 and Th2 epitopes, respectively.

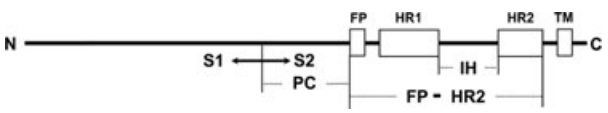

Fig. 1. Schematic diagram of coronavirus S protein and the expressed regions of PC, FP-HR2, and IH proteins. S1, the N-terminal globular head of S protein; S2, the C-terminal membrane-bound stalk of S protein; TM, transmembrane.

MATERIALS AND METHODS

Preparation of recombinant baculovirus-expressing proteins

Genomic RNA was extracted from FIPV KU-2 strain-infected culture fluid. RT of genomic RNA and amplification of cDNA employing PCR were carried out using a standard technique with a DNA thermal cycler. RT-PCR primers were designed for each region: PC region (forward, 5′-GGGGGATCCTTTTGTAACATCCTATACTATGCC-3′; reverse, 5′-GGGGAATTCTTAAGTGTACATAGACATCTTATTACCA-3′), IH region (forward, 5′-GGGAGATCTATGGCCCAAGTTGACCGT-3′; reverse, 5′-GGGGAATTCTTACACAATCTCCTGAAATGTC-3′), and FP-HR2 region (forward, 5′-GGGAGATCTATGGCATCTTTAATTGGCGG-3′; reverse, 5′-GGGGAATTCTTATTTTACATAAGTTTCAATCCTGTT-3′), of the S gene of the FIPV KU-2 strain. The PCR products were cloned into a pVL 1392 plasmid (Pharmingen, San Diego, CA, USA). *Escherichia coli* TOP10 strain was transformed with recombinant plasmid DNA and cultured. The white colonies that grew were selected and recombinant bacmid DNA recovered. SF-9 cells were transfected with recombinant bacmid DNA using cell Cellfectin (Gibco BRL, Grand Island, NY, USA), and the culture supernatant used in further experiments as each protein recombinant baculovirus (Fig. 1). Gene transfer was confirmed employing the PCR method (data not shown). SF-9 cells cultured for a day were inoculated with the recombinant baculovirus. The infected cells were harvested and washed with PBS. One milliliter of RSB buffer (0.01 M NaCl, 0.0015 M $MgCl_2$, and 0.01 M Tris-HCl, pH 7.4) containing 0.2% NP-40 was added to 1×10^7 cells, and the cell suspension kept at 4°C for 15 min with gentle shaking. The suspensions were centrifuged at $800 \times g$ for 10 min and the precipitate washed in PBS. The precipitate was then resuspended in PBS and used for immunization. The specificity and amount of each expressed protein were measured by Western immunoblotting using serum from the FIPV KU-2 strain-infected cat. Wild-type baculovirus-infected SF-9 cells were prepared by NP-40 treatment as control antigens for each expressed protein.

Western immunoblotting assay

Each expressed protein was separated employing 12% SDS-PAGE and transferred to a nitrocellulose membrane. A standard protein marker (Precision Plus Protein Standards) was purchased from Bio-Rad (Hercules, CA, USA). The membrane was blocked with 5% non-fat dry milk powder in TBST (20 mM Tris-HCl, pH 8.0, 0.88% NaCl, and 0.05% Tween-20) for 1 hr at 37°C, incubated for 1 hr at

37°C with serum from the FIPV KU-2 strain-infected cat and then incubated with peroxidase-conjugated goat anti-cat IgG (MP Biomedicals, LLC-Cappel products, Irvine, CA, USA) for 1 hr at 37°C. It was then visualized in substrate for 10 min.

Peptide synthesis

To determine the Th1 and/or Th2 epitope, 30 peptides derived from the S2 domain of the FIPV KU-2 strain were synthesized at Sigma-Aldrich (St Louis, MO, USA) (Table 1). One peptide (FP) was derived from the putative FP region. Twenty-five peptides (HR1-1–HR1-14 and HR2-1–HR2-11) synthesized as 20-mer fragments with a 12-amino-acid overlap were derived from the HR1 and HR2 regions. Four peptides (IH-1–IH-4) were derived from the hydrophobic area of the IH domain. All peptides were purified to purities of more than 70% and supplied as lyophilized powder. The peptides were dissolved in 10% dimethyl sulfoxide at 1 mg/ml, aliquoted, and stored at −80°C.

Table 1. Amino acid sequences of the peptides derived from the S2 domain of the FIPV KU-2 strain

Peptide name	Amino acid sequence	Start position
FP	GNKMSMYTASL	1029
HR1-1	TSAVAVPFAMQVQARLNYVA	1049
HR1-2	AMQVQARLNYVALQTDVLQE	1057
HR1-3	NYVALQTDVLQENQKILANA	1065
HR1-4	VLQENQKILANAFNNAIGNI	1073
HR1-5	LANAFNNAIGNITLALGKVS	1081
HR1-6	IGNITLALGKVSNAITTTSD	1089
HR1-7	GKVSNAITTTSDGFNSMASA	1097
HR1-8	TTSDGFNSMASALTKIQSVV	1105
HR1-9	MASALTKIQSVVNQQGEALS	1113
HR1-10	QSVVNQQGEALSQLTSQLQK	1121
HR1-11	EALSQLTSQLQKNFQAISSS	1129
HR1-12	QLQKNFQAISSSIAEIYNRL	1137
HR1-13	ISSSIAEIYNRLEKVEADAQ	1145
HR1-14	YNRLEKVEADAQVDRLITGR	1153
IH-1	ITGRLAALNAYVSQTLTQY	1169
IH-2	FSLVNSAPEGLLFFHTVLLPTEWEEVTA	1222
IH-3	FFHTVLLPTEWEEVTAWSGIC	1234
IH-4	WSGICVNDTYAYVLKDFDHSIFSYNGTY	1250
HR2-1	TFQEIVIDYIDINKTIADML	1312
HR2-2	YIDINKTIADMLEQYNPNYT	1320
HR2-3	ADMLEQYNPNYTTPELNLLL	1328
HR2-4	PNYTTPELNLLLDIFNQTKL	1336
HR2-5	NLLLDIFNQTKLNLTAEIDQ	1344
HR2-6	QTKLNLTAEIDQLEQRADNL	1352
HR2-7	EIDQLEQRADNLTTIAHELQ	1360
HR2-8	ADNLTTIAHELQQYIDNLNK	1368
HR2-9	HELQQYIDNLNKTLVDLDWL	1376
HR2-10	NLNKTLVDLDWLNRIETYVK	1384
HR2-11	LDWLNRIETYVKWPWYVWLL	1392

Immunization of mice

Four-week-old female BALB/c mice ($n = 3$ or 4 per group) were obtained from Charles River (Hino, Japan) and maintained under SPF conditions. All immunizations were given intraperitoneally every two weeks with each expressed protein (Western immunoblotting: 16 units per dose). Mice were immunized three times in total. Wild-type baculovirus-infected SF-9 cell-derived antigen and PBS-immunized groups were used as negative controls.

Stimulation of splenocytes

One or two weeks after the final immunization, the mice were killed to harvest their spleens. Splenocytes were cultured in RPMI-1640 medium containing 5% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Erythrocyte-depleted splenocytes (4×10^5 cells/well) in 96-well plates were cultured with 30 μg/mL of each peptide, heat-inactivated virus (FIPV KU-2 strain, $10^{4.6}$ $TCID_{50}$/mL; FIPV 79–1146 strain, $10^{5.5}$ $TCID_{50}$/mL; the homology of the amino acid sequence of the S2 domain of these two viruses is 60.7%), or culture medium as a control for three days.

ELISA

The concentrations of IFN-γ, IL-2, and IL-4 in the supernatant of splenocytes cultured with stimulatory substances for three days were measured using commercial mouse IFN-γ, IL-2, and IL-4 ELISA kits (Endogen, Cambridge, MA, USA) according to the manufacturer's protocol, respectively.

Statistical analysis

All results are expressed as means ± standard error of the mean. Student's *t*-test or one-way analysis of variance was employed as appropriate to determine the significance of differences. A P-value of < 0.01 or $P < 0.05$ was considered significant.

RESULTS

Specificity and amount of each expressed protein

The specificity and amount of three expressed proteins, PC, FP-HR2, and IH, were measured by Western immunoblotting assay using serum from the FIPV KU-2 strain-infected cat. In agreement with the size of PC, FP-HR2, and IH proteins, 25-, 37-, and 15-kDa bands, respectively, were visualized (Fig. 2) and detected in up to a 16-fold dilution (16 units). No expressed proteins reacted with the serum of the SPF cat.

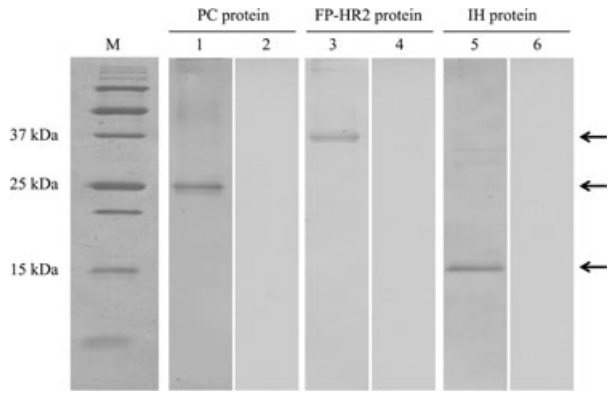

Fig. 2. Analysis of expressed proteins using Western immunoblotting. M, marker: lanes 1 and 2, PC protein: lanes 3 and 4, FP-HR2 protein: lanes 5 and 6, IH protein. Lanes 1, 3, and 5, serum of the FIPV KU-2 strain-infected cat; lanes 2, 4, and 6, serum of the SPF cat. The arrows show expressed proteins, PC, FP-HR2, and IH proteins.

Th1- and Th2-type immune responses against heat-inactivated FIPV antigen of murine splenocytes immunized with each expressed protein

Evaluation of the Th1-type immune response of PC, FP-HR2, and IH proteins was based on the concentrations of IFN-γ and IL-2 in the culture supernatant (Fig. 3a, b). In FP-HR2 protein-immunized mice, the concentrations of IFN-γ and IL-2 in the supernatant of splenocytes cultured with or without FIPV antigen were significantly higher than in that of splenocytes derived from wild-type baculovirus-infected SF-9- or PBS-immunized mice ($P < 0.05$, $P < 0.01$, respectively). In IH protein-immunized mice, the concentration of IL-2 in the supernatant of splenocytes cultured with or without FIPV antigen was significantly higher than in that of splenocytes derived from wild-type baculovirus-infected SF-9- or PBS-immunized mice ($P < 0.05$, $P < 0.01$, respectively). However, in PC protein-immunized mice, the concentrations of IFN-γ and IL-2 in the supernatant of splenocytes cultured with or without FIPV antigen were not significantly higher than in that of splenocytes derived from wild-type baculovirus-infected SF-9- or PBS-immunized mice.

Evaluation of the Th2-type immune response of PC, FP-HR2, and IH proteins was based on the concentration of IL-4 in the supernatant (Fig. 3c). In FP-HR2 protein-immunized mice, the concentration of IL-4 in the supernatant of splenocytes cultured with or without FIPV antigen was significantly higher than in that of splenocytes derived from wild-type baculovirus-infected SF-9- or PBS-immunized mice ($P < 0.05$, $P < 0.01$). In IH protein-immunized mice, the concentration of IL-4 in the supernatant of splenocytes cultured with the heat-inactivated FIPV 79–1146 strain was significantly higher

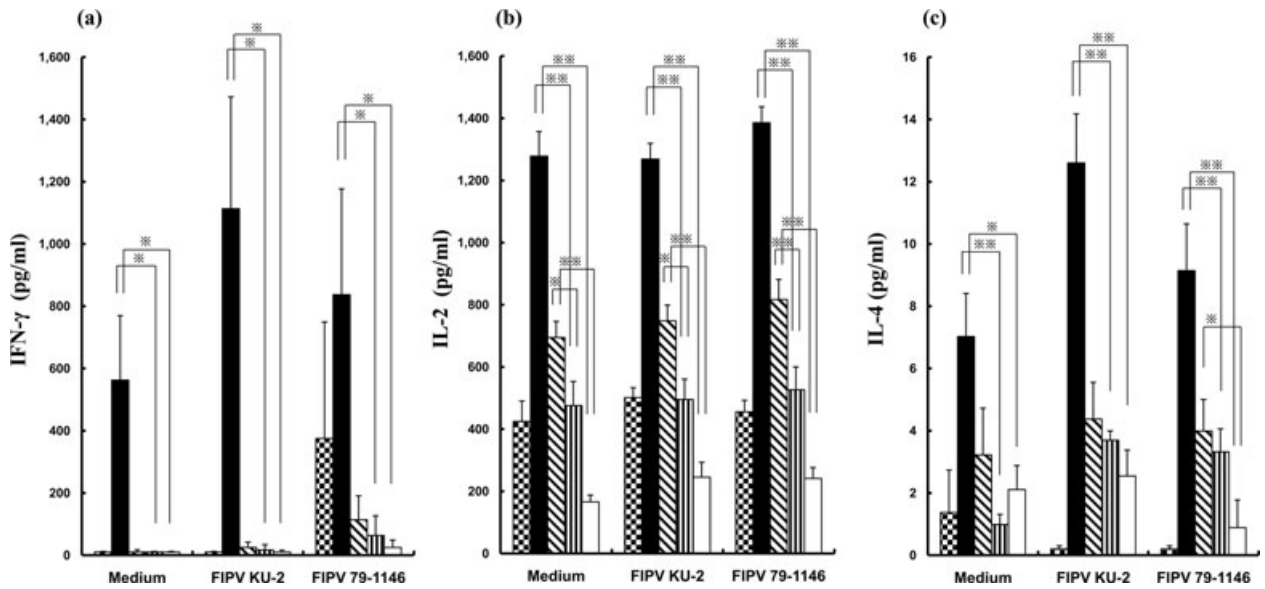

Fig. 3. Splenocytes derived from mice immunized with PC protein, FP-HR2 protein, IH protein, Wild-type baculovirus-infected SF-9 or PBS were cultured with heat-inactivated FIPV (KU-2 or 79–1146 strain) or culture medium as a negative control for three days. The concentrations of (a) IFN-γ, (b) IL-2 and (c) IL-4 in the culture medium were measured using an ELISA kit. *, $P < 0.05$; *, $P < 0.01$ compared with the wild-type baculovirus-infected SF-9- or PBS-immunized group; (▩), mice immunized with PC protein; (■), mice immunized with FP-HR2 protein; (▨), mice immunized with IH protein; (▩), mice immunized with Wild-type baculovirus-infected SF-9; (□), mice immunized with PBS.

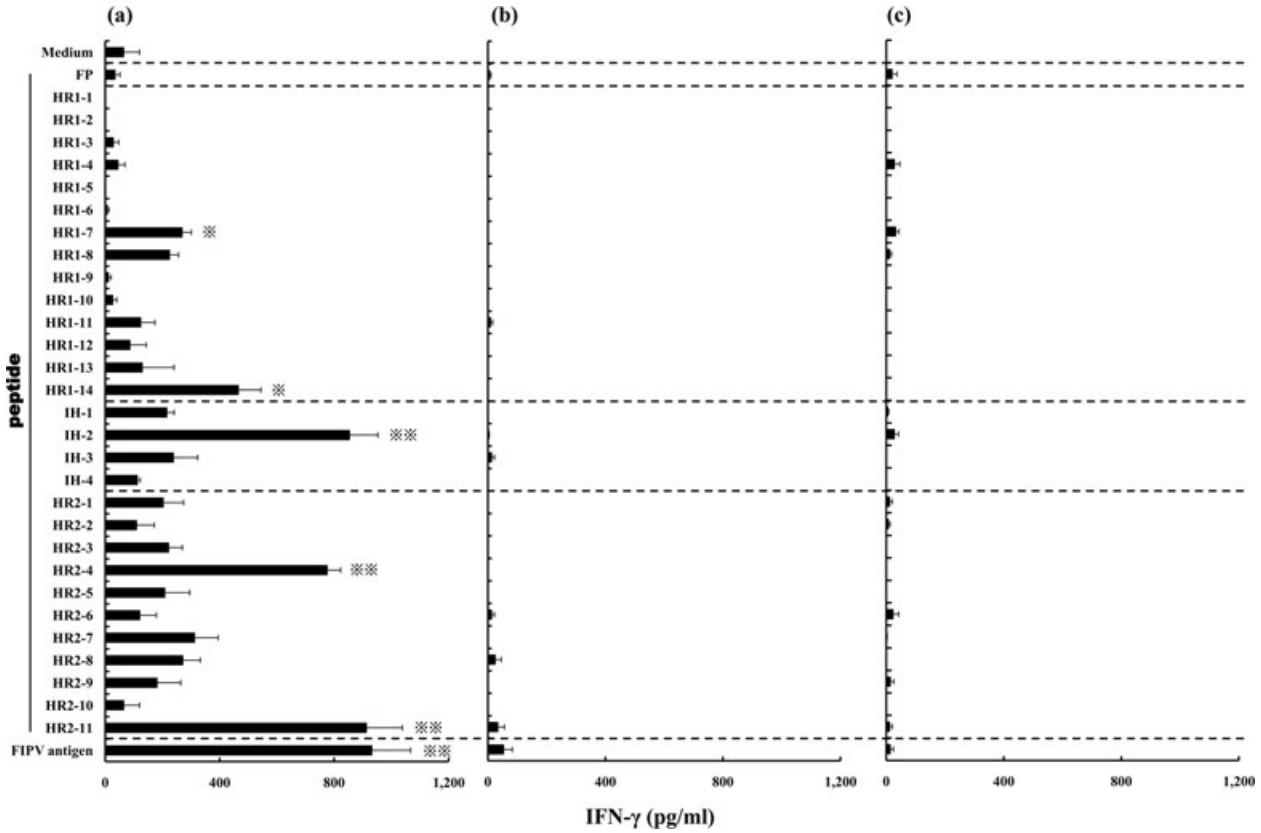

Fig. 4. Splenocytes derived from mice immunized with (a) FP-HR2 protein, (b) wild-type baculovirus-infected SF-9 or (c) PBS were cultured with individual peptides derived from the FP-HR2 region of the FIPV KU-2 strain, heat-inactivated FIPV KU-2 strain as a positive control, or culture medium as a negative control for three days. The concentration of IFN-γ in the supernatant was measured using a Mouse ELISA kit. *, $P < 0.05$; **, $P < 0.01$ compared with the control culture with medium alone.

than in that of splenocytes derived from PBS-immunized mice ($P < 0.05$). However, in PC protein-immunized mice, the concentration of IL-4 in the supernatant of splenocytes cultured with or without FIPV antigen was not significantly higher than in that of splenocytes derived from wild-type baculovirus-infected SF-9- or PBS-immunized mice.

Th1- and Th2-type immune responses against peptides derived from the FP-HR2 region of murine splenocytes immunized with FP-HR2 protein

Evaluation of the Th1- or Th2-type immune response against 30 peptides derived from the FP-HR2 region was based on the concentration of IFN-γ or IL-4 in the supernatant, respectively (Figs 4, 5). In FP-HR2 protein-immunized mice, the concentration of IFN-γ in the supernatant of splenocytes cultured with the HR1-7, HR1-14, IH-2, HR2-4, HR2-11, or FIPV antigen was significantly higher than in that of splenocytes cultured with medium alone ($P < 0.01$: IH-2, HR2-4, HR2-11, and FIPV antigen; $P < 0.05$: HR1-7 and HR1-14) (Fig. 4a). The concentration of IL-4 in the supernatant of splenocytes cultured with the FP, HR1-6, HR1-7, HR1-10, HR1-11, HR1-12, HR1-14, IH-1, IH-2, IH-4, HR2-1, HR2-2, HR2-3, HR2-7, HR2-8, HR2-11, or FIPV antigen was significantly higher than in that of splenocytes cultured with medium alone ($P < 0.01$: HR1-7, HR1-10, HR1-11, HR1-12, HR1-14, HR2-2, HR2-3, HR2-7, HR2-8, HR2-11, and FIPV antigen; $P < 0.05$: FP, HR1-6, IH-1, IH-2, IH-4, and HR2-1) (Fig. 5a). This indicates that four peptides, HR1-7, HR1-14, IH-2, and HR2-11, induce both Th1- and Th2-type immune responses. In wild-type baculovirus-infected SF-9- or PBS-immunized mice, the concentrations of IFN-γ and IL-4 in the supernatant of splenocytes cultured with or without peptide were not increased (Fig. 4b, c; Fig. 5b, c).

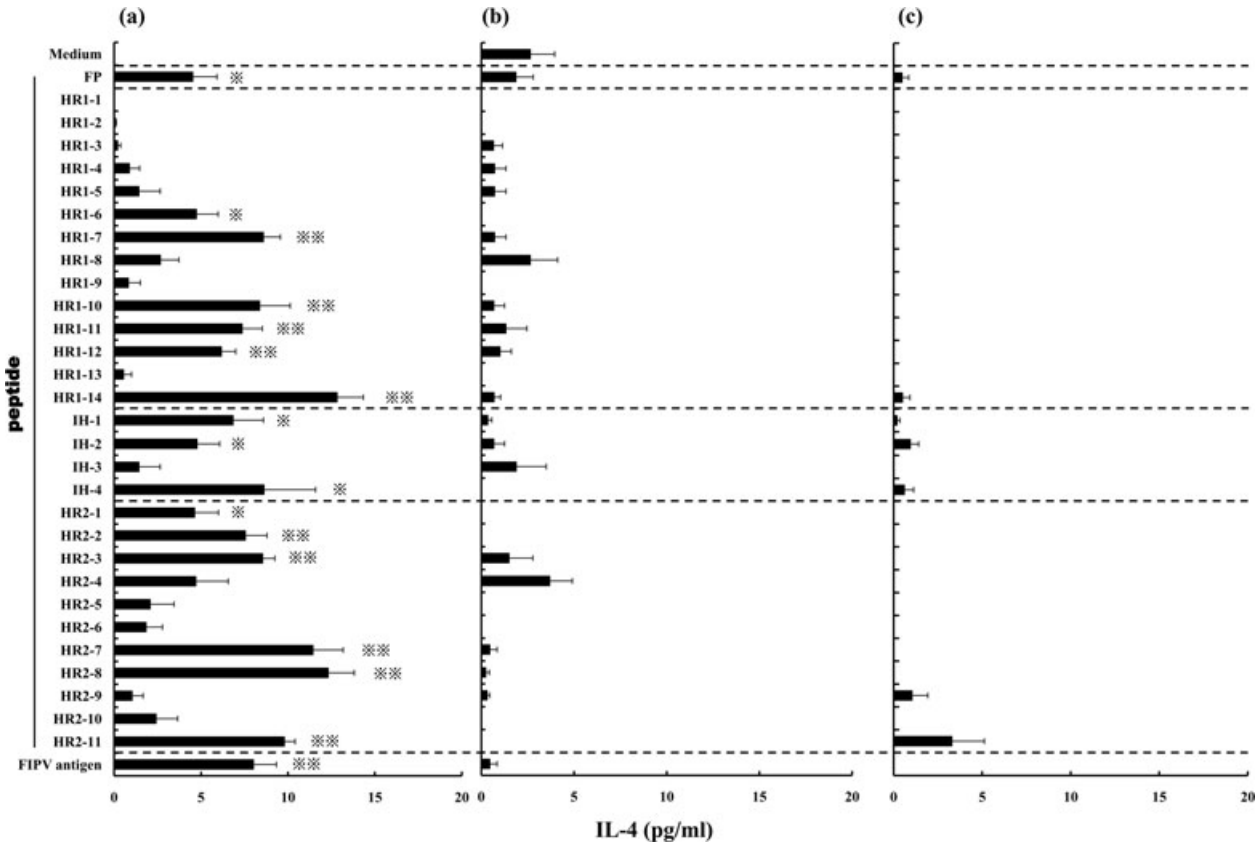

Fig. 5. Splenocytes derived from mice which were immunized with (a) FP-HR2 protein, (b) wild-type baculovirus-infected SF-9 or (c) PBS were cultured with individual peptides derived from the FP-HR2 region of the FIPV KU-2 strain, heat-inactivated FIPV KU-2 strain as a positive control, or culture medium as a negative control for three days. The concentration of IL-4 in the supernatant was measured using a mouse ELISA kit. *, $P < 0.05$; **, $P < 0.01$ compared with the control culture with medium alone.

DISCUSSION

We have shown that FP-HR2 protein derived from the S2 domain, which does not include ADE epitopes, exhibits strong immunogenicity in the mouse. In addition, we showed that 5 and 15 peptides derived from the FP-HR2 region induce Th1- and Th2-type immune responses, respectively. These results suggest that these peptides contain the Th1 and Th2 epitopes, respectively. Of these, four peptides contained both the Th1 and Th2 epitopes.

Comparing the immunogenicity of mouse N-terminal-sided PC protein with that of C-terminal-sided FP-HR2 protein of the S2 domain, IFN-γ, IL-2, and IL-4 concentrations were significantly increased only in FP-HR2 protein-immunized mice. FP-HR2 protein consists of FP, HR1, HR2, and IH domains. The concentrations of IFN-γ, IL-2, and IL-4 in FP-HR2 protein-immunized mice were significantly higher than those of IH protein-immunized mice. This suggests that, except for the IH domain, the FP, HR1, and/or HR2 region contains a highly immunogenic site. IH protein showed less immunogenicity in mice, especially in regard to induction of IFN-γ, than did FP-HR2 protein. However, stimulation with IH-2, a peptide derived from the IH region, induced IFN-γ production, suggesting the presence of Th1 epitopes. Reportedly, SARS-CoV and transmissible gastroenteritis virus produce no neutralizing antibodies after immunization of mice with a peptide containing a neutralizing epitope, but enhance immunogenicity and produce neutralizing antibodies when bound to a peptide with Th1 epitopes (26, 27). The HR1 and HR2 regions contain peptides with the Th1 epitopes identified in this study, (i.e. HR1-7, HR1-14, HR2-4, and HR2-11). Thus, IH protein alone is unlikely to recognize antigen-presenting cells; however, FP-HR2 protein (i.e. IH protein bound to HR1 and HR2) may exhibit greater immunogenicity.

H-2Db, an MHC class I molecule of BALB/c mice, is known to have a structure similar to that of HLA-A24.

Amino acid sequences predicted by the National Institute of Health (http://www-bimas.cit.nih.gov/molbio/hla_bind/) to have a strong capacity to bind to HLA-A24, were explored in the FP, HR1, and HR2 regions. The results demonstrated the presence of epitopes showing a marked capacity to bind to aa1109-1117 (HR1-8) of the HR1 region and aa1319-1327 (HR2-1), aa1337-1345 (HR2-3 and HR2-4), aa1349-1357 (HR2-5), and aa1406-1414 (HR2-11) of the HR2 region. Among these peptides, HR2-4 and HR2-11 induced IFN-γ production in this experiment. This suggests that these two peptides are MHC class I-binding peptides with the Tc1 epitope. The other peptides, HR1-7, HR1-14, and IH-2, have the Th1 epitope.

Like the sites of Th1 epitopes in FIPV identified in this study, SARS-CoV contains Th1/Tc1 epitopes in the HR1, IH, and HR2 regions of the S2 domain (22–24). Among these, Th1/Tc1 epitopes (SSp-1) present in the HR2 region are known to induce strong CTL activity and are regarded as candidates for vaccine development. Thus, the peptides with Th1/Tc1 epitopes identified in this study, particularly HR2-4 and HR2-11 that seem to have Tc1 epitopes, are potential candidates for vaccine development for prevention of FIPV infection.

Although Th1/Tc1 epitopes in the FP-HR2 region appeared to strongly induce the Th1-type immune response in a mouse model, their effects in the cat remain unknown. It is important for an effective peptide-based vaccine against FIPV infection to include Th1/Tc1, but not ADE, epitopes. In the cat, the identification of Th1/Tc1 epitopes in the S2 domain of FIPV has important implications. In the future, Th1/Tc1 epitopes in FIPV will be explored using FIPV-infected cats. The development of a vaccine against FIPV infection based on these epitopes is awaited.

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