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H. 知的財産権の出願・登録状況（予定を含む）

なし

厚生科学研究費補助金（新興・再興感染症研究事業）

分担研究報告書

ヒト型ウシを用いたヒト型抗体の作成

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研究要旨

本研究の目的は、SARS コロナウイルス(CoV)の感染防御、感染後の SARS 発症予防、さらに SARS 発症後の重症化予防と治療のため、ヒト型抗体産生ウシを用いた SARS ウイルス中和ヒト型抗体の開発である。本年度は SARS-CoV ゲノムにコードされる蛋白質のうちヒト型抗体産生動物の免疫のための抗原候補として、spike 蛋白質、membrane 蛋白質および nucleocapsid 蛋白質を選択し、それらの組換え蛋白質のデザイン、発現および精製法の確立を試みた。spike 蛋白質は全長の他、全体を 4 つの断片に分けた設計とした。membrane 蛋白質は C 末端側領域を選択した。nucleocapsid 蛋白質は分子全体を抗原とした。これらを His-Tag 融合蛋白質として大腸菌で発現後、Tag のアフィニティ精製を行い、さらに Tag 除去を実施する計画とした。デザインされた総計 8 個の組換え蛋白質について発現・精製・Tag 除去の各段階の検討を行った結果、すべての蛋白質について発現に成功し、うち 7 個で精製を完了、4 個で Tag 除去を完了し、抗原としての調整を終了した。

A．研究目的

重症急性呼吸器症候群（SARS）は、その致命率の高さ（約 10%）、superspreader の存在などから、人間活動の国際化と相まって、一地方病としてではなく、I 類の国際感染症として各地域・国の経済にも多大の影響を及ぼした。医師・看護師、臨床検査技師などは常に感染の危険にさらされており、いったん患者が発生すると、これら、医療従事者のみならず、一般国民の感染予防・発症予防・重症化予防と治療法の確立は国際的な急務である。

本研究においては、SARS ウイルス感染予防、感染後の発症予防、発症後の重症化予防と治療法確立を目的とし、ヒト型ウシを用いたヒト型中和抗体の開発を目指す。

本分担研究では SARS-CoV 蛋白質の組換え蛋白質を抗原としたヒト型抗体産生動物の免疫を計画しており、本年度は抗原の調整のため、SARS-CoV 蛋白質からの抗原候補の選択、その設計、大量発現系の構築、精製法の確立の検討を実施した。

B．研究方法

1．大腸菌発現系の構築：各組換え蛋白質は大量発現が容易な大腸菌のシステムを選択した。発現用宿主大腸菌として、組換え蛋白質の発現が発現誘導時まで高度に制御される菌株、コドンバイアスを考慮した菌株、組換え蛋白質の分解を抑制するようプロテアーゼを欠損した株などを必要に応じて使用した。

2．発現プラスミドの構築：当該組換え蛋白質の精製を容易にするため、各蛋白質とも His-Tag 融合蛋白質となるように設計した。発現用プラスミドとして、Invitrogen 社の pDEST17 および QIAGEN 社の TAGzyme pQE2 を使用した（図6，7）。His-Tag が除去可能な設計とするため、前者では TEV プロテアーゼというエンドプロテアーゼの認識部位を His-Tag 直下に導入した。

3．組換え蛋白質の可溶化と精製：大腸菌での大量発現の確認後、組換え蛋白質を超音波処理などにより可溶化した。得られた可溶化分画は常法に従い、

ニッケルキレートカラムに添加し、充分に洗浄した後、イミダゾールなどによる溶出を行った。His-Tag 除去は pDEST17 誘導体由来の組換え蛋白質は TEV プロテアーゼにより、pQE2 誘導体由来の組換え蛋白質はジアミノペプチダーゼにより行った。

C、研究結果

1）計画の概略：図１には本研究計画の概略を示した。SARS-CoV の蛋白質を抗原として、ヒト型抗体産生動物（マウスおよびウシ）を免疫し、得られたヒト型抗体の SARS-CoV 中和活性測定後、まずマウス由来抗体で、サルなどを用いた in vivo での SARS-CoV 感染の抑制実験を行う。免疫グロブリン療法などの知見から、サルでの実験にはヒト型抗体産生マウス約 100 匹分の抗体が必要と推定され、その免疫に必要な抗原量は 5-25mg と考えられる。さらにその後のウシの実験では、300mg 程度の抗原が必要と考えている（図２）。そのため大量の純粋な抗原を調整出来るシステムを構築する必要がある。

2）抗原の設計：SARS-CoV ゲノムには 14 個の ORF が存在する。これらのうちウイルス粒子表面に存在すると考えられる spike 蛋白質、membrane 蛋白質、および以前に他のコロナウイルスで中和抗体誘導抗原となることが報告されている nucleocapsid 蛋白質、の３つの蛋白質を抗原候補として選択した（図４）。また他の分担研究者が行った SARS 患者の血清中に存在する SARS 抗原反応性抗体のエピトープ解析の結果を元に、これら３つの蛋白質内のエピトープ部分を考慮しながら、組換え蛋白質のデザインを行った（図５）。membrane 蛋白質については M116 および M63 と名付けた２つの組換え蛋白質、nucleocapsid 蛋白質についてはその全体、spike 蛋白質については、シグナルペプチド領域と膜貫通領域を除いた分子全体、および分子全体を４つに分けた断片、の総計８個の組換え蛋白質を作成することとした。前述の様に大量の抗原が必要となるため、大量発現の容易な大腸菌の発現系を使用することとした。また精製を容易にするためすべての組換え蛋白質に His-Tag というアフィニティタグを融合させることにした。抗原として使用する際にはアフィニティタグの除去が必要と考え、蛋白質分解酵素によりその除去が可能な設計とした（図３）。前述の８蛋白質のうち、M116、N63、N および S(-TM)については Invitrogen 社 pDEST17 という発現用ベクターを利用して発現プラスミドを構築することとし、N 末端側のアフィニティタグ直下に AcTEV というプロテアーゼの認識配列を導入した（図６）。残りの S1-N、S1-C、S791 および S2 の各組換え蛋白質は、ジアミノペプチダーゼという酵素でアフィニティタグが除去可能な QIAGEN 社の TAGzyme pQE2 を使用して発現プラスミドを構築した（図７）。

3）組換え蛋白質の発現と精製：発現に使用する大腸菌はプロテアーゼ欠損株、コドンバイアス対応株、高度発現制御株などを適宜使用し、各蛋白質固有の特性に対応した最適化を行い、最終的に設計したすべての蛋白質の発現に成功したが（図１２）、S(-TM)については大量発現に至らず現在その条件を検討中である。精製については、ニッケルキレートクロマトグラフィーを利用したアフィニティタグによる精製で、その過程を迅速化できた。封入体となる組換え蛋白質も散見されたが、変性剤などを使用した可溶化条件の検討を行い、現在検討中の S1-C 以外では精製条件の構築を完了した。蛋白質分解酵素によるアフィニティタグ除去については、M63、N、S791 および S2 で検討を完了した。これらの発現・精製・アフィニティタグ除去の様子を図９～１１に示した。

D．考察

設計した 8 個の組換え蛋白質のうち、7 個で精製条件の検討を完了し、うち 4 個でアフィニティタグ除去まで完了した。これらの過程を通じて得られた知

見を元に残りの蛋白質についても精製とアフィニティタグ除去の条件を設定できると考えられる。得られた組換え蛋白質は電気泳動的検証では精製度 95%以上であり、抗原としての使用に充分耐えるものである。これらの蛋白質については別途 N 末端配列解析を行い、最終的な抗原標品とする予定である。また電気泳動時に観察される目的蛋白質以外の混在バンドについてはそれらが、目的蛋白質の自己分解による可能性もあるため、これを N 末端配列解析により検証していく予定である。

E. 結論

一連の検討で、標的とした蛋白質の抗原準備を半数の組換え蛋白質で完了した。残りの蛋白質についても調整を進めるとともに、今後はこれらの蛋白質をヒト型抗体産生マウスに投与して、それらの抗原性を検討し、中和抗体作成のための最適抗原を検索していく。

F. 健康危険情報

なし

G. 研究発表

なし

H. 知的財産権の出願・登録状況（予定を含む）

なし

図1 中和抗体作成の戦略

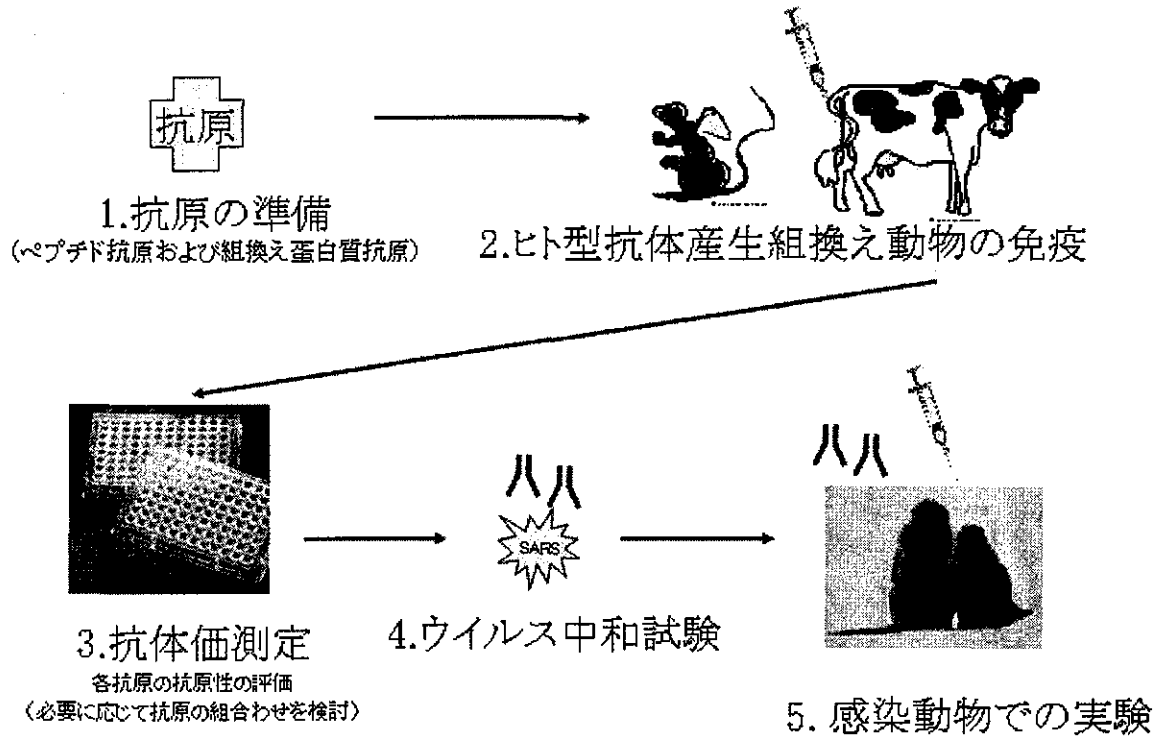


図2 抗原の準備(1)

免疫動物としてマウスおよびウシを使用

1. マウスから得られた抗体を用いて、ウイルス感染動物モデルで中和抗体の有効性を検討する場合、マウス100匹分程度が必要になる。 → 必要抗原量は:
10 - 50μg/匹/感作
×
感作5回
×
100匹= **5 - 25mg**

2. ウシの免疫では一頭あたりの抗体量は充分と考えられるが、その大きさのため抗原の絶対量が必要。 → **300mg**

図3 抗原の準備(2)

1. ペプチド抗原→

- 当センターの解析で、ベトナムSARS患者で同定されたSARS-CoV抗原エピトープを元にデザイン
- ペプチド本体の他に、抗原性の向上を期待して、MAP化やT細胞認識モチーフなどを付加したものも準備

2. 組換え蛋白質抗原→

- 上述のエピトープマッピングの結果を利用した蛋白質のデザイン
- 大量調整の容易な大腸菌の系を利用
- His-Tag化による精製の迅速化
- 蛋白質分解酵素切断サイト導入によるHis-Tag除去

図4 SARS-CoVゲノムにコードされる因子

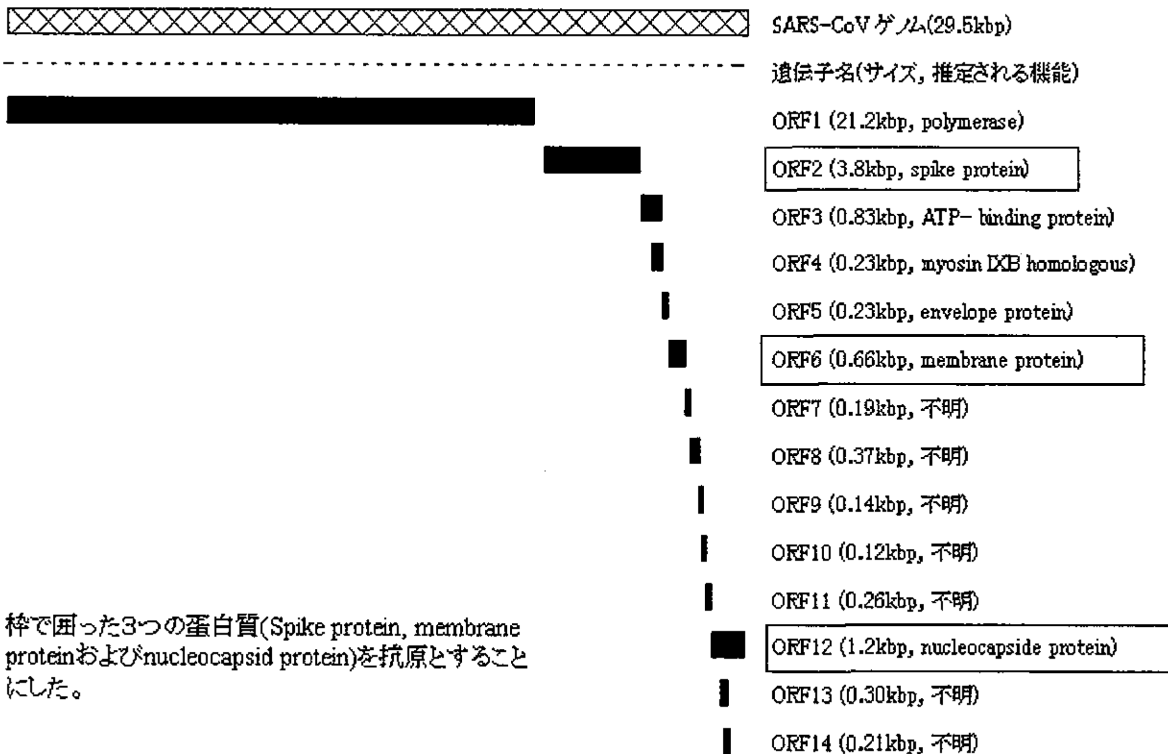


図5 組換え蛋白質抗原のデザイン

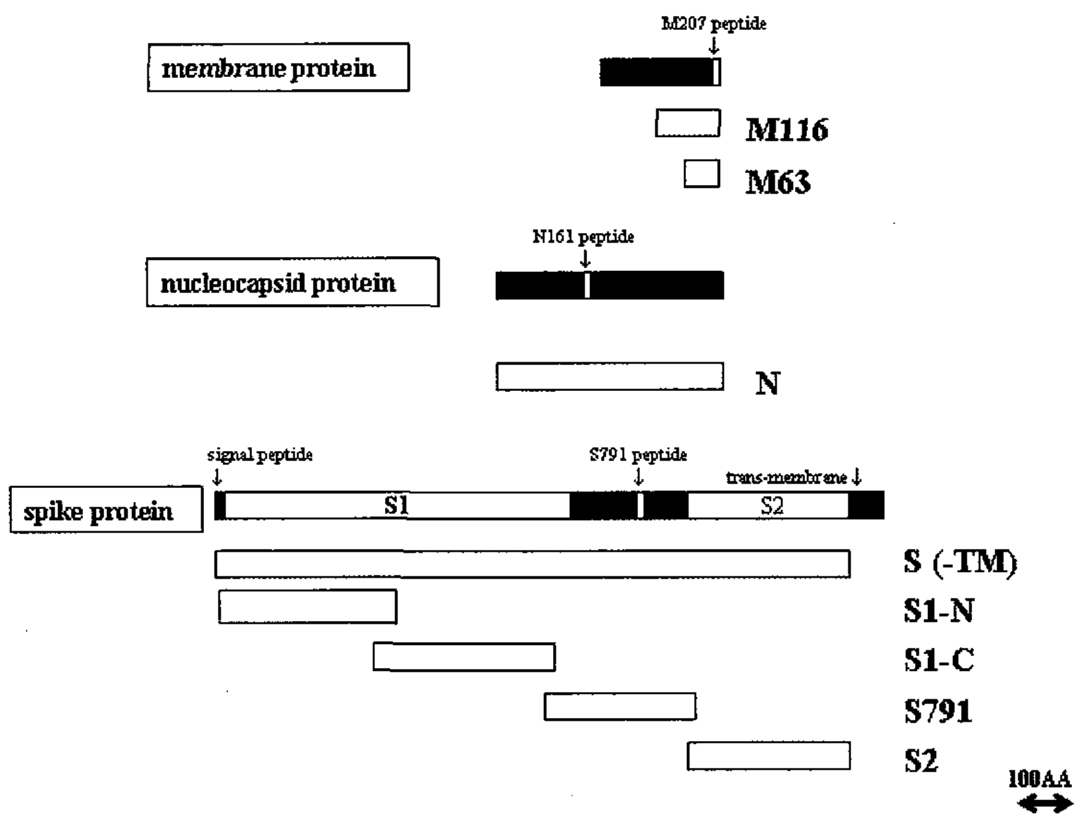
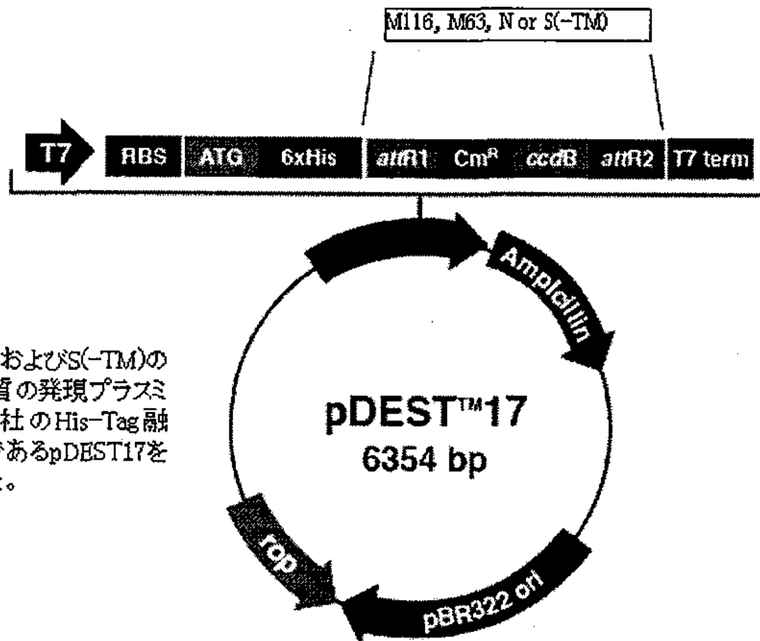


図6　大腸菌発現用ベクターの構築(1)



M116, M63, NおよびS(-TM)の各組換え蛋白質の発現プラスミドはInvitrogen社のHis-Tag融合型ベクターであるpDEST17を用いて構築した。

図7　大腸菌発現用ベクターの構築(2)

S1-N, S1-C, S791およびS2の各組換え蛋白質の発現プラスミドはQIAGEN社のHis-Tag融合型ベクターであるTAGzyme pQEを用いて構築した。

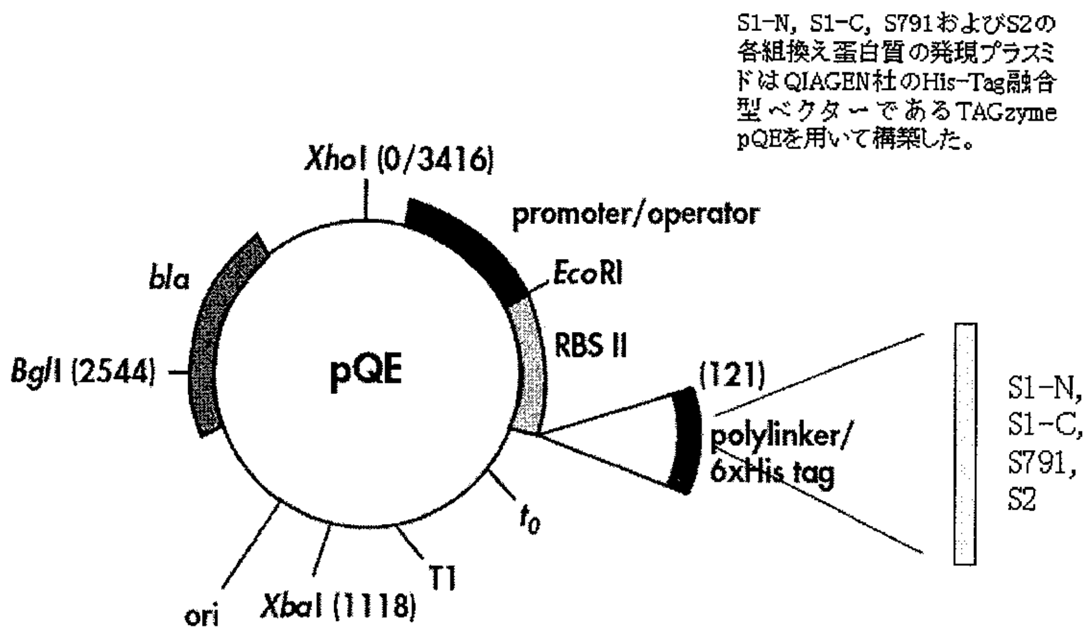


図8　抗原の精製

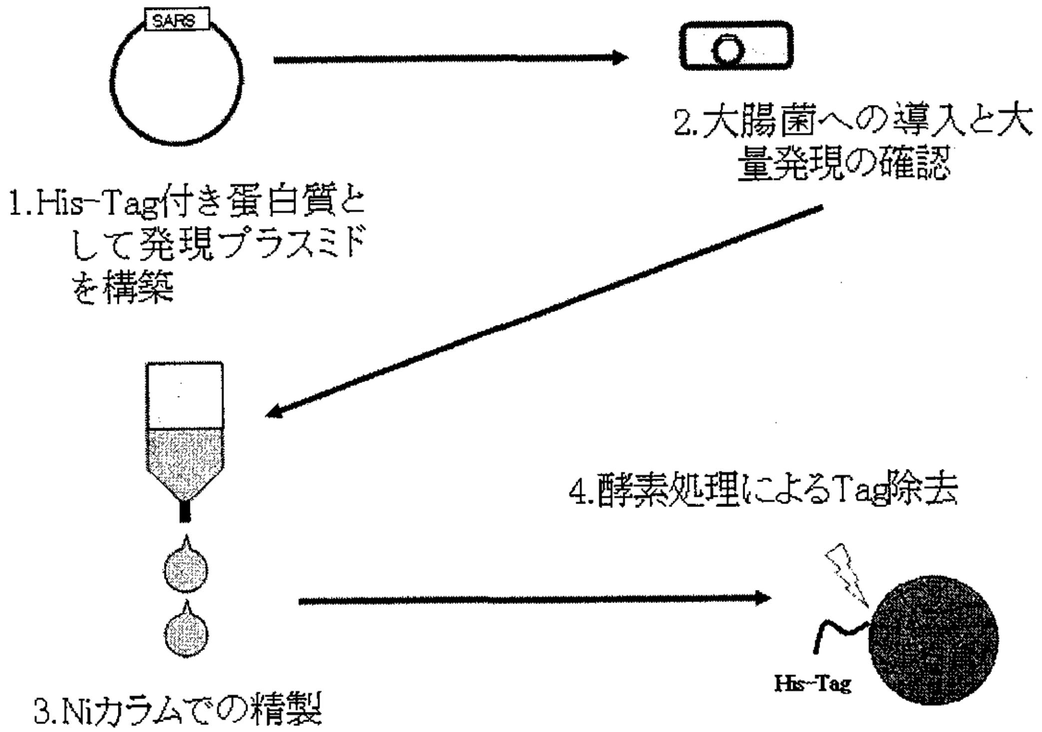


図9　精製例（M63, N protein）

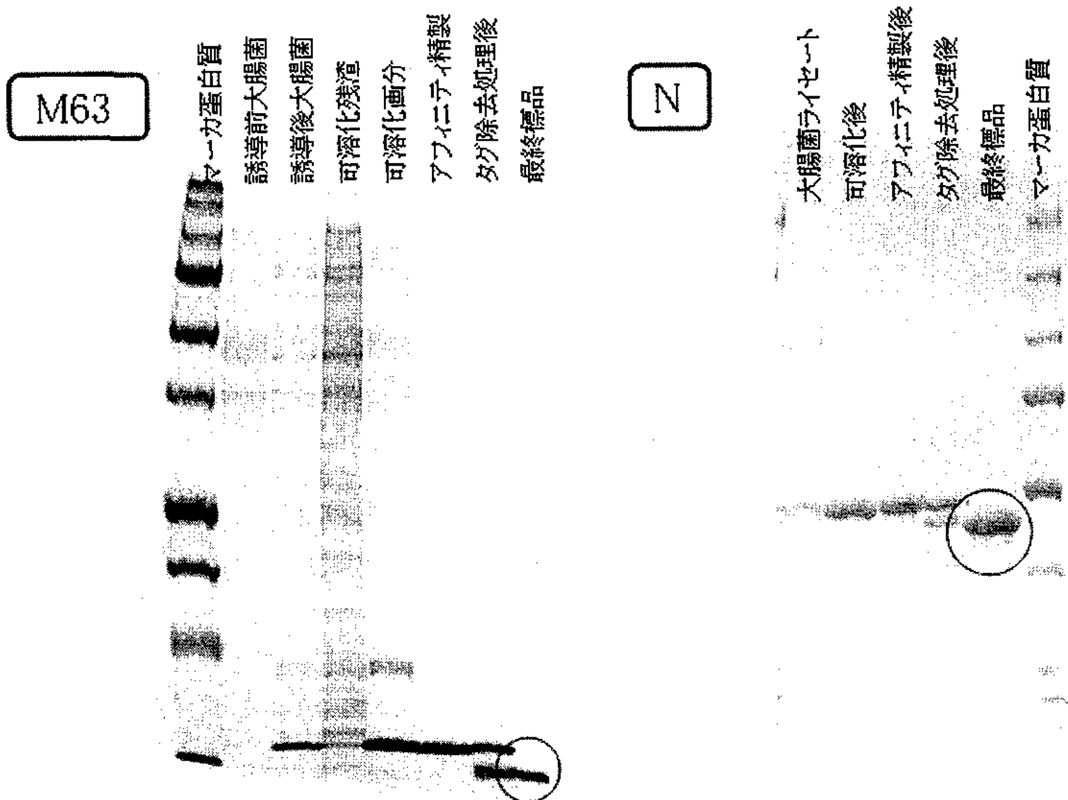


図10 精製例(S(-TM), S1-N, S1-C)

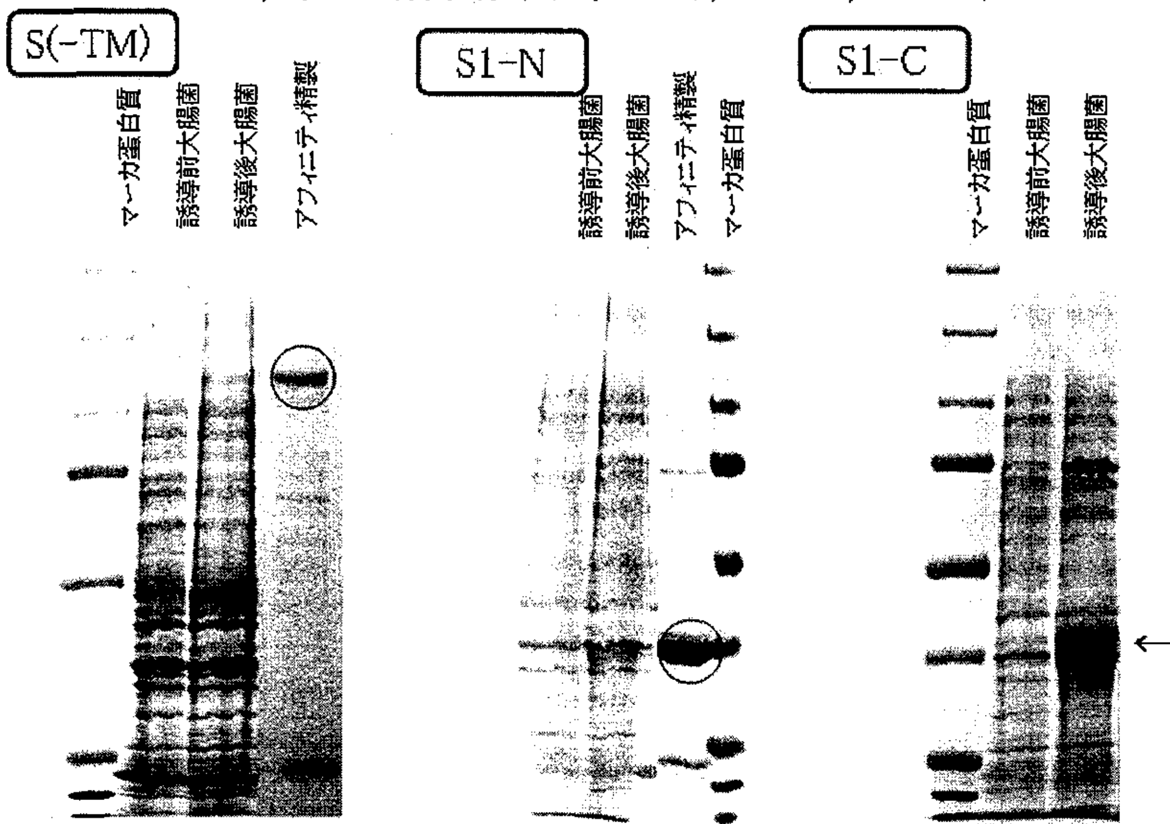


図11 精製例(S2およびS791フラグメント)

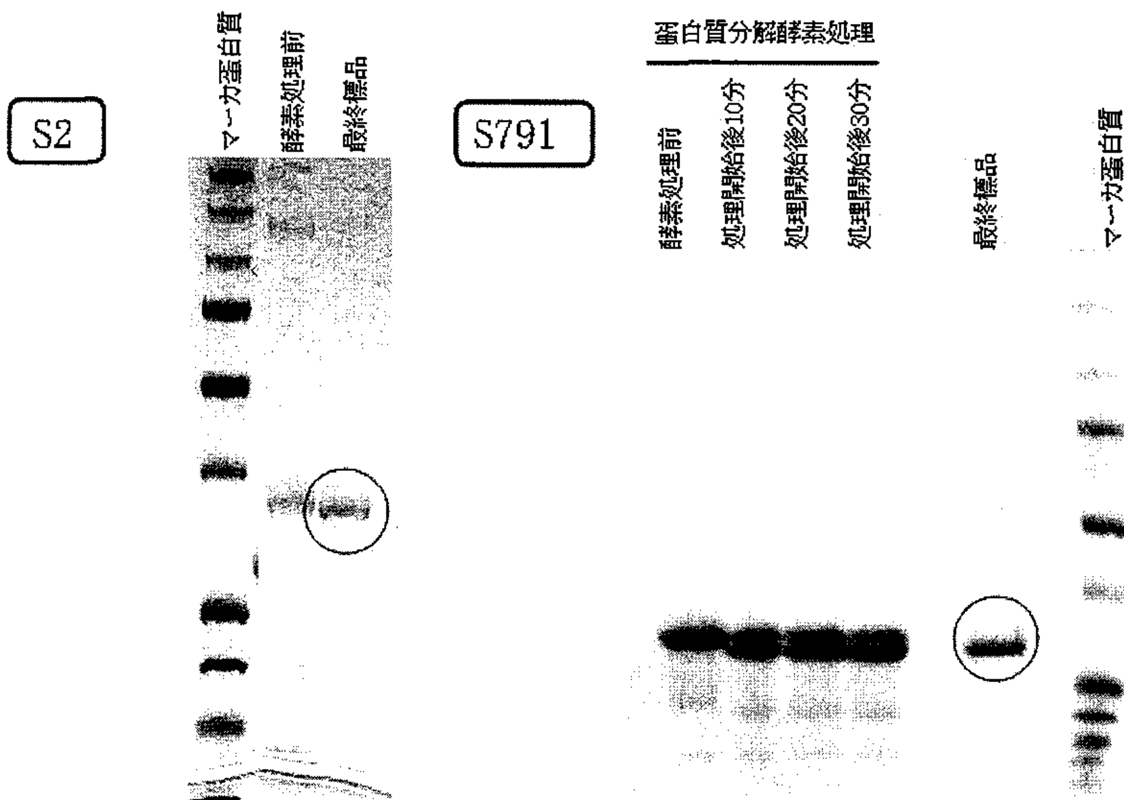
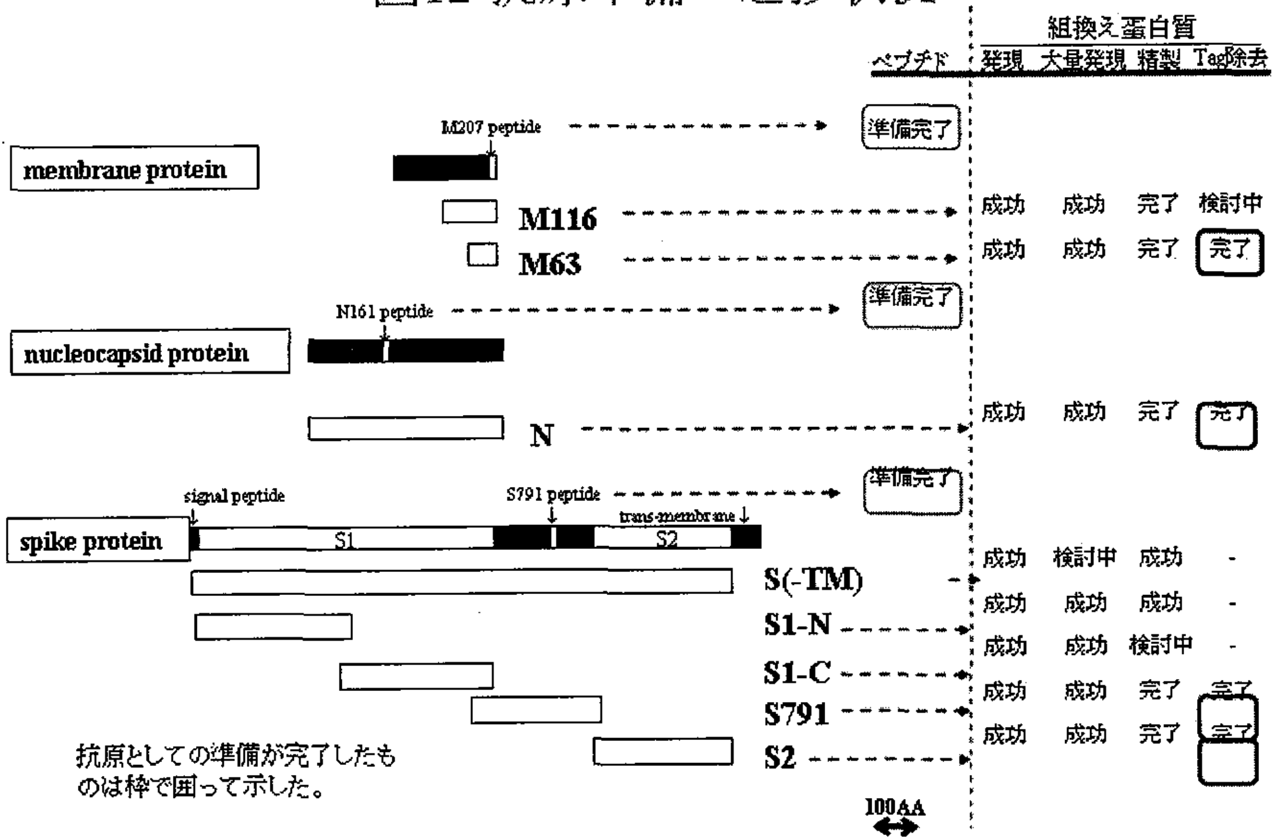


図12 抗原準備の進捗状況



Ⅲ. 研究成果の刊行物・別刷

別紙4

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Shichijo, S., Keicho, N., Long H.T., Quy, T., Phi, N.-C., Ha, L. D., Ban, V.-V. Itoyama, S., Hu, C.-J., Komatsu, N., Kirikae, T., Kirikae, F., Shirasawa, S., Kaji, M., Fukuda, T., Sata,, M., Kuratsuji, T., Itoh, K., Sasazuki, T.	Assessment of synthetic peptides of severe acute respiratory syndrome coronavirus recognized by long-lasting immunity	Tissue Antigens	64	600-607	2004
Itoyama, S., Keicho, N., Long H.T., Quy, T., Phi, N.-C., Long T-H., Ha, L-D., Ban, V.-V., Hu, C.-J., Ohashi, J., Hijikata, M., Matsushita, I., Kawana, A., Yanai, H., Kirikae, T., Kuratsuji, T. Sasazuki, T.	ACE1 polymorphism and progression of SARS	Biochem. Biophysic. Res. Commun.	323	1124-1129	2004
Hamano E, Hijikata M, Itoyama S, Quy T, Phi NC, Long HT, Ha le D, Ban VV, Matsushita I, Yanai H, Kirikae F, Kirikae T, Kuratsuji T, Sasazuki T, Keicho N.	Polymorphisms of interferon-inducible genes OAS-1 and MxA associated with SARS in the Vietnamese population	Biochem. Biophysic. Res. Commun.	329	1234-1239	2005

Haagmans BL, Kuiken T, Martina BE, Fouchier RA, Rimmelzwaan GF, van Amerongen G, van Riel D, de Jong T, Itamura S, Chan KH, Tashiro M, Osterhaus AD.	Pegylated interferon-alpha protects type 1 pneumocytes against SARS *coronavirus* infection in macaques	Nature Med.	10	1-4	2004

Brief communication

Assessment of synthetic peptides of severe acute respiratory syndrome coronavirus recognized by long-lasting immunity

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Key words:
IgG; long-lasting immunity; SARS; structural protein; synthetic peptides

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The authors thank Dr Nguyen Le Hang, Ms. Nguyen Thu Ha and Pham Phuong Thuy for supporting the co-ordination and implementation of this research project in Vietnam, and Drs Masamichi Koujiro and Akira Yamada of Kurume University School of Medicine, Asahi-machi, Kurume, Fukuoka, Japan, for co-ordinating this research.

Abstract: In order to determine highly immunogenic severe acute respiratory syndrome coronavirus (SARS-CoV) epitope peptides capable of inducing long-lasting immunity, we first screened immunoglobulin-G (IgG) antibodies reactive to 197 different overlapping 15-mers from the SARS-CoV proteins in the sera of three infected patients. Forty-two peptides among them were reactive to the sera from all three patients. Consequently, we tested for the reactivity of these 42 peptides to patients' sera ($n = 45$) at 6-month post-infection. The significantly higher levels of IgG antibodies specific to three (S791, M207 and N161) of 42 peptides were detectable in the post-infection sera from 23 (51%), 27 (60%) and 19 (42%) of 45 patients, respectively. These three peptides, recognized by their long-lasting immunity, may provide a better understanding of the immunogenicity of SARS-CoV.

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A novel coronavirus (SARS-CoV) was discovered in association with the cases of life-threatening severe acute respiratory syndrome (SARS) that occurred in March of 2003 (1, 2). The genome of the SARS-CoV is 29,727 nucleotides in length and has 11 open reading frames, and its genome organization is similar to that of other coronaviruses (3). Since March of 2003, studies to determine immunogenic epitopes have been performed at a fast pace, within a short period of time, because of the urgent need to develop both therapeutic and diagnostic modalities for the SARS-CoV (4–8). The results of these studies indicate that both the spike (S) and nucleocapsid (N) proteins of the SARS-CoV contain immunogenic regions. However, further studies are needed in order to identify the hot spots, for which diagnostic and therapeutic tools can be developed. In order to determine highly immunogenic regions, an investigation was performed of SARS-CoV epitope peptides capable of inducing long-lasting immunity, and the three candidate peptides have been reported in this study.

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Forty-two peptides recognized by sera from SARS patients

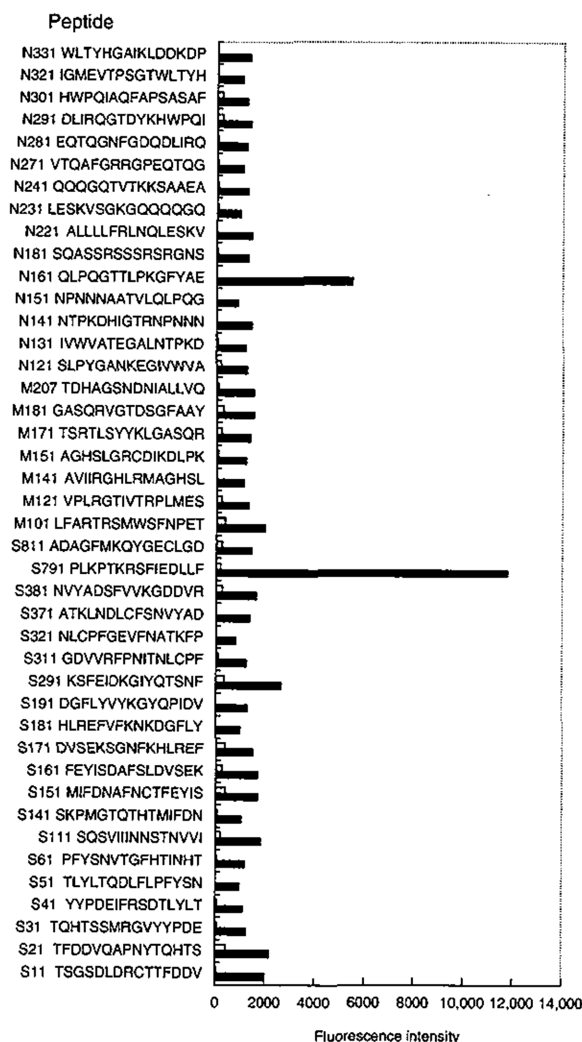

***Fig. 1.* Screening of peptides.** A total of 197 different 15-mer peptides (>70%) possessing 5-amino acid overlap sequences based on the full genomic sequences of the severe acute respiratory syndrome coronavirus (SARS-CoV), including 125 spike (S), 43 nucleocapsid (N), 22 membrane (M) and seven envelope (E) proteins (3), were purchased from American Peptide Company, Inc. (Vista, CA). Each peptide was dissolved in dimethylsulfoxide (DMSO) and was then stored at −20°C until use. These peptides were tested for their reactivity to the sera of early stages of three Taiwanese SARS-CoV-infected patients by using flowmetry analysis with Luminex™ (Luminex Corp., Austin, TX) (9). The sera were collected from Jen-Ai Municipal Hospital, SaAn District, Taipei, Taiwan. The patients' sera showed significantly higher levels of immunoglobulin-G (IgG) ($P < 0.05$) activities reactive to 42 of 197 peptides tested, including 20 spike (S)-, seven membrane (M)- and 15 nucleocapsid (N)-derived peptides, when the means of the scores of fluorescence intensity (FI) from the sera (1000-fold dilution) of the three patients (closed bar) were compared to those of the three healthy donors (HD) (open bar). The peptides were coupled to colour-coded beads, according to the modified manufacturer's instructions (Luminex Corp.). In brief, 100 μl of colour-coded beads were mixed with 100 μl of peptide (1 mg/ml in 0.1 M morpholinoehanesulfonic acid (MES) buffer, pH 4.5). The peptide-loaded beads were then incubated with 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide 2-(N-Morpholino)ethanesulfonic acid (EDC) (1 mg ml^{-1}) at room temperature for 30 min in darkness, and the beads were washed with Tween-20 phosphate-buffered saline (PBS). The beads were treated with 2-aminoethanol for 10 min at room temperature in darkness, washed twice and then re-suspended with 1 ml of 0.05% Block Ace (Snow Brand Milk Products Co., Ltd, Hokkaido, Japan) in Tween-20 PBS. Two microlitres of serum at dilutions of 100–10,000 times was incubated with 25 μl of the peptide-coupled colour-coded beads for 2 h at room temperature on a plate shaker in a 96-well filter plate (MultiScreen™-BV, Millipore Co., Bedford, MA). After incubation, the plate was washed by using a vacuum manifold apparatus and was incubated with 100 μl of biotinylated goat anti-human IgG (gamma-chain-specific: Vector Laboratory Inc., Burlingame, CA) for 1 h at room temperature on a plate shaker. The plate was then washed, and 100 μl of streptavidin-PE (Molecular Probes, Eugene, OR) was added into wells, followed by incubation for 30 min at room temperature on a plate shaker. The bound beads were washed three times followed by the addition of 100 μl of Tween-20 PBS into each well, and the plate was placed for 3 min on a plate shaker. Fifty microlitres of sample was analysed by using the Luminex™ system with the help of the method reported previously (13, 9, 15).

We first measured the levels of immunoglobulin-G (IgG) antibodies reactive to each of the 197 peptides in the sera of the Taiwanese SARS-CoV-infected patients ($n = 3$) and the Japanese healthy donors (HD) ($n = 3$) as negative controls by means of the flowmetry analysis with Luminex™ (Luminex Corp., Austin, TX), a new method that has the great advantage of allowing users to measure a large number of serum samples against a large number of peptide antigens at relatively low cost, time and labour intensity, as recently reported by us (9). The patients' sera showed significantly higher levels of anti-peptide activities ($P < 0.05$) against 42 of 197 peptides tested, including 20 spike (S), seven membrane (M) and 15 nucleocapsid (N)-derived peptides (Fig. 1). The scores, for instance, for the fluorescence intensity (FI) of the anti-SARS-CoV spike protein at positions 791–805 (termed anti-S791) were highest among the peptides tested, and were 1813, 22,964 and 11,240 in the sera of the three patients, whereas those of the controls were 207, 58 and 210, respectively. The scores for the FI of the anti-SARS-CoV nucleocapsid protein at positions 161–175 (termed anti-N161) were 697, 815 and 14,084, whereas those of the controls were 0, 11 and 129, respectively. The results of the dose-dependent curves were obtained in all of the 42 peptides for all three cases. The representative results of the anti-S791 and anti-N161 antibodies have been presented in Fig. 2.

The 42 peptides shown in Fig. 1 were tested for their reactivity to the post-infection (6th month) sera from patients with the Vietnamese SARS-CoV infection ($n = 45$). As negative controls, sera of both Vietnamese HD ($n = 50$) and the contact persons ($n = 230$), who were free from illness but worked in the same hospitals, were simultaneously tested at a serum dilution of 1:100. Both the mean and the median of the FI of sera from Vietnamese HD, contact persons and the patients reactive to each of the 42 peptides have been presented in Table 1. The levels of anti-S791, anti-M207 and anti-N161 activities in the sera of the SARS-CoV patients were significantly ($P < 0.005$) higher than those of both Vietnamese HD and the contact persons as evaluated by means of both Student's t-test and the Mann–Whitney test (Table 1). In contrast, there were no significant differences in the reactivity against any of 42 peptides between the HD and the contact persons.

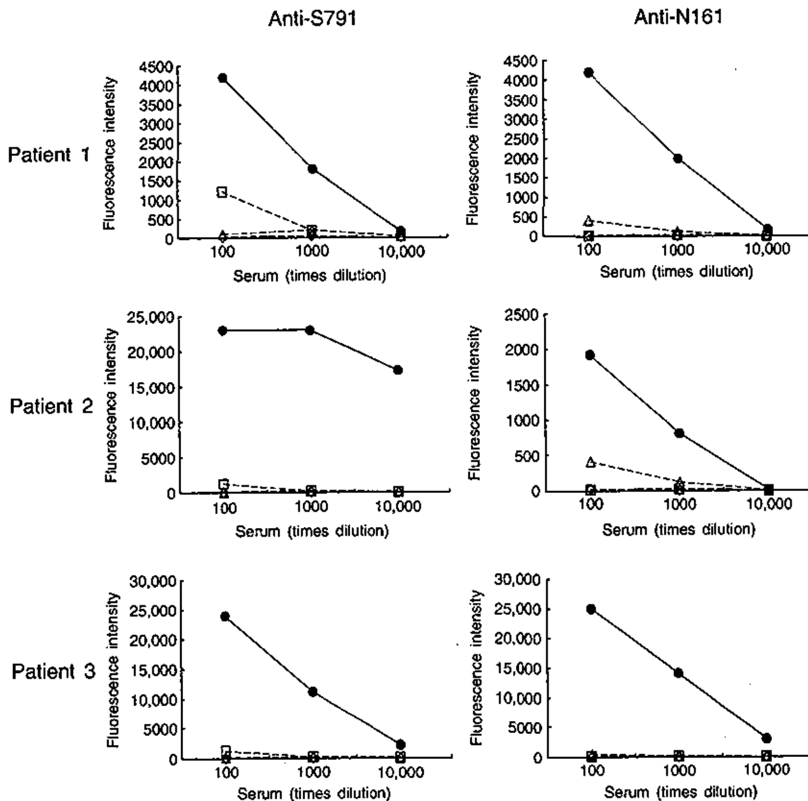

Fig. 2. **Dose dependency.** The dose dependency of the anti-peptide activities was observed in all of the 42 peptides at three different serum dilutions (100-, 1,000- and 10,000-fold). Representative results of the dose-dependent curves of the anti-S791 and anti-N161 activities have been shown in this figure. The levels of immunoglobulin-G (IgG) have been presented as closed circles (patients), and as an open circle, open square and open triangle (three healthy donors), respectively.

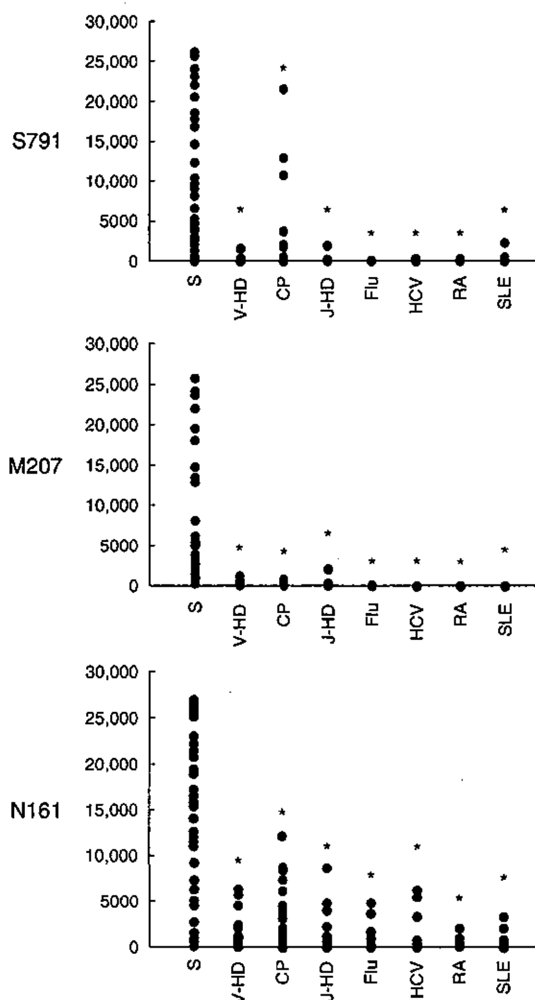

Fig. 3. **Anti-peptide immunoglobulin-G (IgG) at post-infection.** The 42 peptides shown in Fig. 1 were tested for their reactivity to sera from post-infection (6th month) patients with the Vietnamese SARS-CoV infection ($n = 45$). As negative controls, the sera of both Vietnamese healthy donors (V-HD) ($n = 50$) and the contact persons (CP), who were free from illness of SARS-CoV infection but worked in the same hospitals, were tested. Sera from Japanese patients with hepatitis-C virus (HCV), influenza (Flu), rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE), along with Japanese HD (J-HD), were also tested at the same times at a serum dilution of 1:100. Representative results of the fluorescence intensity (FI) scores at a serum dilution of 100:1 of anti-S791, anti-M207 and anti-N161 activities have been shown in this figure. The significance of the differences (*$P < 0.05$) in Luminex reactivities of SARS patients against all of other negative control groups, including V-HD, CP, J-HD, Flu, HCV, RA and SLE, was observed by means of Mann–Whitney test. Plasma from 78 Vietnamese post-infection (6th month, $n = 45$) SARS-CoV patients was collected at Hanoi French Hospital and Bach Mai Hospital in Vietnam. All cases met a modified World Health Organization (WHO) case definition of SARS (16). This study was approved by both the Japanese and Vietnamese Institutional Review Board. Written informed consent was obtained from each of the participants. Sera from Japanese patients with Flu ($n = 12$), HCV ($n = 12$), RA ($n = 15$) and SLE ($n = 10$) and Japanese healthy donors (J-HD, $n = 27$) were provided by Kurume University Hospital and Kurume Medical Center after informed consent was obtained. SARS-CoV, severe acute respiratory syndrome coronavirus.)

Each of the FI scores at a serum dilution of 1:100 of anti-S791, anti-M207 and anti-N161 activities has been plotted in Fig. 3. The cut-off value of the FI scores for anti-S791 peptide activity at a serum dilution of 1:100 was set at 970 (mean: 199 plus 2 SD, 386 × 2 of 50 HD). Under these circumstances, significant levels (>970 at a serum dilution of 1:100) of anti-S791 activity were detected in the sera from 23 of 45 patients (51%), 18 of 230 contact persons (7.8%) and four of 50 HD (8%). When the cut-off value for anti-M207 activity was set at 896 (mean: 356 plus 2 SD, 270 × 2 of 50 HD), significant levels (>896) of anti-M207 activity were detected in the sera from 27 of 45 patients (60%), 10 of 230 contact persons (4.3%) and three of 50 HD (6%). Similarly, when the cut-off value for anti-N161 activity was set at 2705 (mean: 525 plus 2 SD, 1090 × 2 of 50 HD), significant levels (>2705) of anti-N161 activity were detected in the sera from 19 of 45 patients (42%), 21 of 230 contact persons (9.1%) and two of 50 HD (4%) (Fig. 3).

The levels of anti-M181 activity in the sera of the SARS-CoV patients were also significantly higher than those of Vietnamese HD and contact persons as evaluated with the help of both Student's *t*-test and Mann–Whitney test (Table 1). However, the positive cases showing FI scores of greater than the mean plus 2SD were only six of 45 patients (13%). In contrast to these four peptides, significant levels of IgG reactive to the remaining 36 peptides were either scarcely or not detected in the patients (Table 1).

Wang et al. (4) reported four different epitope peptides recognized by the sera of SARS-CoV patients. One of them, the N66 (nucleocapsid protein at positions 161–182) peptide, was 7-amino acids longer at the C-terminal than the N161 (at positions 161–175) peptide reported in this study. These findings suggest that this region of nucleocapsid proteins is one of the most highly immunogenic epitopes of the SARS-CoV when peptides are used. However, the S791 peptide shown to be the other candidate of immunogenic peptides in the present study was not tested by Wang et al. (4), because they selected peptides with relatively high hydrophilicity. They also did not detect the M207 peptide as an immunogenic epitope, although they tested the M206 peptide, which is one N-terminal amino acid longer than the M207 peptide.

In order to determine the cross-reactivity of the patients' sera to the other infectious diseases or auto-immune diseases, sera from Japanese patients with hepatitis-C virus ($n = 12$), influenza virus ($n = 12$), rheumatoid arthritis ($n = 15$) and systemic lupus erythematosus (SLE) ($n = 10$) and the Japanese HD ($n = 27$) were also tested at the same times at a serum dilution of 1:100. However, anti-S791, anti-M207 or anti-N161 activity was not detectable in the

sera of any groups tested, including SLE patients, although the cross-reactivity between the SARS-CoV and SLE was suggested in the study by using an Enzyme-linked immunosorbent assay (ELISA) kit coated by non-purified antigen (10). These results indicate that anti-S791, anti-M207 and anti-N161 activities were largely restricted to the SARS-CoV infection, although sera of Vietnamese patients shall be provided as controls to confirm this issue.

Both the Luminex™ and ELISA were employed for the measurement of anti-peptide antibodies in order to ensure the reliability of the former new method. As expected, both the assays could detect anti-SARS peptide activity reactive to each of the three peptides with relatively higher sensitivity in the Luminex assay for the measurement of anti-M207 antibody. Representative results of the three cases have been presented in Fig. 4.

Reactivity of the synthesized 15-mer peptides with sera from post-infection (6th month) SARS patients with the help of flowmetric analysis by using Luminex™

Name of peptide	Peptide sequence	FI of HD[a] (mean ± SD) (*n* = 50)	FI of CP[b] (mean ± SD) (*n* = 230)	FI of S[c] (mean ± SD) (*n* = 45)	Percentage of positive S samples	FI of HD[a] median[d] (75, 25)	median (75, 25)	median (75, 25)
S11	TSGSDLDRCTTFDDV	767 ± 1505	735 ± 1496	469 ± 631	0	217 (107, 520)	200 (109, 581)	210 (131, 474)
S21	*TFDDVQAPNYTQHTS*	92 ± 396	113 ± 356	117 ± 382	4	18 (13, 38)	24 (12, 56)	18 (9, 30)
S31	TQHTSSMRGVYYPDE	423 ± 766	573 ± 962	493 ± 847	7	231 (158, 376)	257 (164, 478)	207 (162, 288)
S41	YYPDEIFRSDTLYLT	32 ± 103	44 ± 130	43 ± 79	4	8 (0, 27)	15 (0, 32)	23 (7, 37)
S51	TLYLTQDLFLPFYSN	14 ± 64	7 ± 28	7 ± 21	0	0 (0, 0)	0 (0, 0)	0 (0, 0) 0 (0, 3)
S61	PFYSNVTGFHTINHT	17 ± 46	13 ± 45	13 ± 29	2	0 (0, 12)	0 (0, 9)	0 (0, 19)
S111	SQSVIIINNSTNVVI	48 ± 158	34 ± 75	53 ± 159	4	13 (5, 26)	17 (8, 30)	17 (12, 28)
S141	SKPMGTQTHTMIFDN	265 ± 630	290 ± 599	320 ± 686	4	119 (52, 269)	106 (51, 224)	121 (67, 208)
S151	MIFDNAFNCTFEYIS	353 ± 651	806 ± 3057	423 ± 623	7	180 (95, 344)	247 (141, 512)	239 (151, 387)
S161	FEYISDAFSLDVSEK	128 ± 423	168 ± 371	143 ± 279	4	46 (29, 85)	63 (37, 112)	60 (36, 87)
S171	DVSEKSGNFKHLREF	327 ± 610	528 ± 1865	418 ± 697	4	178 (122, 366)	198 (126, 371)	213 (164, 359)
S181	HLREFVFKNKDGFLY	20 ± 54	12 ± 39	10 ± 13	0	0 (0, 15)	0 (0, 7)	4 (0, 16)
S191	DGFLYVYKGYQPIDV	22 ± 65	14 ± 32	39 ± 113	7	0 (0, 13)	0 (0, 11)	0 (0, 35)
S291	KSFEIDKGIYQTSNF	440 ± 1088	604 ± 1321	564 ± 1054	4	165 (109, 331)	207 (121, 428)	229 (149, 371)
S311	GDVVRFPNITNLCPF	171 ± 338	180 ± 345	180 ± 315	2	73 (46, 135)	82 (52, 165)	125 (65, 191)
S321	NLCPFGEVFNATKFP	627 ± 1008	835 ± 2051	508 ± 631	2	322 (175, 596)	341 (202, 664)	333 (212, 481)
S371	ATKLNDLCFSNVYAD	495 ± 1056	875 ± 2035	719 ± 1405	7	231 (157, 474)	289 (189, 607)	237 (179, 521)
S381	NVYADSFVVKGDDVR	376 ± 1047	488 ± 1047	498 ± 1037	7	130 (72, 317)	158 (84, 339)	124 (83, 244)
S791	PLKPTKRSFIEDLLF	199 ± 386	489 ± 2027	3374 ± 5981[e,g,h]	51	39 (6, 214)	37 (9, 124)	1057 (189, 3160)[f,g,h]
S811	ADAGFMKQYGECLGD	268 ± 711	399 ± 970	356 ± 775	4	90 (66, 160)	105 (66, 216)	108 (88, 232)
M101	LFARTRSMWSFNPET	86 ± 224	152 ± 367	151 ± 358	4	42 (18, 76)	48 (18, 101)	51 (32, 117)
M121	VPLRGTIVTRPLMES	69 ± 338	61 ± 207	109 ± 330	4	0 (0, 11)	0 (0, 14)	2 (0, 39)
M141	AVIIRGHLRMAGHSL	17 ± 58	12 ± 32	8 ± 13	0	0 (0, 10)	0 (0, 9)	0 (0, 15)
M151	AGHSLGRCDIKDLPK	781 ± 726	951 ± 1623	1714 ± 3558	16	586 (324, 1035)	562 (296, 951)	627 (368, 1314)
M171	TSRTLSYYKLGASQR	77 ± 372	86 ± 230	114 ± 329	4	8 (0, 27]	14 (0, 51)	18 (7, 39)
M181	GASQRVGTDSGFAAY	904 ± 1655	1470 ± 2614	2596 ± 3007[g]	20	499 (296, 869)	588 (369, 1300)	1306 (706, 3237)[g,h]
M207	TDHAGSNDNIALLVQ	356 ± 270	339 ± 254	2867 ± 4928[g,h]	60	271 (177, 438)	279 (178, 451)	1150 (612, 2224)[g,h]
N121	SLPYGANKEGIVWVA	61 ± 375	101 ± 809	71 ± 268	2	0 (0, 0)	0 (0, 0)	0 (0, 9)
N131	IVWVATEGALNTPKD	169 ± 576	208 ± 576	194 ± 513	7	50 (26, 116)	46 (20, 104)	41 (22, 90)
N141	NTPKDHIGTRNPNNN	276 ± 397	283 ± 393	254 ± 294	2	162 (79, 290)	157 (71, 341)	136 (65, 338)
N151	NPNNNAATVLQLPQG	71 ± 288	189 ± 976	119 ± 411	7	7 (0, 47)	12 (0, 44)	12 (0, 36)
N161	QLPQGTTLPKGFYAE	525 ± 1090	962 ± 2664	6079 ± 7604[g,h]	42	136 (61, 376)	201 (82, 678)	1250 (111, 12099)[g,h]

continued

Name of peptide	Peptide sequence	FI of HD[a] (mean ± SD) ($n=50$)	FI of CP[b] (mean ± SD) ($n=230$)	FI of S[c] (mean ± SD) ($n=45$)	Percentage of positive S samples	FI of HD[e] median[d] (75, 25)	median (75, 25)	median (75, 25)
N181	SQASSRSSSRSRGNS	661 ± 2588	437 ± 1240	388 ± 794	0	111 (63, 207)	87 (44, 231)	79 (49, 245)
N221	ALLLLFRLNQLESKV	181 ± 490	226 ± 505	220 ± 557	4	79 (36, 128)	80 (30, 171)	75 (40, 137)
N231	LESKVSGKGQQQQGQ	314 ± 408	522 ± 1857	312 ± 430	4	150 (109, 431)	209 (100, 370)	181 (99, 362)
N241	QQQGQTVTKKSAAEA	263 ± 594	428 ± 1851	283 ± 496	4	156 (57, 262)	148 (79, 253)	100 (66, 278)
N271	VTQAFGRRGPEQTQG	329 ± 926	681 ± 2088	421 ± 731	4	141 (95, 254)	185 (107, 385)	140 (113, 288)
N281	EQTQGNFGDQDLIRQ	126 ± 344	340 ± 1687	269 ± 375	9	72 (34, 110)	71 (35, 147)	118 (69, 238)[g,h]
N291	DLIRQGTDYKHWPQI	390 ± 865	760 ± 2302	493 ± 949	4	188 (138, 323)	243 (148, 434)	235 (170, 332)
N301	HWPQIAQFAPSASAF	176 ± 397	249 ± 946	155 ± 292	4	34 (17, 146)	40 (18, 97)	56 (37, 131)[h]
N321	IGMEVTPSGTWLTYH	419 ± 2038	307 ± 1782	202 ± 465	0	29 (11, 95)	27 (10, 73)	55 (32, 121)[g,h]
N331	WLTYHGAIKLDDKDP	200 ± 330	225 ± 334	216 ± 292	4	142 (12, 228)	111 (50, 245)	113 (74, 214)

[a]IgG level was determined by using Luminex as described in the legend of Fig. 1. A total of 50, 230 and 45 sera were collected from [a]Vietnamese healthy donors (V-HD), [b]contact persons (CP) and [c]SARS patients (S), respectively, for the experiments.
[d]median (25, 75): the FI at 25 and 75 percentiles were shown.
The significance of the differences (P) in Luminex reactivities between HD and patients was analysed by means of [e]Student's t-test and [f]Mann–Whitney test.
[g]S *vs* HD.
[h]S *vs* CP, $P < 0.005$ (statistical analysis).
SARS, severe acute respiratory syndrome.
FI, fluorescence intensity.

Table 1

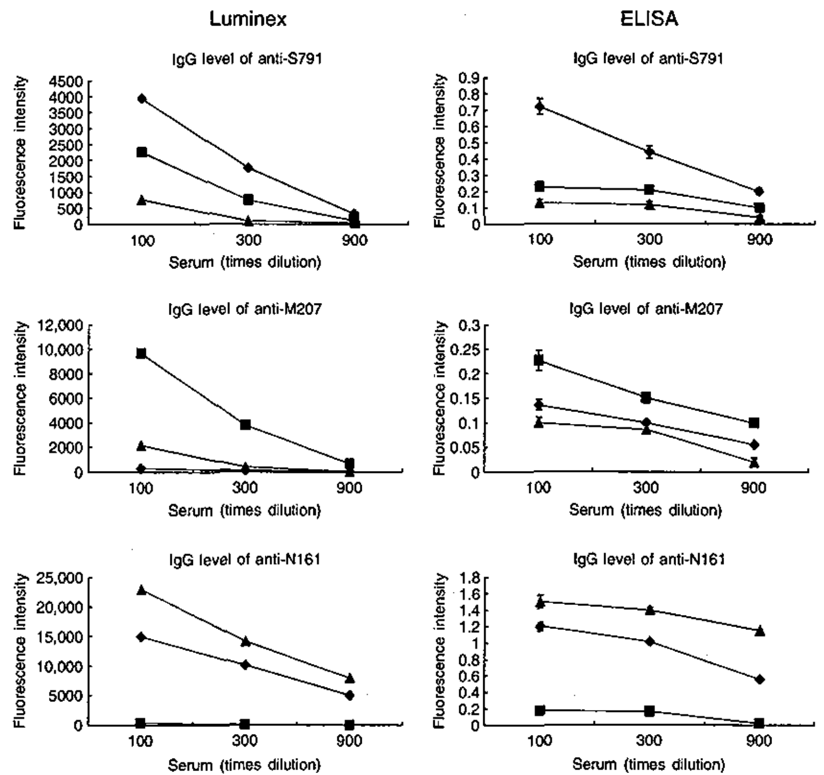

***Fig. 4.* Assayed by conventional ELISA methods.** For the preparation of the peptide immobilized ELISA plate for the antibody absorption test, peptides were diluted in 0.1 M carbonate buffer containing a chemical cross-linker, disuccinimidyl suberate (DSS) (Pierce, Rockford, IL), as reported previously (17). ELISA plates were coated overnight at 4°C with the target peptides at a dose of 200 µg/well. The wells were rinsed three times with 0.05% Tween-20 PBS (PBST). The plates were blocked overnight at 4°C with Block Ace. The representative results have been shown in this figure.