行政院國家科學委員會專題研究計畫成果報告

中文計畫名稱：單純皰疹病毒第一型 UL49.5 基因產物之確認與特性分析

英文計畫名稱：Identification and characterization of HSV-1 UL49.5 gene product

計畫編號：NSC 88-2314-B-040-028

執行期限：87 年 8 月 1 日至 88 年 7 月 31 日

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執行機構：中山醫學院醫事技術學系

一、中英文摘要

本計畫為製造單純皰疹病毒第一型 UL49.5 基因產物，之後利用製造出之蛋白質製備單株及多株抗體，於完成抗體製備再以此確認單純皰疹病毒第一型 UL49.5 基因產物之特性，包括分子量、製造時期、於病毒顆粒位置及轉譯後之修飾作用等。目前已依照研究方法之方略二合成　　，依序為 1. HSV-1a, a.a. 22~43.(22 amino acids) 2. HSV-1b, a.a. 43~57.(15 amino acids) 3. HSV-1c, a.a. 22~57.(36 amino acids)，並將合成之連接 KLH (Keyhole limpet hemocyanin)，之後以 Balb/c mice 及 New Zealand strain rabbits 為動物來源製備抗體。其中單株抗體之製造，第二組及第三組已完成融合瘤之製造，分別獲得 39 及 41 個克隆，但第一組之老鼠於免疫過程中全部死亡。而多株抗體之製造，第三組亦已完成兩次抗原之注射，但第一、二組之兔子亦不幸於免疫過程中死亡。後續之工作包括完成單株及多株抗體之製備與 UL49.5 基因產物之特性分析將持續進行。

The aims of this study are producing poly- and monoclonal antibodies against UL49.5 and characterizing the basic biochemical properties to reconfirm previous findings. According to the hydropathy profiles (Kyte & Doolittle, 1982) of the UL49.5 proteins of HSV-1 (Barnett et al., 1992, Barker & Roizman, 1992), the amino acids fragments chosen to be synthesized are listed below.

HSV-1a. a.a. 22 ~ 43. (22 amino acids)
HSV-1b. a.a. 43 ~ 57. (15 amino acids)
HSV-1c. a.a. 22 ~ 57. (36 amino acids)

In this approach, KLH (Keyhole limpet hemocyanin, Pierce) kit was used. Two fusions were made for HSV-1/b and HSV-1/c respectively and 39 and clones were obtained. Theses clones are under screening. However, unfortunately the mice immunized with HSV-1a and the rabbits immunized with HSV-1a and HSV-1b were died during the immunization process. Except for the identification and characterization of the produced antibodies themselves, the characterization analyses for HSV-1 UL49.5 product include trypsin treatment, PAA (phosphonoacetic acid) treatment, glycosylation inhibitor treatment, reducing agents treatment, lectin binding analysis, immunoprecipitation for modification analysis, and neutralization test are to be continued.

二、計畫緣由與目的

Membrane glycoproteins specified by enveloped viruses are important determinants of viral pathogenicity. They are exposed on the surfaces of virions and on the surfaces of infected cells. They mediate entry of the virus into cells and cell-to-cell spread of infection and also influence tissue tropism and host range. HSV-1 is known to posses at least 11 surface glycoproteins (gB, gC, gH, gK, gL, and gM with genes in the UL, and gD, gE, gG, gI and gJ, encoded in US) and probably other non-glycosylated proteins as well.

In my previous findings, a minor HSV-1 viral structural protein termed p18.5 was recognized by a strain-specific monoclonal antibody CY49. Protein p18.5 contains three major (12K, 13.5K, and 18.5K) and one minor (21.5K) bands on a Western Blot. It

can be detected as early as 4 ~ 6 h post infection and is a true late gene product since the synthesis can be inhibited by DNA synthesis inhibitor treatment. Digestion with trypsin in the presence and absence of 1 % Triton X-100 showed its location to be on the surface of the envelope of purified virus. HSV-1 x HSV-2 intertypic recombinant mapping localised the target gene between map units 0.660 ~ 0.735. These experiments suggested that the most likely candidate gene is UL49.5 (UL49A). Glycosylation of this protein could not be demonstrated through [^{14}C] - glucosamine labelling, immunoprecipitation, glycosylation inhibitor treatment, and lectin binding analysis. The findings by monoclonal antibody CY49 has implied UL49.5 may be a newly identified smallest and non-glycosylated HSV-1 membrane protein (C-C. Yang unpublished data). Unfortunately, CY49 hybridoma cell line was lost due to its unstability. Recently, this UL49.5 protein family has been identified in PRV and BHV-1 (Jons et al., 1996 ; Liang et al., 1996), thus making the need for identification and characterization of HSV-1 UL49.5 gene product more significant and meaningful. Hence we have made the decision to produce mono- and polyclonal antibodies against HSV-1 UL49.5 gene product to reconfirm previous findings. This study includes three stages. Stage 1 is for immunogen production by synthetic peptides. Stage 2 is production of mono- and polyclonal antibodies using the immunogens described above. Stage 3 is characterization of HSV-1 UL49.5 gene product. Except for the identification and characterization of the produced antibodies themselves, the characterization analyses for HSV-1 UL49.5 product contain all the experiments shown by CY49 as described above. These include trypsin treatment, PAA (phosphonoacetic acid) treatment, glycosylation inhibitor treatment, reducing agents treatment, lectin binding analysis, immunoprecipitation for modification analysis, and neutralization test.

The HSV-1 p18.5 is the smallest membrane protein reported so far. However, the findings described above need to be reconfirmed. Further study of this novel and highly conserved viral protein is required to determine its antigenic, functional and biochemical properties. Hence, the aims of this study are producing poly- and monoclonal antibodies against UL49.5 and characterizing the basic biochemical properties to reconfirm previous findings.

三、 結果與討論

The findings by monoclonal antibody CY49 has implied UL49.5 may be a newly identified smallest and non-glycosylated HSV-1 membrane protein. Unfortunately, CY49 hybridoma cell line was lost due to its unstability. Recently, this UL49.5 protein family has been identified in PRV and BHV-1(Jons et al., 1996 ; Liang et al., 1996), thus making the need for identification and characterization of HSV-1 UL49.5 gene product more significant and meaningful. Hence we have made the decision to produce mono- and polyclonal antibodies against HSV-1 UL49.5 gene product to reconfirm previous findings. This study includes three stages.

STAGE 1: IMMUNOGEN PRODUCTION

To achieve the goals of this study, making antibodies against HSV-1 UL49.5 is necessary. In order to make antibodies against HSV-1 UL49.5, below is the approach for immunogen production.

Approach : Synthetic peptides

Synthesis of different fragment of HSV-1 UL49.5 product by a DNA synthesizer is performed according to its DNA sequence (McGeoch et al., 1988). The UL49.5 predicts a hydrophobic protein with 91 amino acids and is separated from UL50 by 23 bp (Barker and Roizman, 1992). According to the hydropathy profiles (Kyte & Doolittle, 1982) of the UL49.5 proteins of HSV-1 (Barnett et al., 1992, Barker & Roizman, 1992), the amino acids fragments chosen to be synthesized are listed below.

HSV-1a. a.a. 22 ~ 43. (22 amino acids)
HSV-1b. a.a. 43 ~ 57. (15 amino acids)
HSV-1c. a.a. 22 ~ 57. (36 amino acids)

The smallest synthetic peptides that will

consistently elicit antibodies that bind to the original protein are 6 residues in length. With peptides of 6 amino acids or slightly larger, the responses vary. Some will generate good antibodies and somewill not. Generally, peptides of approximately 10 residues should be used as a lower limit for coupling. In the literature two strategies are suggested for peptide length. One school suggests using long peptides (up to 40 amino acids long) to increase the number of possible epitopes, while other authors argue that smaller peptides are adequate and their use ensures that the site-specific character of anti-peptide antibodies is retained. Both strategies have been used successfully. The hydrophilic region of UL49.5 is betwenn amino acids 22 and 57. The peptides been synthesized are foucus on this region. Peptides contain hydrophilic amino acids are more likely to be exposed on the surface of the native protein than other sequences and also more likely to be soluble for coupling reactions. In this approach, two of the peptides have Proline at the C-terminus. This causes small complication.The resins which routinely use for peptide synthesis fails if the first residue is Proline. We can synthesize such peptide, using special kind of resin. However, Pro in this place will always react with a small yield, i.e. smaller than any other residue and this resin is a little more expensive. In particular in the case of a 36-mer which falls in to category of difficult peptides and most likely will have to be HPLC purified and will not give very high yield. We can try to improve it, 1) with larger scale and hope for the best, 2) go for "amide resin", or 3) to extend peptide by one residue and have for example -M-S-A-P-G, insted of -M-S-A-P. We tried the last. The peptides were synthesized by Dr. Krystyna B.Piotrowska, Protein Service Laboratory, NAPS Biotechnology Laboratory, University of British Columbia Vancouver, Canada.

HSV-1b was synthesized succesfully and crude peptide yield is almost 300 mg and purity by HPLC close to 90%. HSV-1a caused some problems. On the first attemp, we obtained quite complicated mixture containing not more then 30% of the desired product. HPLC purification of such product is painfull and long and low yielding, that is why we decided to employ more expensive reagents and made another synthesis. The yield of the expected product in the mixture was 58% and HPLC purification gave us 91mg of almost 100% pure product. HSV-1c which is a combination of both previous peptides was expexted to be even more difficult then HSV-1a, and it was. We employed Hmb-Glycine what helped us so much with HSV-1a and it did not give us expected results at first attempt. However, the synthesis was eventually successful. The analysis were also completed (reports included).

In this approach, KLH (Keyhole limpet hemocyanin, Pierce) kit was used. KLH, a copper-containing protein belongs to a group of non-heme proteins called hemocyaninis, which are found in arthropods and molluscs.

STAGE 2: PRODUCTION OF MONO- AND POLYCLONAL ANTIBODIES

The protocols for monoclonal antibodies production had been established (C-C. Yang, Ph.D. thesis, 1994). Briefly, six to twelve weeks old inbred BALB/C mice are used for the production of monoclonal antibodies. Rabbits (new zealand strain) are used for the production of polyclonal antibodies. Intraperitoneal injections (i.p.) are used for the immunization of mice except for the final boost which intravenous injections (i.v.) are used. Subcutaneous injections (s.c.) are used for the immunization of rabbits. Each injection is relatively small, often 50 ~ 100 µl for mice, and up to 800 µl for rabbits. For mice, a dose of 50 ~ 100 µg at each immunization is used ; for rabbit this figure is increased 10-fold to 0.5 ~ 1 mg. For initiation of the primary response, immunogen is mixed with an equal amount of complete Freund's adjuvant (CFA). 18 ~ 24 days after the first injection, the same immunogen is mixed with an equal amount of incomplete Freund's adjuvant (IFA) as a second boost. For fusion, the myeloma cell lines Sp2/0- Ag14 is used. For polyclonal

antibodies, the produced rabbit antisera can be used directly; for monoclonal antibodies either the supernatant of hybridomas culture medium or ascitic fluid produced by injecting hybridomas into the peritoneum of mice can be used as antibodies. Two fusions were made for HSV-1/b and HSV-1/c respectively and 39 and clones were obtained. Theses clones are under screening. However, unfortunately the mice immunized with HSV-1a and the rabbits immunized with HSV-1a and HSV-1b were died during the immunization process.

STAGE 3: CHARACTERIZATION OF HSV-1 UL49.5 GENE PRODUCT

Except for the identification and characterization of the produced antibodies themselves, the characterization analyses for HSV-1 UL49.5 product contain all the experiments shown by CY49 as described above. These include trypsin treatment for the protein localization on the virus particle assay, PAA (phosphonoacetic acid) treatment for molecular weight change, glycosylation inhibitor (tunicamycin and monensin) treatment and lectin binding analysis for glycosylation assay, reducing agents (DTT and β-mercaptoethanol) treatment for disulphide bonding assay, immunoprecipitation for modification analysis, and neutralization test for function analysis. This stage need to be continued.

四、計畫成果自評

This study cost more time and money than expected for the peptide synthesis and antibodies production. For example, the synthesis of the peptides took over six months and spent 10 times more money (NT 224,940) than applied (NT 21,900). As the unexpected difficulties occurred during the peptides synthesis and antibodies production processes, the progress was hence delayed. However, the production of antibodies, either monoclonal or polyclonal, were continued. Hopefully, this study can be finished within next six months.

五、參考文獻

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Mode: Reprocessed Data
Reported On: 07-12-99 14:36:41
Original Results: C:\SPA\SYSTEM1\Data\HSV-1C-P1.RES
Reprocessed Results: C:\SPA\SYSTEM1\Data\HSV-1C-P1R.RES
Notes:
pure

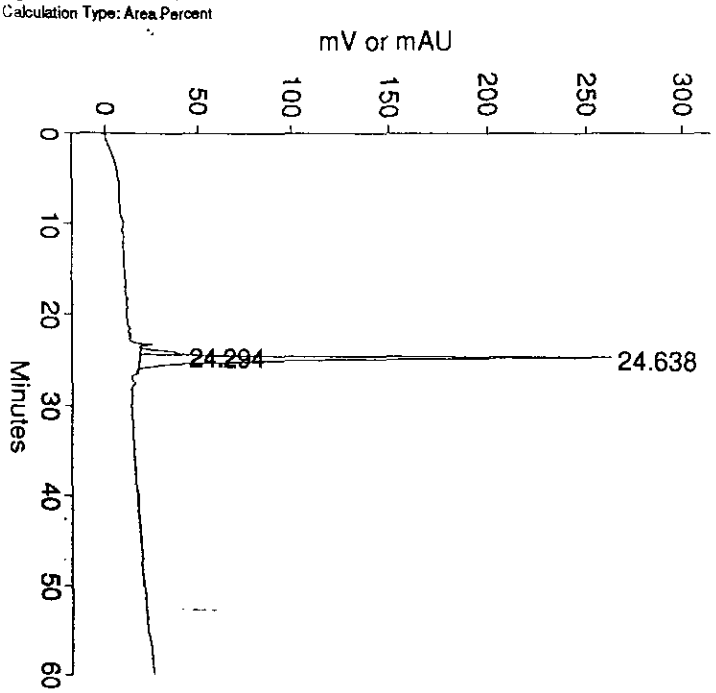
Analysis Report

Name: HSV-1C-P1
Vial: C05
Injection: 1 of 1
Type: Sample
Injected On: 07-09-99 12:37:26

Injection Volume: 20.0 uL

Acquisition Log
Column Pressure (bar): 99
Column Temperature (C): 26
Noise (microAU): 2e+002
Drift (microAU/min): 6e+002
Pump Flow Stability: 1.0
Run-Time Messages: Yes, consult SYSTEM.LOG

Signal 1: FOCUS A 214 nm
Calculation Type: Area Percent



Component	RT(min)	Area	Height	Area%	Peak Type
1	24.294	580056	23334	7.94	Fused
2	24.638	6725806	244666	92.06	Last Fused
Totals		7305862	268000	100.00	

Mode: Acquired Data
Original Results: C:\SPA\SYSTEM1\Data\HSV1arepeat1.RES
Notes:

Reported On: 06-01-99 13:07:43

HSV1b
CRUDE

Analysis Report

Name: HSV1arepeat1
Type: Sample

Vial: C01

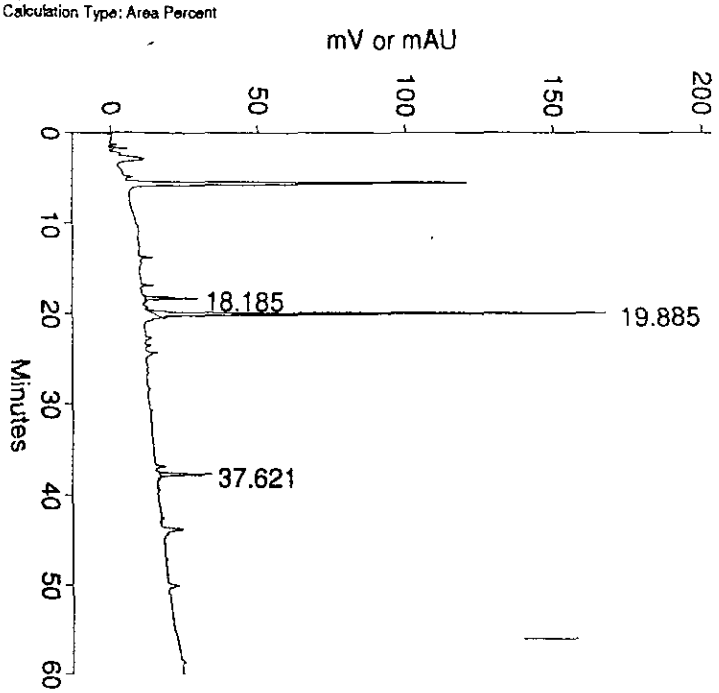
Injection: 1 of 1
Injected On: 06-01-99 11:52:06

Injection Volume: 10.0 uL

Acquisition Log
Column Pressure (bar): 106
Noise (microAU): 3e+002
Pump Flow Stability: 1.0
Run-Time Messages: Yes, consult SYSTEM.LOG

Column Temperature (C): 26
Drift (microAU/min): -5e+001

Signal 1: FOCUS A 214 nm
Calculation Type: Area Percent



Component	RT(min)	Area	Height	Area%	Peak Type
1	18.185	207525	18350	9.33	Resolved
2	19.885	1782206	155346	80.14	Resolved
3	37.621	234201	17676	10.53	Resolved
Totals		2223932	191372	100.00	

Mode: Acquired Data
Original Results: C:\SPA\SYSTEM1\Data\HSV1a1.RES
Notes:

Reported On: 05-27-99 17:45:57

Analysis Report

Name: HSV1a1
Type: Sample

Vial: A01

Injection: 1 of 1
Injected On: 05-27-99 16:40:43

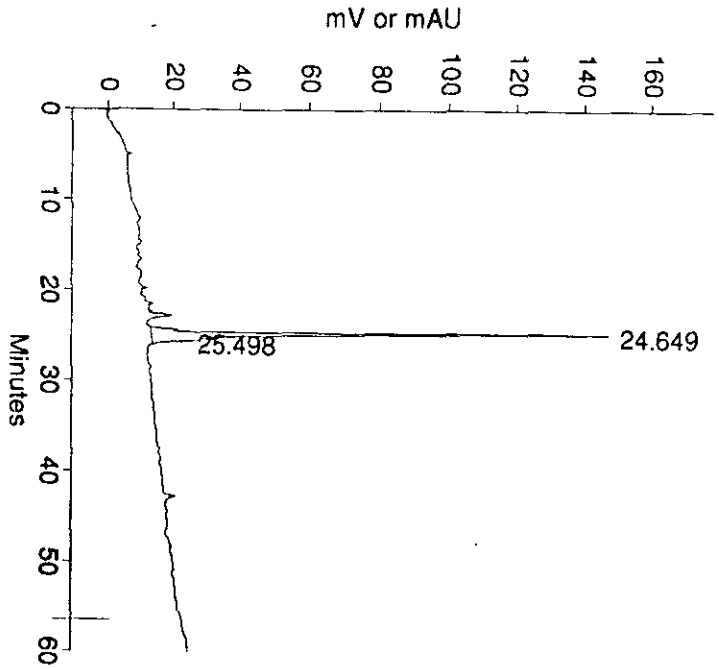
Injection Volume: 10.0 uL

Acquisition Log
Column Pressure (bar): 100
Noise (microAU): 2e+002
Pump Flow Stability: 0.9
Run-Time Messages: Yes, consult SYSTEM.LOG

Column Temperature (C): 26
Drift (microAU/min): -5e+002

Signal 1: FOCUS A 214 nm
Calculation Type: Area Percent

HSV1a
HPLC purified



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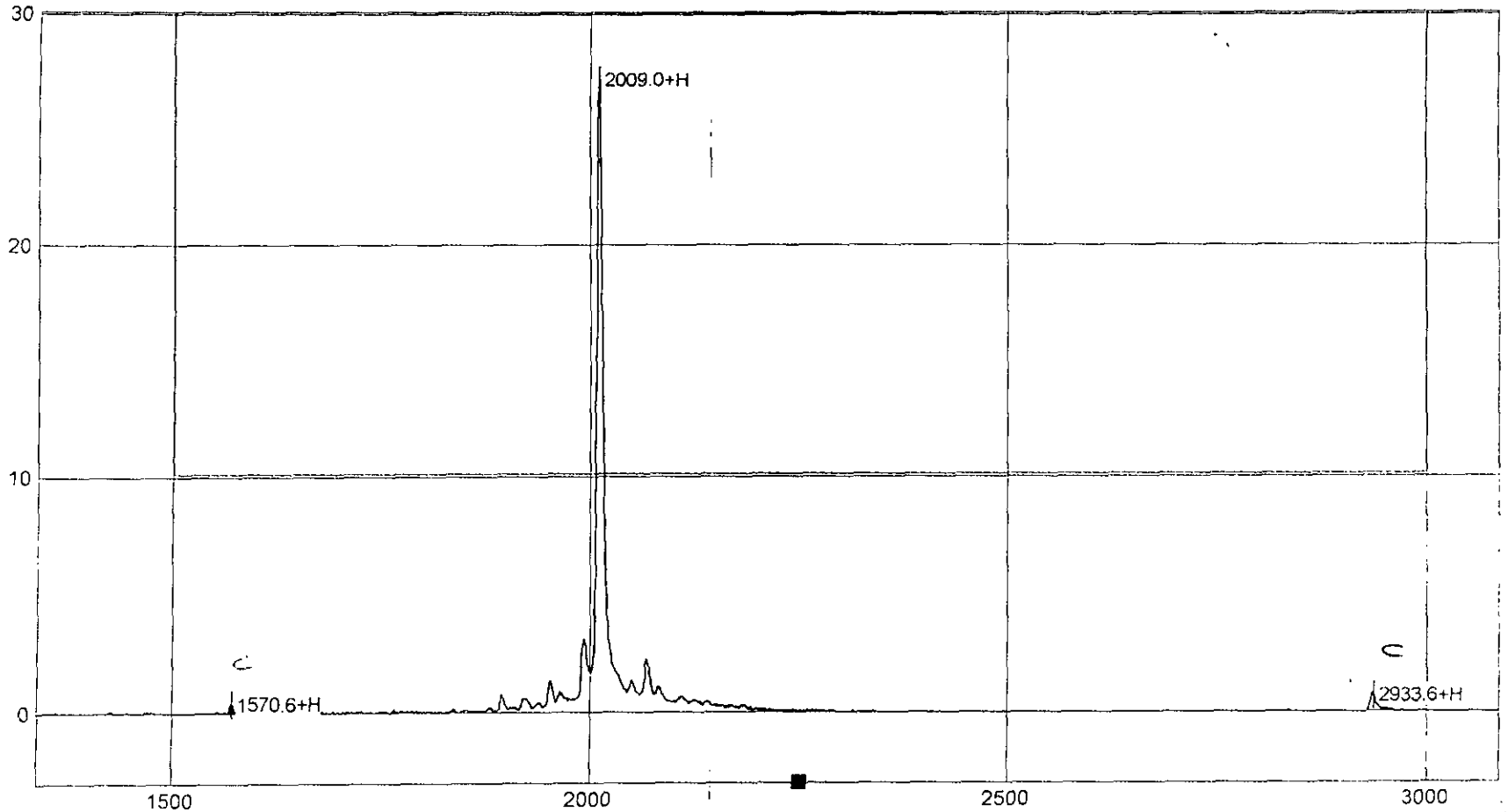
Tuesday, May 11, 1999 15:02

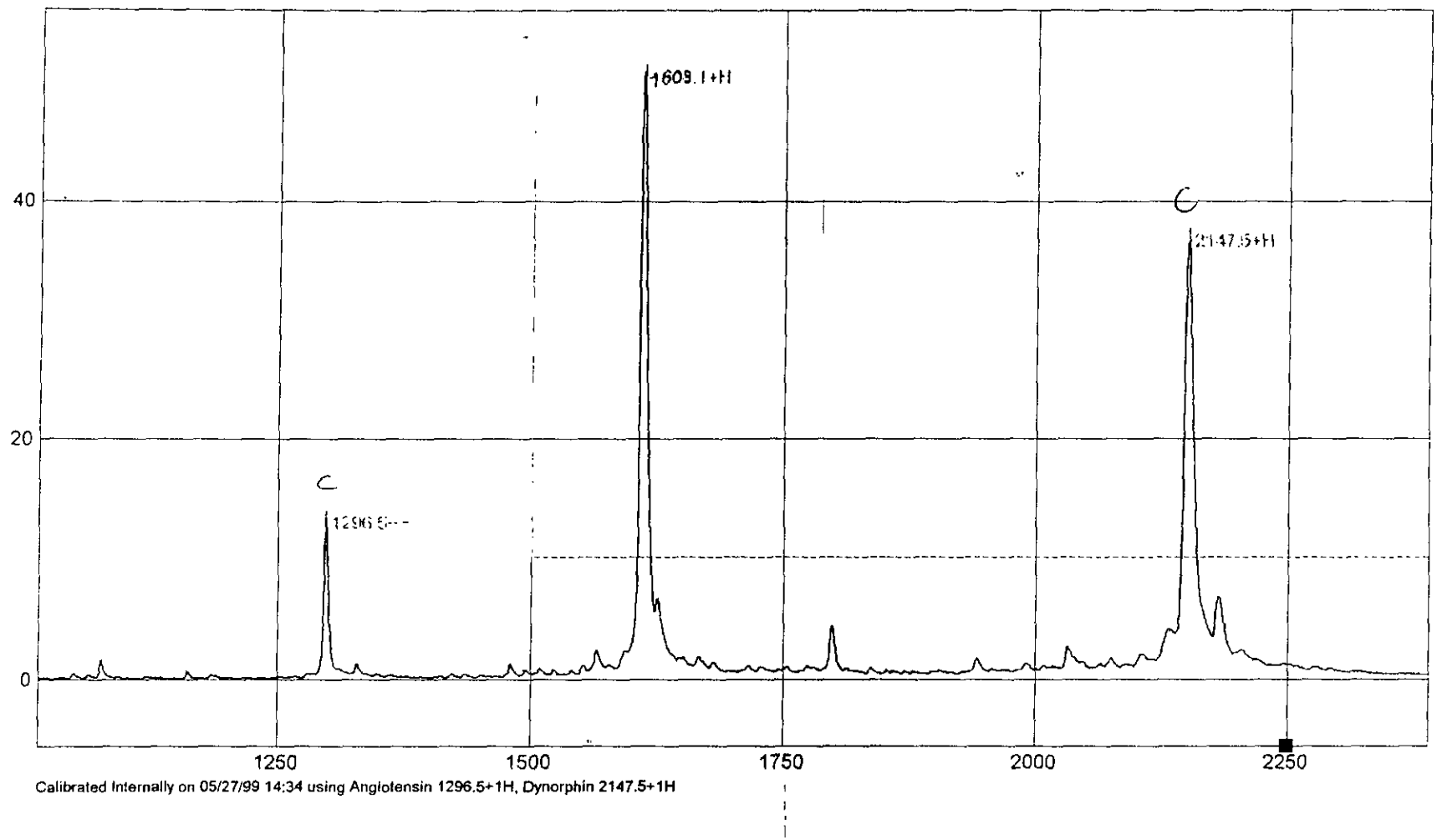


Calibrated Externally on 05/11/99 15:01 from Data Average which is Calibrated Internally on 05/11/99 15:01 using Glu1 fibrinogen peptide 1570.6+1H, ACTH 2933.6+1H

HSV 1a "I" calc MW 2007.94

C + calibrants

Thursday, May 27, 1999 14:30



HSV 1b calc MW 1607.66

FOUND 1608.1

C - CALIBRANTS

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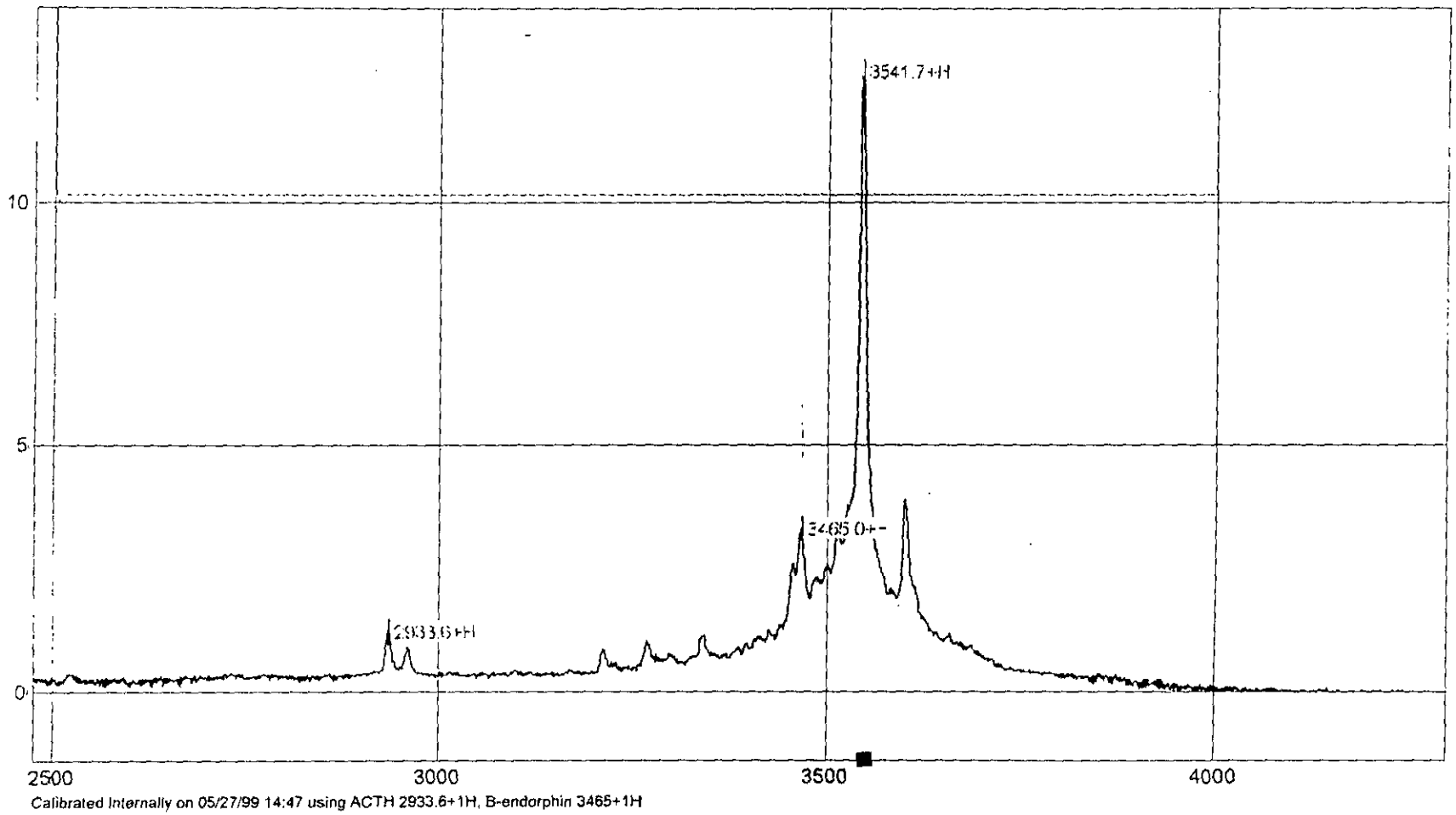

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