AD_____

Award Number: DAMD17-02-2-0012

TITLE: Low Level Exposure to Sulfur Mustard: Development of a SOP for Analysis of Albumin Adducts and of a System for Non-Invasive Diagnosis on Skin

PRINCIPAL INVESTIGATOR: Daan Noort, Ph.D.
Govert P. van der Schans, Ph.D.

CONTRACTING ORGANIZATION: TNO Prins Maurits Laboratory
2280 AA Rijswijk, The Netherlands

REPORT DATE: December 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 2004	Annual (1 Dec 2003 - 30 Nov 2004)

4. TITLE AND SUBTITLE
Low Level Exposure to Sulfur Mustard: Development of a SOP for Analysis of Albumin Adducts and of a System for Non-Invasive Diagnosis on Skin

5. FUNDING NUMBERS
DAMD17-02-2-0012

6. AUTHOR(S)

Daan Noort, Ph.D.
Govert P. van der Schans, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

TNO Prins Maurits Laboratory
2280 AA Rijswijk, The Netherlands

E-Mail: noortd@pml.tno.nl

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

In the third year of the grant period, the fluorescence derivatization of the tripeptide S-(2-hydroxylethylthioethyl)-Cys-Pro-Phe (S-HETE)Cys-Pro-Phe was investigated; the 5/6-carboxyfluoresceine (FAM) derivative of the tripeptide (S-HETE)Cys-Pro-Phe has been prepared. When spiked pronase digests were subjected to derivatization with FAM succinimidyl ester, the FAM-tripeptide could be detected in the digest, albeit after prior clean-up by reversed-phase HPLC. Unfortunately, the FAM-tripeptide could not be determined in a pronase digest of albumin isolated from a highly exposed blood sample.

With regard to the most abundant adduct, i.e., the histidine adduct, attempts to isolate the histidine adduct by means of reversed-phase HPLC were not successful yet.

A definitive SOP for the tripeptide assay was drafted. The inter-individual and intra-individual variation of the in vitro sensitivity of human blood to sulfur mustard was determined, following the albumin – tripeptide (HETE-Cys-Pro-Phe) SOP. Furthermore, the day-to-day variability was determined. The SOP for the tripeptide adduct has been demonstrated to a scientist of USAMRICD, in an independent institute. The method could be set up within one day and the scientist of USAMRICD was able to perform the entire assay on his own after 2 days.

New, stable hybridomas producing antibodies were selected, directed against sulfur mustard adducts to keratin isolated both from previously exposed human callus and from freshly exposed human callus. An alternative method, antibody phage display, was successfully applied for selection of recombinant antibodies which recognized keratin from human callus exposed to sulfur mustard.

14. SUBJECT TERMS
adducts, albumin, biomonitoring, GC-MS, human blood, immunochemical detection, immunoslotblot, keratin, LC-tandem MS, retrospective detection, skin sulfur mustard

15. NUMBER OF PAGES
62

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

_X_ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI - Signature Date

ACKNOWLEDGMENTS

Many thanks are due for the crucial contributions of Alex Fidder and Mrs. Saskia de Kant who were primarily responsible for the organic chemistry and the analytical chemistry, and to Dr. F. Bikker, Mrs. Roos Mars and Mrs. Helma van Dijk for the biochemistry and immunochemistry, as described in this report. The authors are grateful to Albert Hulst, Mrs. Carla Degenhardt, Ad de Jong, Ben van Baar and Eric Wils who carried out the mass spectrometric analyses, to Simon van Krimpen who did the NMR analyses, and to Cees van der Wulp for his assistance to quantitative immunofluorescence microscopy experiments. Dr J.R. Barr (CDC, Atlanta, GA) is acknowledged for his kind hospitality and advice during demonstration of the procedure. A. Woolfitt (CDC, Atlanta, GA) is acknowledged for excellent technical assistance during the demonstration.

SUMMARY

The need for retrospective detection procedures for exposure to low levels of chemical warfare agents has been urgently illustrated by the conflicts in the Gulf Area and, especially, in the attempts to clarify the Gulf War Syndrome. The research within the current cooperative agreement (DAMD17-02-2-0012) aims at:

1. development of a mass spectrometric or fluorescence-based method for retrospective detection of exposure to low doses of sulfur mustard, based on improvement of analysis of an adducted tripeptide in albumin and of adducts to histidine in hemoglobin and albumin (**part A, B** and **C** of revised statement of work).
2. development of immunoslotblot assays for quantitation of protein – sulfur mustard adduct levels, using monoclonal antibodies already available from previous research in this field (**part D** of revised statement of work).

1. The albumin assay (**part A**) is based on our previous finding that upon pronase treatment of sulfur mustard alkylated albumin, a tripeptide S-(2-hydroxyethylthioethyl)cysteine-proline-phenylalanine ((S-HETE)Cys-Pro-Phe) results which has favorable mass spectrometric properties. In the second year of the agreement, a tentative SOP was drafted for this assay.

Detection methods for the tripeptide other than LC tandem mass spectrometry more or less failed. Derivatization of the tripeptide could be affected with carboxymethyl fluoresceine succinimidyl ester (FAM SE), affording a derivative that could be sensitively detected by means of CE-LIF. Although it was possible to demonstrate the presence of the tripeptide adduct in spiked pronase digests, the method could not be applied to digests of albumin isolated from blood that had been exposed in vitro to sulfur mustard.

With regard to analysis of the histidine-sulfur mustard adducts (*i.e.*, the most abundant amino acid adducts formed after exposure to sulfur mustard), a work-up procedure was developed in the first two years of the cooperative agreement for isolation of these adducts from amino acid mixtures resulting from acidic hydrolysis of globin or albumin (**part B**). This procedure afforded purified histidine adducts, which could be further derivatized, inter alia with fluorescent labels for detection with laser induced fluorescence, with Fmoc-Cl for detection with LC-tandem MS, or with trifluoroacetic anhydride for GC-MS analysis. Unfortunately, the isolation methods were labor intensive, and the reproducibility was poor. Attempts to isolate the adduct directly with reversed-phase HPLC were moderately successful: reproducibility was also poor in this case.

In the third year of the agreement, the interindividual variation of the in vitro sensitivity of human blood to sulfur mustard, as determined by the tripeptide assay, was determined (**Part C**). Blood was taken from volunteers, exposed in vitro to sulfur mustard, and albumin was isolated and processed according to the SOP, including the use of the deuterated internal standard. The deviation found was within the range of 10%. Furthermore, the intra-individual variation, determined by sampling three persons two times, was also shown to be acceptable. The day-to-day variability was determined by subjecting a plasma sample from 1 person to pronase digestion at three different time points.
The SOP for the tripeptide assay was demonstrated by a TNO technician to a scientist of USAMRICD in the laboratories of an independent institute. The method could be made fully operational within one working day. After two days the MRICD scientist was able to perform the entire method on his own.
It is envisaged that the scope of this method is not limited to sulfur mustard and that it can become a generic method for diagnosis of exposure to a wide array of alkylating agents.

An immunoslotblot assay was set up for detection of sulfur mustard adducts to keratin (**Part D**). Some problems arose with respect to reproducibility of the accessibility of the sulfur mustard adducts on keratin in the immunoassay and the stability of the antibody producing

hybridomas. We selected new stable hybridomas producing antibodies directed against sulfur mustard adducts to keratin isolated both from previously exposed human callus and from freshly exposed human callus. It appeared that the accessibility of the sulfur mustard adducts decreased after exposure at concentrations above 500 μM sulfur mustard, possibly due to the high degree of crosslinking by the bi-functional agent.
An alternative method, antibody phage display, was successfully applied for selection of recombinant antibodies which recognized keratin from human callus exposed to sulfur mustard. Thirty four culture supernatants, contained with MoPhabs, were able to distinguish untreated keratin from keratin isolated from human callus that was exposed to 1 mM sulfur mustard. A single clone, IAD7, retained its activity, even after repeated culturing, and culturing in larger volumes. For no obvious reason, all other selected clones lost their activity or specificity.

In summary, the following technical objectives, as described in the revised Statement of Work have now been completed after the third year of the grant period:

Part A: Mass spectrometric or fluorescence-based analysis of an adducted peptide from albumin
t.o. 1, 2, 3, 4, 5, 7, 8 and 9

Part B: Analysis of N1/N3-histidine
t.o. 10, 11, 12

Part C: Development of a standard operating procedure
t.o. 14, 15, 16, 17, 18

Part D: Development of immunochemical assays to quantify the adduct levels of sulfur mustard to proteins
t.o. 19, 20 (partly), 21, 22

N.B.
Originally proposed time schedule for revised statement of work

1st year:	technical objectives	1, 2, 3, 4, 6, 10, 19, 20
2nd year:	technical objectives	5, 7, 8, 11, 12, 20, 21
3rd year:	technical objectives	9, 13, 14, 15, 16, 17, 18, 20, 22, 23, 24

TABLE OF CONTENTS

LIST OF FIGURES Page

LIST OF TABLES

I INTRODUCTION

The use of chemical warfare agents in the Iran-Iraq war has learned that reliable methods for identification and verification of exposure to chemical warfare agents in alleged casualties were urgently needed, and not available at that time. Furthermore, experience with the casualties in the Iran-Iraq war and with other incidents learned that biopsies or autopsies of alleged victims often become available several days or even weeks after alleged exposure. Recently, the need for retrospective detection of exposure and, even more demanding, of low level exposure has been vividly illustrated in the attempts to clarify the causes of the so-called "Persian Gulf War Syndrome". Quantitation of low level exposure provides an indispensable basis to study the subtle toxic effects of such exposures. Moreover, the application of reliable procedures to exclude that even trace exposure to chemical agents has occurred will contribute to combat readiness of the soldier.

Within the framework of previous grants (DAMD17-88-Z-8022, DAMD17-92-V-2005 and DAMD17-97-2-7002) we have worked on the development of methods for diagnosis and dosimetry of exposure to sulfur mustard (see Noort *et al.*, 2002; Black and Noort, 2005; Noort and Black, 2005 for extensive reviews). Our approach is based upon the development of monoclonal antibodies against adducts of sulfur mustard with DNA and proteins for use in a variety of immunochemical assays and upon the development of procedures for mass spectrometric analysis of the adducts. The immunochemical assays can be performed on small samples, are highly sensitive, and can be applied "on site" when properly developed. Results obtained from the GC-MS or LC-MS-MS analyses will confirm the immunochemical results and will provide information on the structure of the adducts. In this way, it can be established whether casualties have indeed been exposed to sulfur mustard, whereas dosimetry of the exposure will be a starting point for proper treatment of the intoxication.
Based on results obtained within the context of our previous grant DAMD17-97-2-7002 we decided to continue our investigations on four topics:

A. further development of the mass spectrometric analysis of the tripeptide (S-HETE)Cys-Pro-Phe, resulting from pronase digestion of albumin alkylated by sulfur mustard (t.o. 1-9; year 1-2).
B. perform further research on analysis of the most abundant adduct formed after exposure of proteins to sulfur mustard, *i.e.*, N1/N3-HETE-histidine (t.o. 10-13; year 1-2).
C. develop the most suitable procedure into a SOP (t.o. 14-18; year 2-3)
D. develop immunochemical assays in order to quantify levels of sulfur mustard adducts to proteins (t.o. 19-24; year 1-3).

In the first two years of the current Cooperative Agreement (DAMD17-02-2-0012) the following results were accomplished:

1. A novel, rapid isolation procedure for albumin, based on affinity chromatography, has been introduced into the assay for determination of the alkylated Cys-34 residue in human serum albumin.
2. The lowest detectable exposure level in human blood for determination of the alkylated cysteine-34 residue in albumin has been improved with a factor of 10, by increasing the amount of albumin.
3. The use of an internal standard, *i.e.*, albumin isolated from human blood exposed to sulfur mustard-d_8, has been worked out completely.
4. The particular albumin - sulfur mustard adduct is stable in the marmoset in vivo, at least 28 days after the exposure.
5. The particular albumin sulfur mustard adduct can be detected in the rat *in vivo* at least 7 days after the exposure, with a half-life of approximately 2 days for the albumin adduct, which is in accordance with literature values for rat albumin.
6. The adduct to the N-terminal valine residue in hemoglobin can be detected in the rat *in vivo* at least 28 days after the exposure.

7. The level of N-terminal valine adduct increases during the first 2-3 days after the exposure, indicating the presence of intact sulfur mustard during that period.
8. A (laborious) isolation procedure for the histidine adducts has been developed, based on cation-exchange chromatography.
9. A GC-MS derivatization reaction for the histidine adducts has been developed, which enables sensitive detection of the adduct.
10. Several fluorescent derivatives of the histidine adducts have been prepared. The 5/6-carboxyfluorescein (FAM) derivatives could be detected at a level of 20-25 pg/ml with capillary electrophoresis – laser induced fluorescence detection.
11. A tentative SOP was drafted for the tripeptide assay.
12. Several clones are available producing antibodies which show specificity not only for hemoglobin alkylated with 50 μM sulfur mustard but also for alkylated keratin.
13. An improved screening procedure has been set up for selection of antibodies from clones raised against adducted peptides with amino acid sequences present in human globin.
14. An immunoslotblot assay was set up for detection of sulfur mustard adducts to keratin.
15. The lower detection limit of the immunoslotblot assay for detection of sulfur mustard adducts to keratin was at 25 fmol adducted sulfur mustard using 0.5 μg keratin/slot, corresponding to an adduct level of 1 sulfur mustard adduct/ $5x10^7$ unadducted amino acids. The minimum detectable concentration for *in vitro* treatment of human callus with sulfur mustard was 0.2 μM.
16. An improved screening procedure has been set up for selection of antibodies from clones raised against adducted peptides with amino acid sequences present in human globin.

In the third year of the cooperative agreement work on all 4 subjects was continued, eventually leading to a generally applicable, easy to perform SOP for diagnosis of exposure to sulfur mustard.

II MATERIALS AND INSTRUMENTATION

II.1 Materials

WARNING: Sulfur mustard is a primary carcinogenic, vesicant, and cytotoxic agent. This compound should be handled only in fume cupboards by experienced personnel.

Technical grade sulfur mustard was purified by fractional distillation in a cracking tube column (Fischer, Meckenheim, Germany) to a gas chromatographic purity exceeding 99.5%. The following compounds were synthesized as described previously: Nα-Fmoc-(N1/N3-HETE)histidine and sulfur mustard-d_8 .

The following commercially available products were used:
5/6-Carboxyfluorescein succinimidyl ester (FAM, SE) (Molecular Probes Europe BV, Leiden, The Netherlands). Diethanolamine, acetonitrile (Baker Chemicals, Deventer, The Netherlands); pronase Type XIV from Streptomyces Griseus (E.C. 3.4.24.31), tween 20 (polyoxyethylenesorbitan monolaurate) (Sigma Chemical Co., St. Louis, MO, U.S.A.); immobilized TPCK-trypsin (14 units/ml gel) heptafluorobutyrylimidazole (Pierce, Rockford, IL, U.S.A.); 9-fluorenylmethylchloroformate (Fmoc-Cl), β-mercaptoethanol, Tris.HCl, EDTA (Janssen, Beerse, Belgium); benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; PyBOP (Novabiochem); trifluoroacetic acid, thiodiglycol, trypsin (Aldrich, Brussels, Belgium); fetal calf serum (FCS; LCT Diagnostics BV, Alkmaar, NL); goat-anti-mouse-Ig-alkaline phosphatase, goat anti-mouse-IgG-alkaline phosphatase (KPL, Gaithersburg, USA); microtiter plates (96 wells; polystyrene 'high binding'), microtiter plates (96-wells culture plates) (Costar, Badhoevedorp, The Netherlands); 4-methylumbelliferyl phosphate (MUP), dispase, RNAse T1, protease inhibitor cocktail mini tablets, ampicillin (Amp), kanamycin (Kan) (Boehringer, Mannheim, Germany); rabbit-anti-mouse-Ig-horse radish peroxidase (Dakopatts, Glostrup, Denmark); FITC-labeled 'goat-anti-mouse' (Southern Biotechnology Associates, Birmingham, AL), RPMI-1640 medium (Gibco BRL, Breda, The Netherlands); skimmed milk powder, less than 1% fat, (Campina, Eindhoven, The Netherlands). Carbosorb and Permablend scintillation cocktail were obtained from Canberra Packard (Tilburg, The Netherlands). Tryptone yeast extract (TYE), Polyethyleneglycol 6000
Maxisorp immunotubes (5.0-ml; Nunc, Roskilde, Denmark).
Slyde-A-Lyzer cassettes were obtained from Pierce. Centrex UF-2 10 kDa filters were obtained from Schleicher & Schuell (Dassel, Germany). SepPak Florisil and SepPak C-18 cartridges were obtained from Waters (Bedford, MA). Centrex UF-2 (3 or 10 kDa molecular weight cut-off) centrifugal ultrafilters were procured from Schleicher & Schuell (Keene, NH). Ultrafree (100 kD molecular weight cut-off; 15 ml) centrifugal ultrafilters were obtained from Millipore (Bedford, MA). Albumin affinity chromatography was carried out on HiTrap Blue HP columns (1 ml; Amersham Biosciences, Uppsala, Sweden). Desalting of albumin fractions was carried out on PD-10 columns containing Sephadex G-25 (Amersham Biosciences, Uppsala, Sweden). Dowex 50WX8 was obtained from Fluka. The Tomlinson I & J phage libraries were generous gifts from Greg Winter (Centre for Protein Engineering, Cambridge, United Kingdom). Two *Escherichia coli* strains were used in this study as hosts for bacteriophages: TG1 [K-12 Δ(*lac-pro*) *supE thi hsdD5*/F' *traD36 proA*$^+$ *B*$^+$ *lacI*^{q} *lacZ* ΔM15] and HB2151 [K-12 *ara* Δ(*lac-pro*) *thi*/F' *proA*$^+$ *B*$^+$ *lacI*^{q} *Z* ΔM15].

Venous blood of human volunteers (10 ml, with consent of the donor and approval of the TNO Medical Ethical Committee) was collected in evacuated glass tubes, containing Na_2EDTA (15 mg).
Human callus was obtained from chiropodists. Human skin resulting from cosmetic surgery was obtained from a local hospital with consent of the patient and approval of the TNO Medical Ethical Committee.

II.2 Instrumentation

UV absorbance and UV spectra were recorded on a UV/VIS Spectrometer, Lambda 40 (Perkin Elmer, Breda, The Netherlands).

Microtiter plates were washed using the Skanwasher 300 (Skatron Instruments, Norway; Costar). The fluorescence on microtiter plates (excitation at 355 nm; emission at 480 nm) was recorded with a Cytofluor II (PerSeptive Biosystems, Framingham, MA).

Immunoslotblot assays were carried out with Schleicher & Schuell minifold S (6 mm^2 slots) and nitrocellulose filters (pore size 0.1 µm; Schleicher and Schuell). Protein was immobilized by UV-crosslinking with a GS Gene Linker UV chamber (Bio-Rad Laboratories, The Netherlands). An Enhanced Chemiluminescence Blotting Detection System (Boehringer) was used for the detection of peroxidase activity. The chemiluminescence was recorded with a 1450 MicroBeta Trilux Luminescence Counter (EG & G Wallac, Breda, The Netherlands).

Liquid chromatography experiments were run on an ÅKTA explorer chromatography system (Amersham Pharmacia, Uppsala, Sweden). Columns used were a Pep RPC 5/5 column (Pharmacia, Uppsala, Sweden), a Zorbax SB C-18 column (4.6 mm x 150mm; 5 µm, Zorbax, Mac-Mod Analytical, Chadds Ford, PA, USA) and a Source 15 RPC column (Amersham Pharmacia, Uppsala, Sweden).

LC/electrospray tandem mass spectrometric experiments were conducted on a Q-TOF hybrid instrument equipped with a standard Z-spray electrospray interface (Micromass, Altrincham, UK) and an Alliance, type 2690 liquid chromatograph (Waters, Milford, MA, USA). The chromatographic hardware consisted of a pre-column splitter (type Acurate; LC Packings, Amsterdam, The Netherlands), a sixport valve (Valco, Schenkon, Switzerland) with a 10 or 50 µl injection loop mounted and a PepMap C_{18} (LC Packings) or Vydac C18 column (both 15 cm x 300 µm I.D., 3 µm particles). A gradient of eluents A (H_2O with 0.2% (v/v) formic acid) and B (acetonitrile with 0.2% (v/v) formic acid) was used to achieve separation. The flow delivered by the liquid chromatograph was split pre-column to allow a flow of approximately 6 µl/min through the column and into the electrospray MS interface. MS/MS product ion spectra were recorded using a cone voltage between 25 and 40 V and a collision energy between 30 and 35 eV, with argon as the collision gas (at an indicated pressure of 10^{-4} mBar).

LC/electrospray tandem MS spectra were also recorded on a VG Quattro II triple quadrupole mass spectrometer (Micromass, Altrincham, U.K.). The analyses were carried out with multiple reaction monitoring at a dwell time of 2 s, unless stated otherwise. Operating conditions were: capillary voltage 3.6 kV, cone voltage 25 V, collision energy 15 eV, gas (argon) cell pressure 0.3 Pa, and source temperature 120 °C.
LC-tandem MS experiments during the method demonstration at CDC were recorded on an API4000 triple quadrupole instrument with a standard ionspray interface (Applied Biosystems, Toronto, Canada) and a Shimadzu (Kyoto, Japan) modular liquid chromatograph. In this case the chromatographic hardware incorporated three high pressure pumps, two six-port switching valves, an autosampler with a 50 µL injection loop, and two identical Luna C18 (Phenomenex, Torrance, CA, USA) columns (both 150 mm x 1 mm I.D., 5 µm particles) in parallel. This LC system was configured such that no flow splitting was necessary, and that when the first column was being eluted the second column was being equilibrated in preparation for analysis of the next sample. A gradient of solvents C (H_2O with 1% (v/v) formic acid) and D (80:20 acetonitrile:H_2O plus 1% (v/v) formic acid) was delivered at 50

μL/min. Injections of 50 μL were typically made. The samples were loaded onto the selected analytical column during a 5 min period using Solvent C at 50 μL/min, and then both of the six-port valves were switched. Elution was by means of a linear gradient, ramped from 0% D commencing immediately after the valve switching, to 100% D after a further 25 minutes. During elution of the selected analytical column, Solvent C was used for regeneration of the selected off-line column, at a flow rate of 75 μL/min. The cycle time was 33 minutes. MS/MS (MRM) transitions from *m/z* 470.1 to 105.0 (for the tripeptide) and from *m/z* 478.1 to 113.0 (for the d_8-tripeptide) were recorded at unit resolution on both Q1 and Q3 (i.e., with ion peaks between 0.60 and 0.80 *m/z* units wide at half-maximum height), at a declustering potential of 65 V and a collision energy of 35 eV, with nitrogen as the collision gas.

Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) was performed on a Beckman P/ACE 5000 CE instrument equipped with a Laser Induced Fluorescence detector (Fullerton, CA, USA). Excitation wavelength was 488 nm, emission wavelength 520 nm. A fused silica capillary (i.d. 75 μm) was purchased from Polymicro (Phoenix, AZ, USA). The length of the capillary was 47 cm (40 cm effective length to the detector). Sample introduction was performed by pressure injection for 3 s at 0.5 psi. Separation voltage was 20 kV. The buffer used consisted of 100 mM Borax, pH 9.3.

III EXPERIMENTAL PROCEDURES

III.1 Development of assays for analysis of sulfur mustard adducts to proteins as Standard Operating Procedures: mass spectrometric analysis of an alkylated tripeptide in albumin

III.1.1 Derivatization of (S-HETE)-Cys-Pro-Phe with pyrenyl diazomethane (PDAM)

To a solution of (S-HETE)Cys-Pro-Phe (0.7 μmol; 0.33 mg) in CH_3CN/H_2O, 1/1, v/v, was added pyrenyl diazomethane (PDAM; 0.4 mg; 1.6 umol), dissolved in CH_3CN (0.1 ml). The mixture was mixed with a Vortex and subsequently stirred for 2 h at room temperature, under the exclusion of light. After 2 h, the reaction mixture was analyzed by means of FPLC, and the chromatograms were compared with the chromatograms obtained after analysis of separate solutions of PDAM and tripeptide. The newly formed compound was isolated from the reaction mixture and analyzed by LC-MS. Electrospray MS: *m/z* MH^+ 684.2. Yield: 0.4 mg of a yellowish solid (0.6 μmol).

III.1.2 Derivatization of (S-HETE)-Cys-Pro-Phe with FAM-SE, followed by CE-LIF detection

Derivatization was performed by dissolving synthetic (S-HETE)Cys-Pro-Phe (0.25 mg) in N-methylpyrollidon (100 μl). Next, DIPEA (5 μl) and 5,6-FAM-SE (10 μl of a stock solution of 200 mg/mL in DMSO) was added. After incubation for 2 h, aqueous hydroxylamine solution (1.5 M, adjusted to pH 8.5 with 5 M NaOH, diluted 1/1 with water; 200 μl) was added and the mixture was incubated for another hour.
The fluorescent derivative was purified using a Seppak C18 cartridge. First, the cartridge was equilibrated by rinsing with methanol (2 mL), followed by water (2 mL). The reaction mixture was applied, and the cartridge washed with water (4 mL). Finally the product was eluted using methanol (2 mL). HPLC showed two closely eluting peaks (5- and 6 isomer of FAM-(S-HETE)Cys-Pro-Phe); the correct mass was confirmed by ES-MS; *m/z* 828.3 (MH^+).
Quantification was performed using absorbance measuring at 494 nm at pH 8, according to the protocol of the manufacturer. Yield: 56%, uncorrected for losses for analyses.
Capillary electrophoresis experiments were performed on a Beckman P/ACE 5000 (Beckman, Fullerton, CA, USA) capillary electrophoresis instrument equipped with laser induced fluorescence detection. A fused silica capillary (i.d. 75 μm, Polymicro, Phoenix AZ, USA) was mounted in the capillary cartridge and maintained at 25 °C. The total length of the capillary was 47 cm and the effective length from inlet to detection window was 40 cm. Samples were introduced by pressure injection for 3 s at 0.5 psi. Separation voltage was 20 kV. The running buffer consisted of 100 mM Boric acid adjusted to pH 9.3 with sodium hydroxide. A concentration of 3 nM was easily detected using CE-LIF.

III.1.3 Derivatization of (S-HETE)-Cys-Pro-Phe with FAM-SE in pronase digests

First, pronase digests from albumin (from 0.5 ml of plasma, isolated from blood exposed to 0 or 1 mM sulfur mustard) were purified by using the Sep-pak C18 method (vide infra, SOP). The appropriate fractions were concentrated and the residue dissolved in NMP (0.1 mL) or 0.1 M $NaHCO_3$ (0.1 ml). DIPEA (5 μL; in case of NMP as solvent) and 6-FAM SE (25 μL of a 10 mg/ml solution in DMF) was added to 10 ul of the abovementioned solution. After 2 h, the reaction was quenched with hydroxylamine solution. Half of the reaction mixture was applied to HPLC analysis; the region where FAM (S-HETE)Cys-Pro-Phe elutes was collected and analyzed by means of CE-LIF. Prior to CE-LIF analysis, samples were diluted 10 times.

In spiking experiments, pronase digests (hoeveel were spiked with synthetic (S-HETE)Cys-Pro-Phe (1 mg/ml) and derivatization was carried out in 0.1 M $NaHCO_3$.

III.2 Development of assays for analysis of sulfur mustard adducts to proteins as Standard Operating Procedures: mass spectrometric and fluorescence-based analysis of histidine adducts

III.2.1 Synthesis of N1- and N3-HETE-L-histidine

These compounds were synthesized according to the procedure described in the final report of Cooperative Agreement DAMD17-92-V-2005.
The analytical data were in full agreement with earlier obtained data.

III.2.2 Isolation of N1- and N3-HETE-L-histidine by reversed-phase HPLC

In order to isolate the N1/N3-HETE-histidine adduct, solutions of globin (isolated from blood exposed to 0 and 10 mM sulfur mustard) were prepared in 6 M HCl (10 mg/mL). The globin solutions were subjected to acidic hydrolysis (16 h, 110 °C) and the resulting amino acid mixtures were used to set up isolation procedures based on reversed-phase HPLC.
An acidic hydrolysate (20 mg of blank globin in 6 M HCl (2 mL), spiked with N1/N3-HETE-histidine, both 100 microgram) was neutralized with NH_4OH (concentrated, 1.5 mL). Part (1 mL) was purified using a preparative C18 column (250 mm x 10 mm; 5 u; Alltima).
A low gradient profile was applied in order to separate the N1/N3-HETE-His from the other amino acids in the acidic hydrolysate. Flow rate: 3.3 mL/min. Buffers used: A = 0.1% TFA in H_2O, B = 0.1% TFA in 80% CH_3CN. First, the column was eluted with 0% B for 5 min; next a gradient was applied to 50% B in 20 min and finally the column was washed with 100 % B and equilibrated again to 0 % A with 10 mL of A buffer. N1- and N3-HETE-HIS eluted at about 15 min using this system.
Fractions were collected and analyzed by LC/MS with and without derivatization with Fmoc-chloride. MS confirmed the presence of the adducts in the collected fractions.
Next, globin, isolated from blood exposed to 50 mM sulfur mustard was used to test this isolation procedure. Again, the hydrolysate was fractionated on a preparative Alltima C18 column and the collected fraction was lyophilized to dryness and taken into water (100 microliter). Part (10 microliter) was used for LC-MS analysis, part (45 microliter) was used for FMOC derivatization and part (45 microliter) was used for derivatization with TFAA (GC-MS analysis).

III.2.3 Derivatization with Fmoc-Cl

Derivatization with Fmoc-Cl was performed as described in the previous annual report.

III.2.4 Derivatization with TFAA

Lyophilized hydrolysate (45 microliter) was taken up in HCl/methanol (2 mL, 2 M) and heated at 80 °C. The sample was dried and coevaporated with ethyl acetate (0.5 mL). Next, ethyl acetate (1 mL) and trifluoroacetic anhydride (350 microliter) were added and the mixture heated at 110 °C for 30 min. The samples were dried and dissolved in toluene (100 microliter) and analyzed with GC/MS.

III.3.1 Standard Operating Procedure for albumin – tripeptide assay

The plasma sample (0.5 mL) of interest was spiked with plasma (25-50 µL), isolated from blood exposed to 1 - 100 µM d_8-sulfur mustard. The sample was diluted with buffer A (50 mM KH_2PO_4, pH 7; 2 ml), filtrated over a filter disk (0.45 µm), applied on a HiTrap™ Blue HP cartridge (prepacked with Blue Sepharose High Performance, with Cibacron Blue F3G-A as the ligand; 1 mL) that had been equilibrated with buffer A (10 mL). The cartridge was eluted with buffer A (10 mL) by means of a syringe, within one minute. Subsequently, the cartridge was eluted with buffer B (50 mM KH_2PO_4, 1.5 M KCl, pH 7; 3 mL). The latter effluent was collected. The HiTrap column can be regenerated by washing with buffer B (10 ml) and subsequently with buffer A (10 mL). Next, a PD-10 column (containing 10 mL of Sephadex G 25 material) was equilibrated with 50 mM NH_4HCO_3 (25 mL). The albumin fraction, collected from the HiTrap Blue HP column (3 mL), was applied to the column, and the column was eluted with aqueous NH_4HCO_3 (50 mM; 3 mL). The effluent was collected.

Part of the purified albumin fraction (0.75 ml) was used and subsequently Pronase was added (100 µl of a freshly prepared solution (10 mg/ml) in 50 mM NH_4HCO_3), followed by incubation for 2 h at 37 °C. The digests were filtrated through molecular weight cut-off filters (10 kD) under centrifugation at 2772 g in order to remove the enzyme. The filtrate was analyzed by means of LC/MS/MS.

For low exposure levels, the filtrate (after 10 kD filtration) was processed Seppak C18 cartridge. To this end, the cartridge was conditioned with methanol (5 mL), followed by 0.1% TFA/water (5 mL). The pronase digest was applied, and washed with water (2 mL), containing 0.1 % TFA, 10 % CH_3CN/water (2 mL,0.1 % TFA), 20 % CH_3CN/water (2 mL,0.1 % TFA), and finally eluted with 40% CH_3CN/water (2 mL,0.1 % TFA). The eluate was evaporated to dryness, redissolved in water (0.1 % TFA, 50 microliter and analyzed with LC/MS/MS.

Conditions LC-system

Eluent A: 0.2% formic acid in water. Eluent B: 0.2% formic acid in acetonitrile.

Time (min)	% eluent A	% eluent B	Flow (ml/min)
0	100	0	0.1
5	100	0	0.6
50	30	70	0.6

The flow of 0.6 ml/min was split before the column to 35 µl/min. Column: PepMap C18, 3 µm, 15 cm x 1 mm. Loop: 50 µl.

Conditions triple quad MS

Transitions were monitored of the protonated molecular ions of (S-HETE)Cys-Pro-Phe and (S-d_8-HETE)Cys-Pro-Phe to the most intense fragment (HETE):

MH^+ 470.2 → 105

MH^+ 478.2 → 113

Scan time 1.2 s. Cone voltage 35 V, collision energy 20 eV (Argon pressure 3 x 10^{-3} mBar).

III.3.2 Determination of interindividual variation

Blood was taken from volunteers (with informed consent; 10 persons) and exposed to sulfur mustard (0.1 µM), as described in the previous annual report. After incubation for two hours at 37°C, the plasma layer was separated from the erythrocytes. From each of the abovementioned plasma samples, 0.5 mL was mixed with 25 microliter of "internal standard" plasma (25 µl; isolated from blood that had been exposed to sulfur mustard-d_8, resulting in a

virtual exposure level of approximately 1 μM sulfur mustard-d_8. Each plasma sample was further processed according to the SOP.

III.3.3 Determination of intraindividual variation

Blood was taken from volunteers (3), three times, at three different time points (3 months between). Blood samples were exposed to sulfur mustard (0.1 μM) and further processed as desribed in the SOP.

III.3.4 Day-to-day variability of SOP

A single blood sample was exposed to sulfur mustard (0.1 μM); plasma was isolated. At four different time points (within a time period of 6 months), aliquots of the plasma sample were processed according to the SOP.

III.3.5 Demonstration of SOP to USAMRICD scientist

The demonstration was carried out in April 2004. For details, see the annex.

III.4 Development of immunoslotblot assays for quantitative analysis of sulfur mustard adducts to proteins

III.4.1 Exposure of human skin to sulfur mustard vapor

This was performed as described in the previous annual report.

III.4.2 Preparation of skin cryostat sections

This was performed as described in the previous annual report.

III.4.3 Detection of keratin adducts; isolation of keratin from human callus

Human callus (100 mg) was soaked in Tris.HCl buffer (5 ml, 20 mM, pH 7.4) overnight. After centrifugation (30 min, 400 rpm) the residue was stirred in a buffer (5 ml; pH 7.4) containing Tris.HCl (20 mM) and urea (8 M). After centrifugation (30 min, 400 rpm), the residue was extracted with a buffer (5 ml; pH 7.4) containing Tris.HCl (20 mM), urea (8 M), and β-mercaptoethanol (0.1 M).
The crude keratin was purified on a G 75 column (100 × 2 cm) with a buffer (pH 7.6) containing SDS (0.5%), Tris.HCl (10 mM) and DTT (10 mM); flow, 0.25 ml/min. Appropriate fractions were collected and dialysed against water. The remaining solution was lyophilized, affording 20 mg crude keratin/100 mg callus. In spite of the dialysis step it appeared that the protein content was only 7-22%.
Because the latter procedure resulted in a precipitate upon dialysis, particularly in the case of keratin containing sulfur mustard adducts, several other keratin-purification procedures were applied (see Results section). It appeared that precipitation could be avoided when, before dialysis, the keratin fractions were incubated at 65 °C for 10 min. However, this procedure did not improve the accessibility of the sulfur mustard adducts on keratin.
Positive results were obtained when the crude keratin, in 0.5-ml aliquots, was purified through a PD10 column (Sephadex G-25, Amersham), followed by desalting and concentrating of the pooled appropriate fractions over a Centrex UF-2 filter (molecular cut-off 10 kDa, KD10, 1-ml aliquots, each followed by two 1-ml washings with water). Representative concentrations:

20-30 mg keratin/ml. Effects of still remaining salt and SDS could be eliminated by the strong dilution in PBS before application in the immunoassay.

III.4.4 Bio-Rad assays for protein content

For the assessment of the protein content originally the Bio-Rad assay as described previously (Benschop and Van der Schans, 1995) was applied. However, it appeared that the keratin solutions obtained as described in the previous section still contain components which interfere with this assay. Therefore, in those cases the so-called RC DC protein assay (Bio-Rad) was applied according to the manufacturer. Briefly, 125 µl of RC reagent I was added to 25 µl of sample (or standard protein solution) and incubated for 1 min at room temperature. Then 125 µl of RC reagent II was added, followed by centrifugation for 3-5 min at 15,000g. Supernatant was discarded and 127 µl of reagent A' was added and incubated at room temperature for 5 min until the precipitate was completely dissolved. Next, 1 ml of DC reagent B was added and the mixture was incubated for 15 min at room temperature. Then, absorbance was read at 750 nm.

III.4.5 Exposure of human callus to sulfur mustard

This was performed as described in the previous annual report.

III.4.6 Exposure of human skin to saturated sulfur mustard vapor and extraction of epidermal keratins

This was performed as described in the previous annual report

III.4.7 Preparation of skin cryostat section and immunofluorescence microscopy

This was performed as described in the previous annual report

III.4.8 Immunoassays (ELISA) with hybridoma-supernatants

This was performed as described in the previous annual report

III.4.9 Immunoslotblot procedure for the detection of sulfur mustard adducts to keratin

This was performed as described in the previous annual report. Essentially, the procedure developed for the detection of sulfur mustard adducts to DNA (Benschop et al., 2000) was applied. Briefly, a. solution of keratin (200 µl, 2.5 µg/ml PBS) isolated from a human callus sample exposed to sulfur mustard was spotted on a nitrocellulose filter. All samples were blotted in triplicate on the same filter. After blotting, the slots were rinsed with PBS. The filters were dried on air and the keratin was immobilized by UV-crosslinking (50 mJ/cm^2). Next, the filter was incubated with blocking solution (about 50 ml, 5% milkpowder in PBS + 0.1% Tween 20) at room temperature for 30 min and washed three times with PBS + 0.1% Tween 20. Next, the filter was incubated with 1st antibody (1H10, directed against sulfur mustard adducts to human keratin) diluted 500-fold in 20 ml solution containing 0.5% milkpowder in PBS + 0.1% Tween 20, overnight at 4 °C under continuous shaking, washed 4 times with PBS + 0.1% Tween 20, the last three times for at least 15 min each. Next, the filter was incubated with 2nd antibody (directed against the 1st antibody) diluted 1000-fold in 20 ml solution containing 0.5% milkpowder in PBS + 0.1% Tween 20, for 2 h at room temperature under

continuous shaking, washed 4 times with PBS + 0.1% Tween 20, the last three times for at least 15 min each.
Finally, a 1:100 mixture of solution B with solution A (of the Enhanced Chemiluminescence Blotting Detection System) was preincubated in a water bath for at least 30 min at 25 °C. Then, free (wash) solution was removed from the filter with filter paper and position A12 and H1 marked with ball point (not a felt pen!). The filter was placed in a closely fitting box. After addition of substrate solution (10 ml) incubation was performed for 1 min.
Next, the filter was wrapped in plastic (straight from the substrate solution) without air bubbles. Liquid was pressed out, the filter was transferred in plastic into a luminometer cassette and placed in the luminometer. Luminescence was measured according to the required program. For each sample the mean luminescence of duplicate or triplicate samples was calculated.

III.5. Production of new monoclonal antibodies directed against sulfur mustard adducts to keratin

Because the antibodies directed against sulfur mustard adducts to keratin obtained from a previous study did no longer behave as they should do, we decided to produce new antibodies. Because some modifications were applied the procedures have been described below again.
In addition, a new procedure, the phage display assay, was applied.

III.5.1. Solid phase synthesis of peptides containing an Nω-HETE-glutamine or Nω-HETE-asparagine residue

The following peptides containing modified asparagine or glutamine residues were synthesized as described earlier (Benschop et al 2000):

1. G-V-V-S-T-H-(Nω-HETE)Q-Q-V-L-R-T-K-N-K
2. G-I-Q-(Nω-HETE)Q-V-T-V-N-Q-S-L-L-T-P-L-N-K
3. G-V-M-(Nω-HETE)N-V-H-D-G-K-V-V-S-T-H-E-K

III.5.2 Immunization of mice for generation of antibodies against synthesized haptens

Four mice were immunized (*s.c.*) with 100 µl of a mix of the three antigens (about 1 mg/ml) and stimune in a ratio of 1:1.25. Blood samples of all mice were taken after 7 days to test the serum for antibody response against keratin treated with 0.05 or 1 mM sulfur mustard, with a direct ELISA. A positive response was not observed against sulfur (*s.c.*) mustard treated proteins after 21 days. Therefore, the mice received a second immunization with the same mix of haptens at 7 weeks after the first immunization. After the second immunization still no positive response was observed against sulfur mustard treated proteins. However, there was a positive response against each of the three adducted peptides used for immunization in comparison to the corresponding non-adducted peptides. A booster with antigen (volume up to 0.2 ml) was administered (*i.p.*, according to the manufacturer of stimune) 4 weeks later to two mice, one without and one with stimune. After 3 days the animals were killed with CO_2 anesthesia and the blood was collected by heart puncture. A cell suspension of the spleen was prepared for the production of hybrid cell strains.
The other two mice received a booster, without stimune, after another 2 months and were used for spleen cell collection for the production of hybrid cell strains.
The whole procedure was repeated with another four mice but immunization was carried out with keratin isolated from human callus exposed to 1 mM sulfur mustard.

III.5.3 Production of hybrid cell strains

The spleen cells of the mouse were isolated for fusion with SP2/0 plasmacytoma cells. The SP2/0 plasmacytoma cells were grown in RPMI1640-medium supplemented with 10% FCS, 1 mM sodium pyruvate, 1 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 50 mM ß-mercaptoethanol. Spleen cells and SP2/0 cells were washed twice in RPMI-medium without serum. Next, 10^8 spleen cells were added to 10^7 SP2/0 cells and centrifuged (20 min at 50*g*). The supernatant was removed and the cells were exposed to fusion conditions by brief consecutive incubations of a mixture of these cells in 41% and 25% PEG 4000 as follows. The cell pellets were resuspended for 1 min in 41% PEG-solution (0.5 ml). Next, 25% PEG-solution (0.5 ml) was added and the mixture was shaken slowly for 1 min. RPMI-medium without serum (4 ml) was added twice and the cell suspension was shaken slowly for 2 min. The cell suspension was incubated for 15-30 min at room temperature and then centrifuged (20 min at 50*g*). The supernatant was removed and the pellet resuspended in RPMI-medium with 10% FCS. The cells were seeded in a 75-cm^2 culture flask and incubated overnight. After 24 h of incubation, the cells were centrifuged (20 min at 10g) and were resuspended in complete RPMI-medium (38 ml; the same medium as used for growing of SP2/0 cells) supplemented with HAT-medium, *i.e.*, 0.1 mM hypoxanthine, 16 mM thymidine, and 0.4 mM aminopterine. Hybridomas were selected in HAT-medium because they can grow in this medium whereas SP2/0 cells do not survive; spleen cells cannot be cultured. The cells were seeded in 96-well polystyrene culture plates in HAT-medium. Hybrid cells were cultured and refreshed in this selective HAT-medium and their supernatants were screened for specific antibody production in a direct ELISA. Cells producing specific antibodies against sulfur mustard treated proteins were recloned twice by limiting dilution as will be described in the next subsection.

III.5.4 Cloning of hybridomas by limiting dilution

Cells of the fusion mixture producing specific antibodies against sulfur mustard treated protein (keratin) were counted by light-microscopy and diluted in HAT-medium to a concentration of 50, 10 and 5 cells/ml. Per well of 96-well culture plates, 0.1 ml of one of these solutions was added resulting in 5, 1 and 0.5 cell/well. The plates were incubated for eight days without refreshing the medium. Subsequently, the amount of clones per well was counted. The supernatants of wells with only one clone were tested for specific antibody activity against sulfur mustard treated hemoglobin. Clones showing a positive result were recloned once again by limiting dilution to make sure that monoclonal antibodies would be obtained.

III.5.5 Immunoassays (ELISA) with the polyclonal antisera and hybridoma-supernatants

The polyclonal antisera and hybridoma-supernatants were tested in a direct ELISA against keratin isolated from human callus treated with sulfur mustard (0, 50, 1000 µM) and against the immunogen itself (if available in sufficient amounts). The ELISA was performed as follows. Polystyrene 'high binding' 96-well microtiter plates were coated with adducted and non-adducted keratin or peptides dissolved in PBS to a final concentration of 5 µg/ml. Of these dilutions 50 µl was added per well and incubated for 45 min at room temperature. The plates were washed three times with PBS containing 0.05% Tween 20. (The plates were not incubated with PBS containing 1% FCS). The polyclonal antisera and the hybridoma supernatants were diluted 10-1,000 times and 5-100 times, respectively, in PBS with 0.05% Tween 20 and 1% FCS. Of these dilutions 50 µl was added per well and incubated for 60 min at 37 °C. After washing, the second antibody, viz., goat-anti-mouse-Ig(total)-alkaline phosphatase diluted 1:1,000 in PBS containing 0.05% Tween 20, 0.5% gelatin, and 5% FCS, was added (50 µl/well) and the plates were incubated for 60 min at 37 °C. After three washings with PBS containing 0.05% Tween 20, the plates were washed once with 0.1 M

diethanolamine, pH 9.8 (100 µl). A solution of 4-methylumbelliferyl phosphate (0.2 mM in 10 mM diethanolamine, pH 9.8, 1 mM $MgCl_2$; 50 µl) was added as a substrate for alkaline phosphatase and the mixture was incubated at 37 °C for 1 h.

III.5.6. Production of new monoclonal antibodies directed against sulfur mustard adducts to keratin by phage display

As alternative for the selection of SM-keratin-adduct specific antibodies we applied the so-called phage display technique instead of the classical hybridoma technique. The applied procedures have been described below.

Bacterial strains and growth conditions.
Two *Escherichia coli* strains were used in this study as hosts for bacteriophages: TG1 [K-12 Δ(*lac-pro*) *supE thi hsdD5*/F' *traD36 proA*$^+$ *B*$^+$ *lacI*^{q} *lacZ* ΔM15] and HB2151 [K-12 *ara* Δ(*lac-pro*) *thi*/F' *proA*$^+$ *B*$^+$ *lacI*^{q} *Z* ΔM15]. Both strains were grown on tryptone yeast extract (TYE) plates (containing 1% glucose and antibiotics when required. Cultures were grown in 2× TYE broth (2× TY).

Preparation of antigens.
Keratin was isolated from human callus exposed to various concentrations of sulfur mustard up to 1 mM as described above.

Phage display library.
The Tomlinson I & J libraries (a generous gift from Greg Winter, Centre for Protein Engineering, Cambridge, United Kingdom) were used to select phage antibodies. The Tomlinson I & J libraries each comprise over 100 million different single chain antibody variable region fragments scFv. For general information and references on phage display of antibodies see Winter *et al.* (1994).

Rescue of the phage library.
Rescue of the libraries was performed by inoculating 10^{10} CFU of both I and J libraries in strain TG1 in 2× TY broth (TY, 200 ml) containing glucose (Glu, 1%) and ampicillin (Amp, 100 µg/ml) (2× TY+G+Amp) and incubated at 37 °C with shaking (at 250 rpm) until the optical density at 600 nm (OD_{600}) was approximately 0.4. Fifty milliliters of this culture was infected with KM13 helper phage ($2x10^{11}$) and incubated at 37 °C for 30 min. The infected cells were collected by centrifugation (3000 × *g* for 10 min) and resuspended in 2× TY (100 ml) containing glucose (0.1%), Amp (100 µg/ml) and kanamycin (Kan, 50 µg/ml) (2× TY-0.1% Glu+Amp+Kan). Subsequently, the cells were incubated overnight at 30 °C and with shaking at 250 rpm. Bacterial cells were removed by centrifugation at 3,300 × *g* for 30 min., and phages present in the supernatant were precipitated with 1/5 v/v 20% polyethylene glycol 6000 (1/5 v/v 20%), NaCl (2.5 M) (PEG/NaCl) at icewater for at least 1 hr. After centrifugation at 3,300 *g* for 30 min the pellet was resuspended in PBS (1/50 v/v) and centrifuged again at 11,600 *g* for 10 min to remove bacterial debris. The final concentration of the phage was in the range of 10^{12}-10^{13}/ml.

Selection procedure.
The selection procedure was performed exactly as described previously (Winter et al. 1994). Maxisorp immunotubes (5.0-ml) were coated with 4 ml of 5µg/ml antigen (exposed to 1mM sulfur mustard) in PBS, at 4°C for 16 hr. Subsequently, the immunotubes were blocked with PBS containing 2% skimmed milk (MPBS) for 2 h at room temperature (RT) and washed three times with PBS. The phage library, about 10^{12} -10^{13} phages / 4 ml MPBS, was added, and the tubes were incubated on a turntable for 60 min at RT, followed by a standing incubation for 60 min at RT. Unbound phages were removed by washing the tubes 20 times with PBS containing 0.05% Tween 20 (PT). While being rotated on an under-and-over

turntable bound phages were eluted in 500 µl trypsin (1 mg/ml) at RT for 10 min. To determine the number of eluted phages, *E. coli TG1*, grown to an OD_{600} of 0.4 was infected for 30 min. at 37°C and cells were plated in a serial dilution on TYE-AMP-GLU plates and grown at 16 h at 37°C. After the rescue operation of the eluted phages, this selection procedure was repeated for another two rounds.

Production of monoclonal phage antibodies.
After the third and final round of selection, individual colonies were inoculated into 100 µl 2xTY+G+Amp and grown at 37 °C for 16 hr. Subsequently an inoculum of 5µl was transferred to 200 µl fresh 2xTY+G+Amp and bacteria were grown to an early log-phase. Then, 25 µl 2xTY+G+Amp containing 10^9 trypsin-sensitive helper phage KM13 was added to each well. After an incubation at 37 °C for 1 h, with shaking at 250 rpm, cells were collected by centrifugation at 1,800 *g* for 10 min and resuspended in 200 µl 2xTY+Amp+Kan, and grown overnight at 30 °C at 250 rpm. These culture supernatants were contained with monoclonal phage antibodies (MoPhAbs).
For the production of larger quantities of MoPhAbs the pellet was resuspended in 50 ml 2xTY+Amp+Kan and cells were grown at 30 °C for 16 h. Then, the cells were pelleted by centrifugation at 3,300 *g* for 15 min. The supernatant was mixed with 1/5 v/v PEG/NaCl and by an incubation on ice water for 2 h. The precipitated MoPhAbs were pelleted by centrifugation at 3,300 *g* for 30 min. The pellet was resuspended in 2 ml PBS and stored at 4 °C. For long term storage at -70 °C glycerol (15% end concentration) was added.

Expression of soluble antibody fragments.
For expression of soluble antibody fragments (single chain variable fragments, scFvs) the *E. coli* nonsuppressor strain, HB2151, was infected with phages as described for TG1. Individual HB2151 colonies were transferred into 150 µl of 2×TY+Amp-G. Plates were incubated at 37 °C, with shaking at 250 rpm, until the OD_{600} of the cells was about 0.9. For induction of the scFvs Isopropyl-β-D-thiogalactopyranoside (IPTG) (Eurogentec, Seraing, Belgium) was added to a final concentration of 1 mM. Plates were incubated for 16 to 24 h at 30 °C (with shaking at 250 rpm). Bacteria were centrifuged at 1,800 × *g* for 10 min and cell supernatants were collected for further use.

Screening MoPhAbs and scFvs by ELISA.
High affinity microtiter plates (Costar, Corning, USA) were coated with 100 µl antigen (5µg/ml) as described above for the coating of the immunotubes. Before incubation with the MoPhAbs or scFvs the plates were washed three times with PBS and blocked with 2% MPBS for 2 h at RT. Culture supernatants of superinfected TG1 cells containing MoPhAbs and supernatants containing scFvs were used in 2% MPBS and incubated for 60 min at RT. Plates were washed three times with PBS containing 0.05% Tween 20. MoPhAbs were detected by using the Detection Module Recombinant Phage Antibody system (Pharmacia, Uppsala, Sweden) according to the manufacturer's recommendations, using an HRP-anti-M13 as second antibody, which is specific for the viral major coat protein VIII. Binding of soluble antibody fragments was detected with HRP-anti-Protein A (ICN Biomedicals, Inc., Aurora, U.S.A) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was used as substrate. Emission was measured at 415nm. These experiments were repeated at least once.

Immunoslotblot.
The immunoslotblot assay was essentially the same as described earlier (see section III.4.9) with some modifications. Keratin samples (isolated from human callus unexposed or exposed to 250 µM, 500 µM and 1mM sulfur mustard) were diluted to a concentration of 1-5 µg/ml in PBS. The samples were immobilized on nitrocellulose using a Minifold Dot blot manifold (Schleicher and Schuell). For this 3 pieces of blotting paper were pre-wetted in PBS and 1 piece of nitrocellulose (pore size 0.1 µm) was pre-wetted in aquadest and PBS. Then, 50-200

µl of the samples was pipetted in triplicate on the filter. The dots were then rinsed with 400 µl PBS. The nitrocellulose was removed from the filter support and allowed to dry on air for 15 min. The keratin was crosslinked to the nitrocellulose with UV using a GS Gene Linker (Biorad) at 50 mJ.
To prevent non-specific binding of the MoPhAb, the nitrocellulose filters were incubated with 2% milkpowder in PBS + 0.1% Tween (PT) for 1 h at RT. The nitrocellulose was washed 4 times with PT. Then, 10 ml PBS containing, 2% milkpowder and approximately $1x10^{13}$ MoPhabs was added to the filter and incubated overnight at 4 °C under continuous shaking. Then, the filter was extensive washed four times with PT and incubated with 10 ml PBS contained with 2% milkpowder and 5000-fold diluted horseradish peroxidase (HRP) conjugated to mouse anti-M13 monoclonal antibody (Amersham Pharmacia Biotech, Roosendaal, The Netherlands), during 2 h at RT under continuous shaking. The filter was then extensively washed with PT as stated above. In the mean time substrate (BM Chemiluminescence blotting substrate, POD (Roche)) and solution A (10 ml/filter) were prewarmed to 25 °C. After 30 min 1/100 volume of substrate solution B was added and incubated for another 30 min at 25°C. Subsequently, the filter was incubated with the substrate solution for 1 min. The filter was placed in a plastic bag. The chemiluminescence was measured with a luminometer (MicroBeta Trilux (Wallac)).

PCR screening for full length insert.
Clones producing the desired MoPhabs were checked for the presence of full length VH and Vκ insert. DNA was isolated by using the High Pure PCR Preparation Template Kit (Roche, Almere, the Netherlands) according to the manufacturers' protocol. PCR was performed using the primers LMB3 (CAGAAACAGCTATGAC) and pHEN (CTATGCGGCCCCATTCA) under conditions described elsewhere (Winter et al., 1994). Annealing temperature was set at 55 °C and extension time was 2 min. Primers were purchased at OPERON Biotechnologies (Cologne, Germany). The size of the amplified DNA was estimated on a 2% agarose gel at a current of 100 mA, using the Superladder-low 100bp as reference (Eurogentec, Seraing, Belgium). DNA was visualized using ultraviolet light. Full length VH and Vκ insert should result in a DNA fragment of 935bp. A PCR product without insert would result in a DNA fragment of 329bp.

Annex to Experimental part

Demonstration of tripeptide SOP to USAMRICD scientist

The work plan and protocols had been sent to people involved at USMRICD and CDC, in advance of the actual visit. Also, a list of required items/chemicals had been sent to CDC in advance of our visit. All items/chemicals were present upon arrival.
Scientists/technicians involved:
Dr. J.R. (John) Barr (CDC, Emergency Response & Air Toxicants Branch), analytical chemist, mass spectrometrist.
A. (Adrian) Woolfitt (CDC, Emergency Response & Air Toxicants Branch), analytical chemist, mass spectrometrist.
J.R.(Rick) Smith (USMRICD), analytical chemist, mass spectrometrist.
A. (Alex) Fidder (TNO-PML), bio-organic chemist.
Dr. D. (Daan) Noort (TNO-PML), bio-organic chemist.

Reference samples and plasma samples to be used had been sent, stored on dry ice, to CDC in March, 2004. Since then the samples had been stored at –20 °C
0410AF04: (S-d_0-HETE)Cys-Pro-Phe
0410AF05: (S-d_8-HETE)Cys-Pro-Phe
0410AF06: plasma (5 ml), isolated from blood, exposed to 0 uM HD
0410AF07: plasma (5 ml), isolated from blood, exposed to 100 uM d_0-HD
0410AF08: plasma (5 ml), isolated from blood, exposed to 100 uM d_8-HD

Monday, April 5, 2004.

1. Buffers were prepared for the affinity isolation procedure for albumin. Instead of KH_2PO_4, NaH_2PO_4 was used.
2. Blank plasma was obtained from CDC (as replacement for 0410AF06), just to be sure it was truly blank.
3. Plasma sample 0410AF07 was diluted with blank plasma to a (virtual) 10 uM exposure level.
4. Adrian had already prepared mass spec instrument (Sciex, API 4000), as described in "Retrospective detection of exposure to sulfur mustard: improvements on an assay for liquid chromatography – tandem mass spectrometry analysis of albumin – sulfur mustard adducts". (D. Noort et al., J. Anal. Toxicol., **28**, 333-338, 2004).
5. In contrast to the work plan, we did not start with analyzing the reference standards, because of contamination risks: the instrument had already been used for analysis of the tripeptide numerous times and analytical conditions were clear.
6. Samples prepared, in duplicate, according to protocols:
    1. 500 ul 0 uM d_0 + 0 ul 100 uM d_8 ("double blank")
    2. 450 ul 0 uM d_0 + 50 ul 100 uM d_8 ("blank")
    3. 450 ul 10 uM d_0 + 50 ul 100 uM d_8 ("10 uM exposed")

    Codes of samples obtained after pronase digestion, ready for LC tandem MS analysis:
    0415AF01: double blank
    0415AF02: double blank
    0415AF03: blank
    0415AF04: blank
    0415AF05: 10 uM exposed
    0415AF06: 10 uM exposed
7. Sample 0415AF05 gave satisfactory analytical data; the rest of the samples were analyzed overnight. Erroneously, some samples were indicated with a "0414" code instead of "0415". The analytical data are attached.

Tuesday, April 6, 2004.

1. Plasma dilutions were prepared, ranging from 0, 0.01, 0.1 and 1 uM by dilution of the 0410AF07. To 500 ul of the 0 and 0.01 d_0 uM samples was added 25 ul 1 uM d_8 plasma, resulting in an end concentration of 0.05 uM. To 500 ul of the 0.1 and 1 uM d_0 samples, 25 ul of 10 uM d_8 plasma was added, resulting in an end concentration of 0.5 uM.
2. Processing of the samples was performed according to the protocols (single experiments, no duplicate). Since only a ¼ part of the worked-up, desalted albumin solution is taken for pronase digestion, the other ¾ part was stored at –20 °C for experiments on Wednesday.

Codes of samples obtained after pronase digestion, ready for LC tandem MS analysis:

0415AF07: 0 uM d_0/0.05 uM d_8
0415AF08: 0.01 uM d_0/0.05 uM d_8
0415AF09: 0.1 uM d_0/0.5 uM d_8
0415AF10: 1 uM d_0/0.5 uM d_8

The samples were analyzed the same day. However, severe problems occurred with instrument (leakage within the electrospray probe; needle was clogged). Problems were partially fixed and the samples were run overnight again.

Wednesday, April 7, 2004.

1. The analyses of 0415AF07-10 looked satisfactorily. Sensitivity seemed okay. Instrument was fixed.
2. New pronase digests were prepared, by taking double amounts (1.5 ml) of desalted albumin samples. In this case, the double amount of pronase (200 ul of 10 mg/ml) was added.

   Codes of samples obtained after pronase digestion:
   0415AF11: 0 uM d_0/0.05 uM d_8
   0415AF12: 0.01 uM d_0/0.05 uM d_8
   0415AF13: 0.1 uM d_0/0.5 uM d_8
   0415AF14: 1 uM d_0/0.5 uM d_8

These samples were not analyzed, but half of each sample was further processed on Seppak. The remaining half was stored in the freezer.

3. During the course of the pronase digestion, Rick and Adrian practiced the isolation of albumin from plasma, under supervision of Alex.
4. Eluents for the Sep-Pak cartridge procedure were prepared: 0.1% TFA/water, 0.1% TFA/10% acetonitrile in water, 0.1% TFA/20% acetonitrile in water and 0.1% TFA/40% acetonitrile in water.
5. 850 ul (half of sample) of the filtrated pronase digests were further processed on Sep-Pak C18 classic cartridges. The 40 % acetonitrile fractions were collected and evaporated.

   Codes of samples after Sep-Pak clean-up:
   0415AF15: 0 uM d_0/0.05 uM d_8
   0415AF16: 0.01 uM d_0/0.05 uM d_8
   0415AF17: 0.1 uM d_0/0.5 uM d_8
   0415AF18: 1 uM d_0/0.5 uM d_8

(N.B. By mistake, these codes were not used during the analyses!; on the analytical runs they appear as "0415AF07-10 after Seppak"; these samples were analyzed on Thursday, April 8)

6. Concentration of the samples by using the Savant Speedvac apparatus proceeded too slowly. Therefore, the samples were lyophilized overnight in a Virtis "Genesis" 25 EL lyophilization unit.

Thursday, April 8, 2004.

1. Samples 0415AF15-18 (i.e. with codes "0415AF07-10 after Seppak") had been nicely concentrated. The residues were dissolved in 0.1% TFA in water (55 ul) and analyzed according to the protocol.
2. Analytical data were satisfactory; peak ratios d_0/d_8 were as expected.
3. All LC tandem MS runs were compiled into a Powerpoint file by Adrian.
4. It was decided that it was not necessary to run samples at Friday, April 9.

IV RESULTS

IV.1 Further development of the mass spectrometric or fluorescent analysis of the tripeptide (S-HETE)Cys-Pro-Phe, resulting from pronase digestion of albumin alkylated by sulfur mustard (*Part A, t.o. 1-9*)

IV.1.1 Introduction

Results obtained in the first two years of the cooperative agreement showed that pronase digestion of albumin alkylated by sulfur mustard resulted in the formation of the tripeptide (S-HETE)Cys-Pro-Phe, which could be straightforwardly isolated and determined in a rather sensitive way by micro-LC/electrospray tandem MS with multiple reaction monitoring.
Presently, this is by far the most sensitive method for detection of exposure of human blood to sulfur mustard. Interestingly, we recently showed that this method can also be applied to demonstrate exposure (*in vitro* as well as *in vivo*) to a wide range of nitrogen mustard derivatives (Noort *et al.*, 2002), and also acrylamide (Noort *et al.*, 2003).
In the third year of the grant period, it was investigated whether (S-HETE)Cys-Pro-Phe could be detected after fluorescence derivatization.

IV.1.2 Derivatization of (S-HETE)Cys-Pro-Phe for fluorescence detection

Several reagents were explored for fluorescence derivatization of (S-HETE)Cys-Pro-Phe. Initially,we did not succeed in modification of the NH_2 function of (S-HETE)Cys-Pro-Phe for reasons unknown. We therefore directed our attention towards derivatization of the carboxylic acid moiety of the tripeptide. Reaction with commercially available pyrenyl diazomethane gave rise to a fluorescently labeled compound that was stable enough to allow purification by reversed-phase HPLC. However, the detection limit for analysis of this compound was only in the low µg/ml range, i.e., far less sensitive than mass spec analysis. Based on the positive results obtained for derivatization of the histidine adduct with 5/6-carboxyfluorescein succinimidyl ester (FAM-SE), we re-examined the derivatization of the tripeptide with FAM-SE. It appeared that after derivatization and removing excess reagent with hydroxylamine, the peak for the reagent or byproduct was exactly on the same spot as the derivatized tripeptide. CE-LIF analysis of this peak showed two closely eluting peaks, with equal intensity (see Figure 1). The limit of detection was estimated to be 0.3 nM. Mass spectrometric analysis showed the correct mass (see Figure 2).

Subsequently, a more diluted solution of (S-HETE)Cys-Pro-Phe (7 µg/ml) was derivatized with FAM-SE and the mixture was analyzed by means of CE-LIF (see Figure 3). Comparison with the blank (no (S-HETE)Cys-Pro-Phe added) showed the presence of an extra peak at 5.2 min.

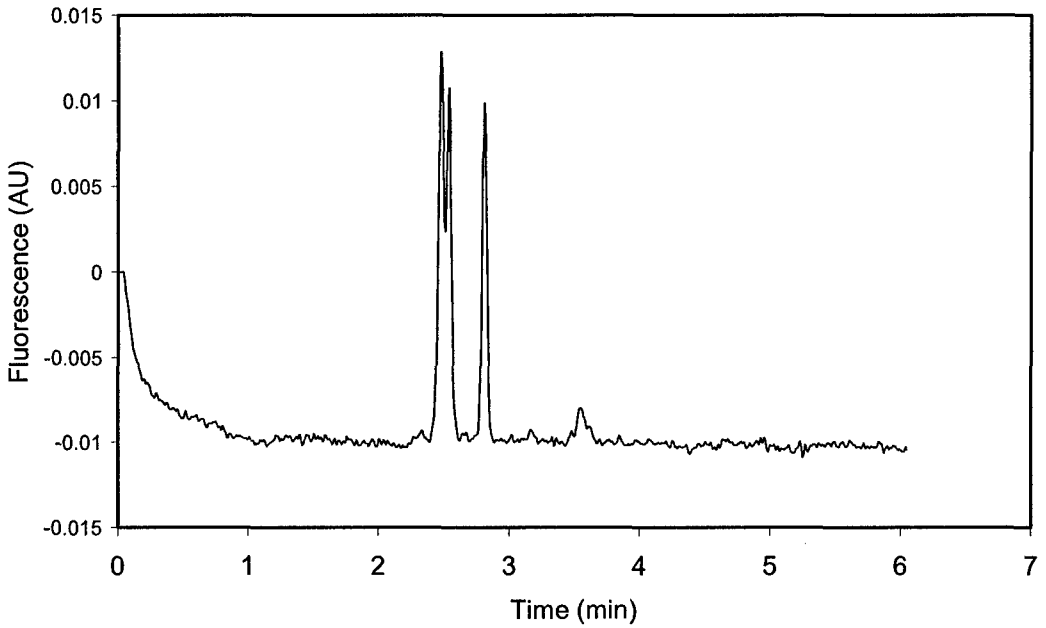


Figure 1. Capillary electrophoresis – laser-induced fluorescence detection of 5/6-carboxyfluoresceine (FAM) derivative of (S-HETE)Cys-Pro-Phe, after derivatization of reference standard with FAM-succinimidy ester (FAM-SE, 5/6-isomer), followed by Sep-Pak C18 clean-up. The double peak left represents the desired derivative (mixture of 5- and 6-isomer); the peak on the right is fluoresceine, at a concentration of 345 pg/ml.

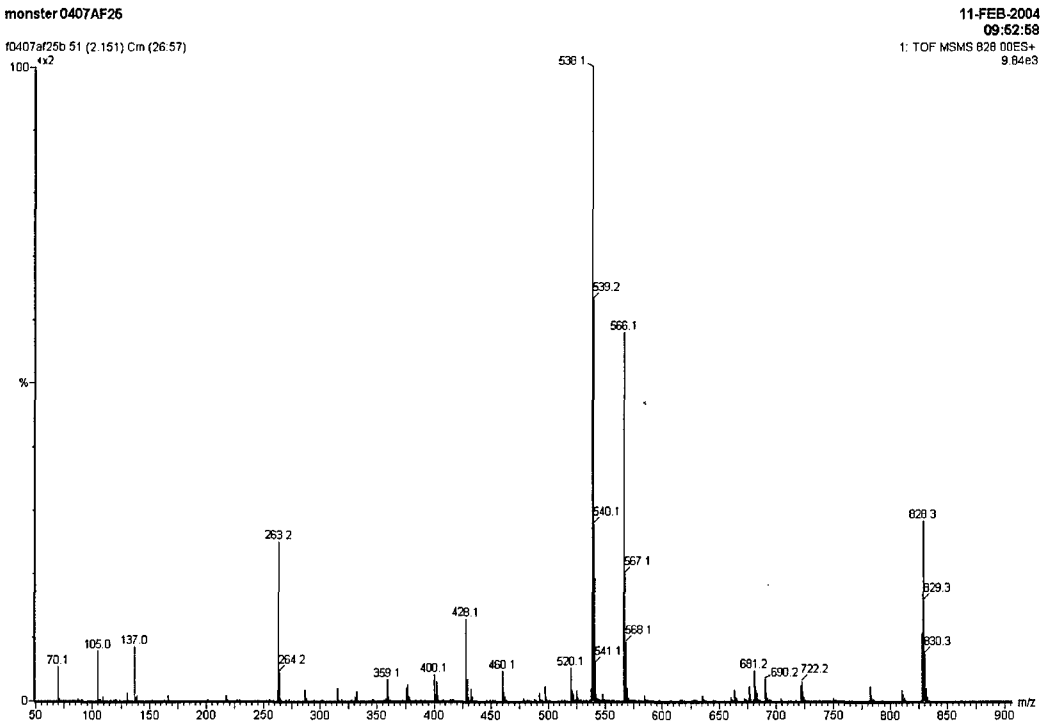


Figure 2. Electrospray MS spectrum of N-5/6-carboxyfluoresceine derivative of (S-HETE) Cys-Pro-Phe.

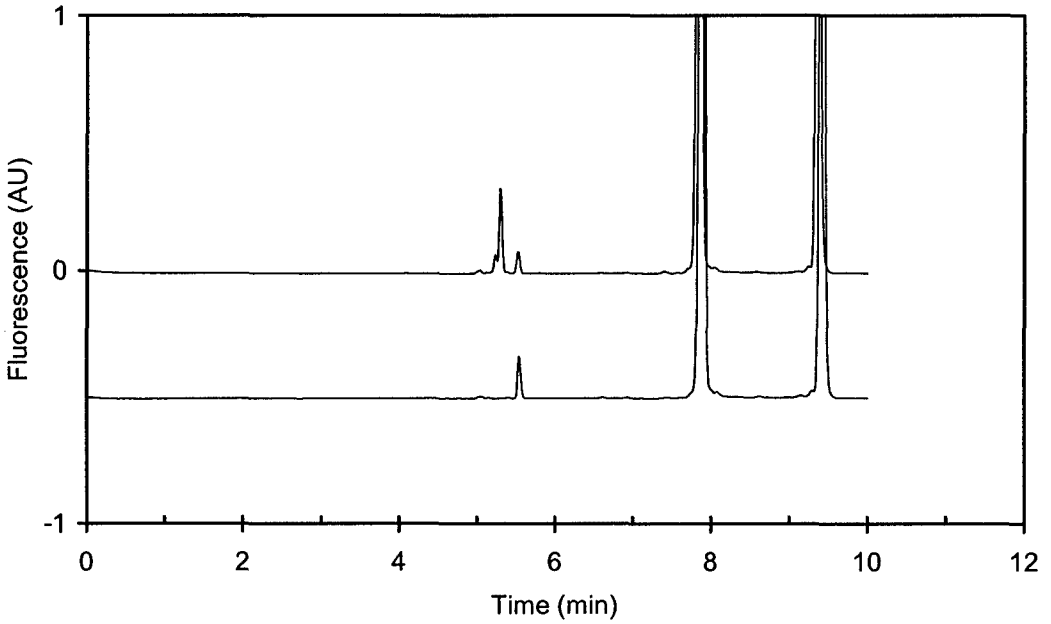


Figure 3. Capillary electrophoresis of 6-FAM derivative of (S-HETE)Cys-Pro-Phe after derivatization at low concentration (7 µg/ml). Synthetic (S-HETE)Cys-Pro-Phe was derivatized with 6-carboxyfluoresceine succinimidyl ester and purified by means of Sep-Pak C18. Upper trace: 6-FAM labeled (S-HETE)Cys-Pro-Phe. Lower trace: blank (no tripeptide added during derivatization). Migration time of 6-FAM(S-HETE)Cys-Pro-Phe 5.2 min

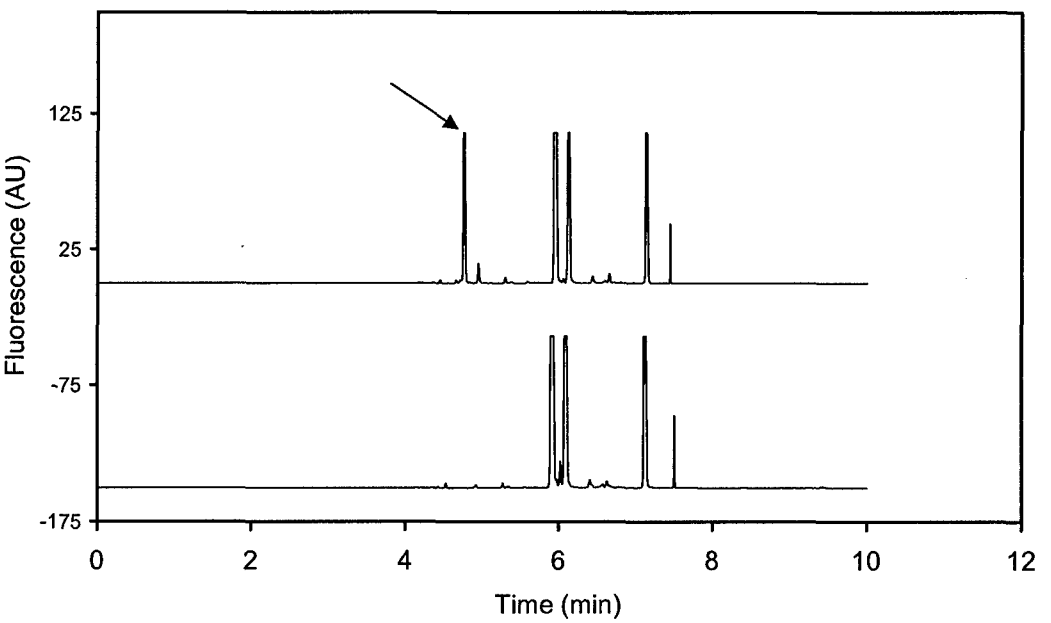


Figure 4. Analysis of (S-HETE)Cys-Pro-Phe in a pronase albumin digest, after derivatization with 6-carboxyfluoresceine succinimidyl ester. Upper trace: albumin pronase digest spiked with synthetic (S-HETE)Cys-Pro-Phe. Lower trace: blank albumin pronase digest.

Subsequently, a pronase digest of albumin was spiked (at 1 mg/ml) with (S-HETE)Cys-Pro-Phe and subsequently derivatized with FAM-SE 6-isomer in 0.1 M $NaHCO_3$. The digest was also derivatized without the addition of (S-HETE)Cys-Pro-Phe. The result is shown in Figure 4. Although the sample was spiked at a very high level, the result was encouraging since it was demonstrated that the tripeptide could be derivatized in a relevant matrix.

Next, pre-purified (Sep-pak C18) pronase digests of albumin that had been isolated from blood that had been exposed to sulfur mustard (1 mM) were derivatized with FAM-SE 6-isomer. The derivatized digest was pre-purified by means of reversed-phase HPLC, and finally analyzed by means of CE-LIF. A similar digest from a non-exposed blood sample was also analyzed. Both NMP and 0.1 M $NaHCO_3$ were used as solvents. A representative result is shown in Figure 5. The trace for the reference compound is also shown. It can be seen that in the area in the electropherogram where the FAM-(S-HETE-Cys)-Pro-Phe derivative elutes an interfering peak is present, which precludes unambiguous analysis.

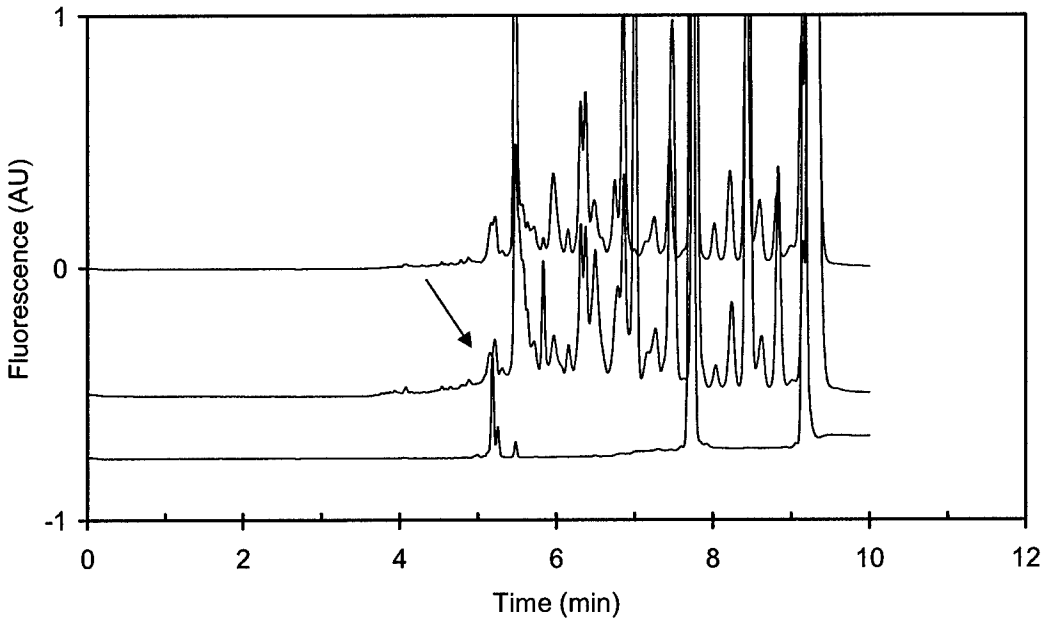


Figure 5. Capillary electrophoresis of albumin pronase digest, after derivatization with 6-FAM-SE. Before and after derivatization the mixture was pre-purified with HPLC.
Upper trace: pronase digest of blank albumin
Middle trace: pronase digest of albumin from blood exposed to 1 mM sulfur mustard
Lower trace: FAM(S-HETE)Cys-Pro-Phe; migration time FAM(S-HETE)Cys-Pro-Phe 5.2 min

IV.2 Further development of methods for sensitive analysis of N1/N3-(2-hydroxyethylthioethyl)histidine (*part B, t.o. 10-13*)

IV.2.1 Introduction

In hemoglobin and albumin from human blood exposed to sulfur mustard, 34% and 28% of total adduct level could be ascribed to N1/N3-HETE-histidine, respectively. In view of this abundance and the stability of the adduct under acidic conditions, which enables its quantitative release from proteins, we investigated the mass spectrometric and CE-LIF analysis of this adduct.
In the first two years it was attempted to further optimize the isolation procedure and subsequent analysis of the histidine adduct after suitable derivatization. In the third year of the cooperative agreement work on these items were continued, with the emphasis on isolation by means of reversed-phase HPLC.

IV.2.2 Direct isolation of histidine adduct by means of reversed-phase HPLC

It was attempted to directly isolate N1/N3-HETE-histidine from acidic digests by means of semi-preparative reversed-phase HPLC, i.e., without derivatization. Subsequent direct LC-tandem MS would circumvent the laborious derivatization of the amino acid mixtures. Figure 6 shows a semi-preparative HPLC run from spiked digests. Appropriate fractions were collected, concentrated and analyzed by LC/MS with and without derivatization with Fmoc-Cl. The adduct could indeed be detected by applying this method, when highly spiked hydrolysates or hydrolysates from highly exposed globin samples were used. Unfortunately, during micro-LC tandem MS analysis the retention time of the underivatized adduct was highly variable, which precluded routine analyses (see Figure 7 and 8 for a successful analysis). Basically, one can argue that this method can be used advantageously for preparative purification of the adduct from acidic or enzymatic digests. Subsequent derivatization of the isolated fraction with either Fmoc-Cl for LC-tandem MS analysis or with TFAA for GC-MS analysis can then be used as an appropriate analytical method. However, this will render the final method very laborious. In the very near future, we will look for micro-bore LC columns on which the histidine adduct might have a longer retention time.

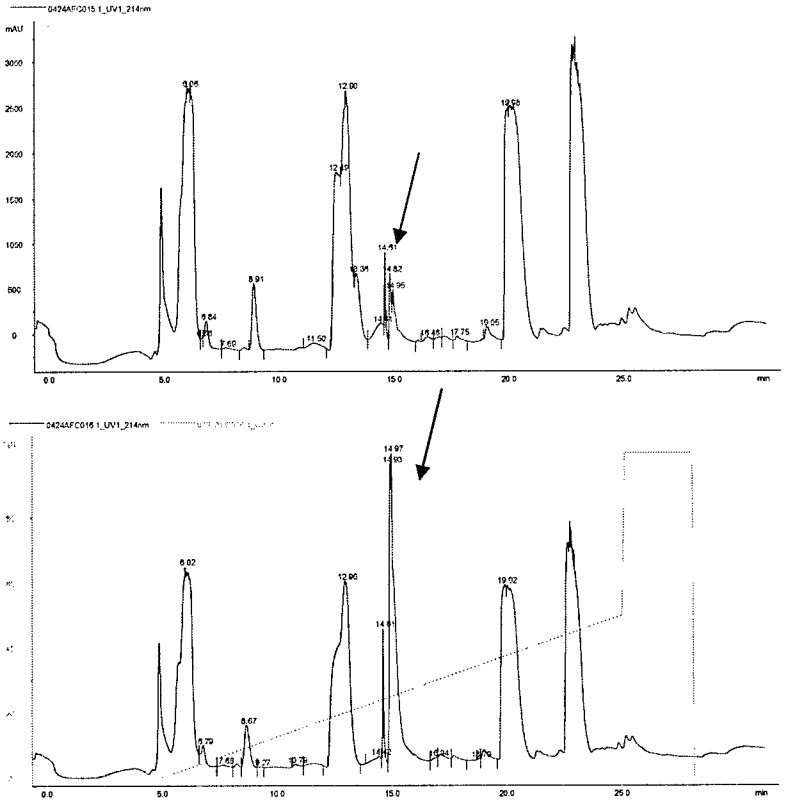


Figure 6. Preparative reversed-phase HPLC purification of acidic hydrolysates of globin (7 mg) spiked with N1/N3-HETE-His, after neutralization with conc. NH_4OH solution.
Trace A: acidic hydrolysate spiked with 30 microgram of each isomer. Trace B: acidic hydrolysate spiked with 90 microgram of each isomer. Fractions of 1 min each were collected and analyzed for N1/N3-HETE-His by means of LC tandem MS.

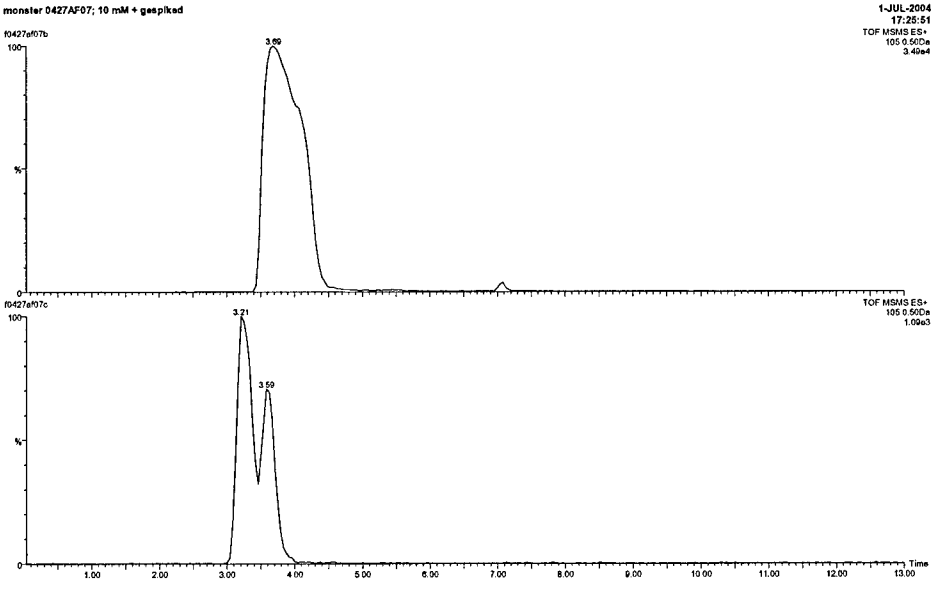


Figure 7. Direct LC-tandem MS analysis of histidine adduct in pre-purified acidic digest of globin, spiked with N1- and N3-(HETE)-histidine. Upper trace: without dilution. Lower trace: diluted sample, showing the individual isomers. Column used: PepMap C18 column.

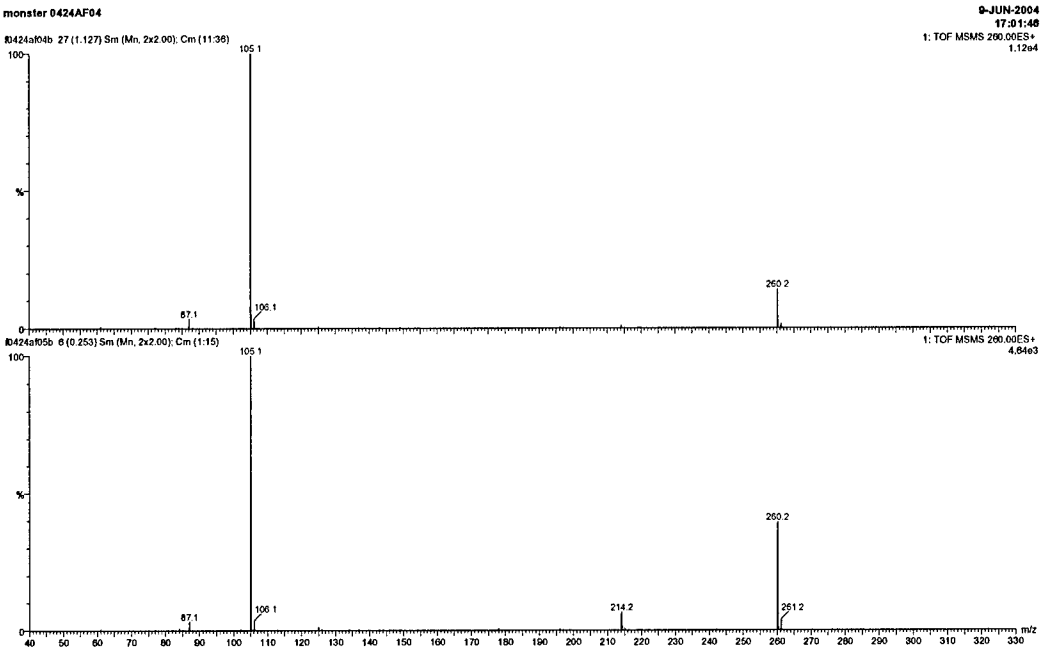
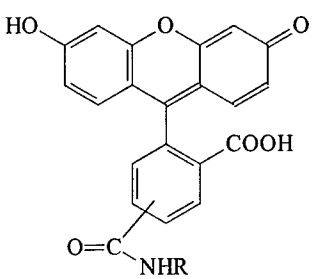


Figure 8. Direct LC-tandem MS analysis of histidine adduct in pre-purified acidic digest of globin, spiked with N1- and N3-(HETE)-histidine; tandem MS spectra of individual isomers. (*m/z* 260, MH^+).

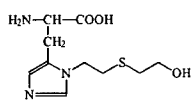
IV.2.3 Derivatization of N1/N3-HETE-histidine for HPLC with laser-induced fluorescence detection

Laser-induced fluorescence has been used for detection of yoctomole amounts of amino acid derivatives. This technique, however, is less selective than mass spectrometry. Various derivatization methods have been explored and several fluorescent derivatives of the histidine adducts have been prepared in the first year of the cooperative agreement (see Figure 9). In the second year of the agreement, it was found that the most promising fluorescein-based reagent was 5/6-carboxyfluorescein (FAM) succinimidyl ester. The detection limits of the FAM N1-HETE- and FAM N3-HETE-histidine derivatives were 24 and 21 pg/ml, respectively.

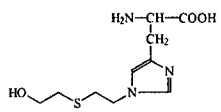
The derivatization of amino acid mixtures (containing the histidine adducts) was studied in more detail in the third year of the grant period. Unfortunately, even in highly spiked samples (microgram/ml level), we could not identify the FAM N1/N3-HETE-histidine derivatives, when the derivatization was carried out as performed for (S-HETE)Cys-Pro-Phe.



FAM derivative



N1-isomer



N3-isomer

R = N1/N3-(2-hydroxyethylthioethyl)histidine

Figure 9. Fluorescent FAM derivative of N1/N3-(2-hydroxyethylthioethyl)histidine.

IV.3 Development of Standard Operating procedure for albumin - tripeptide assay

In the previous report, a tentative SOP for the albumin – tripeptide assay was described. The tentative SOP was converted into a definitive version in the third year of the Cooperative Agreement, after a number of slight modifications. Also, the interindividual and intraindividual variation of adduct formation to Cys-34 in human serum albumin, and the day to day variability of the assay was determined. Finally, the SOP has been demonstrated to a scientist of USAMRICD.

IV.3.1 Interindividual and intraindividual variation of adduct formation of sulfur mustard to Cys-34 in human serum albumin, as determined with the SOP for the albumin – tripeptide assay

Blood samples were drawn from 10 volunteers with informed consent, and subsequently exposed to 0.1 μM sulfur mustard. Work-up of the samples was performed simultaneously. After incubation for 2 h at 37 °C, plasma was isolated. Internal standard plasma (isolated from human blood that had been exposed to d_8-sulfur mustard) was added to a virtual exposure level of d_8-sulfur mustard of 1 μM. The samples were worked up according to the SOP. The results are given in Table 1.

Table 1. Interindividual variation of adduct formation of sulfur mustard to Cys-34 in human serum albumin, as determined with the SOP for the albumin tripeptide assay

volunteer	d_0/ d_8 – tripeptide ratio
1	0.11
2	0.10
3	0.12
4	0.13
5	0.13
6	0.13
7	0.12
8	0.12
9	0.12
10	0.11
Mean ± s.d.	0.12 ± 0.01

Subsequently, the intraindividual variation was determined. From 3 persons blood samples were taken at three different time points (3 months in between) and exposed to sulfur mustard (0.1 μM). Plasma was isolated and the samples were processed according to the SOP and analyzed by LC/tandem MS for the ratio d_0/d_8 (S-HETE)Cys-Pro-Phe. The series at the third time point was lost during processing. The results are shown in Table 2.

Table 2. Intraindividual variation of adduct formation of sulfur mustard to Cys-34 in human serum albumin, as determined with the SOP for the albumin tripeptide assay

volunteer	d_0/d_8 – tripeptide ratio (time point 1)	d_0/d_8 – tripeptide ratio (time point 2)
1	0.13	0.13
2	0.12	0.13
3	0.11	0.13

IV.3.2 Day-to-day variability of SOP for tripeptide assay

The day-to-day variability was determined in the following way. A blood sample from a single volunteer was exposed to sulfur mustard (0.1 μM) and subsequently plasma was isolated. At four different time points (within a time period of 6 months), aliquots of the plasma sample were processed according to the SOP and analyzed by LC/tandem MS for the ratio d_0/d_8 (S-HETE)Cys-Pro-Phe. At one of the time points, the sample was lost during processing. The results are shown in Table 3.

Table 3. Day-to-day variability of SOP for albumin tripeptide assay

Time point	Ratio d_0/d_8
1	0.13
2	0.12
3	0.13

IV.3.3 Demonstration of the SOP for the tripeptide assay to a USAMRICD scientist

As was also the case for SOP's developed in the previous cooperative agreement DAMD17-97-2-7002, i.e., the SOP for modified Edman degradation and the SOP for immunochemical analysis of DNA adducts, the current SOP for the tripeptide assay was demonstrated to a scientist of USAMRICD. The aim was to demonstrate that (1) the assay could be set up in an independent laboratory within a short period of time and that (2) the assay was easy to perform by a person that had no experience with the procedure.

The LC tandem MS system represented in Figure 10 was used for the analyses. Initially, both columns are equilibrating with 100% C at 50 uL/min. Solvent C from pump #3 flows through the autosampler and through column #1. A 50uL injection is carried out, and the sample is loaded onto column #1 for 5 minutes. At this time, the valves V1 & V2 are switched, so that column #1 is connected to the gradient pumps (#1 & #2) and to the MS. A solvent gradient from 0% C to 100% C is delivered over the next 25 mins (i.e., between 5 and 30 mins after the injection), and the sample elutes to the MS. Meanwhile, column #2 is connected to pump #3 and to waste, and is equilibrated at 75 uL/min with 100% solvent C. Between 30 and 32 min after injection, both columns are equilibrated with 100% C at 50 uL/min. At 32 min after injection, the valves V1 & V2 are switched again so that pump #3 is now connected to column #2. At ~33 min, the next sample injection is made and the process is repeated using the alternate column for sample separation.

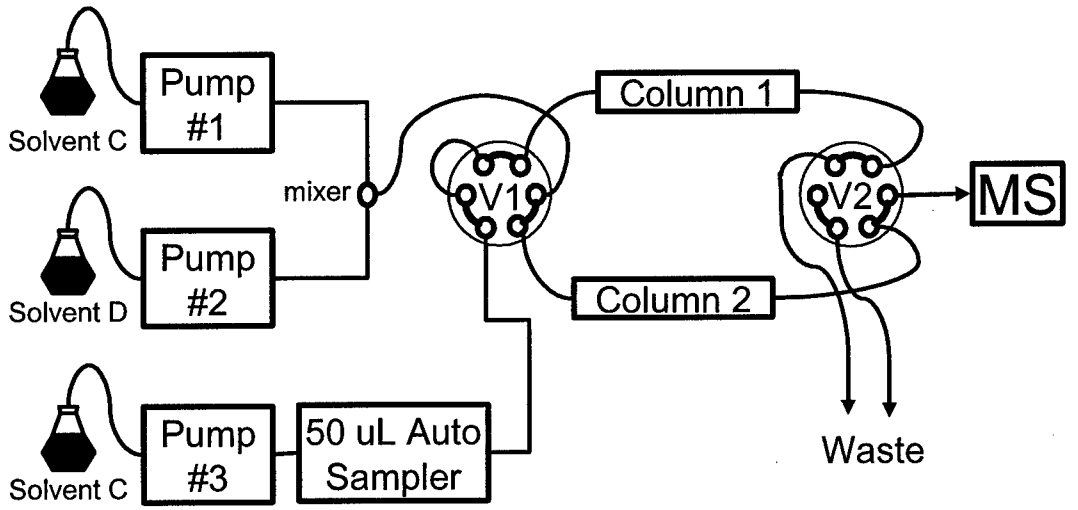


Figure 10. LC tandem MS set-up used during the method demonstration

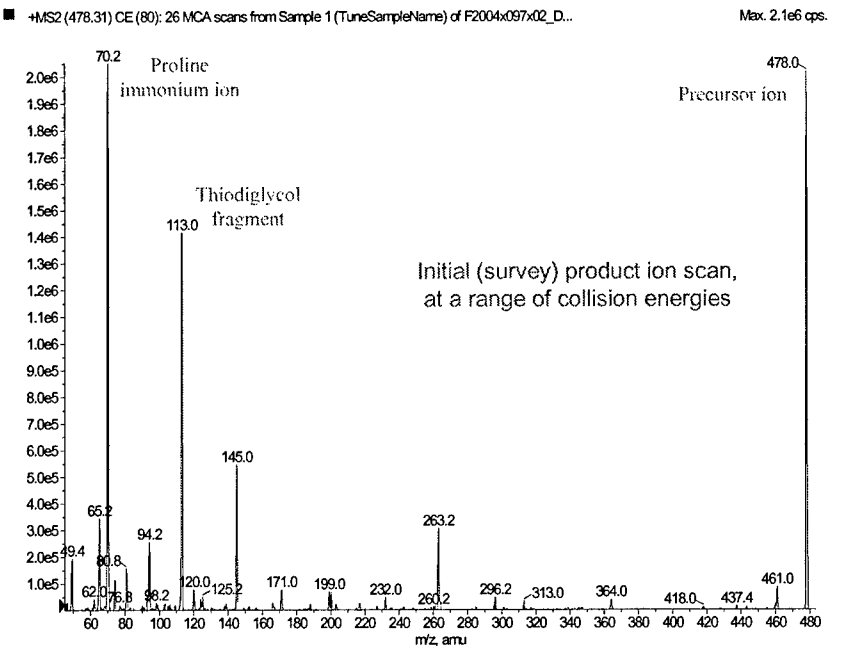


Figure 11. Initial (survey) product ion scan of (S-d_8-HETE)Cys-Pro-Phe (*m/z* 478).

The following experiments were performed:

1. MS/MS optimization on the synthetic (S-d_8-HETE)Cys-Pro-Phe, including a composite initial MS/MS fragment ion scan of *m/z* 478 and optimization of collision energy for the eight best transitions. The deuterated peptide adduct was taken, in order to not contaminate the instrument.
2. Processing (i.e., albumin isolation, pronase digestion) and mass spectrometric analysis of several plasma samples, that had been isolated from human blood that had been exposed to various concentratrions of sulfur mustard.

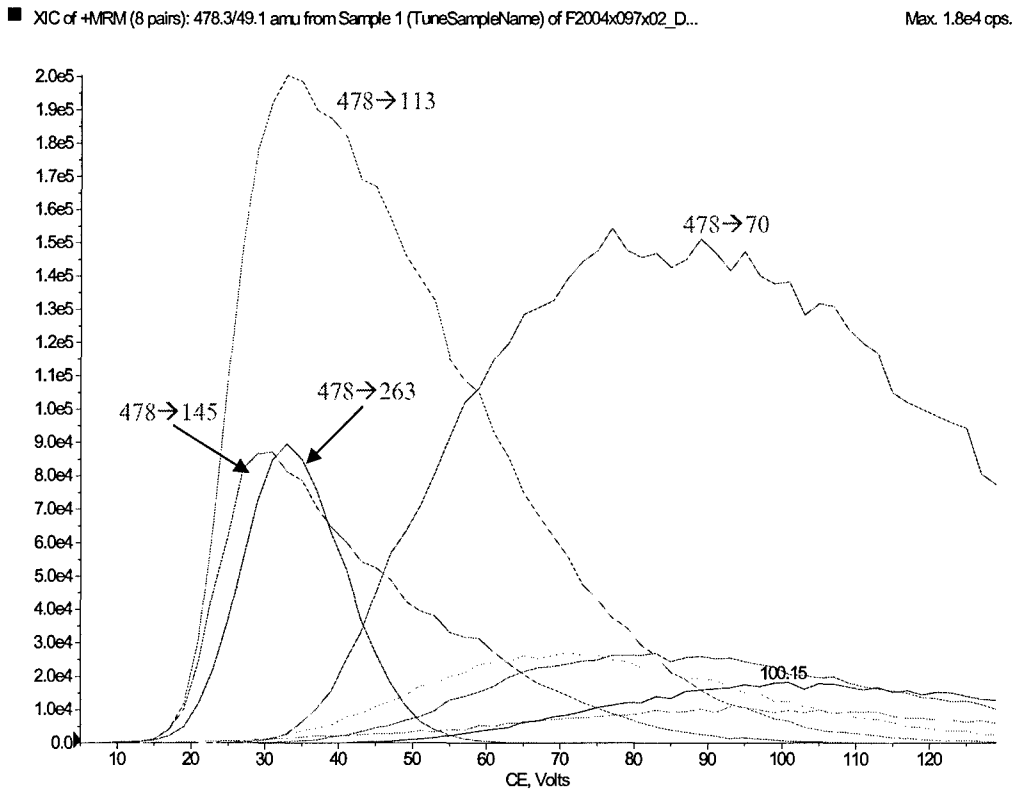


Figure 12. Optimization of collision energies for the best eight MRM transitions of (S-d_8-HETE)Cys-Pro-Phe.

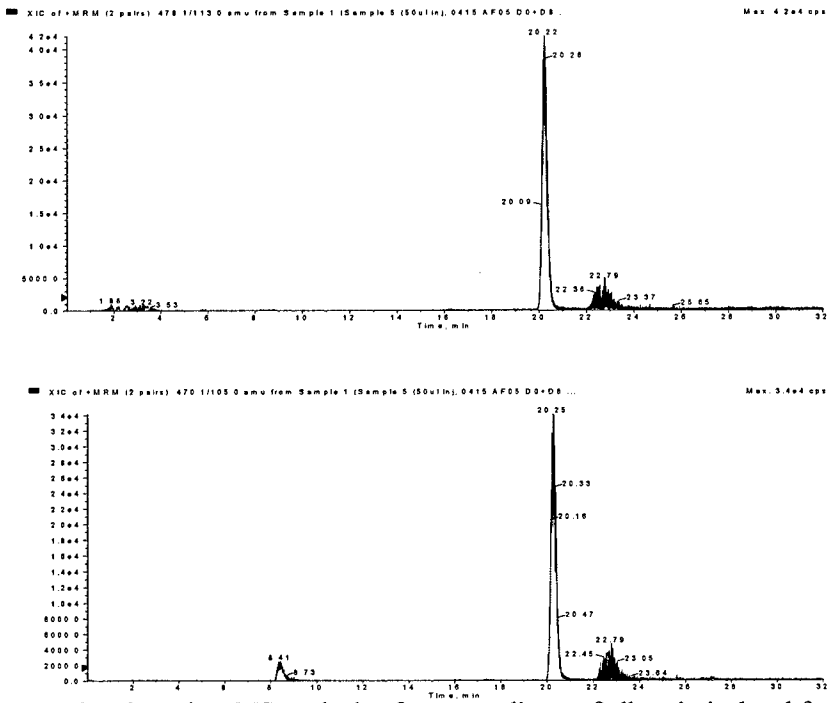


Figure 13. LC tandem MS analysis of pronase digest of albumin isolated from blood that had been exposed to sulfur mustard (10 μM; upper trace); internal standard at 10 μM (lower trace).

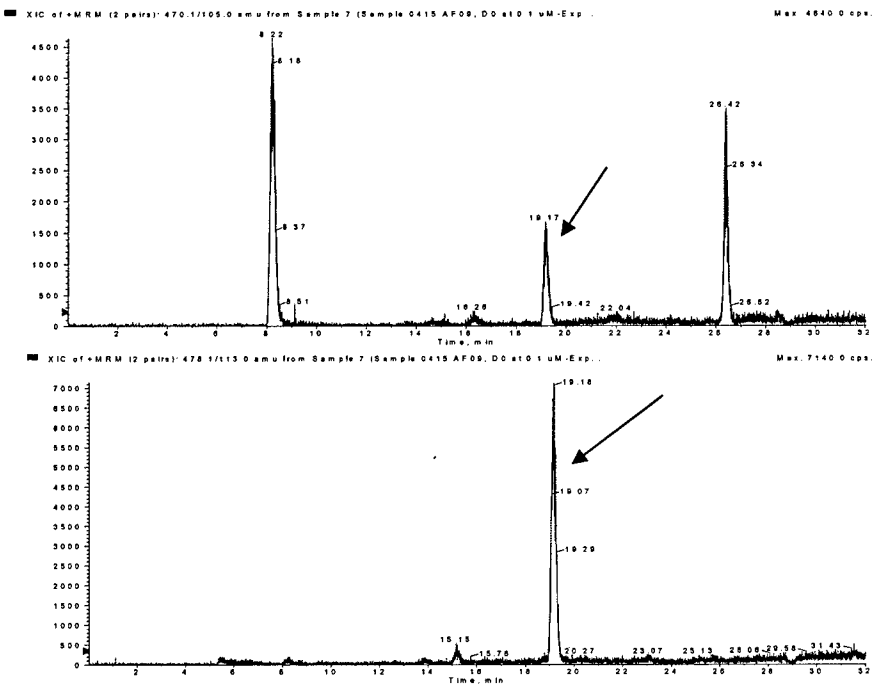


Figure 14. LC tandem MS analysis of pronase digest of albumin isolated from human blood after exposure to sulfur mustard (0.1 μM) (upper trace). The lower trace represents the d_8-internal standard (virtual exposure level: 0.5 μM).

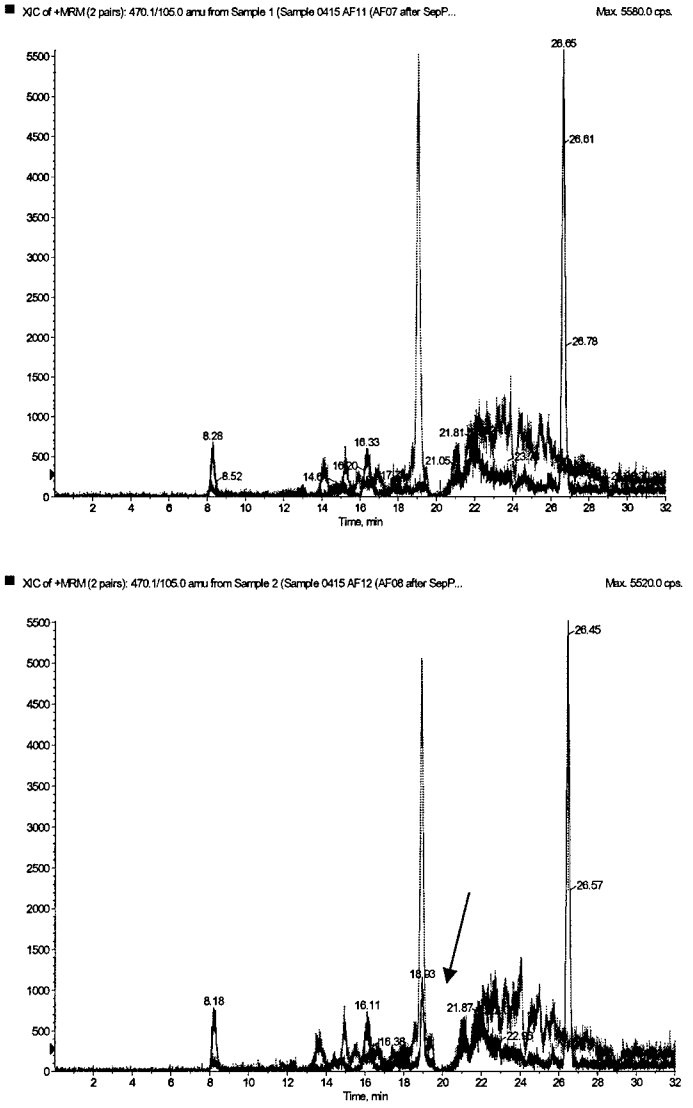


Figure 15. LC tandem MS analysis of pronase digest of albumin isolated from unexposed human blood (upper trace) and exposed human blood (10 nM; lower trace), after Sep Pak cleanup of the digest. The blue trace represents the (S-d_0-HETE)Cys-Pro-Phe, and the red trace represents the d_8-internal standard, spiked at a virtual level of 0.05 μM.

For establishing the right conditions for the instrument, the d_8-tripeptide reference standard was used (see Figures 11 and 12). The use of the corresponding d_0-reference standard was circumvented, because of possible contamination of the instrument. The method could be readily set up within one working day. First, a plasma sample with a relatively high exposure level (10 μM) was used (see Figure 13). The signal for d_0-sulfur mustard and d_8-internal standard were of comparable intensity, as was to be expected. Similar results were obtained for lower exposure levels (0.1 μM); see Figure 14. A 10 nM exposure level could still be detected when a Sep-Pak C18 step was incorporated in the procedure (see Figure 15).
The sensitivity of the instrument used at the CDC laboratory was comparable to that obtained at TNO.

IV.4 Optimization of immunoassay for screening of available antibodies against sulfur mustard adducts to proteins

IV.4.1 Variations in isolation of keratin from human callus

In an attempt to reproduce the screening results with antibodies specific for alkylated keratin, some problems arose. On keratin samples newly prepared from callus (obtained from other local chiropodists) exposed to sulfur mustard no specificity was observed. On the originally prepared keratin samples, all clones tested were still positive. Because this suggested that something was wrong with the isolation of the keratin samples, several isolation procedures described in literature were applied, but all without the desired specificity for adducts of sulfur mustard to the keratin.
Briefly, we applied besides the originally applied method (Sun and Green, 1978) a modification of this method by homogenizing the suspension (10 min), followed by sonication during the extraction phase. Also, the method of Steinert (1975) was applied in which extraction occurred in Tris (20 mM), urea (8 M) and β-mercaptoethanol (0.1 M). Upon extraction all preparations were centrifuged (30 min 3500 rpm) and the supernatants dialysed against water. Because dialysis resulted in the formation of a precipitate this step was substituted by filtration through a KD10 filter followed by lyophilization.
Precipitation of keratin during dialysis could also be prevented by denaturing the crude protein by heating at 65 °C for 10 min, but this did not result in an improved accessibility of the sulfur mustard adducts.

Further studies on the protein samples indicated that assessment of the keratin concentrations in the samples, pipetted from the filters after two washings with water, resulted in erroneous results when the BioRad method was applied. This suggests that in spite of the repeated washings the keratin samples still contained compounds which interfere with the protein measurements. In addition, when applying the more reliable RC DC method a large variation in protein concentration (from 0.2 up to 3 mg/ml) was obtained. Both phenomena may have a confounding effect on the responses obtained in the direct ELISA.

A significant improvement in the isolation procedure appeared to be the application of a brief homogenization step of the human callus suspension in 0.9% NaCl, immediately after sulfur mustard exposure, followed by extraction overnight in Tris.HCl (20 mM), urea (8 M), and β-mercaptoethanol (0.1 M), as described by Steinert (1975) and purification over a PD10 column and finally desalting by filtration through a KD10 filter.

Exposure of purified keratin to sulfur mustard resulted in precipitation of the keratin, even after exposure to only 100 μM sulfur mustard. This suggested that crosslinking by sulfur mustard made the protein insoluble and which also made the sulfur mustard adducts less accessible for the antibodies at higher degrees of crosslinking.
The homogenization of the pieces of human callus treated with sulfur mustard made the sulfur mustard adducts more accessible for the antibodies. However, at high exposure levels (more than 500 μM sulfur mustard) the accessibility of sulfur mustard adducts was still decreased. Exposure to 250 μM sulfur mustard resulted in a higher response than exposure to 1 mM sulfur mustard. A typical experiment is presented in Figure 16.

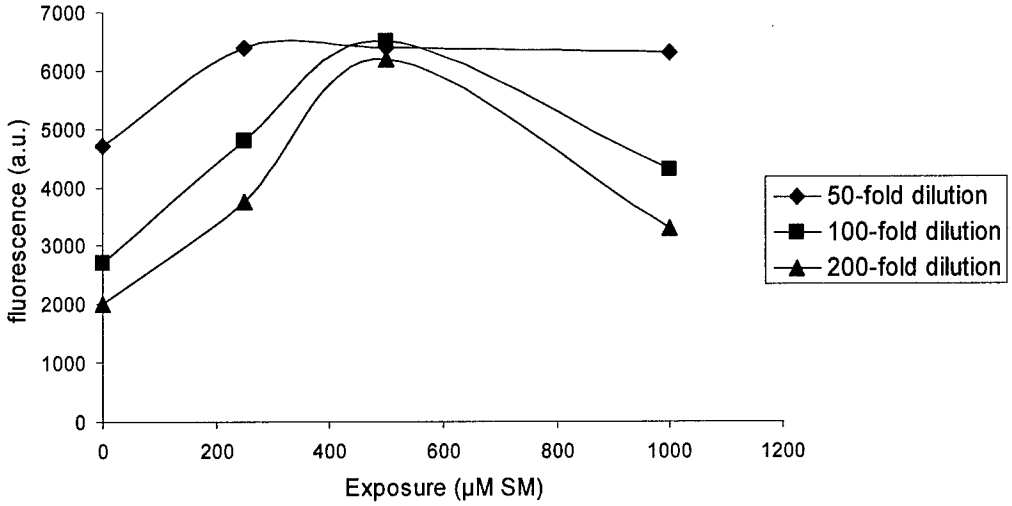


Figure 16. Direct ELISA with keratin isolated from human callus exposed to sulfur mustard (0, 250, 500 and 1000 µM sulfur mustard) with supernatants of clone 5F7 at 50-, 100- and 200-fold dilution. Data points are averages of two independent measurements of the same sample. The range between these points was about 10%.

These data clearly indicate that after high exposure the accessibility of sulfur mustard adducts has been decreased.

IV.4.2 Selection of newly prepared monoclonal antibodies directed against sulfur mustard adducts to keratin in a direct ELISA

The originally obtained primary clones which produced antibodies specific for sulfur mustard adducts to keratin were tested in a direct ELISA. Unfortunately, the 9 primary clones tested did not show any significant specific activity on newly prepared keratin (isolated from human callus treated with 50 µM sulfur mustard). Also, subcloning of 2 of the primary clones did not result in significant specific monoclonals.
For that reason, we decided to produce new antibodies. In total 8 fusions were carried out with spleen cells of mice immunized with a mix of the 3 SM-adducted peptides mentioned before or with keratin isolated from human callus exposed to 1 mM sulfur mustard.
The mice received two immunizations followed by a booster without stimune. One mouse received a booster with stimune. Fusion of spleen cells from all mice resulted in clones producing specific antibodies on keratin isolated from human callus treated with 1 mM sulfur mustard (except mouse RM03). The mouse boostered without stimune did not behave different from the other mice. In total about 4000 clones were tested. An overview of the selection is presented in Table 3.

Table 4. Overview of the selection of hybridomas producing antibodies directed against keratin isolated from human callus treated with 1 mM sulfur mustard

Mouse	immunization	96-well	24-well	6-well	25 cm2	75 cm2	frozen
RM01	mix of 3 SM-peptides	63	25	15	9	4	**3H4**,2D10,**1C6**, 1B8
RM02	mix of 3 SM-peptides, with stimune	72	21	6	4	1	3F9,5A7
RM03	mix of 3 SM-peptides	1					
RM04	mix of 3 SM-peptides	27	8	7			1D10,2D4,**2E4**,3F6,**4F7**,5E8
RM05	SM keratin	22	6	7	6	2	5A8,**5C7**
RM06	SM keratin	12	8	5	7	1	3F8,3F9,4F11
RM07	SM keratin	20	12	8	1		3D5,2H1
RM08	SM keratin	37	15	7	2	1	**1D6**,**1E8**,2F12,3F12,4D3,4E10,5A6

Promising hybridoma-cultures in the 6-well state, or further cultured, were frozen. The most promising frozen clones have been printed in bold (more then a factor of 2 difference between exposed and unexposed keratin at 25-fold or more dilution of culture supernatant).

From mouse RM03 no specific hybridomas could be selected. From all other mice two or more promising hybridoma-cultures have been selected. These are currently subcloned.

IV.4.3. Characterization of the newly prepared antibodies directed against keratin isolated from human callus treated with sulfur mustard.

Antibodies of clone 5C7 were applied in an immunoslotblot assay (Figure 17).

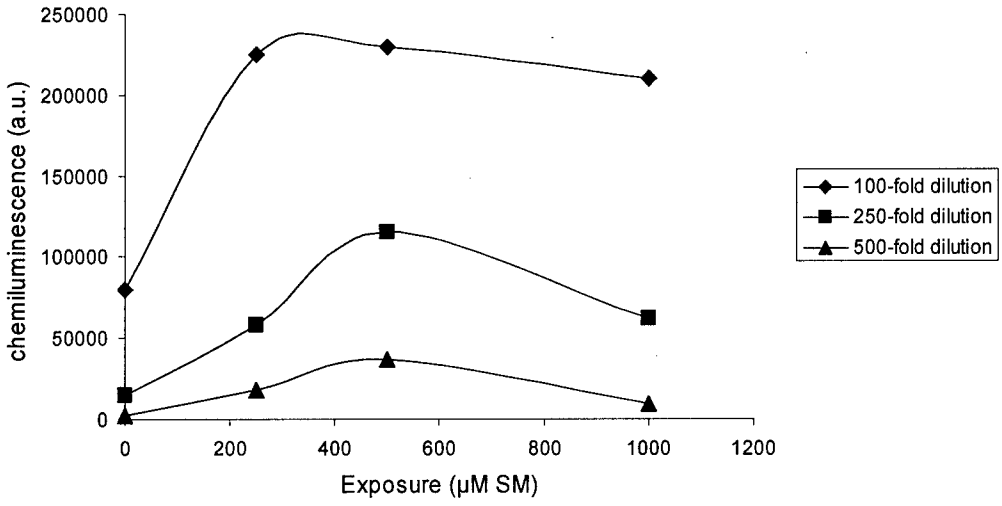


Figure 17. Immunoslotblot assay with keratin isolated from human callus exposed to sulfur mustard (0, 250, 500 and 1000 µM sulfur mustard) with supernatants of clone 5F7 (see Table 3) at 100-, 250- and 500-fold dilution. Keratin was applied in 0.5 µg aliquots in the assay. Data points are averages of two independent measurements of the same sample. The range between these points was about 10%. Chemiluminescence values above 200000 a.u. are less reliable because of the upper limits of the luminometer.

These data showed more or less the same pattern as with the direct ELISA. Again, after 1 mM sulfur mustard the accessibility of the adducts seemed to be decreased, resulting in a lower chemiluminescence.
Also supernatants of the hybridoma cultures, 3H4, 1C6 and 4F7, applied in an immunoslotblot assay showed promising specificities (data not shown).

These data indicate that, albeit not as efficient as found before with 1H10 antibodies, new antibodies are available which recognize SM-adducts to keratin isolated from human callus exposed to sulfur mustard.

Currently, these clones are subcloned to select monoclonal antibodies. Furthermore, it will be attempted to further improve the accessibility of the sulfur mustard adducts on keratin.

IV.5 Selection of recombinant antibodies by antibody phage display

Antibody phage display is a very powerful technique for selecting recombinant antibodies from a large library (Winter et al., 1994). An antibody phage library consists of the variable regions of heavy (VH) and light (Vκ) chains of human antibodies, which are randomly combined and linked together by a polypeptide linker to form a single-chain fragment (scFv). These scFvs are fused to a minor coat protein of bacteriophage M13, pIII, resulting in phages displaying antibody fragments; phage antibodies (PhAbs). The display of scFvs on a filamentous phage offers the possibility to select PhAbs without using hybridoma technology. Phage antibodies are selected by panning the library for several rounds on an immobilized antigen. At present, large synthetic libraries are available, which are created from unrearranged V gene segments from nonimmunized healthy human donors. These libraries can be used to select antibodies against any given antigen, including foreign antigens, self antigens, nonimmunogenic antigens, and toxic antigens. The goal of this study was to select antibodies; PhAbs or scFvs, to detect adducts of sulfur mustard to keratin in human skin.

IV.5.1. Selection of phage antibodies.

The Tomlinson I and J phage libraries were used to select MoPhAbs and scFvs against keratin sulfur mustard adducts. Three rounds of selection were performed on keratin that was exposed to 1mM sulfur mustard as well as keratin that was unexposed keratin (Figure 18). MoPhAbs derived from the successive selection rounds were screened for their capacity to distinguish keratin that was exposed to sulfur mustard from unexposed keratin and *vise versa*. For this, 768 individual, randomly chosen colonies after the final selection round from both libraries (I and J); in total 1536 colonies, were used to prepare culture supernatants contained with MoPhAbs. These MoPhAbs were subsequently tested with an ELISA for antigen binding. Thirty four culture supernatants, contained with MoPhabs, were able to distinguish untreated keratin from keratin that was exposed to 1mM sulfur mustard (Figure 19). All thirty four clones were selected for the production of larger phage quantities as described in the experimental procedures. A single clone, IAD7, retained its activity, even after repeated culturing, and culturing in larger volumes. For no obvious reason, all other selected clones lost their activity or specificity. Clone IAD7 was further characterized in more detail.

IV.5.2. PCR Screening.

Clone IAD7 was checked for the presence of full length VH and Vκ insert. The DNA fragment that was obtained by PCR of DNA isolated from clone IAD7 had an estimate size of approximately 935bp (Figure 20). This result shows that clone IAD7 contains a full length VH and Vκ insert.

IV.5.3. Characterization of MoPhabs by Immunoslotblot.

The MoPhAbs of clone IAD7 were characterized by the ISB assay. Based on experience that we gathered by the development of an ISB assay for measuring sulfur mustard adducts (N_7-GUA) in DNA (Van der Schans et al., 2004) we established optimal conditions for measuring various adduct levels in keratin. 50, 100 and 200 µl of 1-5 µg/ml antigen was spotted on the filter. It was found that optimal resolution for the detection of keratin which had been exposed to various concentrations sulfur mustard, was obtained when spotting 200µl at a concentration of 2.5µg/ml on the nitrocellulose, as shown in Figure 21.

IV.5.4. Expression of soluble antibody fragments.

Clone IAD7 was chosen for expression of scFvs. The *E. coli* nonsuppressor strain, HB2151, was infected with phages from clone IAD7, and induced with IPTG. Culture supernatants containing single chain antibody variable region fragments (scFvs) were collected to be detected by ELISA further use. No signal was obtained by ELISA, suggesting that HB2151 did not express scFvs. Even after repeated attempts, no scFv expression could be observed.

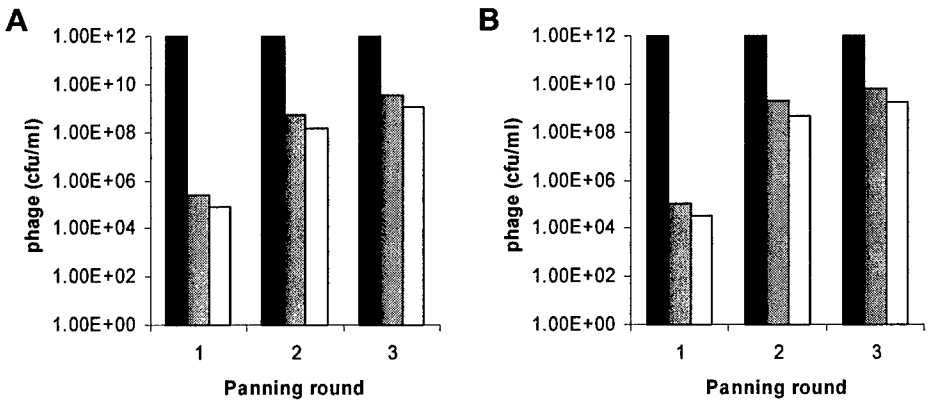


Figure 18. Number of eluted and amplified phages after each of the three rounds of selection. A; Tomlinson I library, B; Tomlinson J library. The black columns indicate the number of amplified phages after each round, which were used for the next round of selection. The grey bars represent the numbers of phages which were eluted from the exposed keratin after each round. The white bars represent the number of phages which were obtained after elution of the exposed antigen and subsequently used for selection and elution of the unexposed antigen. The titer was determined as cfu/ml (cfu= colony forming unit).

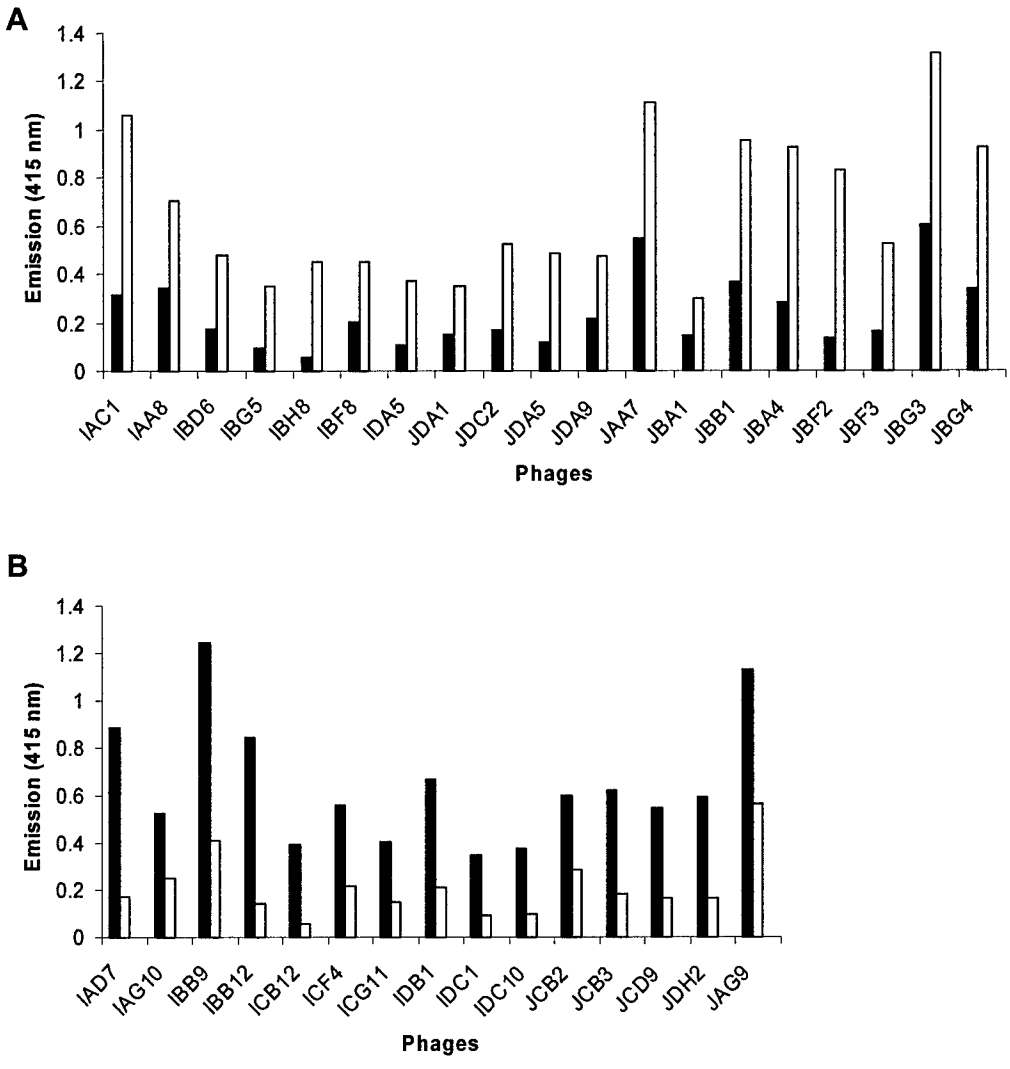


Figure 19. Culture supernatants containing MoPhabs that were able to distinguish unexposed keratin from keratin, which was exposed to 1 mM sulfur mustard. The black bars represent the affinity to unexposed keratin. The white bars represent the affinity for keratin expose to 1 mM sulfur mustard, of the same MoPhAb. A, MoPhabs showing highest affinity for exposed keratin; MoPhabs showing highest affinity for unexposed keratin. Affinity is quantified in emission of ABTS at 415 nm.

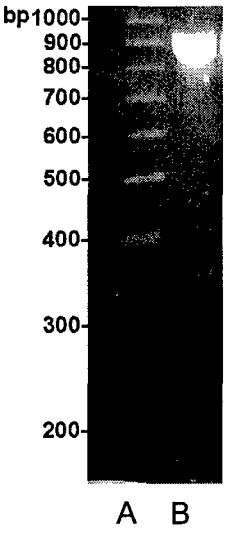


Figure 20. PCR check of candidate IAD7 for the presence of full length Vκ and VH insert. Agarose gel loaded with A; reference DNA Superladder-low 100bp, B; DNA fragment after PCR of DNA from clone IAD7. The size of this DNA fragment was estimated at 935 bp, corresponding to DNA contained with a complete Vκ and VH insert.

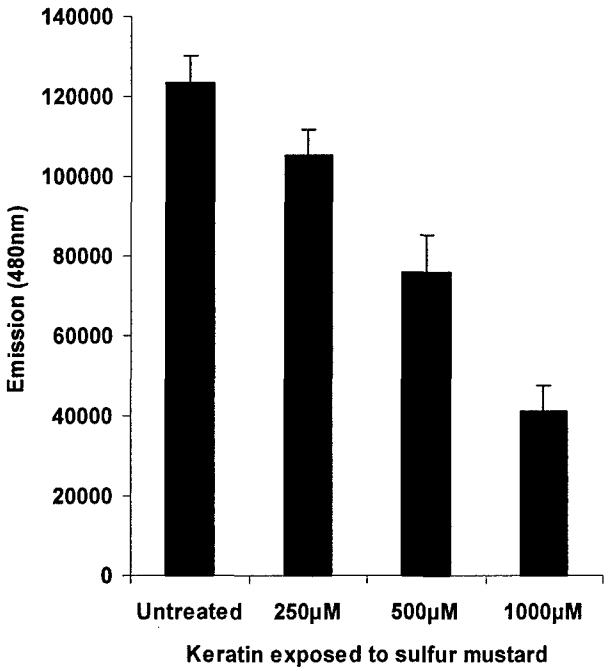


Figure 21. Immunoslotblot showing a typical example of binding activity of MoPhAb IAD7 to untreated keratin and keratin that was exposed to 250 µM, 500 µM and 1000 µM sulfur mustard. Values are mean values + SEM (standard error of the mean), $P \leq 0.05$. For optimal resolution 200 µl of 2,5 µg/ml antigen were spotted on the nitrocellulose filter, as represented in the figure.

V DISCUSSION

Development of a Standard Operating Procedure for determination of sulfur mustard protein adducts; mass spectrometric or fluorescence-based analysis of an adducted tripeptide from albumin *(part A,; t.o. 1- 9)*

Attention has been paid to the fluorescence derivatization of the sulfur mustard peptide adduct (S-HETE)Cys-Pro-Phe, derived from human serum albumin. We could derivatize the reference standard (S-HETE)Cys-Pro-Phe with 5/6 carboxyfluorescein succinimidyl ester (FAM-SE) and succeeded in subsequent analysis by means of capillary electrophoresis with laser-induced fluorescence detection (CE-LIF). Furthermore, we succeeded in detecting the tripeptide adduct in a pronase digest spiked at a high level with the synthetic reference standard, albeit after pre-purification with reversed-phase HPLC. Pre-purification of the samples was required because otherwise proper analysis by means of capillary electrophoresis was not possible (overloading of the capillary). Unfortunately, the adduct could not be detected in a derivatized pronase digest from albumin that had been isolated from blood after exposure to sulfur mustard (1 mM). In the area in the electropherogram where the FAM-(S-HETE)Cys-Pro-Phe derivative elutes an interfering peak is present, which precludes unambiguous analysis. Furthermore, we believe that at low adduct concentrations and in the presence of highly abundant, competing amino acids, the derivatization reaction proceeds too sluggishly. However, even when the latter problems can be solved, we believe that the fluorescence-based method can never surpass the MS-based method because of the quite laborious sample prep, e.g., pre-purification of samples with HPLC before CE-LIF analysis, required for proper CE-LIF analysis.

Development of a Standard Operating Procedure for determination of sulfur mustard protein adducts; analysis of N1/N3-HETE-histidine *(part B, t.o. 10-13).*

Although histidine adducts are the most abundant adducts resulting after exposure of proteins to sulfur mustard, there is no sensitive method for their analysis available. It was attempted in the third year of the agreement to semi-preparatively separate underivatized N1/N3-HETE-histidine by means of reversed-phase HPLC. Unfortunately, this method proved not to be reproducible due to the high variation in retention times during LC-tandem MS analysis.

We also proposed to study the feasibility of fluorescence detection of sulfur mustard-histidine adducts. Although HPLC with fluorescence detection is much less specific than mass spectrometric detection, it has the advantage that yoctomole amounts of amino acid derivatives can be analyzed. In the third year of the grant period the carboxyfluoresceine (FAM) derivative of N1/N3-histidine was chosen for further analyses, because it can be prepared quite easily using a commercially available reagent and it proved to be a stable derivative, in contrast to the FITC derivative that slowly rearranged. Although the detection limit of the FAM derivatives of N1/N3-HETE-histidine was determined to be 20-25 pg/ml, this method could not be applied to processed acidic digests of globin or albumin. As was the case with the tripeptide adduct, we believe that the derivatization of the adduct does not proceed at low concentration levels, probably due to high abundant components like native amino acids. Furthermore, one of the disadvantages of not following an MS-based approach is that it is hard to distinguish between all the fluorescent derivatives, especially when the compound of interest is only present in low amounts compared to other compounds as is definitely the case when analyzing adducts.

Development of a Standard Operating Procedure for determination of sulfur mustard protein adduct; comparison of the albumin – tripeptide method with the histidine adduct method *(part C, t.o. 14-18)*

The procedure for analysis of the tripeptide adduct (S-HETE)Cys-Pro-Phe originating from albumin is a very rapid, straightforward method for diagnosis of exposure to sulfur mustard, and is in our view at this moment superior to the method for determination of histidine adducts. The work-up of the plasma sample is minimal, no derivatization is required, and the method can probably be automated. A definitive SOP has now been drafted, on the basis of the tentative version described in the second annual report. The Standard Operating Procedure for analysis of the tripeptide adduct was demonstrated to a scientist of USAMRICD. In this particular case the demonstration was performed within the laboratories of CDC, because the LC-tandem MS instrument at ICD was not operational (yet) at that particular time. The demonstration was well-prepared: reference compounds, a list with required materials, chemicals etc., protocols and a work-plan were sent in advance to both parties (CDC and USAMRICD). All required items arrived on time and, if needed, were stored at -20 °C. We did not start with the analysis of reference standards, because we did not want to contaminate the instrument with the analyte. The analysis started with a 10 μM exposure level, including isotope dilution by using plasma isolated from blood that had been exposed to d_8-sulfur mustard. Peak ratios d_0/d_8 were as to be expected. There were no problems with contaminated blanks. Next, lower-exposed samples were studied; results were satisfactory. Finally, the Sep-Pak C18 procedure was demonstrated in order to enhance the sensitivity of the procedure. Overall outcome of the method demonstration was that it was possible to set up the SOP at a different, well-equipped laboratory within a relatively short period of time (one working day), and that it was possible to fully transfer the method to an analytical scientist, within two working days.
In the future, we plan to use columns with immobilized pronase. This might enable the construction of a 2-dimensional LC-tandem MS system in which an unprocessed plasma sample can be introduced. This would be highly convenient for use under field laboratory conditions, and more importantly, would speed up the actual diagnosis.
The interindividual variation of the in vitro sensitivity of human blood to sulfur mustard was determined, following the SOP for the tripeptide assay. The deviation found was within the range of 10%. Furthermore, the intra-individual variation and the day-to-day variability of the assay was also shown to be acceptable.
It is envisaged that the scope of this method is not limited to sulfur mustard and that it can become a generic method for diagnosis of exposure to a wide array of alkylating agents.

Immunochemical protein adduct analysis *(Part D, t.o. 19-24)*

The main advantage of detection of adducts to proteins over those to DNA is the expected much longer half-life of the protein adducts. Therefore, antibodies were raised against (S-HETE)-cysteine in partial sequences of human hemoglobin in our previous studies. However, the minimum detectable concentration obtained for *in vitro* exposure of human blood with these antibodies was only 50 μM of sulfur mustard. Consequently, further exploratory research on immunochemical assays of protein adducts was a major topic of the current study. In the third year of the cooperative agreement, the main focus was on adducts to keratin.

Immunochemical analysis of keratin adducts
In addition to the respiratory tract, the skin is a major target for vesicants such as sulfur mustard. Proteins in the skin, particularly those in the stratum corneum, are readily accessible to agents. Since keratin is the most abundant protein in stratum corneum and epidermis, methods for retrospective detection of skin exposure to sulfur mustard were developed in the present study. Keratins (MW 40-70 kDa) form the backbone of the intermediate filaments (IFs) in epithelial tissues. In basal epidermal cells almost 30% of all synthesized proteins are

keratins. For development of an immunochemical assay, two partial end domain sequences of keratin K14 and one partial end domain sequence of keratin K5 were synthesized on a solid support as haptens for raising antibodies, as earlier described (Van der Schans et al., 2002). This approach appeared to be very successful for raising antibodies.
Some problems arose with antibodies specific for alkylated keratin, with regard to the reproducibility of the assay. Unfortunately, the experiments with 1H10 antibodies could not be reproduced with newly prepared keratin from SM-exposed human callus. Nevertheless, newly prepared antibodies could be also successfully applied in the immunoslotblot assay, albeit with a lower sensitivity. Its real limits of detection should still be assessed.

Selection of recombinant antibodies by phage display

The purpose of this part of the study was to generate antibodies to detect sulfur mustard adducts on human keratin. In addition to the classical pathway of antibody generation as performed by the hybridoma technique, we explored the suitability to use phage display technologies for the same purpose. In comparison to phage display, the hybridoma technique is significantly more expensive and time consuming. Moreover, phage display does not require immunizations and the sacrifice of laboratory animals.
By using the Tomlinson I & J libraries (Winter et al., 1994) we have been able to select a single MoPhAb, denoted IAD7, which was able to distinguish unexposed keratin from keratin that was exposed to various concentrations sulfur mustard.
MoPhAb IAD7 is directed against the native, unexposed keratin. Exposure to increased concentrations of sulfur mustard results in a decreased affinity to keratin, as shown in ELISA and ISB. It is plausible that structural changes in the protein, caused by adduct formation and crosslinkin (Byrne et al., 1996; Dillman et al., 2003) may hinder effective antigen recognition. Based on these characteristics MoPhAb IAD7 would be a suitable antibody that may be used in the detection and diagnose of sulfur mustard exposure to the human skin. We argue that MoPhAb IAD7 may be used in parallel with antibodies which recognize the actual sulfur mustard adduct as complementary antibody or negative control.
Despite repeated effort we were not able to detect soluble scFvs after IPTG induction of *E. coli* HB2151. We argue that it might be possible that the affinity of the scFv in ELISA for its antigen is too inefficient to be detected by ELISA soluble form but can bind as phage due to avidity effects. Otherwise, if the scFv genes contain amber (TAG) stop codons expression would be suppressed in *E. coli* TG1 but not in *E. coli* HB2151. Strain TG1 suppresses termination of translation by a stop codon and introduces a glutamate residue at these positions. This means that in TG1 a TAG stop codon between the scFv and the *gIII* gene is suppressed and leads to co-expression of the scFv-pIII fusion gene. HB2151 is a non-suppressor strain which will stop translation between the scFv gene and *gIII,* and is able to generate high levels of scFv upon IPTG induction. HB2151 containing scFv genes that do not contain TAG stop codons will yield high levels of soluble scFvs, but those that contain TAG codons in the scFv gene will not produce any scFv at all.

VI KEY RESEARCH ACCOMPLISHMENTS OBTAINED IN THIS GRANT PERIOD

1. A derivatization procedure was developed for the tripeptide (S-HETE)Cys-Pro-Phe for analysis with CE-LIF.
2. It was determined that the tripeptide can be determined in derivatized pronase digests by means of CE-LIF, but only when spiked at high levels.
3. The tentative SOP for the tripeptide assay has been converted in a definitive version.
4. The interindividual -, intraindividual - , and day-to-day variability of the tripeptide assay was determined to be acceptable.
5. The SOP was successfully demonstrated and transferred to a scientist of USAMRICD.
6. An improved screening procedure has been set up for selection of antibodies from clones raised against adducted peptides with amino acid sequences present in human globin.
7. Although not as efficient as found before with 1H10 antibodies, new antibodies have been prepared which recognize SM-adducts to keratin isolated from human callus exposed to sulfur mustard.
8. An immunoslotblot assay was set up for detection of sulfur mustard adducts to keratin.
9. An alternative method, antibody phage display, was successfully applied for selection of recombinant antibodies which recognized keratin from human callus exposed to sulfur mustard.
10. Thirty four culture supernatants, contained with MoPhabs, were able to distinguish untreated keratin from keratin that was exposed to 1mM sulfur mustard.
11. A single clone, IAD7, retained its activity, even after repeated culturing, and culturing in larger volumes. For no obvious reason, all other selected clones lost their activity or specificity.

VII REPORTED OUTCOMES (from beginning of cooperative agreement)

Publications

NOORT, D., FIDDER, A., HULST, A.G., and LANGENBERG, J.P. (2002) Low level exposure to sulfur mustard: development of an SOP for analysis of albumin and/or hemoglobin adducts. Proceedings of the 2002 Medical Defense Bioscience Review, June 2002, Hunt Valley, MD, USA.

NOORT, D., BENSCHOP, H.P. and BLACK, R.M. (2002) Biomonitoring of exposure to chemical warfare agents. A review. Toxicol. Appl. Pharmacol. **184**, 116-126.

VAN DER SCHANS, G.P., NOORT, D., MARS-GROENENDIJK, R.H., FIDDER, A., CHAU, L.F., DE JONG, L.P.A. and BENSCHOP, H.P. (2002) Immunochemical detection of sulfur mustard adducts with keratins in the stratum corneum of human skin. Chem. Res. Toxicology **15**, 21-25.

VAN DER SCHANS, G.P., NOORT, D., MARS-GROENENDIJK, R.H., FIDDER, A. and LANGENBERG, J.P. (2002) Immunochemical detection of sulfur mustard adducts with keratins in the stratum corneum of human skin. Proceedings of the 2002 Medical Defense Bioscience Review, June 2002, Hunt Valley, MD, USA.

VAN DER SCHANS, G.P., MARS-GROENENDIJK, R.H., NOORT, D., and LANGENBERG, J.P. (2002) Development of immunochemical assays for detection of sulfur mustard-adducts with proteins. Proceedings of the meeting of NATO TG-004, November 2002, Oslo, Norway.

NOORT, D., FIDDER, A., BENSCHOP, H.P., DE JONG, L.P.A. and SMITH, J.R. Procedure for monitoring exposure to sulfur mustard based on modified Edman degradation of globin. J. Anal. Toxicol., **28**, 311-315, 2004.

VAN DER SCHANS, G.P., MARS-GROENENDIJK, R., DE JONG, L.P.A., BENSCHOP, H.P. and NOORT, D. Standard operating procedure for immunoslotblot assay for analysis of DNA-sulfur mustard adducts in human blood and skin. J. Anal. Toxicol., **28**, 316-319, 2004.

NOORT, D., FIDDER, A., HULST, A.G., WOOLFITT, A.R., ASH, D., and BARR, J.R. Retrospective detection of exposure to sulfur mustard: Improvements on an assay for liquid chromatography – tandem mass spectrometry analysis of albumin/sulfur mustard adducts. J. Anal. Toxicol., **28**, 333-338, 2004.

NOORT, D., FIDDER, A., DE KANT, S., and HULST, A.G. Low Level Exposure to Sulfur Mustard: Development of an SOP for Analysis of Albumin Adducts. Proceedings of the Bioscience Review 2004, in press.

BLACK, R.M., and NOORT, D. (2005) Methods for retrospective detection of exposure to toxic scheduled chemicals. Part A: Analsyis of free metabolites. In "Chemical Weapons Convention Chemicals Analysis; sample collection, preparation and analytical methods" Ed. M. Mesilaakso; John Wiley & Sons, Chichester, UK.

NOORT, D., and BLACK, R.M. (2005) Methods for retrospective detection of exposure to toxic scheduled chemicals. Part B: Mass spectrometric and immunochemical analysis of covalent adducts to proteins and DNA. In "Chemical Weapons Convention Chemicals

Analysis; sample collection, preparation and analytical methods" Ed. M. Mesilaakso; John Wiley & Sons, Chichester, UK.

VAN DER SCHANS, G.P., MARS-GROENENDIJK, R.H, BIKKER, F.J. and NOORT, D. (2005) Immunochemical Procedures for Simple Analysis of Exposure to Sulfur Mustard. Contribution to book entitled: "Medical Aspects of Chemical and Biological Terrorism"; "Chemical terrorism and traumatism", edited by Christophor Dishovsky and Professor Alexander Monov, Publ. house of the Union of Scientists in Bulgaria. (in press).

Abstracts

NOORT, D., FIDDER, A., HULST, A.G., and LANGENBERG, J.P. (2002) Low level exposure to sulfur mustard: development of an SOP for analysis of albumin and/or hemoglobin adducts. Book of abstracts of the 2002 Medical Defense Bioscience Review, June 2002, Hunt Valley, MD, USA.

VAN DER SCHANS, G.P., NOORT, D., MARS-GROENENDIJK, R.H., FIDDER, A. and LANGENBERG, J.P. (2002) Immunochemical detection of sulfur mustard adducts with keratins in the stratum corneum of human skin. Book of abstracts of the 2002 Medical Defense Bioscience Review, June 2002, Hunt Valley, MD, USA.

VAN DER SCHANS, G.P., MARS-GROENENDIJK, R.H., NOORT, D., and LANGENBERG, J.P. (2002) Development of immunochemical assays for detection of sulfur mustard-adducts with proteins. Proceedings of the meeting of NATO TG-004, November 2002, Oslo, Norway.

NOORT, D. (2003) Use of LC tandem MS techniques in identification and sensitive detection of covalent adducts of xenobiotics with proteins, Drug Metabolism Reviews, 35 (S 1), 3.

NOORT, D., FIDDER, A., DE KANT, S., and HULST, A.G. Low Level Exposure to Sulfur Mustard: Development of an SOP for Analysis of Albumin Adducts. Abstract Book Bioscience Review, Hunt Valley MD, May 16-21, 2004.

NOORT, D., VAN DER SCHANS, M.J., and BENSCHOP, H.P. (2004) Biomonitoring of exposure to chemical warfare agents. Abstract book 2004 Joint SOFT/TIAFT Meeting, Washington DC, August 30 – September 3, 2004, p. 241.

Presentations

Development of immunochemical assays for detection of sulfur mustard-adducts with proteins. Presented by G.P. Van der Schans at meeting of NATO TG-004, November 2002, Oslo, Norway.

Use of LC tandem MS techniques in identification and sensitive detection of covalent adducts of xenobiotics with proteins". Presented by D. Noort at "8th European ISSX Meeting", Dijon, France. April 27 – May 1, 2003.

Methods for biomonitoring of individuals (involved by destruction of CW's) who may be contaminated with low concentrations of sulfur mustard, lewisite, phosgene and OP's. Presented by G.P. Van der Schans at meeting of CEPA IEX 13.11, Destruction of old chemical munitions, April 2004, Civitavecchia, Italy.

NOORT, D., FIDDER, A., DE KANT, S., and HULST, A.G. Low Level Exposure to Sulfur Mustard: Development of an SOP for Analysis of Albumin Adducts. Bioscience Review , Hunt Valley MD, May 16-21, 2004.

NOORT, D., VAN DER SCHANS, M.J., and BENSCHOP, H.P. (2004) Biomonitoring of exposure to chemical warfare agents. 2004 Joint SOFT/TIAFT Meeting, August 30 – September 3, 2004, Washington DC.

VIII CONCLUSIONS

1. The albumin tripeptide assay, based on our previous finding that upon pronase treatment of sulfur mustard alkylated albumin a tripeptide cysteine(S-2-hydroxyethylthioethyl)-proline-phenylalanine ((S-HETE)Cys-Pro-Phe) results which has favorable mass spectrometric properties, is a very fast and sensitive assay for biomonitoring of exposure to sulfur mustard.
2. For detection with laser-induced fluorescence, various derivatization methods have been explored for the tripeptide. The most convenient derivative turned out to be the 6-carboxyfluoresceine (FAM) derivative.
3. The tripeptide could not be detected in a pronase digest of sulfur mustard alkylated albumin, after derivatization with FAM succinimidyl ester and subsequent CE-LIF detection.
4. Direct isolation of the histidine adduct from pronase or acidic digests by means of preparative reversed-phase HPLC could be effected, but was not yet reproducible due to variation in retention time of the adduct
5. The interindividual and intraindividual variation, and the day-to-day variability of the tripeptide assay lie in an acceptable range.
6. The SOP drafted for the tripeptide assay could be successfully demonstrated to a scientist of USAMRICD in a different laboratory indicating the high utility of the procedure.
7. In the current cooperative agreement the screening procedure for the selection of monoclonal antibodies against adducts of sulfur mustard with proteins (globin, albumin and keratin) has been improved. The most suitable antibodies are used for the development of an immunoslotblot assay for retrospective detection of exposure to sulfur mustard.
8. Unfortunately, some problems arose with antibodies specific for alkylated keratin, with regard to the reproducibility of the assay. These problems have been studied in more detail in the third year of the grant period. It appeared that the accessibility of the sulfur mustard adducts decreased at sulfur mustard exposures above 0.5 mM. We selected new clones producing antibodies specific for exposed keratin, of which several retained their activity upon continued culturing.
9. An alternative method, antibody phage display, was successfully applied for selection of recombinant antibodies which recognized keratin from human callus exposed to sulfur mustard. Thirty four culture supernatants, contained with MoPhabs, were able to distinguish untreated keratin from keratin that was isolated from human callus exposed to 1 mM sulfur mustard.
10. A single clone, IAD7, retained its activity, even after repeated culturing, and culturing in larger volumes. For no obvious reason, all other selected clones lost their activity or specificity.

IX REFERENCES

Benschop, H.P., and Van der Schans, G.P. (1995) Immunochemical and mass spectrometric detection of mustard gas adducts to DNA and proteins: verification and dosimetry of exposure to mustard gas. Final report grant no. DAMD17-92-V-2005.

Benschop, H.P., Noort, D., Van der Schans, G.P., and De Jong, L.P.A. (2000) Diagnosis and dosimetry of exposure to sulfur mustard: Development of standard operating procedures; further exploratory research on protein adducts. Final report Cooperative Agreement DAMD17-97-2-7002. NTIS AD-A381035/XAB.

Black, R.M., and Noort, D. (2005) Methods for retrospective detection of exposure to toxic scheduled chemicals. Part A: Analsyis of free metabolites. In "Chemical Weapons Convention Chemicals Analysis; sample collection, preparation and analytical methods" Ed. M. Mesilaakso; John Wiley & Sons, Chichester, UK.

Byrne, M.P., Broomfield, C.A. and Stites, W.E. (1996) Mustard gas crosslinking of proteins through preferential alkylation of cysteines. *J. Protein Chem.* **15**, 131-136.

Dillman, J.F., McGary, K.L. and Schlager, J.J. (2003) Sulfur mustard induces the formation of keratin aggregates in human epidermal keratinocytes. *Toxicol Appl. Pharmacol.* **193**, 228-236.

Fuchs, E. and Weber, K. (1994) Intermediate filaments: structure, dynamics, function, and disease. *Annu. Rev. Biochem.* **63**, 345-382.

Noort, D., Hulst, A.G., De Jong, L.P.A., and Benschop, H.P. (1999) Alkylation of human serum albumin by sulfur mustard *in vitro* and *in vivo*: mass spectrometric analysis of a cysteine adduct as a sensitive biomarker of exposure. *Chem. Res. Toxicol.* **12**, 715-721.

Noort, D., Hulst, A.G., and Jansen, R. (2002) Covalent binding of nitrogen mustards to the cysteine-34 residue in human serum albumin. *Arch. Toxicol.* **76**, 83-88.

Noort, D., Fidder A., Hulst, A.G. (2003) Modification of human serum albumin by acrylamide at cysteine-34: a basis for a rapid biomonitoring procedure. *Arch. Toxicol.* 77, 543-545.

Noort, D., Benschop, H.P., and Black, R.M. (2002) Biomonitoring of exposure to chemical warfare agents: a review. *Toxicol. Appl. Pharmacol.* **184**, 116-126.

Noort, D., Fidder, A., Hulst, A.G., Woolfitt, A.R., Ash, D., and Barr, J.R. (2004) Retrospective detection of exposure to sulfur mustard: Improvements on an assay for liquid chromatography – tandem mass spectrometry analysis of albumin/sulfur mustard adducts. *J. Anal. Toxicol.*, **28**, 333-338.

Noort, D., and Black, R.M. (2005) Methods for retrospective detection of exposure to toxic scheduled chemicals. Part B: Mass spectrometric and immunochemical analysis of covalent adducts to proteins and DNA. In "Chemical Weapons Convention Chemicals Analysis; sample collection, preparation and analytical methods" Ed. M. Mesilaakso; John Wiley & Sons, Chichester, UK.

Steinert , P.M. (1975) The extraction and characterization of bovine epidermal α-keratin. *Biochem. J.* 149, 39-48.

Steinert, P.M. (1978) Structure of the three-chain unit of the bovine epidermal keratin filament. *J. Mol. Biol.* **123**, 49-70.

Sun, T.-T, and Green, H. (1978) Keratin filaments of cultural human epidermal cells. *J. Biol. Chem.* **253**, 2053-2060.

Van der Schans, G.P., Scheffer, A.G., Mars-Groenendijk, R.H., Fidder, A., Benschop, H.P., and Baan, R.A. (1994) Immunochemical detection of adducts of sulfur mustard to DNA of calf thymus and human white blood cells. *Chem. Res. Toxicol* **7**, 408-413.

Van der Schans, G.P., Noort, D., Mars-Groenendijk, R.H., Fidder, A., Chau, L.F., De Jong, L.P.A., and Benschop, H.P. (2002). Immunochemical detection of sulfur mustard adducts with keratins in the stratum corneum of human skin. *Chem. Res. Toxicol.* **15**, 21-25.

Van der Schans, G.P., Mars-Groenendijk, R., de Jong, L.P., Benschop, H.P. and Noort, D. (2004) Standard operating procedure for immunoslotblot assay for analysis of DNA/sulfur mustard adducts in human blood and skin. *J. Anal. Toxicol.* **28**, 316-319.

Winter, G., Griffiths, A.D., Hawkins,R.E. and Hoogenboom, H.R. (1994) Making antibodies by phage display technology. *Annu. Rev. Immunol.* **12**, 433-455.

X BIBLIOGRAPHY OF PUBLICATIONS AND MEETING ABSTRACTS (from beginning of cooperative agreement)

Publications

NOORT, D., HULST, A.G., and JANSEN, R. Covalent binding of nitrogen mustards to the cysteine-34 residue in human serum albumin. Arch. Toxicol. **76**, 83-88, 2002.

NOORT, D., FIDDER, A., HULST, A.G., and LANGENBERG, J.P. (2002) Low level exposure to sulfur mustard: development of an SOP for analysis of albumin and/or hemoglobin adducts. Book of abstracts of the 2002 Medical Defense Bioscience Review, June 2002, Hunt Valley, MD, USA.

NOORT, D., BENSCHOP, H.P. and BLACK, R.M. (2002) Biomonitoring of exposure to chemical warfare agents. A review. Toxicol. Appl. Pharmacol. **184**, 116-126.

VAN DER SCHANS, G.P., NOORT, D., MARS-GROENENDIJK, R.H., FIDDER, A., CHAU, L.F., DE JONG, L.P.A. and BENSCHOP, H.P. (2002) Immunochemical detection of sulfur mustard adducts with keratins in the stratum corneum of human skin. Chem. Res. Toxicology **15**, 21-25.

VAN DER SCHANS, G.P., NOORT, D., MARS-GROENENDIJK, R.H., FIDDER, A. and LANGENBERG, J.P. (2002) Immunochemical detection of sulfur mustard adducts with keratins in the stratum corneum of human skin. Proceedings of the 2002 Medical Defense Bioscience Review, June 2002, Hunt Valley, MD, USA.

VAN DER SCHANS, G.P., MARS-GROENENDIJK, R.H., NOORT, D., and LANGENBERG, J.P. (2002) Development of immunochemical assays for detection of sulfur mustard-adducts with proteins. Proceedings of the meeting of NATO TG-004, November 2002, Oslo, Norway.

NOORT, D., FIDDER, A., BENSCHOP, H.P., DE JONG, L.P.A. and SMITH, J.R. Procedure for monitoring exposure to sulfur mustard based on modified Edman degradation of globin. J. Anal. Toxicol., **28**, 311-315, 2004.

VAN DER SCHANS, G.P., MARS-GROENENDIJK, R., DE JONG, L.P.A., BENSCHOP, H.P. and NOORT, D. Standard operating procedure for immunoslotblot assay for analysis of DNA-sulfur mustard adducts in human blood and skin. J. Anal. Toxicol., **28**, 316-319, 2004.

NOORT, D., FIDDER, A., HULST, A.G., WOOLFITT, A.R., ASH, D., and BARR, J.R. Retrospective detection of exposure to sulfur mustard: Improvements on an assay for liquid chromatography – tandem mass spectrometry analysis of albumin/sulfur mustard adducts. J. Anal. Toxicol., **28**, 333-338, 2004.

BLACK, R.M., and NOORT, D. (2005) Methods for retrospective detection of exposure to toxic scheduled chemicals. Part A: Analsyis of free metabolites. In "Chemical Weapons Convention Chemicals Analysis; sample collection, preparation and analytical methods" Ed. M. Mesilaakso; John Wiley & Sons, Chichester, UK.

NOORT, D., and BLACK, R.M. (2005) Methods for retrospective detection of exposure to toxic scheduled chemicals. Part B: Mass spectrometric and immunochemical analysis of covalent adducts to proteins and DNA. In "Chemical Weapons Convention Chemicals

Analysis; sample collection, preparation and analytical methods" Ed. M. Mesilaakso; John Wiley & Sons, Chichester, UK.

NOORT, D., FIDDER, A., DE KANT, S., and HULST, A.G. Low Level Exposure to Sulfur Mustard: Development of an SOP for Analysis of Albumin Adducts. Proceedings of the Bioscience Review 2004, in press.

VAN DER SCHANS, G.P., MARS-GROENENDIJK, R.H, BIKKER, F.J. and NOORT, D. (2005) Immunochemical Procedures for Simple Analysis of Exposure to Sulfur Mustard. Contribution to book entitled: "Medical Aspects of Chemical and Biological Terrorism"; "Chemical terrorism and traumatism", edited by Christophor Dishovsky and Professor Alexander Monov, Publ. house of the Union of Scientists in Bulgaria. (in press).

Meeting Abstracts

NOORT, D., FIDDER, A., HULST, A.G., and LANGENBERG, J.P. (2002) Low level exposure to sulfur mustard: development of an SOP for analysis of albumin and/or hemoglobin adducts. Book of abstracts of the 2002 Medical Defense Bioscience Review, June 2002, Hunt Valley, MD, USA.

VAN DER SCHANS, G.P., NOORT, D., MARS-GROENENDIJK, R.H., FIDDER, A. and LANGENBERG, J.P. (2002) Immunochemical detection of sulfur mustard adducts with keratins in the stratum corneum of human skin. Proceedings of the 2002 Medical Defense Bioscience Review, June 2002, Hunt Valley, MD, USA.

VAN DER SCHANS, G.P., MARS-GROENENDIJK, R.H., NOORT, D., and LANGENBERG, J.P. (2002) Development of immunochemical assays for detection of sulfur mustard-adducts with proteins. Proceedings of the meeting of NATO TG-004, November 2002, Oslo, Norway.

NOORT, D. Use of LC tandem MS techniques in identification and sensitive detection of covalent adducts of xenobiotics with proteins, Drug Metabolism Reviews, 35 (S 1), 3 (2003).

NOORT, D., FIDDER, A., DE KANT, S., and HULST, A.G. Low Level Exposure to Sulfur Mustard: Development of an SOP for Analysis of Albumin Adducts. Abstract Book Bioscience Review, Hunt Valley MD, May 16-21, 2004.

NOORT, D., VAN DER SCHANS, M.J., and BENSCHOP, H.P. (2004) Biomonitoring of exposure to chemical warfare agents. Abstract book 2004 Joint SOFT/TIAFT Meeting, Washington DC, August 30 – September 3, 2004, p. 241.

XI LIST OF PERSONNEL RECEIVING PAY UNDER THIS COOPERATIVE AGREEMENT

Dr. D. Noort
Dr. G.P. van der Schans
Dr. B.L.M. van Baar
Dr. F.J. Bikker
Mr. A. Fidder
Mrs. R.H. Mars-Groenendijk
Mr. A.G. Hulst
Mrs. S. de Kant
Mrs. C.E.A.M. Degenhardt-Langelaan
Mr. E. Wils
Mr. S.H. van Krimpen