AD_____

Award Number: DAMD17-03-1-0613

TITLE: Inhalation and Percutaneous Toxicokinetics of Sulfur Mustard and Its Adducts in Hairless Guinea Pigs and Marmosets. Efficacy of Nasal Scavengers.

PRINCIPAL INVESTIGATOR: Jan P. Langenberg, Ph.D., Pharm.D.

CONTRACTING ORGANIZATION: TNO Prins Maurits Laboratory
2288 GJ Rijswijk, The Netherlands

REPORT DATE: August 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 (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2004	Annual (1 Aug 2003 - 31 Jul 2004)

4. TITLE AND SUBTITLE
Inhalation and Percutaneous Toxicokinetics of Sulfur Mustard and Its Adducts in Hairless Guinea Pigs and Marmosets. Efficacy of Nasal Scavengers

5. FUNDING NUMBERS
DAMD17-03-1-0613

6. AUTHOR(S)
Jan P. Langenberg, Ph.D., Pharm.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
TNO Prins Maurits Laboratory
2288 GJ Rijswijk, The Netherlands

E-Mail: langenberg@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)

As a follow-up to DAMD17-94-V-4009, the inhalation toxicokinetics of sulfur mustard are studied in more detail in the hairless guinea pig as well as in a species more relevant for man, i.e., the marmoset. Furthermore, its percutaneous toxicokinetics are studied in the hairless guinea pig at a lower exposure level than previously. The aspect of toxicologically relevant time period of *in vivo* persistence of sulfur mustard is integrated in these investigations.
Hairless guinea pigs were nose-only exposed to a dose of sulfur mustard vapor in air corresponding with 0.3 LCt50. At various time points (up to 24 h) after ending the exposure the animals were sacrificed and DNA-adducts were measured in various regions of the respiratory tract. Within this time period the number of DNA-adduct increased and subsequently decreased with time. The highest numbers were measured in the upper airways. No adducts were found in the lungs, which is in agreement with previous findings. Animals were also exposed to 1 LCt50 sulfur mustard vapor in air. The results of these experiments are not available yet. A 'chair' was developed for fixation of marmoset monkeys in order to expose this species nose-only to toxicant vapor in an anatomically natural position.

14. SUBJECT TERMS
Sulfur mustard, inhalation, percutaneous, toxicokinetics, DNA-adducts, hairless guinea pig, marmoset

15. NUMBER OF PAGES
36

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 authors and are not necessarily endorsed by the US 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 and official Department of Army endorsement or approval of the products or services of these organizations.

_____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).

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

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

_____In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH guidelines for Research Involving Recombinant DNA Molecules.

_____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

02-02-2005
Date

ACKNOWLEDGEMENTS

The authors wish to thank Mr. Willem C. Kuijpers and Mrs Wendy Kaman for performing the animal experiments for this project, and Dr. Govert P. van der Schans, his successor Dr. Floris Bikker and Mrs. Roos Mars-Groenendijk for performing the DNA-analyses. Mr. Martin C. Neeleman and Mrs. Bianca P.C. Beckers are thanked for taking good care of the hairless guinea pigs and marmosets.

SUMMARY

The management of casualties of sulfur mustard has not evolved much since its first use in 1917, most likely due to the lack of understanding of its mechanism of action and its fate in the body. The toxicokinetic studies we performed in hairless guinea pigs within the context of Cooperative Agreement DAMD17-94-V-4009 have provided insight into the behavior of sulfur mustard after intravenous administration and respiratory and percutaneous exposures, especially with regard to the relative importance of local and systemic effects for such exposures.

In the current study we extend the toxicokinetic studies of sulfur mustard by studying the respiratory toxicokinetics in more detail in the hairless guinea pig as well as in a species which is more relevant for man, i.e., the marmoset. Furthermore, we propose to study the percutaneous toxicokinetics of sulfur mustard in the hairless guinea pig at a lower exposure level than previously. The aspect of toxicologically relevant time period of *in vivo* persistence of sulfur mustard will be integrated in these investigations.

Hairless guinea pigs were nose-only exposed to a dose of sulfur mustard vapor in air corresponding with 0.3 LCt50 (240 $mg.min.m^{-3}$). At various time points (up to 24 h) after ending the exposure the animals were sacrificed and samples were taken from the respiratory tract for measurement of the number of DNA-adducts. Within this time period the number of DNA-adducts increased and subsequently decreased with time. The highest numbers were measured in the upper airways. No adducts were found in the lungs, which is in agreement with previous findings. Animals were also exposed to sulfur mustard in air at a dose corresponding with 1 LCt50 (800 $mg.min.m^{-3}$). The results of these experiments are not available yet.

A 'chair' was developed for fixation of marmoset monkeys in order to expose this species nose-only to toxicant vapor in an anatomically natural position. Experiments to determine the toxicokinetics of sulfur mustard during and after a 5-min nose-only exposure to 160 $mg.m^{-3}$ of sulfur mustard vapor in air, corresponding with 1 LCt50 (96-h) in the hairless guinea pig, are about to start.

Intentionally left blank.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

I. INTRODUCTION

In spite of intensive research there is still no specific and causal therapy available for the local and systemic effects of intoxication with sulfur mustard, although promising results have been obtained very recently (*vide infra*). In general, treatment of casualties will have to be restricted to sustaining the vital functions of the patient, enhancing the healing process of the lesions and preventing secondary infections. In order to provide a quantitative basis for pretreatment and therapy of intoxications with sulfur mustard the toxicokinetics of this agent as well as its major DNA-adduct (N7-HETE-gua) were studied for the intravenous, respiratory and percutaneous routes, within the framework of Cooperative Agreement DAMD17-94-V-4009 (Langenberg *et al.* 1998). The experiments were performed in the hairless guinea pig, which is considered to be a suitable animal model in studies of sulfur mustard since the thickness of the epidermis and the effect of sulfur mustard on the skin is reasonably comparable to that in man (Mershon *et al.* 1990).

For the analysis of intact sulfur mustard in blood and tissues we developed and validated a method using gas chromatography with mass-spectrometric detection, with a detection limit of 5 pg sulfur mustard/ml blood. The DNA-adducts of sulfur mustard in various relevant tissues were analyzed immunochemically, with a detection limit of 1 adduct per $4*10^8$ nucleotides. The combination of these analyses provides a unique insight into the relationship between the blood and tissue levels of intact sulfur mustard and the resulting DNA-damage.

The intravenous toxicokinetics of a dose of sulfur mustard corresponding with 1 LD50 (96-h, 8.2 mg/kg) and 0.3 LD50 in the hairless guinea pig were characterized by a very rapid distribution phase and a very slow elimination phase. The concentration of sulfur mustard in tissues (lung, spleen, liver, and bone marrow) exceeded that in blood shortly after i.v. administration of the agent, indicating extensive partitioning of sulfur mustard from blood into the tissues. A rapid DNA-adduct formation occurred in blood and lung, and subsequently in other tissues. Repair of adducts was considerable within 6 h. However, at 2 days after administration of sulfur mustard, adducts were still detectable in most of the tissues studied. Nonlinearity of the toxicokinetics with the dose was observed.

Next, the respiratory toxicokinetics were studied. Rather surprisingly, the intact agent could not be detected in blood during and after 5-min exposure of animals to 1 LCt50 sulfur mustard (96-h, 800 $mg.min.m^{-3}$). Low concentrations of N7-HETE-gua were detectable in blood and lung tissue. However, sulfur mustard was measurable in the blood upon an 8-min nose-only exposure of hairless guinea pigs to 3 LCt50 of the toxicant at concentrations up to 1.8 ng/ml. Toxicokinetic evaluation of the concentration-time profile was only possible by assuming a very rapid absorption process in combination with a slow absorption process. In this case, measurable concentrations of N7-HETE-gua in DNA were found in blood, lung tissue, and spleen.

The respiratory tract was isolated from animals that were nose-only exposed to 1 LCt50 sulfur mustard in 5 min, at 4 h after ending the exposure. The tract was divided into 6 regions. Most adduct formation had occurred in the larynx and trachea, whereas hardly any N7-HETE-gua was found in the lung. Thus, the observation that sulfur mustard was not detected in the blood of animals exposed to 1 LCt50 sulfur mustard may be explained from the almost exclusive distribution of the agent within the respiratory tract. Evidently, respiratory exposure to sulfur mustard leads predominantly to local damage in the respiratory tract, at least in (hairless) guinea pigs.

The percutaneous toxicokinetics of sulfur mustard in the hairless guinea pig were also studied. During and after a *ca.* 45-min percutaneous exposure of the animals to a Ct of 10,000 $mg.min.m^{-3}$, concentrations of sulfur mustard in blood up to *ca.* 12 ng/ml could be detected. As in the case of nose-only exposure to a Ct of 2,400 $mg.min.m^{-3}$, the existence of a rapid and a simultaneous slow absorption process was suspected. Concentrations of intact sulfur mustard exceeding those in blood were measured in the tissues shortly after ending the exposure. Rather low concentrations of N7-HETE-gua were measured in DNA of most tissues. We concluded tentatively that systemic intoxication from sulfur mustard is more likely to occur from a percutaneous exposure than from a respiratory exposure.

Based on the amount of DNA-adducts in the skin, Topical Skin Protectants 1511 and 2701 (obtained from the U.S. Army Medical Research Institute of Chemical Defense) protected hairless guinea pigs almost completely against skin damage caused by sulfur mustard vapor. TSP 2701 performed somewhat better against liquid sulfur mustard than TSP 1511.

Results of our investigations on diagnosis/retrospective detection of exposure to sulfur mustard, within the context of Cooperative Agreement DAMD17-97-2-7002 (Benschop *et al.* 2000) showed, surprisingly, that adduct levels of sulfur mustard to hemoglobin in guinea pigs and marmosets increase for several days after intravenous bolus administration of the toxicant. Subsequently, the level of hemoglobin adducts decreases, still being detectable at 56 and 92 days after sulfur mustard administration in guinea pigs and marmosets, respectively. Therefore, protein-adduct formation can be regarded as cumulative on a relevant time scale. This result stands in sharp contrast with the concentration-time profile of DNA-adducts, due to repair and/or removal of such adducts.

At least two aspects of this result should be taken into consideration. Firstly, it can have consequences for treatment of systemic effects of intoxication with sulfur mustard, since hemoglobin-adduct formation will probably be parallel with adduct formation to other proteins which play a role in the etiology of damage due to exposure to sulfur mustard (Mol, 1999). For example, it may become relevant to attempt removal of sulfur mustard from circulation. Secondly, the late formation of hemoglobin-adducts can be used as a measure for the time period in which toxicologically relevant levels of sulfur mustard are present in the body. The definition of this time period has been elusive sofar and should now be investigated on the basis of 'post-exposure hemoglobin-adduct formation'.

In summary, our toxicokinetic studies of sulfur mustard as performed within the context of the abovementioned Cooperative Agreements have provided elucidating data on the fate of this agent in hairless guinea pigs, especially with regard to the relative importance of local and systemic effects upon respiratory and percutaneous exposure.

It seems difficult to reconcile our conclusion that respiratory exposure leads predominantly to local damage with the results of our measurements of N7-HETE-gua in DNA from blood of Iranian casualties of sulfur mustard exposure (Benschop *et al.* 1997). As judged from their overall condition, these patients were exposed to rather small amounts of sulfur mustard vapor via the respiratory route, since skin damage was hardly noticable. Nevertheless, in blood samples taken 3 to 4 weeks after exposure we found blood levels of N7-HETE-gua in DNA of these patients corresponding with those in hairless guinea pigs immediately after nose-only exposure to up to 3 LCt50 of sulfur mustard vapor. Furthermore, we measured hemoglobin-adduct levels corresponding with those found in human blood after *in vitro* treatment with 0.9 μM sulfur mustard. These contrasting results suggest that species differences may play a major role in determining the relative importance of local and systemic damage upon respiratory exposure.

Although our previous experiments indicate, rather surprisingly, that skin exposure may lead to more systemic intoxication than respiratory exposure, such a conclusion needs further confirmation by skin exposure to less extreme Ct-values than 1 percutaneous LCt50, i.e., preferably to a Ct-value used for the respiratory exposure. Such experiments will also provide information on the (non)linearity with dose in the case of percutaneous exposure.
Anyhow, our results stress the overriding importance of local damage both from respiratory and from percutaneous exposure. Since our methodology of quantitation of epithelial tissue damage by means of DNA-adduct formation is uniquely applicable to test the efficacy of protective measures, we proposed to study the therapeutic efficacy of scavengers that can be applied to the respiratory tract upon exposure to sulfur mustard. To the best of our knowledge, this approach has not been investigated previously.

Shortly, we proposed to address items as indicated above:

(i) Comparative inhalation toxicokinetics of sulfur mustard and N7-HETE-gua in the hairless guinea pig and in a species more relevant for man, i.e., the marmoset;

(ii) The percutaneous toxicokinetics of sulfur mustard and N7-HETE-gua in the hairless guinea pig at a dose comparable with that of the respiratory exposure as investigated in the previous Cooperative Agreement;

(iii) The time period during which toxicologically relevant levels of sulfur mustard are present in marmosets, as based on late formation of stable hemoglobin-adducts;

In the following the various aspects of the proposed research are further elucidated.

(i) Inhalation toxicokinetics of sulfur mustard and of N7-HETE-gua in DNA

Initially, we were rather surprised that the concentrations of sulfur mustard in blood were below the detection limit during and after nose-only exposure to 1 LCt50. However, the observation that N7-HETE-gua could not be detected in spleen, bone marrow and small intestine, while rather low concentrations of N7-HETE-gua were measured in the lung at 10 min and 48 h after ending the exposure is in agreement with this outcome. Most clarifying was the distribution of N7-HETE-gua within the respiratory tract at 4 h after ending the 5-min nose-only exposure. Adduct formation occurred mainly in the upper airways, whereas almost no sulfur mustard appeared to have reached the lung.

From these findings it was concluded that sulfur mustard apparently causes lethal damage in the respiratory tract without a systemic uptake to such an extent that systemically lethal concentrations can build up. This mechanism stands in contrast with that for nerve agents, which are absorbed in the (upper) respiratory tract to cause mainly systemic toxicity.

Cameron *et al.* (1946) have also observed that hardly any sulfur mustard reaches the lung in rabbits, based on measurements of the concentration of sulfur mustard in air sampled from the trachea via a cannula. They hypothesized that absorption of lethal doses of sulfur mustard occurs via the nasal mucosa. However, since we could not detect sulfur mustard in blood the present results seem to suggest that the respiratory toxicity of sulfur mustard is overwhelmingly of a local rather than of a systemic nature, at least in the (hairless) guinea pig.

Within the context of Cooperative Agreement DAMD17-92-V-2005, Benschop and Van der Schans (1995) established exposure to sulfur mustard of two Iranian victims of the Iran-Iraq conflict in 1988 by measurement of DNA-adducts in lymphocytes and granulocytes as well as adducts to hemoglobin (Benschop *et al.*, 1997). The blood samples in which the exposure was verified were taken at 22 and 26 days after the alleged exposure. One victim suffered from injuries compatible with sulfur mustard intoxication but did not have lung injuries; the symptoms of the other victim were only vaguely compatible with sulfur mustard intoxication. Interestingly, the N7-HETE-gua levels and adduct levels to hemoglobin measured in these Iranian victims are comparable with those measured in hairless guinea pigs immediately after nose-only exposure to 0.3-3 LCt50. As deduced from the clinical signs, the Iranian victims appeared not to be severely intoxicated, whereas the hairless guinea pigs were. Furthermore, one has to take into account that a long period of time had passed between exposure of the Iranian victims and collection of the blood samples. Since DNA-adducts will disappear with time due to repair mechanisms and/or removal of the damaged white blood cells, there seems to be a large discrepancy between adduct formation resulting from respiratory exposure between humans and guinea pigs.

A tentative explanation for this discrepancy is the high complexity of the nasal region of the guinea pig (Schreiber and Raabe 1981). Obviously, in species with a less complex nasal system such as man, a larger fraction of the inhaled sulfur mustard may reach the lung. This may lead to lung damage by direct reaction of sulfur mustard with the lung tissues and/or indirectly via systemic uptake. We propose to study the inhalation toxicokinetics of sulfur mustard in the marmoset monkey, of which the anatomy of the naso-pharyngeal airway is much more similar to that in man, in order to test this hypothesis. The marmosets will be nose-only exposed to a Ct of 800 $mg.min.m^{-3}$ in 5 min, which corresponds with 1 LCt50 (96-h) in the hairless guinea pig. The concentration-time course of sulfur mustard in blood will be determined, and the concentrations of intact sulfur mustard and N7-HETE-gua in blood and tissues will be measured at various time points after ending the nose-only exposure.

Furthermore, the distribution of N7-HETE-gua within the respiratory tract of the marmoset will be studied, and the tract will be examined histopathologically, as was previously performed for hairless guinea pigs (Langenberg *et al.* 1998).

In addition, the time course of the distribution of N7-HETE-gua within the respiratory tract of the hairless guinea pig will be studied, by measurement of the adduct at various time points after ending the nose-only exposure to 1 and 0.3 LCt50.

(ii) Percutaneous toxicokinetics of sulfur mustard and N7-HETE-gua in DNA

As already stated above, we observed a strikingly similar phenomenon in the concentration-time profiles of sulfur mustard in blood for percutaneous and respiratory exposure (Langenberg *et al.* 1998). As in the case of inhalation toxicokinetics, the percutaneous toxicokinetics of sulfur mustard in the hairless guinea pig appeared to be characterized by the existence of two absorption phases, one relatively rapid and one relatively slow. The slow absorption phase may be due to absorption of sulfur mustard into the blood from skin depots which were 'loaded' with sulfur mustard during the exposure. Furthermore we concluded that, since the concentration build-up of sulfur mustard in blood proceeded more rapidly for the nose-only exposure, that absorption via the respiratory route is more rapid than via the percutaneous route. Nevertheless, we concluded that at an equitoxic level, systemic intoxication from sulfur mustard is more likely to occur in the hairless guinea pig from a percutaneous exposure than from a respiratory exposure. However, it needs to be stressed that 1 percutaneous LCt50 requires a more than tenfold higher Ct of exposure than 1 nose-only LCt50, i.e., 10,000 mg.min.m^{-3} versus 800 mg.min.m^{-3}. One may doubt whether an exposure to 10,000 mg.min.m^{-3} can be considered to be realistic under battlefield conditions. Therefore, we now proposed to study the percutaneous toxicokinetics of sulfur mustard and its major DNA-adduct for an exposure which is militarily relevant and will lead to measurable sulfur mustard concentrations in blood. We have chosen for an exposure to a Ct of 2,400 mg.min.m^{-3} in 45 min. This will allow a comparison with the results obtained for 45-min percutaneous exposure to a Ct of 10,000 mg.min.m^{-3} and for 8-min nose-only exposure to a Ct of 2,400 mg.min.m^{-3}. Moreover, this approach will allow evaluation of the degree of (non)linearity with dose for a percutaneous exposure to sulfur mustard vapor.

(iii) Post-exposure adduct formation to hemoglobin as a means to determine the time period during which toxicologically relevant levels are present

In the Final Report of Cooperative Agreement DAMD17-94-V-4009 we have made some speculations with respect to the toxicologically relevant level of sulfur mustard in blood and/or tissues and the time course thereof (Langenberg *et al.* 1998). Obviously, the strategy of (pre)treatment of intoxication with sulfur mustard would benefit from definition of such a time period. For the nerve agent soman the toxicologically relevant level was defined as the AUC which would be able to (re)inhibit the minimum essential concentration of acetylcholinesterase with a half-life of 1 h (Benschop and De Jong, 1990). The blood concentration of C(±)P(-)-soman which corresponded with this threshold value was calculated to be 150 pM (*ca.* 30 pg/ml).

For soman the calculation of lowest concentration of toxicological relevance is relatively simple, since the toxic effect is well defined, which is not the case for sulfur mustard. Reduction of the viability of cells within 24 h of exposure can be used as a criterion for defining the lowest concentration of toxicological relevance. Exposure of cells to 75 µM (*ca.* 12 µg/ml) of sulfur mustard reduces cell viability within 24 h, whereas 50 µM has no apparent effect (Mol, 1996). This concentration is more than 2-fold higher than the retrapolated blood concentration of sulfur mustard at time 0 after i.v. administration of 8.2 mg/kg sulfur mustard. Since this dose corresponds with 1 LD50, it seems unlikely that 75 µM is a realistic value for the lowest level with toxicological relevance. It is also known that DNA adducts can be detected after exposure of white blood cells to 10 nM (*ca.* 1.6 ng/ml) sulfur mustard for 1 h. This adduct formation can be considered to be an adverse effect, although the correlation between adduct formation and cytotoxicity is unclear, and some repair of the DNA damage may occur. A concentration of 1.6 ng of sulfur mustard per ml of blood will already be reached at *ca.* 18 min after i.v. administration of 1 LD50 sulfur mustard. However, our measurements show that the concentrations of sulfur mustard in tissues exceed the concentration in blood for a considerable period of time after administration. This underscores the need to follow the toxicokinetics of sulfur mustard in blood and tissues for several hours instead of 18 min, until the concentrations of intact sulfur mustard in relevant compartments can no longer induce toxic effects. Since sulfur mustard is designated as a primary carcinogenic compound, one could also advocate defining the toxicologically relevant concentration as the lowest concentration which is carcinogenic. However,

such a concentration is unknown for sulfur mustard. The most conservative approaches to defining carcinogenic concentrations are based on the one-hit model, assuming that even 1 molecule of a carcinogenic compound can cause cancer (Cornfield, 1977). Obviously, our method is not suitable for detection of 1 molecule sulfur mustard in the body of the hairless guinea pig.
Measuring the time course of adduct formation to hemoglobin may help in defining a toxicologically relevant level for sulfur mustard. Although such an adduct formation has no direct relationship with the toxic effects of sulfur mustard, it can be argued that hemoglobin-adduct formation can serve as a relevant marker for adduct formation with proteins that actually play an important role in the etiology of sulfur mustard-induced tissue damage. Following this reasoning, the time period in which toxicologically relevant concentrations of sulfur mustard are present in the body can be defined as the time period during which adducts to hemoglobin can still be formed. Since - due to the absence of repair mechanisms - adducts of sulfur mustard to hemoglobin are highly stable, these adducts are most suitable for purposes of establishing the time period during which toxicologically significant levels of sulfur mustard are present. By following the build-up of these adducts with time after exposure to sulfur mustard, and correlating these data with concentrations of intact sulfur mustard in blood, it should be possible to define such a time period.
We proposed to follow the concentration-time course of the sulfur mustard adduct to hemoglobin in the experiments to determine the toxicokinetics of sulfur mustard in marmosets nose-only exposed to sulfur mustard vapor in air (Ct 800 $mg.min.m^{-3}$) and in hairless guinea pigs percutaneously exposed to sulfur mustard vapor in air (Ct 2,400 $mg.min.m^{-3}$), in order to obtain some insight into post-exposure hemoglobin-adduct formation for these relevant exposure routes, and hopefully into the time period during which toxicological concentrations of sulfur mustard are circulating. In addition, a more controlled experiment will be performed, in which sulfur mustard will be administered intravenously to three marmosets at a dose corresponding with 0.3 LD50 in the hairless guinea pig (2.46 mg/kg) after which the concentration-time course of the hemoglobin-adduct will be followed for as long as necessary.

As stated above, very recently promising results have been obtained in the search for a causal antidote for sulfur mustard. Within the context of Cooperative Agreement DAMD17-02-1-0206 Dr. Marijke Mol from our laboratory is studying the mechanism of action of sulfur mustard using proteomics, which has led to new hypotheses concerning how the toxic effects of sulfur mustard come to be. Based on her hypothesis that matrix metalloproteases are involved in the blister formation process, she has tested various types of inhibitors of these proteases. Of these the compounds Ilomastat (QuickMed Technologies, USA) and BB94 (British Biotechnologies, UK) appeared to be highly effective in preventing blister formation on human skin exposed to saturated sulfur mustard vapor, also when applied several hours after exposure to the vesicant, at least *in vitro* (Mol 2004). So there is justified hope that a causal antidote for sulfur mustard will become available in the near future.

II. EXPERIMENTAL PROCEDURES

II.1. Materials

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

Technical grade sulfur mustard (SM) was purified by fractional distillation in a cracking tube column (Fischer, Meckenheim, Germany) to a gas chromatographic purity exceeding 99.5 %. The internal standard, D8-sulfur mustard (D8-SM) was obtained as described elsewhere (Benschop and Van der Schans, 1995). Ethyl acetate ('zur Rückstandsanalyse') was procured from Merck (Darmstadt, Germany) and was distilled over a column packed with Dixon rings (plate number 80; NGW, Wertheim, Germany) before use. Isopropanol (purity > 99.5 %) was purchased from Fluka (Buchs, Switzerland).
The following products were obtained commercially and were used without further purification: heparin (Vitrum, Stockholm, 5000 IU/ml), ketamine hydrochloride (Vetalar®, Parke-Davis, Morris Plains, NJ, USA), buprenorfine hydrochloride (Temgesic®, Schering-Plough, Amstelveen, The Netherlands), HPLC-grade water (Fisons, Loughborough, UK), Tenax TA, 60-80 mesh (Chrompack, Middelburg, The Netherlands). Disodium edetate (EDTA), Triton® X-100, sodium chloride, potassium chloride, disodium hydrogen phosphate dihydrate, potassium dihydrogen phosphate, phenol, phosphoric acid (85 %), chloroform and ethanol were obtained from Merck (Darmstadt, Germany); sodium dodecyl sulfate (SDS), Tris.HCl from J.T. Baker (Phillipsburg, NJ, USA), and isoamyl alcohol from UCB (Brussels, Belgium); calf thymus DNA, proteinase K (EC 3.4.21.14, activity ca. 20 units/mg protein) and RNase T1 (EC3.1.27.5, activity ca. 40 units/mg protein) from Boehringer (Mannheim, Germany); RNase A, and Tween 20 from Sigma Chemical Co. (St. Louis, MO, USA); and skimmed milk powder, less than 1 % fat, from Campina (Eindhoven, The Netherlands).

Immunoslotblot assays were carried out with Schleicher & Schuell minifold S (6 mm^2 slots; Schleicher & Schuell, Dassel, Germany) and nitrocellulose filters (pore size 0.1 µm; Schleicher and Schuell). DNA was immobilized by UV-crosslinking with a GS Gene Linker UV chamber (Bio-Rad Laboratories, Hercules, CA, USA). The enhanced chemiluminescence blotting detection system (Boehringer) was used for the detection of peroxidase activity. The developed film was scanned with a densitometer (Ultroscan XL, Pharmacia). In later experiments the chemiluminescence was recorded with a 1450 MicroBeta Trilux Luminescence Counter (EG & G Wallac, Breda, The Netherlands).

II.2 Determination of N7-HETE-gua in DNA

DNA isolation
Mucosal epithelia from different locations in the respiratory tract of the hairless guinea pig; nasal cavity, nasopharynx, larynx, trachea and carina, were collected by scraping off the mucosal surfaces. Lung tissue was homogenized prior to DNA isolation.
For DNA isolation all samples were incubated overnight in 300 µl cell lysis solution supplemented with Proteinase K (100µg/ml), under continuous slow shaking at room temperature. Then, RNAse A treatment (50 µg/ml) was carried out for 15 min at 37 °C, followed by cooling on ice for 5 min. To precipitate cellular debris 100 µl Protein Precipitation Solution was added and incubated on ice for 5 min. After centrifugation at 5,200g for 10 min the supernatant was transferred to a new vial containing 300 µl isopropanol to precipitate the DNA. After centrifugation at 5,200g for 5 min the pellet was washed with 70% ethanol, centrifuged at 5,200g for 5 min and dried on air. The DNA was dissolved in 50 µl 0.1TE buffer under continuous shaking at room temperature.

DNA denaturation
Double-stranded calf thymus DNA and DNA samples isolated from respiratory organs from the hairless guinea pig after sulfur mustard vapor exposure were denatured to single-stranded DNA in 0.1 TE buffer containing 4.1% formamide and 0.1% formaldehyde (50 µg DNA/ml) at 52 °C for 15 min, followed by

rapid cooling on ice and storage at -20 °C. It is essential that, after denaturation, the DNA samples were frozen at least one time before application in the immunoslotblot assay.

Immunoslotblot procedure for N7-HETE-Gua
Two-hundred µl single-stranded DNA (5 µg/ml) containing N7-HETE-Gua was slot blotted onto a nitrocellulose filter. As a reference, calibration samples of calf thymus DNA with adduct levels in the range of 0-10 N7-HETE-Gua/10^7 nucleotides were included. All samples were blotted in duplicate on the same filter. After rinsing the slots with PBS the filters were air dried. Subsequently, DNA was immobilized by UV crosslinking (50 mJ.cm^{-2}).
The next steps in the procedure, i.e., treatment with blocking solution, 1st antibody (2F8, directed against N7-HETE-Gua in DNA) and 2nd antibody (rabbit-anti-mouse-Ig-horse radish peroxidase), were performed as previously described (Benschop and Van der Schans, 1995). Solutions A and B of the chemiluminescence blotting detection system were mixed (100:1) and equilibrated for 1 h at 25 °C. The filters were incubated for 1 min in substrate solution and placed in a plastic bag. Excessive substrate was pressed out. Next, the filters were placed in a luminometer and the chemiluminescence was measured. All experiments were performed at least in duplicate.

The Standard Operating Procedure for determination of sulfur mustard adducts to DNA in respiratory tract of the hairless guinea pig is described in Annex B.

II.3. Gas chromatography of sulfur mustard (TDSA-GC-MSD)

Sulfur mustard in biological samples is determined by gas chromatography with mass-selective detection and large volume injection.
The GC-MS configuration consisted of an Agilent 6890A GC, equipped with a Gerstel Thermo Desorption Autosampler device (TDSA) and an Agilent 5973 Mass Selective Detector (MSD) (Agilent Technol. Inc., Wilmington, DE, USA).
The GC was fitted with a 30 m x 0.25 mm I.D. VF-5MS fused silica column with a film thickness of 1.00 µm (Chrompack Varian), inserted directly into the mass spectrometer source.
The desorption tubes for the TDSA were filled with 300 mg Tenax TA (60-80 mesh) and a loosely packed plug of dimethyldichlorosilane-treated glass wool of about 3 to 4 cm to promote evaporation of the large volume of the solvent. Before use, the Tenax tubes were preconditioned by heating under a stream of helium or nitrogen at 220°C for 8 h.
Tenax tubes were loaded with 100 to 400 µL of the sample extracts in portions of 100 µL using a Hamilton 100 µL syringe with a loading interval time of 5 min. During this step, a gentle stream of nitrogen (250 mL/min) flowed through the tubes to evaporate the solvent. Fifteen min after the final loading step, the Tenax tube was installed into the TDSA. The TDSA oven temperature was started at 30 °C and then ramped at 60 °C/min to 220 °C where it was held for 5 min. During desorption, the helium flow rate through the tube was 30 mL/min. The TDSA-GC transfer line was set at 220°C.
The desorbed sample components were trapped in splitless mode by a CIS which was cooled to –50°C with liquid nitrogen. After desorption, the trapped components were transferred in splitless mode to the capillary column by rapid heating of the CIS at 12°C/s to 220°C where it was held for 5 min. Splitless time was 1 min and purge flow was 50 mL/min. The initial GC oven temperature was 65°C for 4 min, then ramped at 20°C/min to 260°C and held at this temperature for 11 min. The helium column flow during analysis was set at 1 mL/min in constant flow mode. The GC-MSD transfer line was heated at 220°C. The MSD was operated in the selected ion mode (SIM) using electron impact ionization (70 eV) and the ions monitored were m/z 109 for SM and 115 for D8-SM, with a SIM dwell time of 80 ms. Temperature of the quadrupole was maintained at 150°C, temperature of the ionization source was 230°C.

II.4 Vapor exposure of animals

Apparatus for generation of sulfur mustard vapor
The apparatus for controlled generation of SM vapor in air is described in detail elsewhere (Langenberg *et al.* 1998).

Apparatus for nose-only exposure of hairless guinea pigs to sulfur mustard vapor
The exposure apparatus was is also decribed in detail in Langenberg *et al.* (1998).

Apparatus for nose-only exposure of marmosets to sulfur mustard vapor
Development of such an apparatus is one of the Technical Objectives of this Cooperative Agreement, and is described in paragraph III.3.

II.5 Animal experiments

Animals
Male hairless guinea pigs [300-400 g; species identification Crl:IAF(HA)BR] were purchased from Charles River USA (Wilmington, MA, USA). The animals were allowed to eat and drink *ad libitum*. They were allowed to acclimatize to their new environment for at least 1 week before they were used in any experiment.
Two Standard Operating Procedures are applicable to the housing and care of the hairless guinea pigs, i.e., 'Ordering and Housing of Experimental Animals' (SOP Q213-W-039) and 'Cleaning and Maintenance of Animal Facilities' (SOP Q213-W-040).
Male and female marmoset monkeys [*Callithrix jacchus*, 150-250 g], are obtained from the Biomedical Primate Research Center (Rijswijk, The Netherlands) which is located next to TNO-PML. In accordance with SOP Q215-W-058 the animals are housed individually in stainless steel wire cages at a temperature (25 °C) and humidity (50 %) controlled room. They are allowed to drink *ad libitum*. Food supply is according to the aforementioned SOP.
The protocols for the animal experiments were approved by the TNO Committee on Animal Care and Use.

Nose-only exposure of hairless guinea pigs to sulfur mustard vapor
Hairless guinea pigs were anesthetized with a combination of racemic ketamine hydrochloride (Vetalar®, 50-100 mg/kg, i.m.) and acetylpromazine maleate (Vetranquil®, 0.05-0.1 mg/kg, i.m.). Next, they were immobilized in the modified Battelle-tube (see Langenberg *et al.* 1998) and nose-only exposed to sulfur mustard vapor in air for 5 min. At various time points after ending exposure (10 and 30 min, 1, 2, 4 and 24 h) groups of anesthetized animals (n=4 per time point) were euthanized by intraperitoneal administration of an overdose (0.4 ml) of sodium pentobarbital (Nembutal®),after which the respiratory tract was isolated for analysis.

III. RESULTS AND DISCUSSION

III.1 TIME COURSE OF N7-HETE-GUA IN THE RESPIRATORY TRACT OF HAIRLESS GUINEA PIGS AFTER 5-MIN NOSE-ONLY EXPOSURE TO 1 LCT50 SULFUR MUSTARD IN AIR (T.O. 1)

The progress of DAMD17-94-V-4009 was hampered considerably by problems concerning the availability of hairless guinea pigs. At Charles River in the USA, an eye infection ruined the whole colony, and in Europe the situation became critical when the company decided to move their breeding facility from Germany to Hungary. Finally, we were able to obtain sufficient numbers of hairless guinea pigs from BRL (Switzerland) via Harlan NL.
Unfortunately, BRL could not help us out this time, since a major customer of theirs in France is using up nearly all of these animals. Increasing the number of animals to be bred to meet our demands would take quite some time. Moreover, their prices for these animals have risen to an outrageous level.
However, meanwhile in the USA, Charles River has succeeded in rebuilding their colony, and is now able to deliver sufficient numbers of animals for our purposes. So far, the quality of the animals is excellent.

Animals were weighed, anesthetized and 5-min nose-only exposed to sulfur mustard vapor in air, at a concentration of 160 $mg.m^{-3}$. This results in an exposure dose of 800 $mg.min.m^{-3}$, which corresponds with 1 LCt50 (96-h), as determined within the context of the abovementioned Cooperative Agreement (Langenberg *et al.* 1998). Animals were euthanized at various time points, i.e., at 10 and 30 min, and at 1, 2 and 24 h after ending the 5-min nose-only exposure. The 4-h time point was obtained previously (Langenberg *et al.* 1998). For each time point n=4. Samples from the mucosa of various sites of the respiratory tract, i.e., from the nasal cavity, nasopharynx, larynx, trachea and carina, were collected by scraping off the mucosal surfaces. Furthermore, the lungs were sampled. In all of these samples the amount of adducts of sulfur mustard to guanine in the DNA was determined. In addition samples were taken for histopathological evaluation.

These animal experiments have recently been concluded very recently. Results are not available yet, but will be reported in due course.

III.2 TIME COURSE OF N7-HETE-GUA IN THE RESPIRATORY TRACT OF HAIRLESS GUINEA PIGS AFTER 5-MIN NOSE-ONLY EXPOSURE TO 0.3 LCT50 SULFUR MUSTARD IN AIR (T.O. 2)

Hairless guinea pigs were weighed, anesthetized and 5-min nose-only exposed to sulfur mustard vapor in air, at a concentration of 48 $mg.m^{-3}$. This results in an exposure dose of 240 $mg.min.m^{-3}$, which corresponds with 0.3 LCt50 (96-h), as determined within the context of the abovementioned Cooperative Agreement (Langenberg *et al.* 1998). Animals were euthanized at various time points, i.e., at 10 and 30 min, and at 1, 2, 4 and 24 h after ending the 5-min nose-only exposure. For each time point n=4. Samples from the mucosa of various sites of the respiratory tract, i.e., from the nasal cavity, nasopharynx, larynx, trachea and carina, were collected by scraping off the mucosal surfaces. Furthermore, the lungs were sampled. In all of these samples the amount of adducts of sulfur mustard to guanine in the DNA was determined. In addition samples were taken for histopathological evaluation.

The vapor of sulfur mustard in air was generated, monitored semi-continuously on-line and fed into the exposure apparatus using the equipment developed within the context of Cooperative Agreement DAMD 17-94-V-4009 (Langenberg *et al.* 1998). In short, a number of mass-flow controllers in combination with specially-designed glass tubings and a storage vessel for the neat mustard were used to control and influence the vapor concentration. Several valves and pressure gauges were installed for safety reasons. Where possible, the equipment was thermostatted. The exposure module was equipped with a three-way valve in combination with pressure monitoring, activated carbon canisters and needle valves to allow the operator to handle the equipment in a safe way.
After surgery, needed to insert the carotid cannula, the animal was positioned in the modified Battelle-tube and connected to the exposure module.

In order to perform the animal experiments in a time-efficient way, a schedule was made. An example of such a schedule is shown in Figure 1. This approach appeared to work very well.

The concentration of the sulfur mustard vapor in air was monitored semi-continuously by means of a gas chromatograph equipped with a fixed volume gas sampling valve, a capillary column (BP-1, length *ca.* 8 m and an internal diameter of 0.32 mm with a film thickness of 1 µm) and an FID. For calibration purposes this column is also connected to an on-column injector by means of a glass press-fit connector and an uncoated fused silica column (*ca.* 1 m length and 0.32 mm i.d.). This allows the operator to calibrate the complete system without disconnecting the column from the gas sampling valve.
Standard solutions of sulfur mustard in ethyl acetate were prepared for the calibration. Data-acquisition was performed with a combination of an amplifier (constructed in-house at TNO-PML), a National Instruments Daq-board installed in a Windows™ PC and a 'home-made' written software program for analyzing the data.

Due to the limitations (in terms of resolution) of this 12 bits Daq-board, the reported vapor concentration seems to fluctuate quite a bit, in reality the concentration cannot vary that much, as verified by manual reintegration of the chromatographic peaks. The relatively small standard deviation of these calculations of the vapor concentration emphasizes the overall quality of the analytical configuration. An example of the time course of the sulfur mustard vapor concentration can be seen in Figure 2. In Table 1 the individual averaged vapor concentrations as well as the deviation of these concentrations are presented.

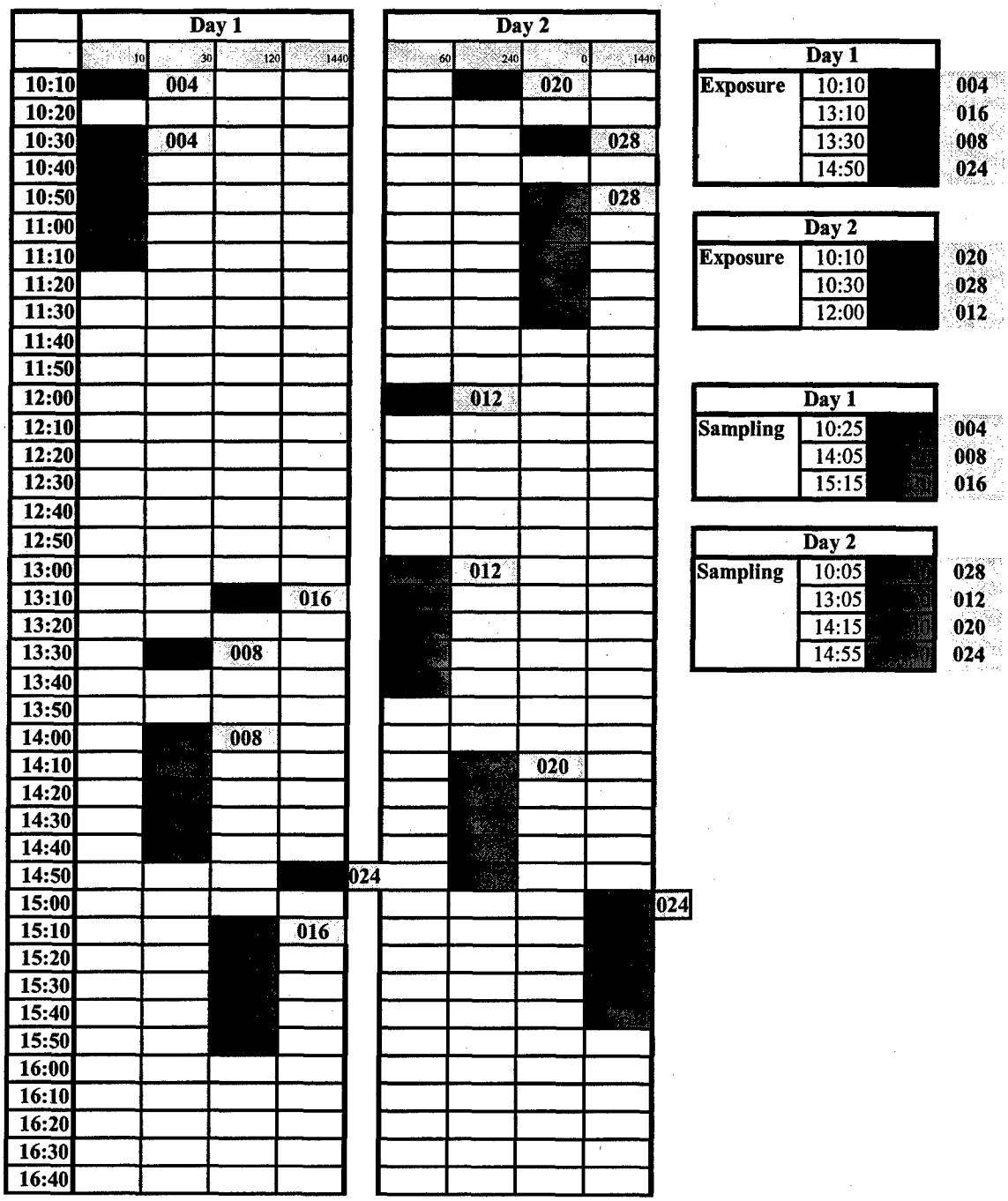

Figure 1. Example of the schedule for the nose-only exposures.

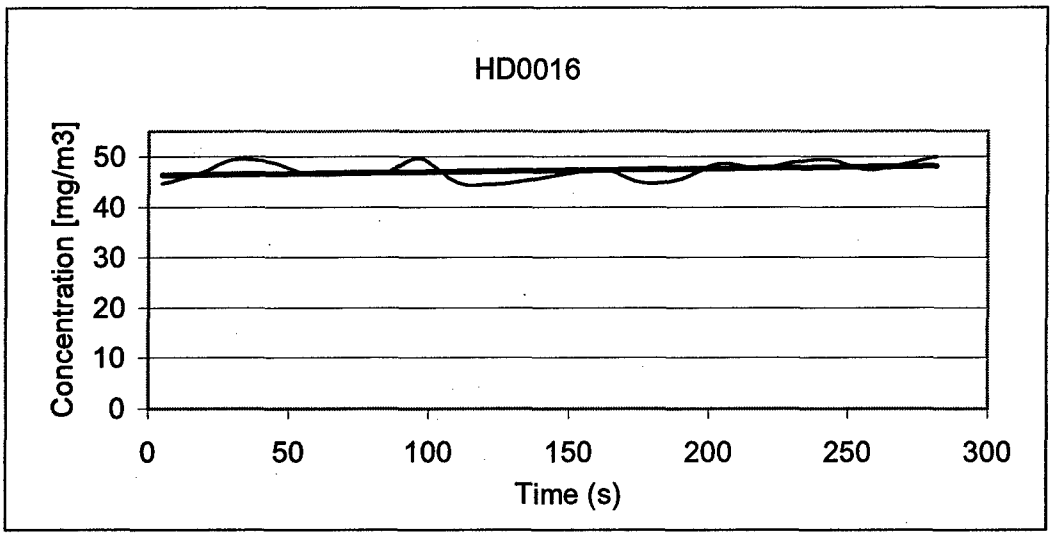

Figure 2. Time course of the sulfur mustard vapor concentration during animal experiment HD0016.

Distribution of N7-HETE-Gua within the respiratory tract of the hairless guinea pig after 5-min nose-only exposure to sulfur mustard vapor in air of 0.3 LCt50.

The concentration of N7-HETE Gua was measured in the respiratory tract samples with the immuno-slot-blot procedure described in paragraph II.2 and Annex B. Immunodetection of N7-HETE-Gua has proven to be a sensitive, reproducible and reliable method to quantify damage caused by sulfur mustard (Benschop *et al.* 1997, van der Schans *et al.* 1996, Langenberg *et al.,* 1998).

The concentrations of N7-HETE-gua measured in the respiratory tract samples of the individual animals as well as the averaged values with s.e.m. per group of 4 animals for each time point are presented in Tables 2 and 3, respectively. The distribution of N7-HETE-Gua within the respiratory tract at the various time points after exposure is presented in Figure 3.

N7-HETE-Gua could be detected in the nasal cavity, nasopharynx, larynx, trachea and carina, but not in lung tissue following 5-min nose-only exposure to 0.3 LCt50 of sulfur mustard vapor in air. The highest adduct levels were found at 10 and 30 min after exposure in the nasopharynx (up to 18 ± s.e.m. ($p \leq 0.05$, n=4) adducts per 10^7 nucleotides) and trachea (up to 19 ± s.e.m. adducts per 10^7 nucleotides). The lowest adduct levels were found in the nasal mucosa at all time points after exposure (2-5 ± s.e.m. adducts per 10^7 nucleotides), and in all respiratory organs, except for lung tissue, at 1440 min after exposure.

In the nasopharynx the highest adduct level is measured at 10 min after ending the exposure, after which the adduct level decreases with time, most likely due to repair of the damage to DNA. Deeper in the respiratory tract, i.e., in the trachea the maximum adduct level is observed at 30 min after the level decreases.

In general the adduct levels appear to build up relatively rapidly after nose-only exposure to sulfur mustard vapor in air and decrease quite rapidly within the first 24 h after ending the exposure. This observation is in agreement with previous findings (Langenberg *et al.* 1998).

For efficiency reasons, the histopathological evaluation of the samples from this T.O. will be done together with those of T.O., and will be reported in due course.

Table 1. The concentration of sulfur mustard vapor to which the individual animals were exposed.

Guinea pig	Date	File	Concentration (mg/m3)	Stdev (mg/m3)	Time after exposure
HD001	2/Mar/04	HD0203A	50.10	2.91	10 min
HD002	9/Mar/04	HD0903A	47.14	2.22	10 min
HD003	16/Mar/04	HD1603A	48.99	1.62	10 min
HD004	23/Mar/04	HD2303A	49.58	2.36	10 min
		Average	**48.95**		
		Stdev	**1.29**		
HD005	2/Mar/04	HD0203C	49.53	2.03	30 min
HD006	9/Mar/04	HD0903C	47.44	2.92	30 min
HD007	16/Mar/04	HD1603C	48.91	1.47	30 min
HD008	23/Mar/04	HD2303C	48.43	3.09	30 min
		Average	**48.58**		
		Stdev	**0.88**		
HD009	3/Mar/04	HD0303B	48.52	3.12	60 min
HD010	10/Mar/04	HD1003B	48.94	1.68	60 min
HD011	17/Mar/04	HD1703B	47.96	1.49	60 min
HD012	24/Mar/04	HD2403B	47.28	1.75	60 min
		Average	**48.17**		
		Stdev	**0.72**		
HD013	2/Mar/04	HD0203B	50.69	1.60	120 min
HD014	9/Mar/04	HD0903B	50.51	2.04	120 min
HD015	16/Mar/04	HD1603B	47.93	1.89	120 min
HD016	23/Mar/04	HD2303B	48.60	2.01	120 min
		Average	**49.43**		
		Stdev	**1.38**		
HD017	3/Mar/04	HD0303A	47.70	3.36	240 min
HD018	10/Mar/04	HD1003A	47.88	1.53	240 min
HD019	17/Mar/04	HD1703A	50.00	2.39	240 min
HD020	24/Mar/04	HD2403A	49.30	2.45	240 min
		Average	**48.72**		
		Stdev	**1.12**		
HD021	2/Mar/04	HD2303D	48.33	1.89	24 h
HD022	9/Mar/04	HD0903D	47.93	1.45	24 h
HD023	16/Mar/04	HD1603D	47.63	1.73	24 h
HD024	23/Mar/04	HD0203D	50.22	1.67	24 h
		Average	**48.53**		
		Stdev	**1.17**		
		Average	**48.73**		
		Stdev	**1.1**		

Table 2. N7-HETE-gua (expressed as number per 10^7 nucleotides) in respiratory tract regions of individual hairless guinea pigs at various time points after 5-min nose-only exposure to 48 mg.m-3 of sulfur mustard vapor in air, corresponding with 0.3 LCt50.

10 min 30 min 60 min 120 min 240 min 24 h control

	HD001	HD005	HD009	HD013	HD017	HD021	HD025
Nasal mucosa	6.65	0.14	7.3	2.31	3.76	17.33	0
Nasopharynx	57.64	42.43	3.92	7.32	2.86	0	0
Larynx	48.06	16.78	23.42	27.44	2.77	2.67	0
Trachea	31.51	33.04	13.99	13.55	1.34	4.2	0
Carina	n.g.	18.77	n.g.	8.27	3.85	2.7	0
Lung	0	0	0	0	0	0	0

	HD002	HD006	HD010	HD014	HD018	HD022	HD026
Nasal mucosa	0	8.54	0	0.03	0	0	0
nasopharynx	5.75	11.32	8.07	6.5	27.19	3.6	0
larynx	0.13	1.87	1.55	0	15.73	3.3	0
trachea	3.32	0	1.41	2.78	0	2.58	0
carina	2.34	3.08	8.42	5.25	n.g.	4.57	0
Lung	0	0	0	0	0	0	0

	HD003	HD007	HD011	HD015	HD019	HD023	HD027
Nasal mucosa	0.7	0	0	0	0	0	0
nasopharynx	0	0	n.g	0	n.g	n.g	
larynx	0	0.16	n.g	n.g.	n.g	0	0
trachea	0.38	11.24	0.42	1.12	9.66	2.63	0
carina	8.38	8.19	4	0.56	10.01	1.25	
Lung	0	0.01	0	0	0	0	0

(remeasured)	HD003	HD007	HD011	HD015	HD019	HD023	HD027
Nasal mucosa	0	2.74	9.76	5.02	5.22	2.25	0
Lung	0	0	0	0	0	0	0

	HD004	HD008	HD012	HD016	HD020	HD024	HD028
Nasal mucosa	0.08	14.81	0.21	0.28	0	0	0
nasopharynx	10.38	9.47	16.21	8.53	12.65	2.71	0
larynx	6.77	13.61	3.26	3.77	3.13	0.65	0
trachea	7.58	33.21	5.77	4.34	5.69	2.96	0
carina	9.48	4.6	3.35	2.76	3.19	0	0
Lung	0	0	0	0	0	0	0

Table 3. Mean concentrations of N7-HETE-gua (expressed as number per 10^7 nucleotides) with s.e.m. in respiratory tract regions of individual hairless guinea pigs at various time points after 5-min nose-only exposure to 48 mg.m-3 of sulfur mustard vapor in air, corresponding with 0.3 LCt50.

	10	30	60	120	240	1440	control
Nasal mucosa	1.49	5.25	3.45	1.53	1.80	3.92	0.00
Nasopharynx	18.44	15.81	9.40	5.59	14.23	2.10	0.00
Larynx	13.74	8.11	9.41	10.40	7.21	1.66	0.00
Trachea	10.70	19.37	5.40	5.45	4.17	3.09	0.00
Carina	6.73	8.66	5.26	4.21	5.68	2.13	0.00
Lung	0.00	0.00	0.00	0.00	0.00	0.00	0.00

	SEM						
	10 min	30 min	60 min	120 min	240 min	24 uur	control
Nasal mucosa	1.30	2.85	2.11	0.97	1.12	3.38	0.00
nasopharynx	13.24	9.21	3.61	1.91	7.07	1.08	0.00
Larynx	11.55	4.16	7.02	8.59	4.26	0.79	0.00
trachea	7.09	8.26	3.09	2.78	2.20	0.38	0.00
Carina	2.22	1.51	1.59	1.35	2.17	1.36	0.00
Lung	0.00	0.00	0.00	0.00	0.00	0.00	0.00

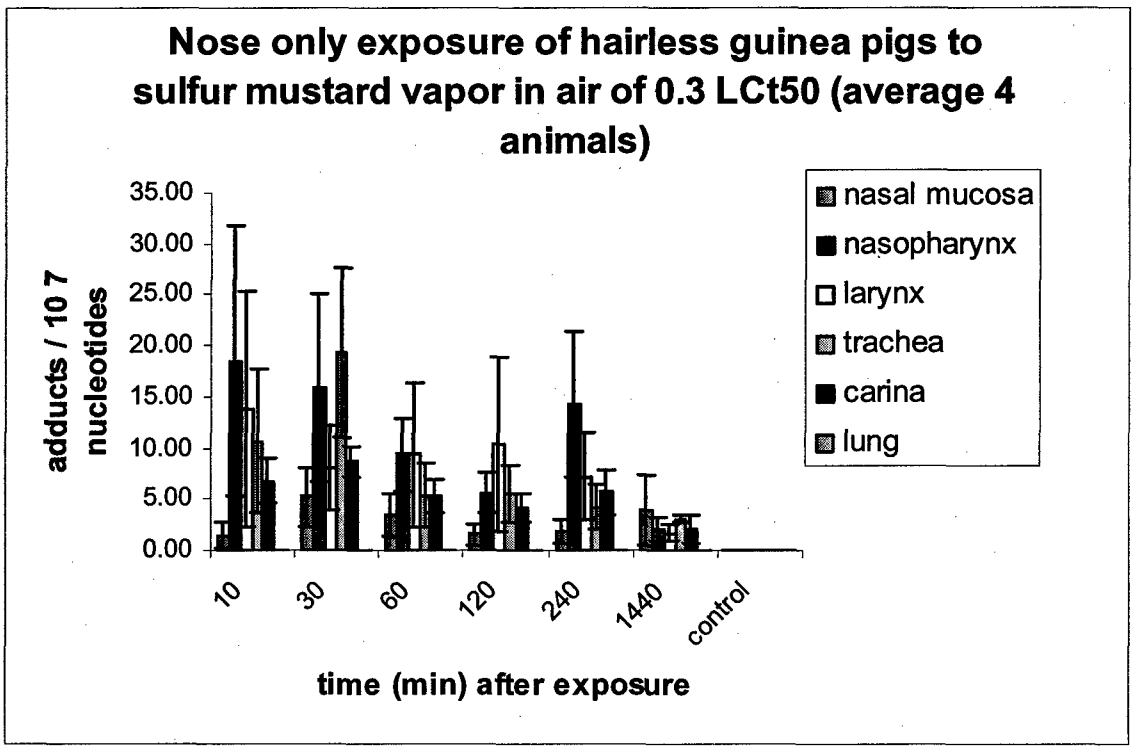

Figure 3. Mean concentration of N7-HETE-Gua (expressed as number of adducts per 10^7 nucleotides) in nasal cavity, nasopharynx, larynx, trachea and carina and lung tissue of hairless guinea pigs at various time points after exposre of sulfur mustard vapor in air (48 $mg.m^{-3}$, corresponding to 0.3 LCt50 (96-h)). Error bars represent the s.e.m. (standard error of the mean at $p \leq 0.05$, n=4).

III.3 APPARATUS FOR NOSE-ONLY EXPOSURE OF MARMOSETS TO SULFUR MUSTARD VAPOR IN AIR (T.O. 3)

In view of the anatomy of the marmoset, the modified Battelle tube for nose-only exposure of hairless guinea pigs as developed within the context of Cooperative Agreement DAMD17-94-V-4009 (Langenberg *et al.* 1998) is not suitable for this species. Consequently, a specially designed stainless steel 'chair' was developed. In this chair the animal can be restrained in such a way that it can breathe freely and can not free its arms or legs. The chair is adjustable for smaller and larger animals. The armrest, the chair support and the leg support are designed so that it is easy to adapt it to the individual animal. Special attention has been paid to the head support. Due to the physiology of the animal to breathe freely the angle between the mouth-head and head-rest of the body is slightly less than 90°, but can be adapted as desired. In Figures 4 to 8 the photographs of the chair can be seen.

Figure 4. Photograph of a marmoset monkey restrained on the chair, front view.

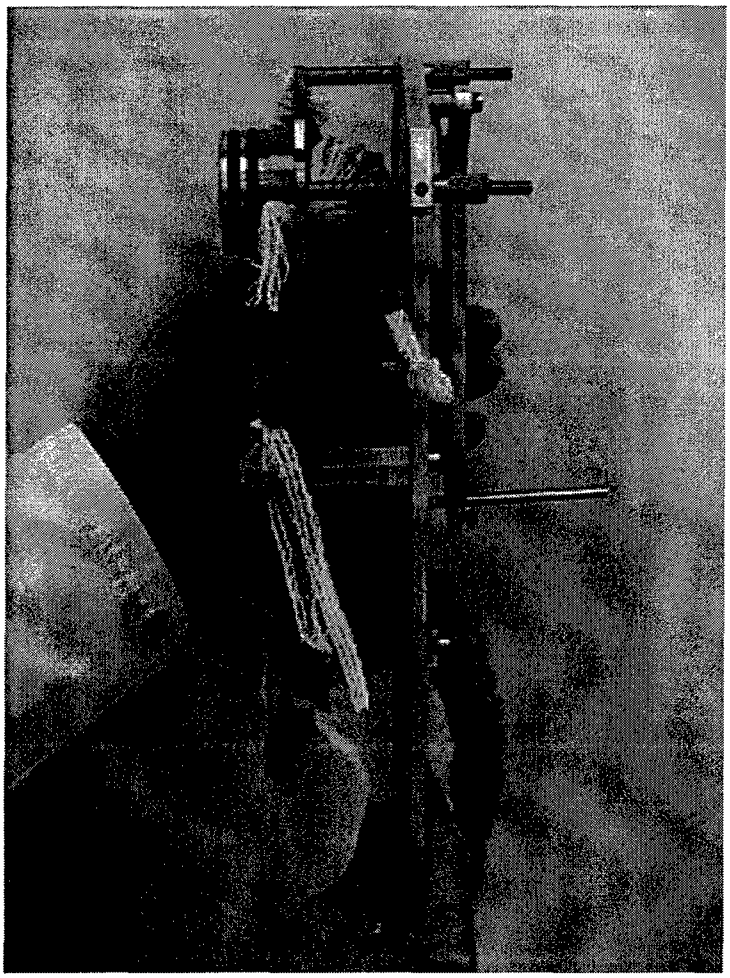

Figure 5. Photograph of a marmoset monkey restrained on the chair, side view.

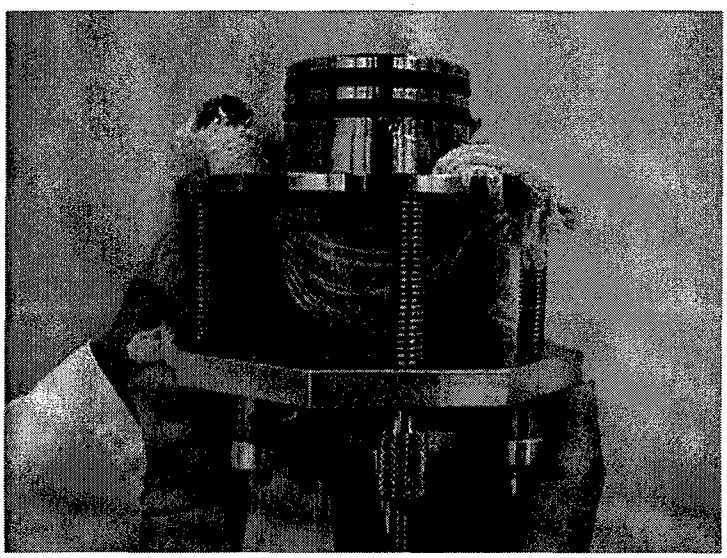

Figure 6. Photograph of a restrained marmoset on the chair, top view. The angle of the mouthpiece (top) to the body is adjustable.

Figure 7. Photograph of a marmoset monkey fixated in the 'chair' and connected into the 'mouth-piece' of the exposure module, taken from the left hand side.

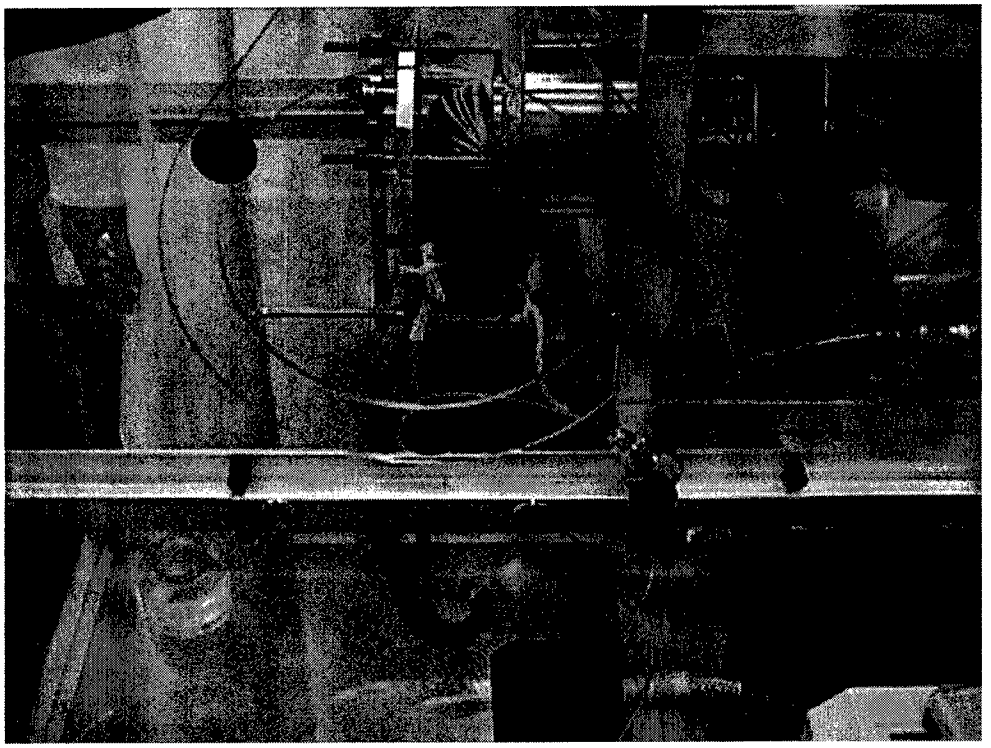

Figure 8. Photograph of a marmoset monkey fixated in the 'chair' and connected into the 'mouth-piece' of the exposure module, taken from the right hand side.

CONCLUSIONS

1. Within a time period of 24 h after 5-min nose-only exposure of hairless guinea pigs to 48 $mg.m^{-3}$ sulfur mustard vapor in air (corresponding with 0.3 LCt50 (96-h)) the number of adducts in the respiratory tract are increasing and subsequently decreasing, the latter most likely due to repair of the damage to DNA.

2. The highest number of DNA-adducts are found in the nasopharynx, followed by the larynx, trachea and the carina.

3. No adducts were found in the lungs, which is in agreement with previous findings at 4 h after ending a 5-min nose-only exposure to 160 $mg.m^{-3}$ (1 LCt50).

4. A 'chair' was developed for fixation of marmosets in an upright position, in order to enable nose-only exposure of this species to toxicant vapor in air. In this way, the animals can be exposed in a natural position.

5. REFERENCES

Benschop, H.P. (1991) Verification, dosimetry and biomonitoring of mustard gas exposure via immunochemical detection of mustard gas adducts to DNA and proteins. Final report grant no. DAMD17-88-Z-8022.

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 of Cooperative Agreement DAMD17-92-V-2005.

Benschop, H.P., Van der Schans, G.P., Noort, D., Fidder, A., Mars-Groenendijk, R.H. and De Jong, L.P.A. (1997). Verification of exposure to sulfur mustard in two casualties of the Iran-Iraq conflict. J. Anal. Toxicol., 21, 249-251.

Benschop, H.P., Noort, D., De Jong, L.P.A., and Van der Schans, G.P. (2000). Diagnosis and dosimetry of exposure to sulfur mustard: development of Standard Operating Procedures; Further exploratory research on protein adducts. Final report for Cooperative Agreement DAMD17-97-2-7002.

Cameron, G.R., Gaddum, J.H., and Short, R.H.D. (1946). The absorption of war gases by the nose. J. Pathol. Bacteriol., 58, 449-455.

Crodddy, E. (1995). Urban terrorism – Chemical warfare in Japan. Jane's Intel. Rev., November, 520-523.

Fidder, A., Noort, D., and Benschop, H.P. (1999) A convenient synthesis of [^{14}C]1,1'-thiobis(2-chloroethane), [^{14}C]sulfur mustard. J. Lab. Comp. Radiopharm. 42, 261-266.

Langenberg, J.P., De Vette, H.Q.M. and Benschop, H.P. (1995). Objective skin sensitivity assessment towards H: Determination of the efficacy of protective clothing by analysis of sulfur mustard adducts in skin. Proc. 5th Int. Symp. on Protection against Chemical and Biological Warfare Agents, Stockholm, Sweden, 11-16 June 1995, Supplement, 119-124.

Langenberg, J.P., Van Dijk, C., Sweeney, R.E., De Jong, L.P.A., Maxwell, D.M. and Benschop, H.P. (1997). Development of a physiologically based model for the toxicokinetics of C(±)P(±)-soman in the atropinized guinea pig. Arch. Toxicol., 71, 320-331.

Langenberg, J.P., Benschop, H.P. and Van der Schans, G.P. (1998). Toxicokinetics of sulfur mustard and its DNA-adducts in the hairless guinea pig – DNA adducts as a measure for epithelial damage. Final Report for Cooperative Agreement DAMD17-94-V-4009.

Mershon, M.M., Mitcheltree, L.W., Petrali, J.P., Braue, E.H., and Wade, J.V. (1990). Hairless guinea pig bioassay model for vesicant vapor exposure. Fundam. Appl. Toxicol., 15, 622-630.

Mol, M.A.E. (2000). Implications of protein alkylation and proteolysis on vesication caused by sulfur mustard. Final report for Cooperative Agreement DAMD17-97-2-7018.

Mol, M.A.E. (2004). New approaches towards the elucidation of epidermal-dermal separation in sulfur mustard exposed human skin and directions for therapy. Second Annual Report for Cooperative Agreement DAMD17-02-1-0206.

Ogston, A.G., Holiday, E.R., Philpot, J.St.L. and Stocken, L.A. (1946). The replacement reactions of β-β'-dichlorodiethyl sulphide and of some analogues in aqueous solution: the isolation of β-chloro-β'-hydroxy diethyl sulphide. Trans. Farad. Soc., 44, 45-52.

Papirmeister, B., Feister, A.J., Robinson, S.I., and Ford, R.A. (1991). Medical Defense Against Mustard Gas. Toxic Mechanisms and Pharmacological Implications., CRC Press, Boca Raton, USA.

Schreiber, J.P. and Raabe, O.G. (1981). Anatomy of the naso-pharyngeal airway of experimental animals. Anatomical Rec., 200, 195-205.

Trap, H.C., Spruit, W.E.T., Oostdijk, J.P. and Langenberg J.P. (1996). Trace analysis of sulfur mustard in biological samples by means of an automated large volume injection onto a two dimensional GC system. Proceedings 18th International Symposium on Capillary Chromatography, Riva, Italy, pp. 1002-1012.

Van der Schans, G.P., Mars-Groenendijk, R.H., Noort, D., De Jong, L.P.A., Bruijnzeel, P.L.B and Benschop, H.P. (1996) Immunochemical detection of adducts of sulfur mustard to DNA and proteins as a tool for biological dosimetry of sulfur mustard exposure. Proceedings of the 1996 Medical Defense Bioscience Review, May 1996, Baltimore, MD, USA, pp. 1146-1155.

Van Helden, H.P.M., Langenberg, J.P. and Benschop, H.P. (1999). Low level exposure to GB vapor in air: Diagnosis/dosimetry, lowest observable effect levels, performance-incapacitation, and possible delayed effects. Second Annual Report for Grant DAMD17-97-1-7360.

ANNEX A. STATEMENT OF WORK/TECHNICAL OBJECTIVES FOR COOPERATIVE AGREEMENT DAMD17-03-1-0613

The goals that are set for the proposed research are:

1. Comparison of the inhalation toxicokinetics of sulfur mustard and its DNA-adducts in the hairless guinea pig and a species more relevant for man, i.e., the marmoset monkey.
2. Establishment of the percutaneous toxicokinetics of sulfur mustard and its DNA-adducts in the hairless guinea pig at a dose comparable with that of the respiratory exposure as studied in Cooperative Agreement DAMD17-94-V-4009.

Technical Objectives

Toxicokinetic studies on respiratory exposed animals

1. Determination of the concentration of N7-HETE-gua in DNA from samples of the mucosa of various sites of the respiratory tract (nasal mucosa, nasopharynx, larynx, trachea, carina, and lung) in groups of 4 hairless guinea pigs at 10 and 30 min, and at 1, 2 and 24 h after ending a 5-min nose-only exposure to sulfur mustard vapor in air with a concentration of 160 $mg.m^{-3}$, which corresponds with 1 LCt50 (96-h). Furthermore, these sites will be subjected to histopathological examination.

2. Determination of the concentration of N7-HETE-gua in DNA from samples of the mucosa of various sites of the respiratory tract (nasal mucosa, nasopharynx, larynx, trachea, carina, and lung) in groups of 4 hairless guinea pigs at 10 and 30 min, and 1, 2, 4 and 24 h after ending a 5-min nose-only exposure to sulfur mustard vapor in air with a concentration of 48 $mg.m^{-3}$, which corresponds with 0.3 LCt50 (96-h) Furthermore, these sites will be subjected to histopathological examination

3. The apparatus designed within the context of Cooperative Agreement DAMD17-94-V-4009 for nose-only exposure of hairless guinea pigs to sulfur mustard vapor in air and used in the experiments as described in items 1 and 2 will be modified to allow the use of marmosets as an animal model.

4. Determination of the time course of the concentration of sulfur mustard in blood during and after a 5-min nose-only exposure of anesthetized, restrained marmosets to sulfur mustard vapor in air with a concentration of 160 $mg.m^{-3}$. Such an exposure corresponds with 1 LCt50 (96-h) in the hairless guinea pig.

5. Determination of the concentration of sulfur mustard in lung, liver, spleen, bone marrow and abdominal fat of 3 marmosets at halfway and at the end of the toxicokinetic experiment described under T.O. 4. Additional experiments will be performed to obtain tissue samples at 10 min after ending the 5-min nose-only exposure to sulfur mustard.

6. Determination of the concentration of N7-HETE-gua in DNA from blood, lung, liver, spleen, bone marrow and small intestine of 3 marmosets at 10 min after ending the 5-min nose-only exposure to sulfur mustard, as well as halfway and at the end of the toxicokinetic experiment described under T.O. 4. Additional experiments will be performed to obtain tissue samples at 24 h after ending the 5-min nose-only exposure to sulfur mustard.

7. Determination of the concentration of the terminal N-valine adduct of sulfur mustard to hemoglobin in the marmoset blood samples taken in the toxicokinetic experiment described under T.O. 4, and in the blood samples taken at 24 h after ending the 5-min nose-only exposure, described under T.O. 6.

8. Determination of the concentration of N7-HETE-gua in DNA from samples of the mucosa of various sites of the respiratory tract (nasal mucosa, nasopharynx, larynx, trachea, carina, and lung) of 3

marmosets at the end of the toxicokinetic experiment described under 4. Furthermore, these sites will be subjected to histopathological examination.

9. If a toxicokinetic profile of sulfur mustard in blood cannot be determined in two subsequent animal experiments under the conditions described under T.O. 4, marmosets will be nose-only exposed to sulfur mustard vapor in air with a concentration of 300 $mg.m^{-3}$ for 8 min. Such an exposure results in a Ct which is three times the LCt50-value for 5-min exposure in the hairless guinea pig. In that case, all experiments described under T.O. 4-8 will be performed with this higher exposure level.

Toxicokinetic studies on percutaneously exposed animals

10. Determination of the time course of sulfur mustard concentrations in blood during and after a 45-min whole-body exposure of anesthetized, restrained hairless guinea pigs to sulfur mustard vapor in air with a concentration of ca. 53 mg/m^{-3} (Ct of 2,400 $mg.min.m^{-3}$). During the experiment, the animals breathe clean air.

11. Determination of the concentration of sulfur mustard in lung, liver, spleen, bone marrow, abdominal fat and dorsal skin of 4 hairless guinea pigs at halfway and at the end of the experiment described under T.O. 10. Additional experiments will be performed to obtain tissue samples at 10 min after ending the 45-min whole-body exposure to sulfur mustard vapor.

12. Determination of the concentration of N7-HETE-gua in DNA from blood, lung, liver, spleen, bone marrow, small intestine and skin of 4 hairless guinea pigs at 10 min after ending the 45-min whole-body exposure to sulfur mustard vapor, as well as halfway and at the end of the experiment described under T.O. 10. Additional experiments will be performed to obtain tissue samples at 24 h after ending the 45-min whole-body exposure to sulfur mustard vapor.

13. Determination of the concentration of the terminal N-valine adduct of sulfur mustard to hemoglobin in the hairless guinea pig blood samples taken in the toxicokinetic experiment under T.O. 10, and in the blood sample taken at 24 h after ending the whole-body exposure to sulfur mustard vapor, described under T.O. 12.

Time Schedule

The technical objectives will be performed over a period of three years:
1st year: technical objectives 1 to 6
2nd year: technical objectives 7 to 13
3rd year: technical objectives 14 to 19

ANNEX B STANDARD OPERATING PROCEDURE FOR DETERMINATION OF N7-HETE-GUA IN DNA

Materials

DNA isolation

Cell lysis solution	10 mM Tris, 1mM EDTA, 1% v/v SDS
DNA isolation kit	PureGene kit, Biozym
PBS (sterile)	Phosphate Buffered Saline (Dulbecco's)

Capped Eppendorf tubes (1.5 ml)
Gilson pipets (20, 200, 1000 µl) and tips
Eppendorf centrifuge, model 5417 C
Proteinase K
Rnase A
Isopropanol
70% Ethanol
Filterpaper
Water bath at 37 °C
Rotating wheel shaker
Incubator at 37 °C

Vibrator	Titertek (Flow)
0.1TE buffer	1 mM Tris-HCl, 0.1 mM Na2EDTA, pH 7.4
UV/VIS spectrometer	E.g., Lambda 40, Perkin Elmer, Breda, The Netherlands

DNA denaturation

Calf thymus DNA calibration samples	Calf thymus DNA exposed to 0, 2.5, 5, 10 and 20 nM sulfur mustard
TE buffer	10 mM Tris-HCl, 0.01 mM Na2EDTA, pH 7.4
MQ water	Purified tapwater, classification I, ISO 3696
Formamide	99%
Formaldehyde	36.5%

52 °C water bath
Gilsonpipets (20, 200, 1000 µl) and tips

Immunoslotblot assay

Nitrocellulose	Protran BA 79, nitrocellulose transfer membrane, 0.1 µm, Schleicher and Schuell
Blotting paper	Gel-blotting-paper GB 002 (0.8 mm), Schleicher and Schuell
Blotting device	Minifold 1 Dotblot manifold, Schleicher and Schuell

Vacuum pump
Glass vacuum flask
12-channel pipet and tips
Gilsonpipets (20, 200, 1000 µl) and tips

Flat forceps	e.g. Millipore

Gloves

PBS (sterile)	Phosphate Buffered Saline (Dulbecco's)
MQ water	Purified water, classification I, ISO 3696
Milkpowder	ELK, skimmed milkpowder, less than 1% fat, Campina, Eindhoven, The Netherlands
1st Antibody, 2F8	Directed against against N7-HETE-Gua, culture supernatant, TNO-PML, Rijswijk, The Netherlands
2nd Antibody	Rabbit-anti-mouse-Ig-horse radish peroxidase, Dakopatts, Glostrup, Denmark

Enhanced Chemiluminescence

Blotting Detection System	Solution A and B, Boehringer Mannheim, Germany

Incubation boxes	
UV-gene cross-linker	E.g., GS Gene Linker UV chamber, Bio-Rad Laboratories, The Netherlands
Waterbath at 25 °C	
Luminometer	E.g., MicroBeta Trilux 6 detector system, Wallac, EG & G Berthold
Plastic sheets	For packing blots
Shaking plate	
Stopwatch	
Filter paper	

Procedure for immunoslotblot asssay to detect sulfur mustard adducts to DNA in respiratory tissue of the hairless guinea pig

Sampling

DNA from mucosal scrapings of the nasal cavity, nasopharynx, larynx, trachea and carina was isolated and treated as described below. Lung tissue was homogenized prior to DNA isolation.

DNA isolation

1. Mucosal scrapings/homogenized lung tissue are transferred into 1.5-ml Eppendorf tubes.
2. Add Cell lysis solution (300 µl), supplemented with Proteinase K (100µg/ml).
3. Incubate 16 h at 37 °C under continuous shaking.
4. Treat with RNase A (1.5 µl, 50 µg/ml) for 15 min at 37 °C, followed by cooling on ice for min 5 min.
5. Add Protein Precipitation Solution (100 µl), mix on a high speed vortex (20 s).
6. Cool on ice for 5 min.
7. Centrifuge at 14,000g for 10 min.
8. Transfer the supernatant to a tube containing isopropanol (300 µl) in order to precipitate the DNA, and centrifuge at 7,000 rpm (5,200g) for 5 min.
9. Wash the pellet with 70% ethanol (300 µl), centrifuge (7,000 rpm, 5 min), and dry on air for *ca.* 15 min.
10. Dissolve the pellet in 0.1TE buffer (50 or 100 µl depending on the size of the pellet) under continuous vibration overnight at room temperature. (Dissolution can be accomplished within 1 to 2 h when performed with fresh blood)
11. Determine DNA concentration by diluting the DNA solution (4 µl) 20-fold with 0.1TE buffer and measure A_{260} in a 1-cm quartz microcuvette in a UV/VIS spectrometer (1000 * A260 = DNA concentration in µg/ml of the undiluted solution). Measure also A_{280} as indication for the purity of the DNA solution. (The A_{260}/A_{280} ratio should be between 1.6 – 1.9)

DNA denaturation

1. Make up solutions (100 µl) with final concentrations of DNA (50 µg/ml), formamide (4.1%), and formaldehyde (0.1%) in 0.1TE buffer, incubate at 52 °C for 15 min, and cool rapidly on ice. Store at
   –20 °C (freezing of the samples at least once is essential). Treat the calf thymus DNA calibration samples in the same way.

Immunoslotblot procedure

1. Dilute the denatured DNA samples in PBS to a final concentration of 5 µg/ml (including the calf thymus DNA calibration samples)
2. Assemble the blotting manifold: connect with vacuum flask and place 2 pieces of blotting paper (wear gloves); make a nitrocellulose filter, cut in a 96-well format, wet (with water and PBS) and place it on the upper part of the manifold (without air bubbles); place the upper part on the other parts and fix the clamps. Switch on the vacuum pump.
3. Spot the DNA solution (200 µl) in duplicate. Do not use positions A12 and H1. (These positions are needed as markers for the positioning of the filter in the luminometer cassette.)
4. Wash each dotted sample with PBS (400 µl) by suction through the filter.

5. Take the nitrocellulose filter from the blotting manifold and dry on air for 10-15 min.
6. Cross-link the DNA to the filter by means of illumination with the UV-gene-cross-linker (50 mJ/cm^2).
7. Incubate the filter with blocking solution (about 50 ml, 5% milkpowder in PBS + 0.1% Tween 20) at room temperature for 30 min.
8. Wash three times with PBS + 0.1% Tween 20.
9. Incubate the filter with 1st antibody diluted 500-fold in 20 ml solution containing 0.5% milkpowder in PBS + 0.1% Tween 20, overnight at 4 °C under continuous shaking.
10. Wash 4 times with PBS + 0.1% Tween 20; the last three times for at least 15 min each.
11. Incubate the filter with 2nd 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.
12. Wash 4 times with PBS + 0.1% Tween 20; the last three times for at least 15 min each.
13. Incubate solution A (of the Enhanced Chemiluminescence Blotting Detection System) in a waterbath at 25 °C until temperature equilibrium is reached. Mix solution B with solution A in a ratio 1:100 and preincubate the substrate solution for at least 30 min at 25 °C.
14. Remove free (wash) solution from the filter with filter paper, mark position A12 and H1 with ball point (not a felt pen!).
15. Place the filter in a closely fitting box, add 10 ml of substrate solution and incubate for 1 min.
16. Wrap the filter, straight from the substrate in plastic, without air bubbles. Press out liquid, transfer the filter in plastic into the luminometer cassette and place it in the luminometer.
17. Measure luminescence according to the required program. Collect data in a file and calculate for each sample the level of N7-HETE-Gua/10^7 nucleotides (in an Excel worksheet).

ANNEX C ACRONYMS AND ABBREVIATIONS

A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
Ct	Product of concentration and exposure time
CW	Chemical warfare
DNA	Deoxyribonucleic acid
ε	Molar extinction coefficient
ELISA	Enzyme linked immunosorbent assay
FID	Flame ionization detector
GC	Gas chromatography
ISB	Immunoslotblot
LC	Liquid chromatography
LCt50	Product of concentration and exposure time that produces 50 % mortality in a population
LD50	Dose that produces 50 % mortality in a population
LIF	Laser-induced fluorescence
MS	Mass spectrometer
N7-HETE-gua	N7-hydroxyethylthioethyl guanine
NBC	Nuclear, Biological, Chemical
PBS	Phosphate buffered saline
PML	Prins Maurits Laboratory
TDSA	Automated thermodesorption sampler
TNO	Netherlands' Organization for Applied Scientific Research
USAMRICD	U.S. Army Medical Research Institute of Chemical Defense
USAMRMC	U.S. Army Medical Research and Materiel Command
UV	Ultraviolet