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Development of an antibody that binds sulfur mustard

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1. Summary

An antibody that binds *bis*(2-chloroethyl)sulfide (sulfur mustard) was developed. The immunizing antigen was prepared from the hapten 4-(2-chloroethyl)benzoic acid covalently bound to keyhole limpet hemocyanin (KLH). The antibody was monitored by a solid phase enzyme-linked immunosorbent assay (ELISA). The test antigen consisted of a second hapten, 8-chlorocaprylic acid, covalently bound to bovine serum albumin (BSA). The test antigen was absorbed to the wells of 96-well plates. The immunizing and test antigens contain a common chloroethyl moiety. Thiodiglycol, the principal hydrolysis product of sulfur mustard, does not react with the antibody. This antibody, because of its specificity, has the potential to be a valuable tool for mustard research and forensic detection.

2. Introduction

Studies on sulfur mustard, or *bis*(2-chloroethyl)sulfide, date back to 1822 [1]. Studies of the compound were also reported by Riche in 1854 [2], Guthrie in 1860 [3], and Niemann in 1860 [4]. The first synthesis of a relatively pure product of known structure was reported by Meyer in 1886 [5, 6]. Meyer discontinued further work in the area because of the hazards involved. His findings were resurrected about 30 years later by German scientists searching for an effective chemical warfare agent. Sulfur mustard was first used on the battlefield by the Germans near Ypres, Belgium, in July 1917 [7]. Sulfur mustard is an alkylating agent that produces severe burns on exposed skin and tissues. Erythema and blistering occur with relatively low dose exposures. Sulfur mustard burns heal slowly and are susceptible to infection. From the end of World War I to the present time there have been no less than 11 purported uses of sulfur mustard [7].

A number of investigators have used various methods for the analysis of sulfur mustard in biological fluids and tissues [8]. None of these are entirely satisfactory and most require a sophisticated and dedicated experimental set-up (gas chromatography coupled with mass spectroscopy, etc.). To overcome these difficulties we decided to explore the development of an enzyme-linked immunosorbent assay (ELISA) for sulfur mustard. ELISAs offer good sensitivity and simplicity of operation. From the onset the relative instability of sulfur mustard in aqueous solution [9 – 12]* at room temperature was recognized as a possible obstacle in the development of an anti-mustard antibody for an ELISA. That is, one must use a stable hapten that will survive the coupling and immunization procedures. Because of the instability of sulfur mustard in aqueous solution, it was decided to use haptens containing only the chloroethyl moiety of sulfur mustard. Haptens containing the sulfur atom that facilitates mustard hydrolysis were not considered. This article is the first documentation of the production of an antibody that binds the sulfur mustard molecule.

Key words: Sulfur mustard; Sulfur mustard antibody; Antibody inhibition; Haptens

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* In the cold the stability is considerably enhanced, as a half-life of 158.0 min has been reported in water at 0.6 °C [13]. Also, hydrolysis under physiological conditions would be expected to be somewhat slower in biological fluids because of the chloride ions present.

3. Materials and Methods

3.1. *Reagents and chemicals*

Sulfur mustard (purity > 96%) (HD), *bis*(2-chloroethyl)disulfide (CEDS), and *bis*(3-chloropropyl)sulfide (CPS) were obtained from the Chemical Research, Development and Engineering Center (CRDEC), Aberdeen Proving Ground, MD. 4-Chlorobutanol-1 (CB), *cis*-4-chloro-2-buten-1-ol (*cis*-CB), 4-(2-chloroethyl)benzoic acid (4-CBA), 1,3,5-tris-2′-chloroethylbenzene (1,3,5-B), and 8-chlorocaprylic acid (8-CCA) were purchased from Ash-Stevens Inc., Detroit MI. 2-Chloro-*N*,*N*-diethylethylamine (SANM) was purchased from Pfaltz and Bauer Inc., Flushing, NY. 2-Chloroethyl methyl sulfide (CEMS) and 2-chloroethyl ethyl sulfide (CEES) were purchased from Aldrich Chemical Company, Milwaukee WI. Cyclophosphamide (CYTOXAN) was purchased from Mead Johnson Inc., Evansville, IN. Thiodiglycol (TDG) was purchased from Pierce Chemical Company, Rockford, IL. 2,2′-Azino-di(3-ethylbenzthiazoline sulfonate) was purchased from Sigma Chemical Co., Saint Louis, MO. Keyhole limpet hemocyanin (KLH) was purchased from Pacific Biomarine Supply, Monterey, CA.

3.2. *Animals*

Two healthy New Zealand White female Buk:NZWfBR rabbits (*Oyctolagus cuniculus*) weighing 2.5–3.5 kg were used in this study. The animals were quarantined on arrival and screened for evidence of disease.

3.3. *Stratagem*

4-(2-Chloroethyl)benzoic acid (4-CBA) was the hapten used for the preparation of the immunizing antigen. 8-Chlorocaprylic acid (8-CCA) was the hapten used for preparation of the test antigen. The haptens were coupled to unrelated proteins, keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). 1-(Dimethylaminopropyl)-3-ethylcarbodiimide (DMEC) was used to covalently bind the haptens to their distinct carrier proteins.

3.4. *Immunizing antigen preparation*

The molar ratio of the starting materials, 4-CBA, KLH, and DMEC, was 1000:1:2000, respectively. KLH (500 mg) in 59.5 ml of water was placed in a 100-ml beaker and slowly stirred on a magnetic stirrer at room temperature. 4-CBA (24.0 mg) was added to the KLH solution. The resultant pH was 6.15. DMEC (12.0 mg dissolved in 10.0 ml of water) was added dropwise over a 30-min period. During the course of the addition the pH dropped, denoting coupling. The pH was maintained for 4 h at pH 6.0 with occasional additions of 0.1 N NaOH. Following overnight incubation in the refrigerator the pH was readjusted to 6.0 with 0.01 N NaOH and the solution centrifuged at $2000 \times g$ for 30 min. The supernatant was exhaustively dialyzed against water and finally against 0.01 M phosphate-buffered saline (PBS) of pH 7.2. A protein determination was performed on the antigen using Pierce's BCA method [14].

3.5. *Test antigen preparation*

The molar ratio of the starting materials, 8-CCA, BSA, and DMEC, was 90:1:10, respectively. Like 4-CBA in the preparation of the immunizing antigen, the solubility of 8-CCA is marginal so a saturated solution in water was used. The coupling and dialysis procedures were the same as used for the immunizing antigen. The following amounts of reactants were used: 8-CCA, 25 mg; BSA, 500 mg; DMEC, 300 mg. A protein determination was performed on the antigen using Pierce's BCA method [14].

3.6. *Immunization regimen and antiserum collection procedures*

The immunizing antigen was dissolved in PBS, pH 7.2, and combined with an equal volume of Freund's complete adjuvants (FCA). A 20-gauge needle that has another Luer Lok fitting welded on the distal end was used to make the antigen-Freund's emulsion. Syringes were fitted on both ends of the modified needle. The emulsion was made by forcing the antigen mixture back and forth through the modified needle. Two female New Zealand White rabbits weighing approximately 3 kg

each were injected intradermally in 25 sites on their shaved backs. Each site was aseptically administered 25 μl of the emulsion that contained 9.5 μg of protein/ml [15]. Two more inoculations were given at 14-day intervals with the antigen combined with Freund's incomplete adjuvants. On day 35 the animals were bled from the median artery of the ear with a sterile 18-gauge needle. The blood was allowed to clot and the serum was poured into centrifuge tubes and centrifuged at 2000 × *g* for 20 min. The serum was decanted and stored frozen at −20 °C until assayed for the presence of antibodies that bind sulfur mustard.

3.7. *Antibody detection and evaluation procedures*

We confirmed antibody production using an ELISA test system [16]. The test antigen consisted of 8-CCA covalently bound to BSA. This antigen (100 μl/well) was adsorbed to the bottom of "Immulon 4" 96-well polystyrene plates (Dyanatech Inc., Chantilly, VA) with 0.01 M carbonate buffer, pH 9.6. The final protein concentration of the test antigen in carbonate buffer was 200 ng/ml. The plates were allowed to incubate overnight in the refrigerator. After absorption the plates were washed 4 times with 0.01 M phosphate-buffered saline (PBS) containing 0.2% BSA (wash buffer). The plates were blocked for 1 h with PBS containing 0.5% BSA. A checkerboard range finding plate was used to find the optimum dilutions of test antigen and antiserum. The antiserum was diluted 40 times with PBS and applied to the previously prepared plates, and incubated overnight in the refrigerator. The plates were then washed 4 times with the wash buffer and peroxidase-labeled goat-anti-rabbit antibody (Sigma Chemical Co., St. Louis, MO) diluted 1:1000 (100 μl/well) was added and incubated for 1 h at room temperature. The plates were washed 4 times with wash buffer. A color-producing substrate consisting of 2,2'-azino-di(3-ethylbenzthiazoline sulfonate) (0.15 mg/ml) in pH 4.0, 0.05 M citrate buffer containing 0.0004% of H_2O_2 was prepared just prior to use. The substrate (100 μl/well) was added and after 30 min the plate was read in a plate reader (Molecular Devices, Menlo Park, CA) set at 405 nm.

3.8. *Antibody inhibition procedures*

To confirm the presence of specific anti-mustard antibodies, inhibition reactions were done with sulfur mustard and related compounds. Inhibitions were performed by serially diluting 10^{-3} M starting concentrations of the inhibitor twice (12 concentrations total) in a separate 96-well plate. Final concentration from the serial dilution procedure was 2.4×10^{-7} M. In the case of 8-CCA and 4-CBA the starting concentrations were significantly less due to the limited solubility of these two compounds*. The dilutions of inhibitor were mixed with a 20-times dilution of antiserum and allowed to incubate on ice for 10 min. Aliquots of 100 μl were transferred to a 96-well plate that was previously coated with 100-μl amounts of 200 ng/ml of 8-CCA-BSA test antigen. After overnight incubation in the refrigerator the plate was developed as in section 3.4. The names and structures of the compounds tested for inhibition are shown in Fig. 1.

4. Results

Both animals produced antiserum that binds sulfur mustard. Day 75 antiserum from a single rabbit was used. The specificity of the antiserum was determined by inhibition with the compounds whose structures are shown in Fig. 1. The results of the specificity studies with the compounds shown in Fig. 1 are shown in Table 1. Inhibition was noted with HD, CEMS, CEES, and SANM. The other compounds showed no cross-reactivity. Fig. 2 is a plot of $\log_{10}$ concentration of sulfur mustard versus percent uninhibited activity.

5. Discussion

Research on sulfur mustard that has been done for the past 75 years has failed to produce: (1) a useful prophylactic compound, (2) an effective treatment compound, and (3) a simple diagnostic or forensic test for indicating the presence of low

* In water at 25.0 °C the solubility of 8-CCA is 3.4×10^{-6} M and that of 4-CBA is 2.3×10^{-5} M. As these concentrations would be further decreased in the cold (conditions for the antibody inhibition procedure), their lack of expected response in the ELISA test system is not an unusual phenomenon but simply the result of solubility limitations.

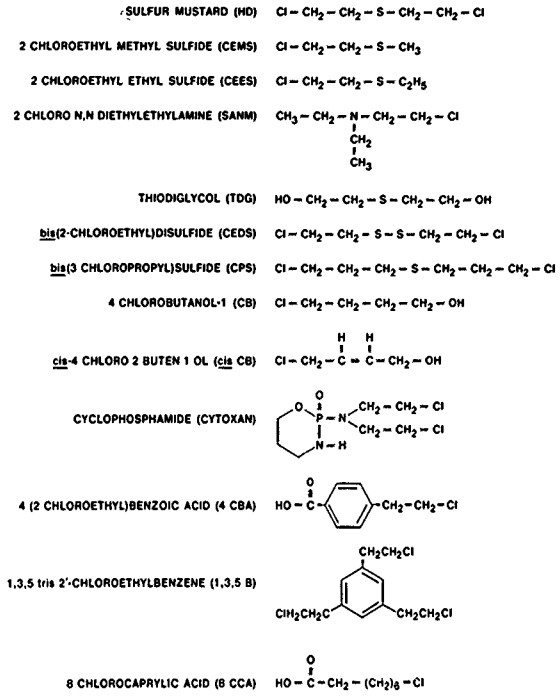

Fig. 1. Structures of compounds tested for specificity.

levels of sulfur mustard. We have produced the first antibody capable of binding sulfur mustard. We envision the utilization of antibodies binding sulfur mustard to address the deficiences in mustard research cited above.

Because of the relative instability of sulfur mustard in aqueous solution we decided to use hapten antigens containing only the chloroethyl moiety. Haptens containing the sulfur atom that facilitates mustard hydrolysis were not considered because such analogs would not survive the coupling or immunization processes. Our choice for an immunizing hapten was 4-chloroethyl benzoic acid. The chloroethyl moiety alone would not be expected to elicit an antibody response. However, the adjacent benzene ring of 4-chloroethyl benzoic acid would lend immunogenicity to the chloroethyl moiety. This fact was noted in Landsteiner's classic publication "The Specificity of Serological Reactions" [17]. The para-positioned carboxyl group provided a convenient attachment site. The benzene ring also provided adequate spacing from the carrier protein. The test antigen for the ELISA testing procedure was 8-chlorocaprylic acid. This hapten contained the needed chloroethyl moiety, adequate spacing from the unrelated carrier protein, and a convenient attachment site to the carrier protein. This hapten was chosen rather than 4-chloroethyl benzoic acid because antigenicity was not required.

Antibody assessment was accomplished by testing the cross-reactivity of the rabbit anti-mustard antiserum with sulfur mustard and related com-

TABLE 1

Percentage inhibition observed for compounds tested[a, b].

Compound	% Inhibition		
	(5.0×10^{-4} M)[c]	(3.1×10^{-5} M)	(4.9×10^{-7} M)
1. HD	97	43	0
2. CEMS	100	58	19
3. CEES	100	83	16
4. SANM	36	37	15
5. TDG	0	0	0
6. CEDS	0	0	0
7. CPS	0	0	0
8. CB	0	0	0
9. *cis*-CB	0	0	0
10. CYTOXAN	0	0	0
11. 4-CBA	0	0	0
12. 1,3,5-B	0	0	0
13. 8-CCA	0	0	0

[a]Response compared to control. [b]Approximate IC_5 values: HD, 3.5×10^{-5} M; CEMS, 2.0×10^{-5} M; CEES, 5.6×10^{-6} M. [c]Inhibitor concentration.

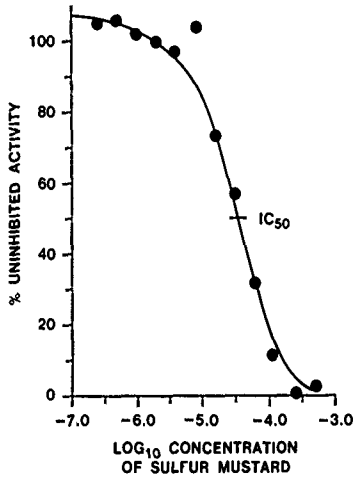

Fig 2 Plot of $\log_{10}$ concentration of sulfur mustard versus percent uninhibited activity.

pounds. We successfully produced a mustard specific antiserum that is inhibited by sulfur mustard, chloroethyl ethyl sulfide, and chloroethyl methyl sulfide. Single arm nitrogen mustard produces a much weaker inhibition. As is evident from Table 1, the strongest inhibition reaction occurs when the inhibitor's chloroethyl moiety is attached to a sulfide sulfur. The subtle substitution of a disulfide group (CEDS) for a sulfide group (HD, CEMS, CEES) destroys specificity. The lack of response by test solutions of the haptens (8-CCA and 4-CBA) in the ELISA test system is not an unusual departure from expected immunochemical response, but simply the result of solubility limitations. Of particular importance is the negative response of thiodiglycol, the principal hydrolysis product of sulfur mustard. The estimation of thiodiglycol in biological fluids cannot serve as reliable evidence of exposure to mustard. Wils and co-workers [18, 19] investigated the presence of thiodiglycol in urine as related to exposure to sulfur mustard. The endogenous presence of this compound in some biological fluids precludes its use as a marker to provide unequivocal verification of exposure to sulfur mustard. An anti-mustard antibody that does not cross-react with thiodiglycol offers the basis for a direct test for low levels of intact sulfur mustard.

It was interesting to note that inhibition of the antiserum by sulfur mustard was negligible below a concentration of 5×10^{-6}M. This is in contrast with the results with CEMS and CEES in which antibody inhibition is noted at concentrations as

low as 5×10^{-7} M. This suggests that sulfur mustard is only partially saturating the two specific sites of the antibody until the concentration of 5×10^{-6}M is reached. The ELISA response from this point to higher concentrations is more sensitive to changing concentrations of sulfur mustard than those observed for CEES and CEMS. The greater inhibitory response to changing concentrations of sulfur mustard may be attributed to the dual valency of the antigen and subsequent intermolecular antibody binding, a phenomenon similar to a precipitin reaction involving a multivalent antigen.

Our work provides the first demonstration of the production of antibodies that bind sulfur mustard. A logical extension of this achievement is the production of a standard monoclonal anti-mustard antibody. A standard monoclonal antibody will have a significant role in future mustard research. Mustard antibodies can be employed to: (1) elucidate the mechanism of mustard injury, (2) immunodirect treatment and prophylaxis of mustard exposure, and (3) detect low levels of mustard deployment.

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