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CHARACTERIZATION OF IMMUNE RESPONSE TO MUSTARD GAS USING A NEW FLUOROIMMUNOASSAY*

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ABSTRACT

One way to confront chemical warfare in order to reduce their harmful effects, is to use the body's defense mechanisms. In order to do so firstly mustard gas (HD) was injected in an animal model in the form of an immunogen and the induced humoral immune response was investigated by two different immunological methods such as the Ouchterlony test and a new liquid phase fluoroimmunoassay.[7] It was proved that the animal model had produced specific antibodies against mustard gas which could specifically interact with the chemical gas.

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INTRODUCTION

Based on chemical and physical properties, mustard gas is considered a hapten. Landsteiner was the one who first coupled the small molecules to different proteins and synthesized a variety of conjugates which after injection could mount specific immune responses.[5]

In order to induce immune responses against mustard gas, which is considered a hapten, it was necessary to modify the hapten to an immunogen by covalently conjugating the small molecule to a large foreign protein carrier.

The vesicant agent (HD) can react with sulfhydryl and amino groups in different aminoacids under physiological conditions.[1] The reactions with SH and basic aminoacids are more rapid in comparison with NH_2 groups and acidic aminoacids. Mustard gas can react with different macromolecules via its halogen part, liberating a Cl^-ion, producing HCl after coupling with a H^+ion.[4] Ball and Northrop in 1942 showed that mustard gas could modify macromolecules after reaction with protein acidic groups. Davis and Ross showed that by addition of sulfur atoms, after the sulfur mustard reaction with different proteins, the alkalinity of treated proteins had been increased causing higher isoelectric points.[2] Fleming coupled mustard gas and its related compounds to different protein molecules and after injection in an animal model, characterized specific immune responses against different conjugates of mustard gas using the precipitation test.[3]

In this report, specific immune responses against mustard gas, after induction, were characterized by a liquid phase fluoroimmunoassay using a new synthesized fluorescent mustard gas.[7]

MATERIALS & METHODS

Fluorescent mustard gas conjugation

Fluorescent mustard gas was synthesized using a new liquid phase fluoroimmunoassay which was introduced in 1982.[6] One gram of AmNS (1-amino-4-naphthalene sulfonic acid) (Jenssen chemical) was dissolved in 100 mL distilled water and 147 microliters of mustard gas was added to it and reaction mixture was left in room temperature for at least four hours, keeping the pH between 6-7 using NaOH (2N).

Purification and characterization of AmNS-HD

The reaction mixture was centrifuged and the supernatant was then transferred to silica gel thin layer

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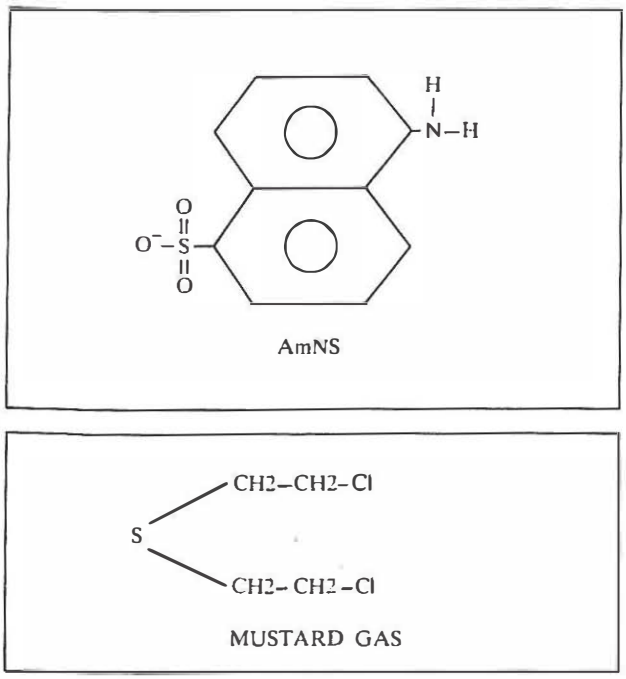

Figure 1. Thiodiglycol. Mustard Gas.

chromatograms using 22.5 mL butanol, 7.5 mL acetic acid and 1.8 m L distilled water as a solvent. Different bands were resolved (Fig. 4) and used for spectrophotometrical analysis such as max, excitation maximum and emission maximum analysis.

Fluoroimmunoassay

Synthesized AmNS-HD was used to search for specific antibodies produced against mustard gas using the new liquid phase-fluoro-immunoassay. A specified concentration of fluorescent mustard gas was added to a quartz cuvette and the final volume adjusted to 1.0 mL was with P B S (PH = 800). Emission spectra were scanned at 415 nm after excitation at 320 nm, using a fluorescence spectrophotometer (Hitachi MPF-4). Then a specified volume of the purified antimustardgas antiserum (either as gamma globulin or IgG fraction) was added to the mixture and emission spectra obtained by scanning.

Immunological specificity of the reactants in the fluoroimmunoassay was verified by modifying the fluorescence assay to a ligand inhibition (competitive) assay using non-fluorescent thiodiglycol (which is very similarto mustard gas) (Fig.1) as an inhibitor (I). In the inhibition assay, different concentrations of (I) were titrated with a constant amount of the other reagent. After each addition of (I), the relative fluorescence intensity was measured. Proper controls such as buffer, protein solution, etc. were also tested as inhibitors in a competition assay.

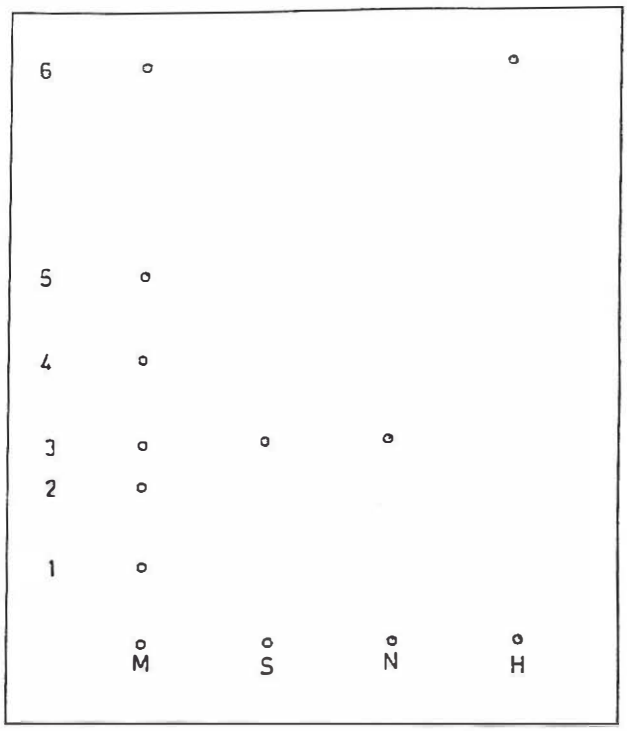

Figure 2. Silica gel TLC using butanal, acetic acid and water (22.5 and 1.8 ml) as the solvent system.
M : Refers to conjugation mixture supernatant after centrifugation
S : Refers to conjugation mixture precipitate after centrifugation
N : Refers to free AmNS in distilled water
H : Refers to free mustard gas

RESULTS

Characterization of fluorescent conjugate

The results of silica gel thin layer chromatography is shown in Figure2. As it is indicated in this figure, there are four bands, (Bands No. 1, 2, 4, and 5) which differ from free AmNS and free mustard gas.

These bands show possible and different conjugation between AmNS and mustard gas.

All different bands were resolved and used for

Table I . Characterization obtained by TLC

Fraction No.	RF	Color of Bands	λ max[a]	Excitation[b] max.	Emission[c] max.
1	0.07	Yellow	329	310	420
2	0.17	"	329	828	420
3	0.45	Orange	317	326	415
4	0.15	Yellow	327	330	415
5	0.62	"	327	330	415
6	0.88	Colorless	191	--	--

a : λ max was measured by UV-260 (Shimadzu Corp.)
b: Excitation max. was measured by spectrophotometer fluorescence (Hitachi MPF-4)
C: Emission max. was measured by spectrophotometer fluorescence (Hitachi MPF-4).

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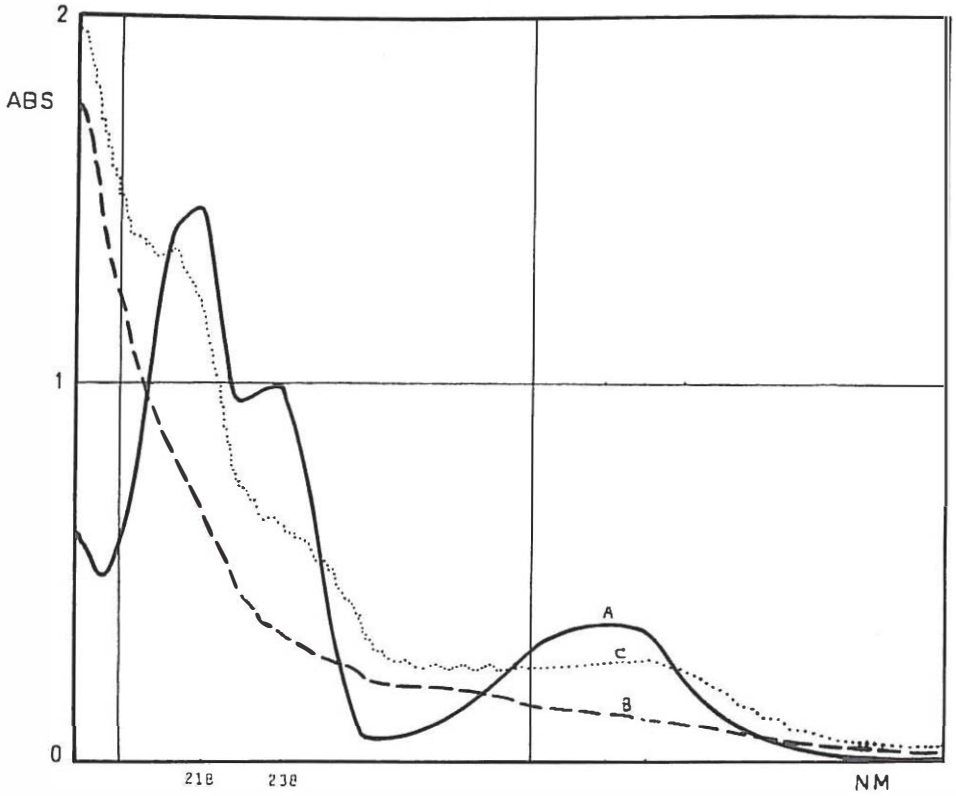

Figure 3. Comparative max analysis using spectrophotometer UV 2e0 (Shimadzu)
Curve A : free AmNS
Curve B : free mustard gas
Curve C : Conjugate of AmNS-mustard gas (band No.2)

spectrophotometrical analysis (Table I). Figure3 shows a comparative max.analysis between AmNS (curve A), free mustard gas (curve B) and band No. 2 (possible conjugate) (curve C).

Fluoroimmunoassay

Bands No. 1,2,4 and 5 which differ from free AmNS and mustard gas and showed possible conjugates of AmNS-HD, were mixed. The excitation and emission max. of the mixture were around 320 nm and 415 nm respectively.

Upon binding of the fluorescent mustard gas with induced antimustard gas antibody (verified in the Ouchterlony test), fluorescent enhancement was observed.[7] The degree of fluorescent enhancement was directly proportional to antibody concentration (Fig.4). Anti-HD antisera used in fluoroimmunoassay were tested as either enriched γ-globulin fractions or as pure IgG. Fluorescence enhancement was indicative of a highly specific reaction, since normal γ-globulin or normal IgG fractions, normal rabbit serum and B.S.A. had no effect on the fluorescence of AmNS-HD conjugate mixture.

Fluorescence enhancement which resulted from the specific interaction between antibody and the mustard gas moiety within the conjugate mixture was inhibitable by addition of non-fluorescent thiodiglycol, a similar compound to mustard gas (Fig. 1) as an inhibitor (Fig .5).

DISCUSSION

In order to perform a new liquid phase fluoroimmunoassay, a fluorescent mustard gas was synthesized using AmNS as a fluorescent dye.[6] Both TLC and spectrophotometrical analysis indicated that there were conjugates of AmNS-HD in the reaction mixture (Figure 2 and 3). The success of conjugation was also approved by mass spectroscopy (data not shown).

Upon specific interaction between AmNS-HD conjugates and homologous antibody populations (activity verified in the Ouchterlony test), fluorescence enhancement was observed. The specificity of the enhancement was approved by using proper controls such as normal rabbit sera, BSA, etc.

Immunological specificity of the interaction between the antibody population and the fluorescent mustard gas was further established by ligand inhibition studies using non-fluorescent thiodiglycol as inhibitor (Fig.5).

Yarbrough suggested that fluorescence enhance-

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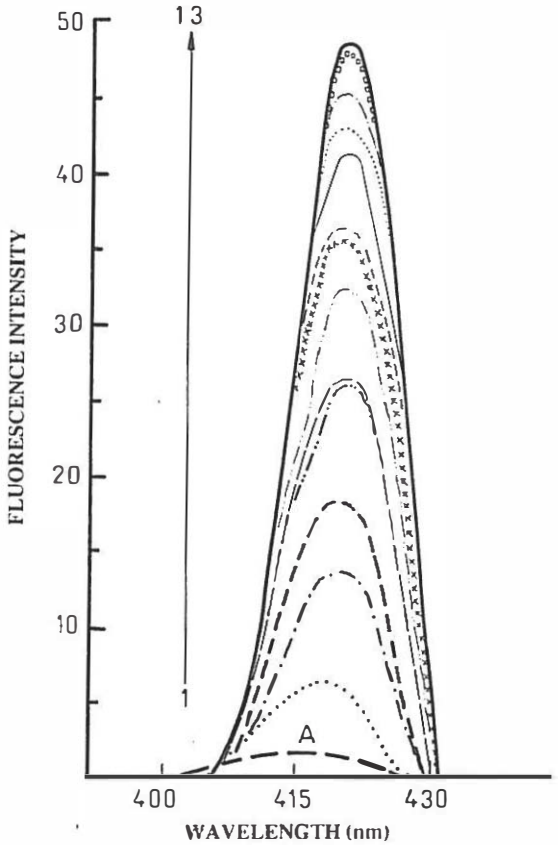

Figure 4. Titration of fluorescent conjugate with increasing concentration of specific antibody population. A specified concentration of AmNS - mustard gas conjugate mixture (curve A) was titrated with increasing concentration of purified rabbit antimustard gas IgG (Curves No. 1-13).

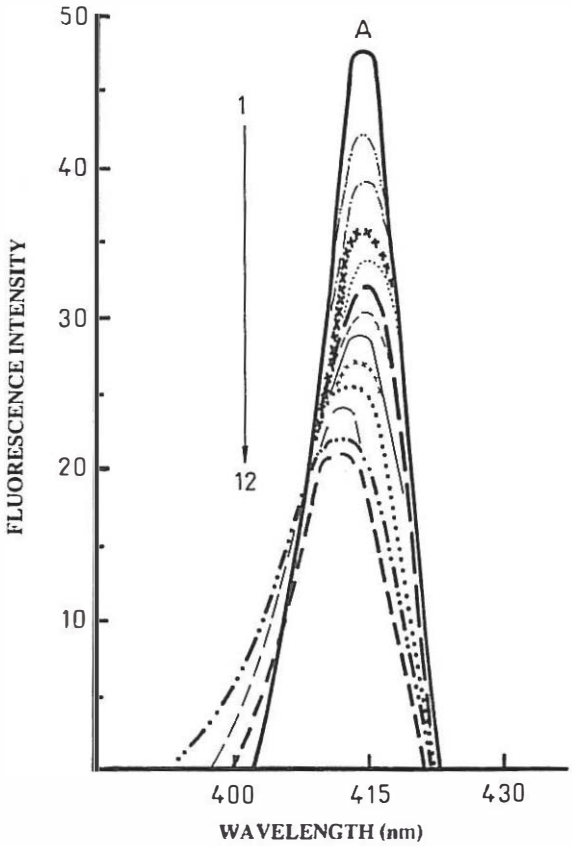

Figure 5 .Ligand inhibition assay Curve A : relative fluorescence intensityof AmNS-mustard gas conjugate in the presence of purified specific IgG.
Curves No. 1-12 : Relative fluorescence intensity of curve A in the presence of increasing concentration of thiodiglycol (inhibitor).

ment observed with AmNS ligands may be due to the resonance energy transfer from tryptophan moieties within protein, to AmNS ligand which possess excitation maxima corresponding to the region in which tryptophan molecules emit fluorescence.[8]

In summary, this study, indicated that it was possible to induce specific humoral responses against mustard gas and the specificity of the induced antibody population was verified with both the Ouchterlony test and the new fluoroimmunoassay which is a sensitive and useful method for studying primary interactions between a hapten and antibody molecule.

Finally, the results of this study indicate that it may be possible to manufacture new vaccines against chemical war gases or to produce therapeutic sera especially via monoclonal hybridoma technology.

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Notes: The results of this research weresubmittedas an abstract in the first international medical congress on chemical warfare agents in Iran, Mashhad University of Medical Sciences (13-16 June 1988).

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PROPOSAL FOR SURGICAL CLASSIFICATION OF CHRONIC SUPPURATIVE OTITIS MEDIA

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ABSTRACT

The authors presented a proposal for surgical classification of chronic suppurative otitis media which may be used for evaluating the surgical outcome of the disease.

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INTRODUCTION

The purpose of our study is to present a proposal for surgical classification of chronic suppurative otitis media which could be applied for evaluation of success of surgical treatment basing this on preoperative and operative objective condition.[1]

MATERIALS AND METHODS

We have analysed 1261 medical records of patients surgically treated in our clinic for chronic suppurative otitis media. All the significant factors affecting the outcome of surgery were systematized and classified including age of the patient, contents and condition of the external auditory canal, localization of perforation on the tympanic membrane, precise hearing assessment, localization of cholesteatoma, ossicular damage, localization of tympanosclerosis, occurrence of possible otogenic complications and the presence of some general diseases or disorders influencing the clinical course and success of surgical treatment of chronic suppurative otitis media. All these elements were coded for comprehensive presentation.

RESULTS

The resulting classification was formulated and proposed as follows:

I. Age
1. < 10 years
2. 11-20
3. 21-40
4. 41-60
5. > 60 years

OTOSCOPIC FINDINGS

II. External auditory canal
1. Pus
2. Polyp
3. Granulation
4. Other

III. Tympanic membrane (perforation)

Marginal	Central
1. Attic	a) Anterior
2. Posterior	b) Posterior
3. Attic and posterior	c) Inferior
4. Large	d) Large
5. Other	e) Other

IV. Audiometric investigations of hearing loss (deafness)

Type	Degree	"Gap"
1. Conductive	a) 20	A. 10 dB
2. Mixed	b) 21-40	B. 11-20
3. Sensorineural	c) 41-60	C. 21-30
4. Profound	d) 60 dB	D. 31-40 dB

SURGICAL FINDINCS

V. Cholesteatoma (localisation)
1. Attic
2. Posterior part of the cavum
3. Attic and posterior part of the cavum
4. Other

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VI. Ossicular damage
1. Stapes
2. Incus
3. Incus and stapes
4. Incus and malleus
5. Incus, stapes and malleus
a) Fixation of the footplate
b) Absence of the footplate

VII. Tympanosclerosis (localisation)
1. Tympanic membrane
2. Stapes
3. Attic
4. Enlarged
5. Other

VIII. Mastoid process (damage)
1. Pneumatized and osteitis
2. Non-pneumatized and osteitis
3. Other

IX. Eustachian tube
1. Recanalized
2. Non-passable

X. Otogenic complications
1. Paralysis facial nerve
2. Fistula of the labyrinth
3. Labyrinthitis
4. Meningitis
5. Sepsis
6. Extradural abscess
7. Subdural abscess
8. Cerebellar abscess
9. Cerebral abscess

XI. General diseases
1. Allergy
2. Diabetes mellitus
3. Upper respiratory infection
4. Immunologic disorders
5. Other

Example of the surgical classification:
I3- patient is between 21-40 years old,
II1- Pus is present in the external auditory canal,
III2- Posterior marginal perforation of the tympanic membrane,
IV2cB- Mixed deafness between 41-60 d B with cochlear reserve between 11-20 dB,
V2- Cholesteatoma in the posterior parts of the cavum tympani,
VI3- Incus and stapes are damaged,
VII1- Tympanosclerosis is localized on the tympanic memnrane,
VIII2- Mastoid process is non-pneumatized and osteitic,
XI2- Patient is suffering from diabetes mellitus
Formula is: I3, II1, III2, IV2cB, V2, VI2, VII1, VIII2, XI2.

DISCUSSION

According to our knowledge, there is no generally accepted surgical classification of chronic otitis media. Thus it is very difficult to estimate the success of surgical treatment of chronic otitis media and to compare the results obtained using different methods by various authors. Pratt has proposed the surgical classification for chronic otitis media including types of surgical methods.[2]

Our proposed classification for chronic suppurative otitis media does not include surgical methods of treatment, because it is paractically impossible to classify surgical methods, since a majority of otolaryngologists combine different types and also add their own modifications.

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