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The Allergenicity of Complex Cations

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A homologous series of eight quaternary ammonium salts (quats) were used as complex cations in a survey of contact hypersensitivity in guinea pigs. Two of the quats tested were found to be strong allergens which was due to stable association with membrane lipids at the surface of epidermal cells. This surface complexation reaction was studied in detail by using a spin-labelled quat of intermediate allergenicity. Electron spin resonance was used to show that stable "ion pairs" are formed between membrane receptor sites and the two strong allergens. Information was obtained on the specificity and kinetics of immunogenic complex formation as well as on the position and orientation of these haptens on epidermal receptor sites *in vivo*.

A large number of chemical substances are known to cause irritant and allergic contact dermatitis in humans (1), and many of these skin reactions can be duplicated in experimental animals. The guinea pig has been the animal of choice for most of these experiments due to the similarity between guinea pig and human skin. Delayed hypersensitivity skin reactions of this kind occur as a result of the body's defense against those foreign substances which are capable of penetrating the outer horny layer of the skin to form immunogenic complexes which can stimulate the immune response. An understanding of such skin reactions is complicated by the great variety of substances which are capable of causing contact dermatitis. Reactive compounds include reagents which form covalent complexes with cell surface proteins (1); metal ions which form a variety of coordination complexes with cell surface ligands (2), and simple cations which are capable of forming stable "ion-pairs". Both the covalent reagents and metal ions lack specificity in the complexes which they form, and therefore many cell surface groups on membrane proteins react in addition to those receptor sites which produce antigens. This lack of specificity in the random labelling of epidermal cell membrane

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proteins has made it difficult to identify both the structure and the location of the original contact antigenic complex (3).

We chose to study the quaternary ammonium compounds (quats) as possible inducers of contact dermatitis for two reasons: (1) these substances are in widespread use as disinfectants, and they are often applied to the skin directly in cosmetics and in other medications (4-15), and (2) this homologous series of compounds has a uniform structure with a single active site of a quaternary ammonium cation which can form ionic complexes with negatively charged groups (16).

MATERIALS AND METHODS

The following hydrophilic quats were chosen for this study: Quat 1 (acylaminopropyl-dimethyl-2-hydroxyethyl ammonium chloride); Quat 3 (alkyl-benzyl-di-2-hydroxyethyl ammonium chloride); Quat 72 (alkyl-di-polyethoxymethyl ammonium chloride). Hydrophobic quats selected for this study included: Quat 4 (alkyl-benzyl-dimethyl ammonium chloride); Quat 8 (alkyl-dimethyl-ethyl-benzyl ammonium chloride); Quat 16 (aryl-trimethyl-ammonium chloride); Quat 20 (benzyl-dimethyl-2-methyl-4 (1,1,3,3, tetramethyl-butyl-phenoxy-ethoxyethyl ammonium chloride); Quat 42 (alkyl-pyridinium chloride). (Obtained from Lonza Chemical Co., New Jersey, and from Aldrich.)

Preparation of Spin-Labelled Quat.

1.0 gr of dimethybenzylamine (Aldrich) was dissolved in 50 ml of dry methanol and 0.8 g 4-bromoacetamido 2,2,6,6,piperidine-N-oxyl was added. The reaction mixture was refluxed for one hour, cooled down and 250 ml of dry diethylether added. Pale yellow crystals were formed immediately. They were filtered off, washed with diethylether and dried over P_2O_5. The 360 MHz ^1H nuclear magnetic resonance spectrum of this spin-labelled quat confirmed the synthesis of acetamido-2,2,6,6 tetramethylpiperidine-N-oxyl-benzyl-dimethyl ammonium bromide. Electron spin resonance experiments were performed on a Varian E4 spectrometer at 25°C.

The EPR spectrum of this spin-labelled quat gave ^aN values of 17.2 gauss as compared with 17.0 gauss for the free reagent 4 bromoacetamido 2,2,6,6,, tetramethylpiperidine-N-oxyl (ATTEMPTO). Line widths of 1.87, 1.87 and 2.20 gauss were recorded with the high field line being broadest. This high field line decreased in intensity and broadens further upon complexation to cell surfaces.

RESULTS AND DISCUSSION

A preliminary systematic investigation was made on the role of quaternary ammonium compounds in initiating the delayed hypersensitivity reaction in guinea pigs (16,17). Systematic studies had not been done in relating the structure of a homologous series of quats to the delayed hypersensitivity response. Using the Freunds Complete Adjuvant Test (FCAT) (18) and the Guinea Pig Maximization Test (GPMT) (19) with groups of ten and twenty animals, respectively, we found that only two of eight quats functioned as strong allergens (17). These two compound quats (16 and 20) were structurally similar.

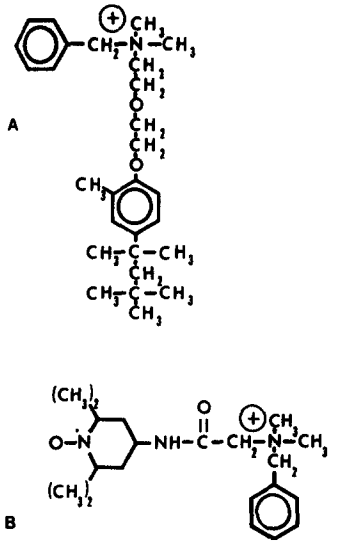


Figure 1: (A) The structure of quat 20 (Benzyl dimethyl-2-methyl-4 (1,1,3,3) tetramethyl-butyl phenoxyethoxyethyl ammonium chloride). Note the presence of a long hydrophobic tail which confers strong allergenicity. (B) The structure of the spin labelled quat.

(Quat 20 = Benzyl-dimethyl-2-methyl-4 (1,1,3,3) tetramethyl-butyl-phenoxyethoxyethyl ammonium chloride. Quat 16 = aryl-trimethyl ammonium chloride). For quats 16 and 20, one of the four covalently attached groups to the quaternary ammonium ion, has a long hydrocarbon tail capable of partitioning into lipid bilayers (Figure 1). Quats with smaller hydrophobic groups (e.g. benzyl) were found to be weak allergens, and those with hydrophilic groups were not active in the standard FCAT and GPMT tests (17). These initial experiments were indicative of the importance of quat-lipid interactions in presenting the quat-cation to cell surface receptor sites in the process of immunogenic complex-formation. To test this hypothesis of stable "ion-pair" formation at the surface of epidermal cells, we synthesized a spin-labelled quat (20) (Fig. 1) and found intermediate allergenicity. Next we took advantage of the properties of this molecule to examine the specificity of the receptor-quat interaction. Spin-labelled quat was applied topically to guinea pig skin and biopsies were taken at different times to follow the fate of the label in the epidermis. To this end, these biopsies were first washed with isotonic saline (0.9% NaCl) to remove any residual label remaining in the dead horny layer. Therefore only those spin-label molecules which react with live epi-

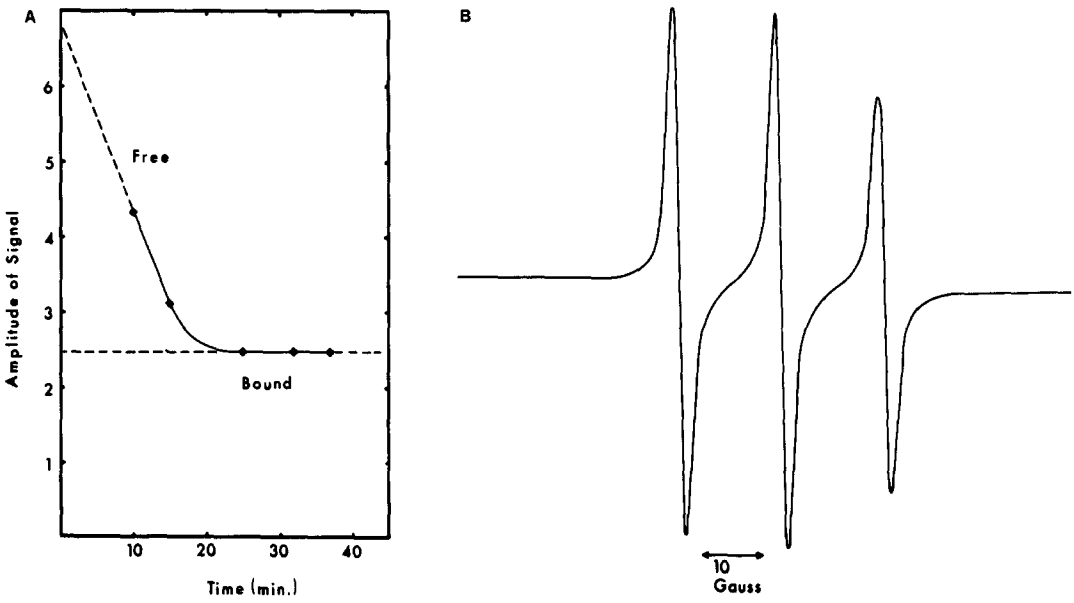


Figure 2: (A) The enzymatic reduction of free spin-labelled quat by guinea pig epidermis *in vivo*, the residual signal represents bound spin-labelled quat. (B) The EPR spectrum of bound spin-labelled quat on guinea pig epidermis. This bound EPR spectrum shows line broadening from 11 gauss (free spin-label) to 18 gauss (bound spin-label) in the central resonance. The high field line is broadened and decreases in amplitude which is indicative of surface complexation with some freedom for rotation of the spin-labelled group.

dermis were monitored by electron spin resonance spectroscopy (EPR). It was quickly recognized that guinea pig epidermis contains an extremely reactive nitroxide reductase which can reduce uncomplexed spin-label (Fig. 2a). This catalytic activity was shown to be enzymatic because the reduction of spin-label was inhibited upon topical application of thioenzyme inhibitors. The time course for the reduction of the free radical on the spin-labelled quat is presented in (Fig. 2a). After this enzymatic conversion of the paramagnetic nitroxide, to the reduced secondary amine product, the remaining nitroxide EPR spectrum represents only cell-surface bound spin-labelled quat (Fig. 2b).

This complexed spin-label appears to be inaccessible to the active site of the nitroxide reductase *in vivo*. The resulting bound nitroxide radical signal has spectral parameters consistent with attachment of the molecule at the membrane surface with some

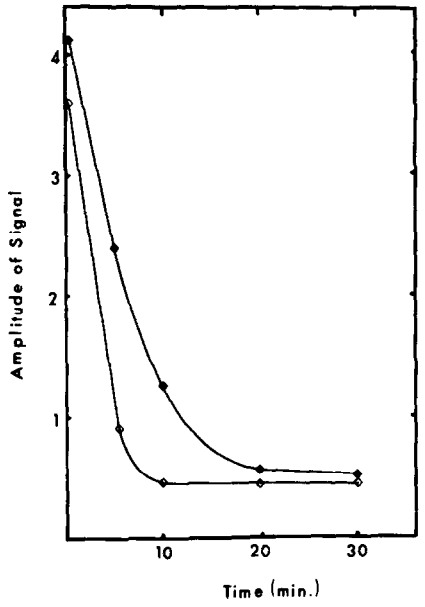
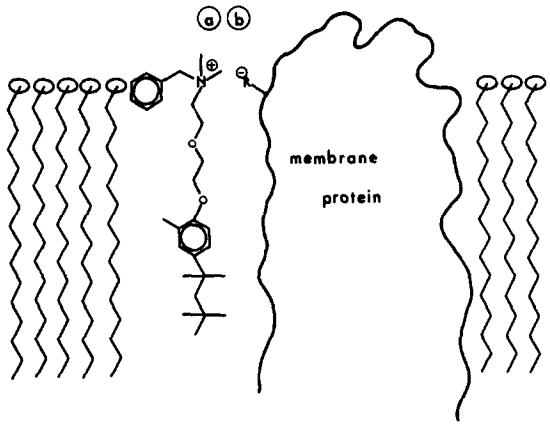


Figure 3: The rate of exchange of bound spin-labelled quat (intermediate allergenicity) with strongly allergenic quats 16 (◆-◆) and 20 (◇-◇).

freedom for rotation of the nitroxide functional group from its point of attachment on the cell membrane (21,22) (Fig. 2b). There was no evidence for strong immobilization of the radical due to membrane/lipid or membrane/protein partitioning (23,24). We took advantage of this technique of surface receptor site labelling to determine the ionic nature of complex formation. This was accomplished by measuring the exchange of bound spin-labelled quat with the two stronger allergens Quat 16 and Quat 20. Both of these quats compete effectively for spin-label receptor sites, indicating that thermodynamically more stable "ion-pairs" are formed with the stronger allergens on the same receptor sites. The kinetics for these exchange reactions demonstrated a fast exchange of greater than 90% of surface complexed spin-labelled cations (Fig. 3). The possibility of some kind of metabolic alteration of the few residual non-exchanged molecules leading to a possible covalent attachment was ruled out because complete exchange from epidermal tissue was accomplished with trichloroacetic acid. This experiment indicates that immunogenic complex formation occurs only through the stabilization of "ion-pairs" in the guinea pig epidermis (Scheme 1).

Recent work from Japan (13), using the strong allergen Trinitrochlorobenzene (TNCB), has shown that the intact epidermal cell is not necessary in presenting the



Scheme 1: Proposed structure for the stable "ion-pairs" in the formation of antigens of ionic origin: (a) membrane complexed quat 20 (b) anionic receptor site which forms a stable antigen with this strong allergen.

initial immunogenic complex to the immune system. Model liposomes containing extracts of TNCB sensitized epidermal cell proteins were shown to illicit contact hypersensitivity. This work represents the first example of a chemical model system containing an intact and active immunogenic complex. Unfortunately, all of the cell membrane proteins react with TNCB to produce a large number of labelled proteins, and this makes identification of the immunogenic complex difficult in such a mixture.

In our study we have found that quats can be much more selective haptens which react rapidly with receptor sites. We have obtained information on the location and on the distance of receptor sites on the surface of epidermal cells in vivo by the direct technique of spin-labelling. The next phase of this research is the determination of how these "ion-pairs" present their antigenic properties to the pathway which imprints the T-lymphocytes.

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