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The specificity of murine polyclonal and monoclonal antibodies to the haptenic drug chlorhexidine induced by chlorine-generated chlorhexidine-protein conjugates

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SUMMARY

Polyclonal and monoclonal antibodies to the antibacterial agent chlorhexidine (1,1′-hexamethylene bis [5-(*p*-chlorophenyl)]biguanide, mol. wt = 505) were raised using a chlorine-generated N-chloro chlorhexidine-keyhole limpet haemocyanin (NCC-KLH) conjugate as the immunogen. Antibodies were detected by ELISA, using a semi-chlorhexidine derivative conjugated to human serum albumin (SC-HSA) as the antigen. Free chlorhexidine could completely inhibit both polyclonal and monoclonal antibody binding to SC-HSA. Direct binding and inhibition ELISA studies revealed that the N-chlorination of chlorhexidine does not significantly alter its specificity as an immunogen or antigen and that chlorhexidine has two identical epitopes. Each epitope consists of the *p*-chlorophenyl biguanide structure of which the terminal *p*-chlorophenyl group appears to be immunodominant. Chlorhexidine is, therefore, a symmetrical divalent hapten and this implies that it may be capable of eliciting immediate hypersensitivity reactions by divalent interaction with antibodies induced by chlorine-generated N-chloro-chlorhexidine-protein immunogens. The clinical significance of these findings is discussed.

Keywords chlorhexidine drug hapten ELISA monoclonal antibody

INTRODUCTION

The immunagenicity of haptenic drugs and chemicals depends largely on their capacity to bind covalently to macromolecular carriers, usually proteins (Landsteiner, 1945; Mitchison, 1971). In this respect, haptenic drugs may be divided into three main categories. Those which intrinsically possess functional groups capable of undergoing covalent protein binding, e.g. the penicillins (Dudley, Butler & Johnson, 1971), those which are converted *in vivo* into protein-binding metabolites, e.g. practolol (Amos, Lake & Atkinson, 1977) and those which may be converted by external, environmental agents into covalently-binding derivatives. Examples of drugs acting in this latter category are difficult to cite and equally difficult to predict since there are many possible converting agents: e.g. enzymes or reactive chemicals. Recently, however, we have described a murine model which has facilitated studies into the immunogenicity of the antibacterial agent chlorhexidine (1,1′-hexamethylene bis [5-(p-chlorophenyl)] biguanide) and its chemically-converted derivative, N-chloro chlorhexidine (Layton, Stanworth & Amos, 1986; 1987). This model is clinically relevant since chlorhexidine-specific IgE-mediated hypersensitivity reactions in humans

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have recently been described (Ohtoshi *et al.*, 1986). Under normal conditions chlorhexidine interacts only electrostatically with proteins, by salt bridging with acidic groups, and is non-immunogenic. When diluted in water containing as little as 25 nM chlorine (approx. 2 ppm), however, the secondary amide groups are attacked and N-chloro biguanide derivatives form which will covalently conjugate with added protein, probably via nucleophilic groups on methionine, tyrosine, lysine and tryptophan residues. The injection of such conjugates (with alum adjuvant) into mice leads to the production of IgE and IgG antibodies which recognize and bind native chlorhexidine. In this present study, we have attempted to define the valency and fine specificity of epitope recognition by murine polyclonal and monoclonal IgG antibodies to chlorhexidine, raised using an N-chloro chlorhexidine-keyhole limpet haemocyanin immunogen.

MATERIALS AND METHODS

Animals. Female Balb/c mice, aged 5–10 weeks were obtained from the animal breeding unit, Birmingham University Medical School.

ELISA materials. Falcon microtitre plates (Microtest III) were obtained from Becton-Dickinson Ltd, Oxford, UK, hydrogen peroxide (100 vols, 30% w/v) from BDH Chemicals Ltd, Dorset, UK and orthophenylene diamine dihydrochloride (OPD) from Sigma Chemical Company, Dorset, UK. All antisera and horseradish peroxidase conjugated antisera were obtained from Nordic Immunoglobulins Ltd, Maidenhead, UK.

Reagents. All chlorhexidine reagents and derivatives (see Fig. 1) were kindly supplied by ICI Pharmaceuticals PLC, Macclesfield, Cheshire, UK. *Bordetella pertussis* vaccine was obtained from the Wellcome Foundation Ltd, London and aluminium hydroxide 1·3% $Al(OH)_3$ (Alhydrogel) from Superfos export company A/S, Copenhagen, Denmark. Keyhole limpet haemocyanin (KLH) ovalbumin (Oval) and human serum albumin (Fraction V, HSA) were obtained from Sigma. All other chemicals were analar grade from BDH or Sigma.

Preparation of semi-chlorhexidine-protein (SC-protein) conjugates. The SC-HSA ELISA antigen and SC-OVAL immunogen were kindly prepared by S. Walsh (ICI Central Toxicology Labs) and had molar ratios of 16:1 and 36:1 (SC:protein) respectively. The conjugates were prepared as follows. ^{14}C-labelled succinic anhydride was prepared by refluxing ^{14}C-labelled succinic acid (Amersham plc, Bucks, UK) with acetic anhydride containing 'cold' succinic anhydride. The ^{14}C-labelled succinic anhydride was then stirred with 1-(*p*-chlorophenyl) biguanide hexylamine dihydrochloride (ICI plc) using dimethyl amino pyridine to catalyse the acylation. The resulting ^{14}C-labelled semi-chlorhexidine (SC) succinamic acid derivative (see Fig. 1) was then reacted with dicyclohexyl carbodiimide and N-hydroxylsuccinimide (NHS) to form the reactive SC-NHS intermediate which was then added to either HSA or OVAL. The conjugates were then desalted on a Sephadex G25 column (Pharmacia, UK).

ELISA procedure for detecting antibodies to chlorhexidine and KLH. Microtitre plates were coated by overnight incubation at 4°C with either the SC-HSA conjugate or KLH (both 10 μg/ml protein in pH 9·6 carbonate buffer (0·05 M), 100 μl per well). These concentrations were optimal. Coated plates were washed in phosphate buffered saline containing 0·05% Tween 20 (PBS/T) and then sera or tissue culture supernatants were added (100 μl per well). Samples were incubated for 2 h at room temperature (RT) then the plates were washed, and aliquots (100 μl) of a goat anti-mouse IgG (Fc) antiserum, diluted 1/500 in PBS/T, were added to each well. After 2 h at RT, plates were washed and a rabbit anti-goat IgG horseradish peroxidase conjugate, diluted 1/2000 in PBS/T, was added (100 μl per well). Plates were then re-incubated for 2 h at RT before washing. Freshly prepared substrate (OPD, 20 mg; 30% w/v hydrogen peroxide, 250 μl; in 50 ml of distilled water) was added (100 μl per well). After 15 min, the reaction was stopped by the addition of 25 μl H_2SO_4 (25%) to each well. Plates were read at 492 nm using a Multiskan MC plate reader interfaced to a BBC Acorn model B computer. Control mean OD values (normal mouse serum or tissue culture medium) were subtracted from all other values. Samples from individual experiments were assayed on the same plate to allow direct comparison of results. Where appropriate, data were subjected to Student's test.

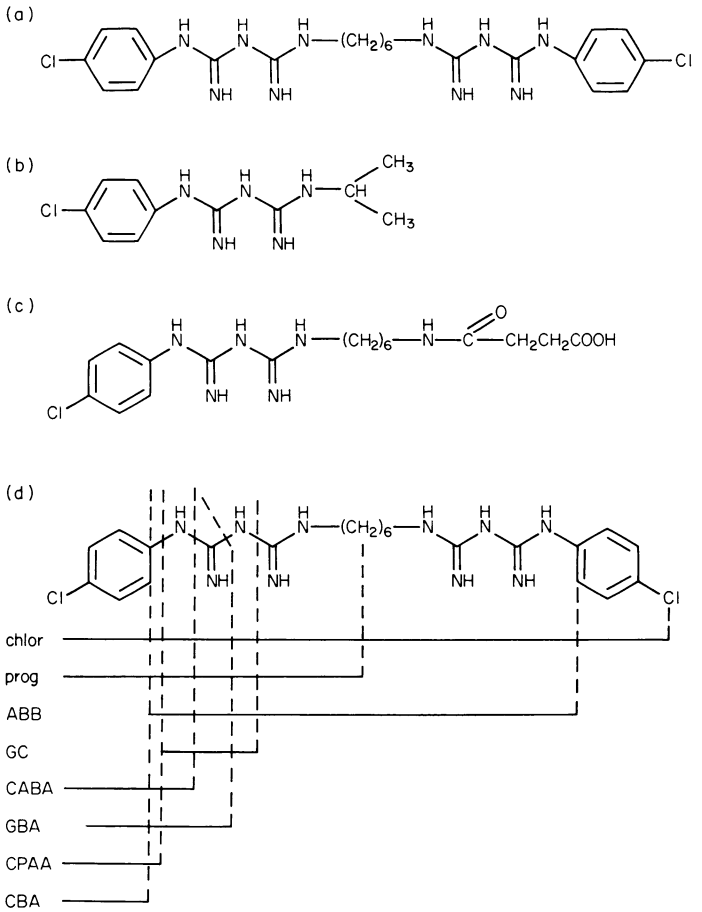

Fig. 1. (a) 1.1′-hexamethylene bis [5-(*p*-chlorophenyl) biguanide], chlorhexidine. (b) 1-(*p*-chlorophenyl) 5-isopropyl biguanide, proguanil. (c) 1-(*p*-chlorophenyl) 5-hexamethylene succinamic acid biguanide, semi-chlorhexidine. (d) Chlorhexidine hapten analogues: chlorhexidine (chlor), proguanil (prog.), aliphatic bis biguanide (ABB). Compounds with structures in common with chlorhexidine: guanidine carbonate (GC), 2-chloro 4-amino benzoic acid (CABA), 4-guanidino benzoic acid (GBA), *p*-chlorophenyl acetic acid (CPAA), 3-chlorobenzoic acid (CBA). Solid lines show common structures.

ELISA inhibition assay. Aliquots of pooled mouse antiserum (diluted 1/10 or 1/20 in PBS/T) or hybridoma culture supernatant (diluted 1/2) were pre-incubated with an equal volume of a range of concentrations of inhibitors for 1 h at RT. The samples were then centrifuged at 600 *g* for 10 min and the supernatants tested as described above. The maximum concentration of inhibitor employed was either 200 μM (at a serum dilution of 1/20), 400 μM, (serum diluted 1/10) or 1 mM (culture supernatant diluted 1/2). These concentrations were selected to minimize non-specific serum protein precipitation by chlorhexidine and the aliphatic bis biguanide. Where precipitation did occur it did not involve immunoglobulins. The inhibitors used are described in Table 1 and their structures in common with chlorhexidine shown in Figure 1.

Preparation of the N-chloro chlorhexidine-KLH immunogen. Chlorine water was prepared by bubbling chlorine gas through distilled water for 2 min. The concentration of chlorine was determined by volumetric analysis. Chlorhexidine digluconate (2 mg/ml in distilled water) was N-chlorinated by the dropwise addition of an equal volume (1 ml) of 1 mM chlorine water. After 10

Table 1. Chlorhexidine, derivatives and inhibitors

Chemical name	Common name	Abbreviation	Mol. wt
1,1′-hexamethylene bis-[5-(*p*-chlorophenyl) biguanide]	Chlorhexidine digluconate	Chlor	505
1-(*p*-chlorophenyl) 5-isopropyl biguanide $(HCl)_2$	Proguanil	Prog	254
1,1′-hexamethylene bis-[5- (lactate) biguanide]	Aliphatic bis biguanide	ABB	461
4-guanidinobenzoic acid (HCl)	—	GBA	179
2-chloro 4-aminobenzoic acid	—	CABA	171
3-chlorobenzoic acid	—	CBA	156
p-chlorophenyl acetic acid	—	CPAA	170
Guanidine carbonate	—	GC	180

min, 2 ml of KLH (100 μg/ml in distilled water) was added slowly. No attempt was made to separate free from bound chlorhexidine at this stage. However, in an identical preparation, using radiolabelled (^{14}C) chlorhexidine (specific activity = 18·3 μCi/mg, ICI plc) the extent of covalent binding of chlorhexidine to KLH was established by precipitating the KLH protein with 0·8 N perchloric acid (PCA), and washing the precipitate three times with isotopically 'cold' chlorhexidine digluconate (1 mg/ml) to remove free chlorhexidine. The radioactivity in the washed protein deposits and in the original supernatants was measured by mixing samples (200 μl) with 2 ml of scintillation fluid (Fisofluor. Fison Ltd) and counting for 2 min in a β-counter (Beckman LS 7500). The average molar ratio of chlorhexidine: KLH (molecular weights = 505 and 3×10^6, respectively) was calculated as 78:1. An N-chloro chlorhexidine-HSA (NCC-HSA) ELISA antigen was also prepared, using the conditions described above, and free chlorhexidine was removed by the PCA procedure. The average molar ratio of chlorhexidine: HSA (molecular weight of HSA = 68,000) was calculated as 2·6:1).

Immunizations. Prior to injection, 1 ml of alum (13 mg) was added to the NCC-KLH preparation (4 ml) described above. The alum-absorbed material was washed once in distilled water to remove free chlorhexidine and resuspended to 5 ml with PBS. A group of eight mice were injected intraperitoneally (i.p) with 0·1 ml NCC-KLH in alum (NCC = 50 ng, KLH = 4 μg/mouse) and 0·1 ml of BP vaccine. On days 22 and 35, mice were injected with 0·2 ml NCC-KLH in alum plus 0·1 ml of BP vaccine. On day 40, the mice were killed and blood taken by cardiac puncture. Sera were pooled and stored at −20°C. until required. Spleens were aseptically-removed, following exsanguination, for hybridoma preparation. A separate group of eight mice were injected i.p. with the SC-OVAL conjugate in alum (4 μg/mouse) with 0·1 ml of BP vaccine, on days 0 and 22. The mice were killed on day 30 and the serum samples obtained were pooled and stored, as above.

Somatic cell fusion and hybridoma screening. Hybridoma cell lines were prepared by a modification of the method of Kohler & Milstein (1975). Briefly, 10^7 X63-Ag8-653 mouse myeloma cells (Kearney *et al.*, 1979) were fused with 10^8 immune spleen cells, from NCC-KLH injected mice, in the presence of 40% polyethylene glycol (PEG 1450). The cells were washed in RPMI 1640 (Gibco Ltd) and cultured in 96-well culture plates (Falcon, Becton-Dickinson Ltd) in RPMI containing 20% fetal calf serum (FCS, Gibco Ltd) hypoxanthine, aminopterin and thymidine (HAT medium). On day 15, the supernatants (diluted 1/5) from wells showing visible clones were tested for antibodies to chlorhexidine and KLH by ELISA using a goat anti-mouse Ig's antiserum (diluted 1/1000) as the first antibody. Hybridoma cells from positive wells were subcloned by limiting dilution. Isotype analysis of monoclonal antibodies were performed by ELISA, using class and subclass-specific goat antisera. Hybridoma cell lines were maintained in RPMI containing 15% FCS.

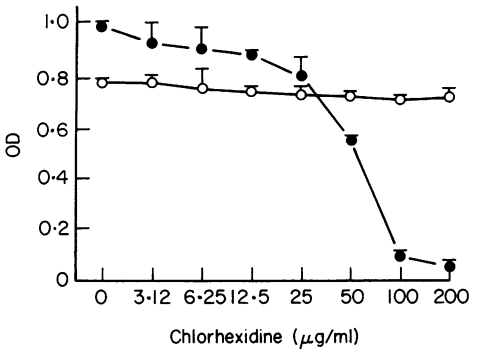

Fig. 2. Chlorhexidine inhibition ELISA. IgG anti-chlorhexidine (●) and anti-KLH (○) levels in a serum pool (1/80 final dilution) from NCC-KLH immunized mice. Mean OD of triplicates +1 standard deviation (SD).

RESULTS

Hapten specificity of serum IgG antibodies induced by N-chloro chlorhexidine-KLH (NCC-KLH). Aliquots of the anti-NCC-KLH serum pool (diluted 1/10) were incubated with aliquots of a range of concentrations of chlorhexidine and the supernatants tested for IgG antibodies to chlorhexidine and KLH. As can be seen from Fig. 2, chlorhexidine inhibited IgG antibody binding to the SC-HSA ELISA antigen, but did not affect binding in the IgG anti-KLH assay. Aliquots of the same serum pool (diluted 1/20) were then incubated with a range of concentrations of various substances (see Table 1) possessing structural features in common with chlorhexidine, as shown in Fig. 1. The results of the ELISA inhibition assays are shown in Fig. 3a. IgG anti-chlorhexidine antibody binding was inhibited only by chlorhexidine itself, proguanil (half the chlorhexidine molecule) and the highest concentration (200 μM) of the aliphatic bis biguanide, which has aliphatic lactate 'end' groups in place of the *p*-chlorphenyl groups of chlorhexidine. The concentrations of chlorhexidine and proguanil which gave 50% inhibition of binding were 28 μM and 182 μM, respectively. The aliphatic bis biguanide inhibited binding by only 20% (significance $P < 0{\cdot}05$) at 200 μM.

Pooled sera from groups of mice injected with either NCC-KLH (three injections) or SC-OVAL (two injections) were assayed for IgG antibodies on microtitre plates coated with either SC-HSA (molar ratio = 16:1), NCC-HSA (molar ratio = 2·6:1) or HSA alone. As Fig. 4 shows, both the SC-OVAL and NCC-KLH conjugates induced IgG antibodies which recognized and bound to epitopes on SC-HSA, NCC-HSA but not to HSA alone. As would be expected, the triple-injected NCC-HSA conjugate induced higher levels of antibody synthesis than the double-injected SC-HSA conjugate. However, the similar relative binding patterns of the NCC and SC antisera on either the NCC or SC antigens suggests that the N-chlorination of chlorhexidine does not significantly alter the epitope(s) which these antibodies recognize. The greater affinity of the two antisera for the SC-HSA compared to the NCC-HSA antigen probably reflects the difference in haptenic substitution onto HSA (16 moles SC compared to 2·6 moles NCC).

The fine specificity of a monoclonal antibody against chlorhexidine. Hybridomas were prepared using splenocytes from mice immunized with NCC-KLH. Of the 576 wells seeded, 14 were shown to be producing anti-KLH antibodies, whilst three produced antibodies recognizing the semi-chlorhexidine determinant on the SC-HSA antigen. One of these (G3) was successfully cloned and isotype analysis showed the hybridoma to be secreting IgG1 antibodies. Monoclonal G3 gave a linear titration curve in the chlorhexidine-specific ELISA (Fig. 5). When aliquots of G3 culture supernatant (diluted 1/2) were incubated with the inhibitors described previously only chlorhexidine, proguanil and the aliphatic bis biguanide significantly inhibited antibody binding as Fig. 3b shows and the 50% inhibitory concentrations were 2·5 μM, 28 μM and 235 μM, respectively. The 10-fold difference between chlorhexidine and proguanil inhibition strongly suggests a divalent

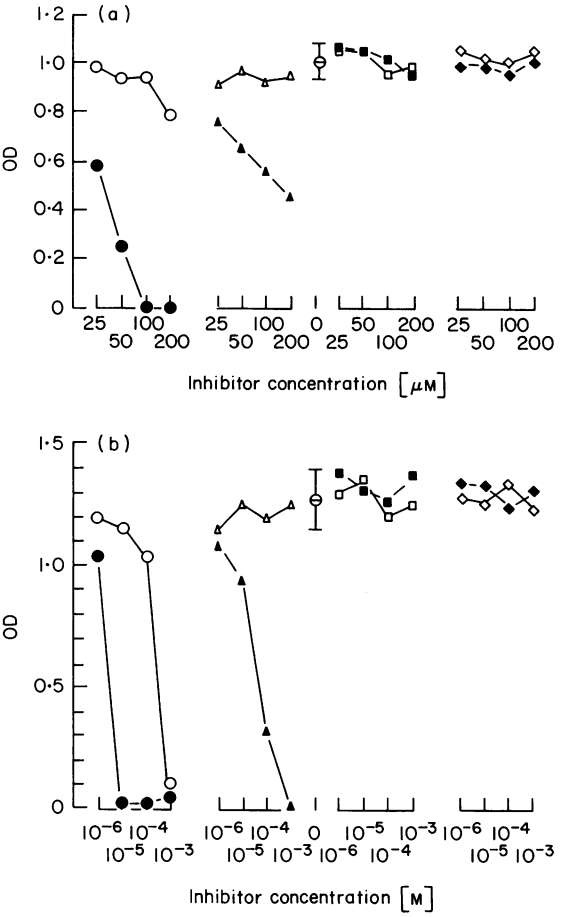

Fig. 3. IgG anti-chlorhexidine inhibition assays. Results = mean OD of duplicates. No inhibitor values (○) = mean + 1 s.d. of four. (a) Polyclonal antiserum (1/80 final dilution) from mice injected with NCC-KLH in alum. (b) Monoclonal G3 anti-chlorhexidine (culture supernatant 1/4 final dilution). Chlor (●), Prog (▲), ABB (○), GC (♦), CABA (■), GBA (△), CPAA (◊), CBA (□).

interaction between G3 and chlorhexidine. To ascertain the relative antigenicity of free compared to protein-bound mono *p*-chlorophenyl biguanide derivatives, inhibition assays were performed using proguanil and semi-chlorhexidine-HSA (SC-HSA) as inhibitors of G3 binding to SC-HSA coated microtitre plates. The structural similarities between proguanil and semi-chlorhexidine are shown in Fig. 1 and the results of the ELISA inhibition assay can be seen in Fig. 6. The protein-conjugated, multivalent derivative was approximately four times more inhibitory, in molar terms, than the free monovalent form. This effect may be solely due to the increase in valency, since chlorhexidine (divalent proguanil) was found to be approximately ten times more inhibitory than proguanil. It may also be that the antibody has a higher affinity for the covalently-linked SC hapten conformation compared to that of the free proguanil hapten. HSA alone, used at the same concentration as in the SC-HSA conjugate, had no significant effect of G3 binding. Penicillin-HSA (18:1) and trinitrophenyl-HSA (12:1) conjugates also had no significant effect on G3 binding to SC-HSA coated plates (data not shown).

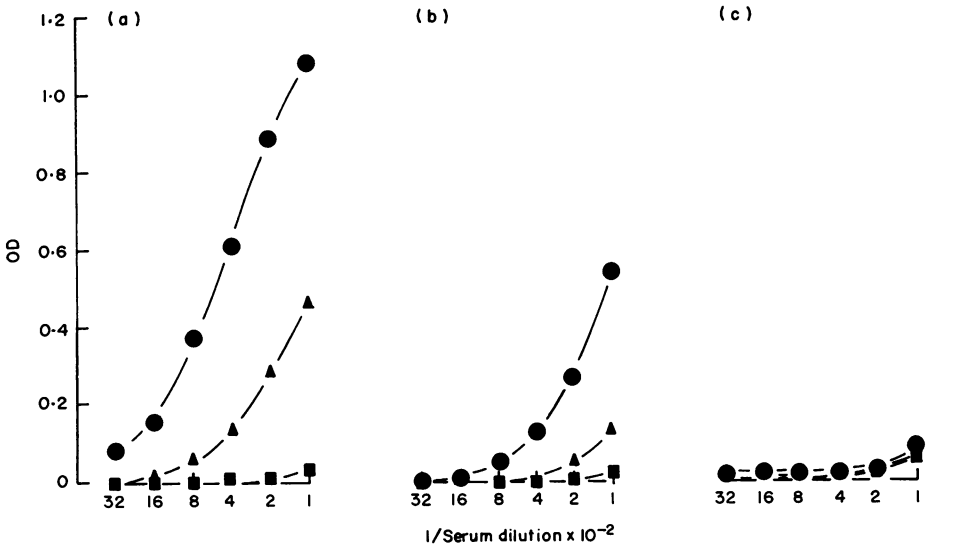

Fig. 4. Antigenicity studies. IgG antibody binding (mean OD of duplicates) in pooled sera from normal mice (■) or from mice immunized with either NCC-KLH (●) or SC-OVAL (▲). Microtitre plate wells coated with either (a) SC-HSA (b) NCC-HSA or (c) HSA (all 10 μg/ml protein).

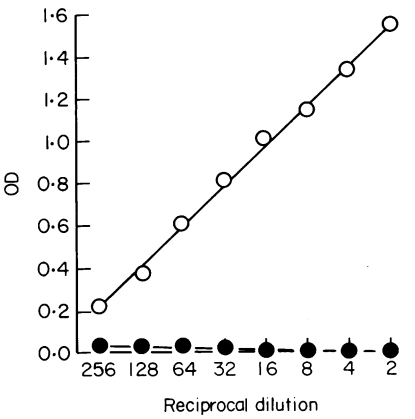

Fig. 5. IgG anti-chlorhexidine ELISA. Titration of culture supernatants from hybridomas secreting the monoclonal G3 anti-chlorhexidine (○) and a monoclonal anti-KLH (●). Mean OD of duplicates.

DISCUSSION

Our previous studies (Layton *et al.*, 1986) and those reported here demonstrate that N-chloro chlorhexidine-KLH conjugates can induce the synthesis of IgE and IgG antibodies which bind to the semi-chlorhexidine-HSA ELISA antigen. Binding of both IgE and IgG antibodies can be totally inhibited by the free native drug chlorhexidine. This is not always the case in drug immunogenicity studies. For example, antibodies induced by a metabolically-generated paracetamol-KLH conjugate did not recognize native paracetamol (Chesham & Davies, 1985) and the penicillin hapten appears to have three main epitopes, as mapped by monoclonal antibodies, consisting of the side chain (e.g. benzylpenicillin), the thiazolidin ring (common to all penicillins) and the new antigenic determinant (NAD) formed by the binding of the penicillin hapten to the carrier protein (de Haan *et al.*, 1985). The SC-HSA ELISA antigen, therefore, may not detect antibodies directed

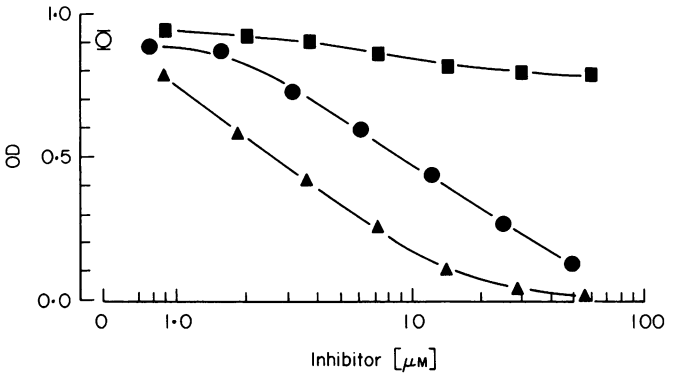

Fig. 6. Monoclonal G3 inhibition ELISA. G3 culture supernatant (1/4 final dilution) incubated with either HSA (■), proguanil (●) or SC-HSA (▲). Results = mean OD of duplicates. No inhibitor (○) value = mean + 1 s.d. of four samples.

against NAD formed by the conjugation of NCC to KLH or by the N-chlorination of chlorhexidine, but the results obtained using the NCC-HSA antigen suggest that such NAD are not formed. Furthermore, antibody binding to the NCC-HSA antigen could also be totally inhibited by free chlorhexidine (data not shown). These findings indicate that the N-chlorination of chlorhexidine does not affect its specificity as an immunogen or antigen and that the *p*-chlorophenyl biguanide sequences form dual epitopes on the symmetrical chlorhexidine molecule. Further evidence in support of these claims was provided by the ELISA inhibition data which demonstrated the relative importance of the *p*-chlorophenyl groups (e.g. prog) compared to the biguanide hexamethylene structure (e.g. ABB), in epitope recognition, although the biguanide structure is required for optimal recognition. This may be a consequence of the spatial conformation of the chlorhexidine hapten on both the immunogen and the ELISA antigen. In this respect, the N-chlorination of chlorhexidine results in the formation of N-chloro biguanide derivatives which can bind covalently to certain nucleophilic functional groups on proteins. This may partially obscure most of the biguanide hexamethylene structures or render them sterically inaccessible, whilst projecting the *p*-chlorophenyl groups in a more immunological favourable conformation. The hydrophobic *p*-chlorophenyl groups, however, may also bind to KLH (Verschueren, Gielens & Lontie, 1980). The semi-chlorhexidine derivative, on the other hand, is attached via a carbodiimide-linked succinamic acid spacer, so that the *p*-chlorophenyl biguanide hexamethylene structure is projected outwards from the carrier. It is conceivable, therefore, that the SC-HSA antigen is inefficient in binding antibodies which recognize the biguanide hexamethylene structure because of steric hindrance. In addition, both hydrophobic (*p*-chlorophenyl) and electrostastic (basic biguanide) SC-protein interactions may occur which could further reduce antibody binding.

The ELISA inhibition studies also revealed that both monoclonal and polyclonal IgG antibodies, raised using the NCC-KLH immunogen, have virtually identical inhibition patterns with the various chlorhexidine analogues and other substances tested. Antibody responses to the negatively charged phthalate hapten are known to be clonally restricted (Luo & Bankert, 1985) whereas hydrophobic haptens, such as trinitrophenol, normally induce clonally-heterogeneous responses. A greater number of different hapten analogues need to be tested before the clonotypic variation of antibodies to the *p*-chlorphenyl biguanide hapten can be fully assessed.

Chlorhexidine was chosen as a model drug hapten because it does not bind covalently to proteins, it is not metabolized *in vivo* into protein-reactive derivatives (Case, 1977) and yet chlorhexidine-specific IgE antibodies have recently been demonstrated in individuals who have experienced immediate-type hypersensitivity reactions following treatment with chlorhexidine digluconate (Ohtoshi *et al.*, 1986). Our animal model studies demonstrate that chlorine-generated N-chloro chlorhexidine-protein immunogens can induce both IgE and IgG antibodies which interact with the native drug. IgG antibody interaction has been shown to be divalent and since

chlorhexidine can totally inhibit both IgE and IgG antibody-binding in the ELISA, this suggests that epitope recognition is the same for both isotypes. This implies that chlorhexidine *per se* would be capable of eliciting immediate hypersensitivity reactions in sensitized individuals by cross-linking specific IgE antibodies on mast cells or basophils. Evidence in support of this is being sought both in our murine model and in humans.

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