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In Vivo Standardization of Cutaneous Bactericidal Activity of Antisepsis by Using Monoxenic Hairless Mice

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Received 6 December 1988/Accepted 8 May 1989

This study was designed to investigate the bactericidal activities of antisepsis on the cutaneous flora of hairless mice monoxenic to Staphylococcus epidermidis, Staphylococcus aureus, or Pseudomonas aeruginosa in vivo. A standardized method for testing such antisepsis to compare their bactericidal effectiveness in humans in described. Seven antisepsis belonging to seven different chemical groups (iodine derivatives, alcohols, mercury compounds, quaternary ammonium salts, biguanides, phenols, and carboxamides) were used as recommended by the manufacturers (conditions of contact or prolonged contact time followed by washing with distilled water). Germfree hairless mice were infected with various bacterial strains by gastric intubation, producing levels of about $10^7$ CFU/cm² of skin. The anti-septic under test was placed on the right or left side of the ventral region. The contralateral side served as a control. In order to standardize the method, a number of crucial parameters were controlled: the amount of anti-septic applied, the area of skin treated, the duration of treatment, and the washing procedure. Skin samples were obtained by cutaneous biopsy, which effectively removed all the bacteria along with the sample. The bacterial populations were counted before and after application of the anti-septic. Reductions of between 0.5 and 1.9 log units were obtained; these are comparable to those observed in humans. The standardization of our procedure and the use of animals with a strictly controlled flora eliminated much of the variability and sources of error inherent in human studies. This model could be of value for the study of resistant bacterial strains responsible for nosocomial infections and for investigations of damaged skin.

A number of difficulties are encountered in the evaluation of the bactericidal activity of antisepsis on the cutaneous microbial flora in humans. There is considerable variability in this flora (both qualitative and quantitative) from one site to another and from one individual to another, and there is a lack of standardized methods for testing bacterial agents in vivo.

We used the hairless mouse, whose hair system is absent and whose epidermal structure resembles that of humans (11, 25–27, 32). The cutaneous flora was determined on cutaneous biopsy samples (6), which is more accurate than the scraping methods (20, 33) generally used in humans, which cannot remove all of the cutaneous flora. However, this holoxenic mouse model (normal flora) has a skin flora different from that of humans, and the flora is also subject to marked variations (3; M. C. Barc, Ph.D. thesis, 1981). The model was thus adapted to the gnotoxenic hairless mouse (controlled flora). In previous studies (7–9), we implanted various bacterial strains into axenic animals to produce monoxenic animals with stable and reproducible flora. This effectively circumvents the qualitative and quantitative variations observed with holoxenic mice.

We investigated the bactericidal activities of various antisepsics on the controlled cutaneous flora of hairless mice monoxenic to Staphylococcus epidermidis, Staphylococcus aureus, and Pseudomonas aeruginosa. The efficiencies of their activities were compared with those reported for humans. The aim of this study was to standardize a method for determining bactericidal activity in vivo.

MATERIALS AND METHODS

Experimental model. (i) Hairless mouse. The hairless mice were supplied by the Centre d’Elevage et de Sélection des Animaux de Laboratoire (Orleans, France). The male and female animals (age, 4 to 8 weeks) were maintained in a sterile isolator with access to standard food (radioisotopically labelled R03 chow).

(ii) Bacterial strains. The reference strains used were S. epidermidis CIP 53124, S. aureus CIP 91144, and P. aeruginosa CIP A22. Before each experiment, the strains were inoculated twice into nutrient broth (at 24-h intervals).

(iii) Inoculation of mice. The mice were inoculated by gastric intubation of 0.5 ml of the strain at $10^7$ CFU/ml. Within 24 h, animals monoxenic to S. epidermidis, S. aureus, and P. aeruginosa were obtained with a level of implantation close to $10^3$ CFU/cm² of skin. This level remained stable with time (8, 9).

Groups of four animals were used for each experiment. The mice were killed by cervical dislocation, and the abdominal surface was marked from top to bottom. The left or right side (chosen at random) was then treated with the anti-septic under test. The untreated contralateral side served as a control. The various antisepsics were also tested in a random order.

Antisepsis tested. We used seven antisepsics representing the main chemical groups. They were either prepared in the laboratory or used in their commercial formulation (Table 1). The bactericidal activity of these antisepsics was determined previously in vitro by using French standard methods (Association Française de Normalisation [AFNOR]).

Procedure. Antiseptic (10 μl) was applied to a 3-cm² area of skin. The anti-septic was left in contact for 2 to 5 min. The
skin was then washed with 3 ml of sterile distilled water for 30 s (Table 1). A control group of mice subjected to the washing procedure only was used to evaluate the quantity of microorganisms eliminated by washing.

**Sampling and counting of cutaneous flora.** After treatment with the antiseptic, the two skin areas were cut out with a sterile, calibrated punch. Each biopsy sample was homogenized (Polytron S10) in 2 ml of dilution fluid, to which was added a neutralizing agent specific to the particular antiseptic used (Table 1). The neutralizing activity was previously tested in vitro (10).

Fractions were taken from this suspension, diluted 1/10 and 1/100, and inoculated in triplicate onto the counting medium (Trypticase soy agar [BBL Microbiology Systems, Cockeysville, Md.]). Neutralizing agent was added immediately before the inoculation. The media were incubated for 48 h at 37°C. The colonies were counted and expressed in CFU per square centimeter of skin or the \( \log_{10} \) of this value.

**RESULTS**

The efficiencies of the antiseptics were expressed as the difference in the number of organisms before and after application of the antiseptic \( \log_{10} \text{before} - \log_{10} \text{after} \). The experiments were carried out in triplicate for each of the four animals in each group. The results were compared by analysis of variance with one independent factor, the antiseptic. Student’s \( t \) test adapted for multiple comparisons was used to calculate the least significant difference between the pairs of samples for each antiseptic. Antiseptics with similar efficiencies, i.e., in groups whose values were not significantly different but whose mean values were significantly different from those of the other groups, were classified by the Neuman Keuls test. An antiseptic was considered to be more active than another one if the mean difference was higher than the least significant difference (95% confidence limit).

In our study, least significant differences of 0.32, 0.36, and 0.27 were found for animals monoxenic to *S. epidermidis*, *S. aureus*, and *P. aeruginosa*, respectively. The activities of the antiseptics among the various monoxenic groups of mice could thus be compared. The different bactericidal activities are represented in Fig. 1, 2, and 3 for the three monoxenic groups of mice, respectively.

For the mice monoxenic to *S. epidermidis*, three groups of activity were distinguished (Fig. 1): (i) Sterlane, Betadine, Septivon, and Hibiscrub; (ii) 70% alcohol and Phisohex; and (iii) Merseptyl and control wash. For the mice monoxenic to *S. aureus*, five groups of activity were found (Fig. 2): (i) Hibiscrub; (ii) Betadine, Septivon, 70% alcohol, and Phisohex; (iii) Septivon, 70% alcohol, Phisohex, Sterlane, and Merseptyl; and (iv) Sterlane, Merseptyl, and control wash.

For the mice monoxenic to *P. aeruginosa*, three groups of activity were distinguished (Fig. 3): (i) Septivon, Betadine, and Hibiscrub; (ii) 70% alcohol; and (iii) Merseptyl and control wash.

The antiseptics in a given activity group had comparable efficiencies. In most cases Betadine, Hibiscrub, Septivon, and Sterlane were nearly always found in the first two groups. Merseptyl had only weak activity against the three strains. Its activity was close to that of the control wash. The control wash had some effect since it led to a reduction in the bacterial population of about 0.6 log units. The copious washing recommended by the manufacturers of Hibiscrub, Septivon, and Phisohex increased the activity of these three products.

We selected *S. epidermidis*, a major cutaneous aerobic organism in humans, and we included *S. aureus* and *P. aeruginosa*, which are often responsible for serious infections in hospitals. Since Phisohex is no longer commercially available, we were unable to test its activity on mice monoxenic to *P. aeruginosa*.

To make this in vivo method as reproducible as possible,

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**TABLE 1. Antiseptics tested, method of application, and their corresponding neutralizing agents**

<table>
<thead>
<tr>
<th>Antiseptics</th>
<th>Product name</th>
<th>Method of application</th>
<th>Neutralizing agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Povidone-iodine solution (96%)</td>
<td>Betadine</td>
<td>Direct contact for 5 min</td>
<td>0.5% Sodium thiosulfate</td>
</tr>
<tr>
<td>Alcohol (70%)</td>
<td></td>
<td>Direct contact for 5 min</td>
<td>5% Egg yolk</td>
</tr>
<tr>
<td>Mercurothiolic acid solution (1%)</td>
<td>Merseptyl</td>
<td>Direct contact for 5 min</td>
<td>5% Egg yolk, 0.5% sodium thioglycolate</td>
</tr>
<tr>
<td>Benzalkonium chloride</td>
<td>Sterlane</td>
<td>Direct contact for 5 min</td>
<td>5% Egg yolk, 0.5% sodium thiosulfate</td>
</tr>
<tr>
<td>Chlorhexidine gluconate</td>
<td>Hibiscrub</td>
<td>Wash, 2-min contact, wash with distilled H2O</td>
<td>0.5% Sodium; thiosulfate, 0.5% histidine</td>
</tr>
<tr>
<td>Hexachlorophene</td>
<td>Phisohex</td>
<td>Wash, 2-min contact, wash with distilled H2O</td>
<td>5% Egg yolk, 3% Tween 80</td>
</tr>
<tr>
<td>Trichlorocarbanilide (0.5%)</td>
<td>Septivon</td>
<td>Wash, 2-min contact, wash with distilled H2O</td>
<td>5% Egg yolk, 3% Tween 80</td>
</tr>
</tbody>
</table>

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**FIG. 1. Activity of the various antiseptics on flora of mice monoxenic to *S. epidermidis*. Numbers at the bottom indicate the different groups of bactericidal activity.**
FIG. 2. Activity of the various antiseptics on flora of mice monoxenic to S. aureus. Numbers at the bottom indicate the different groups of bactericidal activity.

The following parameters were standardized: the amount of antiseptic applied, the area of skin treated, the duration of treatment, and the washing procedure. These parameters were based on those used in previous studies with normal mice (10) and on the recommendations for the use of such antiseptics in humans. Although they were arbitrary to some extent, identical conditions were used for each of the antiseptics.

The neutralizing agents used in a previous study (10) were also used in the present study. Their activities were based on results of a standard in vitro test (AFNOR NFT 72 150) and those of a preliminary in vivo test. Residual bactericidal activity was neutralized in both the homogenates and the suspensions used to count the bacterial colonies. They were used as described previously (12, 13, 15, 17, 23, 29).

The bactericidal activities against the three monoxenic strains (S. epidermidis, S. aureus, and P. aeruginosa) were compared. Most of these antiseptics produced a 75 to 99% reduction in bacterial numbers. The most efficient antiseptics, Hibiscrub, Betadine, Septivon, and 70% alcohol led to similar reductions in the three strains.

Most investigators (12, 13, 16, 23, 29) have found that maximum activity is exerted at different times, depending on the antiseptics and conditions used. Unfortunately, our method of using cutaneous biopsy samples could not be used to study the time course of bactericidal activity. Our animals were sacrificed 5 min after application of the substance. However, this method did enable total recovery of the bacterial flora.

DISCUSSION

Although the activity of antiseptics is readily determined in vitro by standard procedures (AFNOR standards), bactericidal activity in humans is not codified, which accounts for the variety of methods currently in use.

We compared our in vivo results with hairless monoxenic mice with those reported for humans (Table 2). The variability of the results reported for humans depends on the nature of the product, the concentration of its active ingredient, the particular procedure used (hand or skin flora), and individual differences in flora.

For Septivon we were unable to find data in the literature, and for Merseptyl the comparison was not valid because of differences in the formulations. For Sterlane (16-31) we found considerably less activity in our mice than that reported for humans (1.5 to 2 log units).

It appeared that the derivatives of povidone-iodine, chlorhexidine, and 70% alcohol are the most effective both against human flora and those in our monoxenic mice, which is what is required of an antiseptic agent in practice.

This method of using a controlled flora enabled us to evaluate the specific activity of antiseptics toward well-
defined microbial strains. Most of the studies carried out in humans have evaluated the activity against the overall cutaneous bacterial flora. However, most of the constituent organisms are not pathogenic. In the hospital environment, antisepsis is aimed at preventing dangerous infections essentially caused by staphylococcal enterobacteria and *Pseudomonas* species. In fact, there have been few studies on the activity of antiseptics toward these organisms in humans, probably because of practical evaluation difficulties. There have also been few qualitative or quantitative studies on the reduction of bacterial flora by antiseptics.

The skin flora is normally resistant to colonization from most bacteria and acts as a barrier against infection. Various factors play a role in preventing colonization, including the integrity of the stratum corneum affording mechanical protection; the continuous desquamation of the epidermis, which eliminates the outer layer of skin along with the bacteria on that skin; and the formation of a lipid film on the skin surface from the secretion of fatty acids by the resident flora that are under the influence of lipases. *Propionibacterium acnes* also plays a role by secreting propionic acid, which produces an acidic pH that is hostile to transient invading flora.

The immune system, including both humoral (immunoglobulin A secreted in sweat) and cell-mediated (T cells and Langherhans cells) immune systems, also plays a role. Secretion of bacteriocins and site-dependent interactions between bacterial strains and species also play a role. This bacterial equilibrium can also be influenced by environmental factors such as temperature and humidity, the particular strains present in the hospital environment, drugs (skin creams), UV radiation, soaps, and detergents. Pathogenic strains can take advantage of such imbalances and can infect and colonize the epidermis. The advantages of our method with gnoxicenic animals (controlled flora) are that it (i) avoids the variability observed in humans because of interindividual differences in both the nature of the flora and the relative proportions of the constituent organisms in the flora by use of a stable, reproducible flora (3 to 4 log units); (ii) avoids intersite differences by taking samples from identical areas of skin; (iii) avoids differences in sampling methods by using a standardized cutaneous biopsy procedure; and (iv) can use antibiotic-resistant strains, which are responsible for nosocomial infections, and can be used to study damaged skin.

This method with gnoxicenic animals, which is intermediate between the standardized in vitro methods and tests in humans, thus represents a valuable method for testing the cutaneous bacterial activity of antiseptics.

**LITERATURE CITED**


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