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Comparative Enumeration of Lipophilic and Nonlipophilic Cutaneous Diphtheroids and Cocci

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Swabbing skin to collect bacteria for enumeration revealed that a single washing or rinsing of the swab in buffer removed between 90 and 95% of the bacteria collected. Further removal of the remaining bacteria from the initial swab by repeated washings of the swab produced plate counts that showed nearly proportional decreases in the numbers and types of bacteria retained or eluted from the swab. None of the commonly isolated cutaneous bacteria was retained or eluted from the single swab in numbers that misrepresented measurement of their proportions present in the sample. Populations of nonlipophilic bacteria on skin when present were mainly gram-positive catalase-producing cocci. Diphtheroids when present were chiefly lipophilic. Media containing furoxone were selective for cutaneous diphtheroids. The concentration of furoxone used affected isolation and enumeration of the diphtheroids. Lipophilic diphtheroids from the stratum corneum were basically aerobic and anaerobic incubation reduced or eliminated this group from enumeration studies. Lipophilic furoxone-resistant aerobic diphtheroids from several areas, particularly the nasal passages, occurred in numbers inversely proportional to the numbers of staphylococci for periods up to 11 months. The cocci-diphtheroid relationships occurred independently of the total number of aerobic bacteria present, the transient appearance of yeasts or gram-negative bacilli, the required use of certain antibiotics by the subjects, climate, age, and sex.

Limitations in current sampling techniques for studying the microbial flora of human skin exist, but considerable information has been sought concerning the intrinsic or extrinsic factors that regulate cutaneous bacteria. Diphtheroids comprise a predominant group of human autochthonous skin bacteria (8). Among this group are the lipophilic strains that require fatty acids for growth in vitro (6). Use of the term lipophilic is relative because few investigators have distinguished obligate-lipophiles from bacteria whose growth is merely enhanced by fatty acids or substitutes of Tween 80 (9). Nonlipophilic diphtheroids are commonly referred to as "large colony" diphtheroids because growth and colony size are relatively constant on all media with or without lipid supplements, and they are easily enumerated. Since most commercial peptone media contain some lipids, obligate lipophiles may develop as pinpoint colonies, but their enumeration is difficult and impractical.

Lipophilic diphtheroids appear to affect growth and colonization of cutaneous sites by other pathogenic bacteria and may have an important ecological function in relation to the host (5). Cultural methods that measure the occurrence and numbers of lipophilic bacteria relative to nonlipophiles on skin have not been developed, but are essential to all aspects of dermoecology. An experimental procedure for this purpose and the results of its application are reported.

MATERIALS AND METHODS

Swabbing techniques. Sterile cotton wool swabs were moistened in phosphate buffer containing 0.01% Triton X-100(13) and rubbed over cutaneous sites 10 to 12 times while rotating the swab. The swab was placed in 5 ml of buffer and agitated mechanically on a tube mixer for 30 sec. The tube was designated the first swab rinse. The swab was removed with sterile forceps and placed in a second 5-ml buffer tube and mixed again. The swab was removed from the second buffer tube (second swab rinse) and washed again to produce a third swab rinse. Each of the three rinses was serially diluted in 9 ml of buffer, and 0.1-ml amounts of the dilutions were spread on media with glass rods. This procedure was used initially to deter-

mine the retention or elution of skin bacteria from the swabs and the effect this might have on enumerations. A group of 13 male and 2 female adults were used as subjects for all of the studies conducted.

Media. Total aerobic counts were made by using Trypticase soy glucose agar (BBL) supplemented with 0.5% Tween 80 to support the growth of lipophilic diphtheroids. Lipophilic and nonlipophilic diphtheroids were selectively enumerated on FTO agar (10), which contains furoxone as the selective agent. Other media included Mitis Salivarius Agar (BBL) for streptococci, L B S agar (BBL) for lactobacilli, Littman agar (BBL) for nonlipophilic yeasts, and Herellea agar (Difco) for gram-negative bacilli. Mannitol salt agar (BBL) was adjusted to contain 6% NaCl and used to isolate staphylococci, micrococci, or other gram-positive cocci. Cutaneous diphtheroids grew on mannitol salt agar but were distinguished from cocci by Gram stain and colonial morphology. To enumerate nonlipophilic bacteria, Nutrient Broth (Difco) was made lipid-deficient by extracting the lipids from the medium with petroleum ether(9). Five grams per liter of glucose was added to enhance growth, and 1.5% Noble Agar (Difco) was added. The final *p*H was 7.0. Lipophilic bacteria did not initiate growth on this agar unless it was supplemented with sodium oleate or Tween 80 (9). The addition of 10 μg/ml of furoxone (Norwich Pharmacal Co., Norwich, N.Y.) to this medium rendered it selective for nonlipophilic diphtheroids (10). Estimation of nonlipophilic diphtheroids on medium without furoxone was made by Gram staining representative colonies from highest-dilution-countable plates of samples. Aerobic plates were incubated and examined at 1-, 3- and 5-day intervals. Plates were incubated anaerobically for 7 days by using Brewer jars with Gaspaks (BBL). All plates were incubated at 34 C.

Identification of bacteria. Gram-positive cocci were classified by the methods of Baird-Parker(1). Lipophilic diphtheroids were grouped by the scheme of Smith (9).

RESULTS

Swabbing technique. Total aerobic plate counts were made of various cutaneous sites. Emphasis was placed on determining the numbers and types of bacteria that were washed or eluted from a single cotton swab used for each sample when the swab was rinsed for 30 seconds in buffer. Total counts from each of the first swab washings varied with the particular site sampled (Fig. 1). Second and third washings of the swabs revealed linear decreases in the numbers of bacteria that remained adherent to the swab after these additional washings. Combined counts of bacteria from the second and third rinses did not exceed 10% of the total count obtained from the first rinse. Enumeration of specific bacterial groups showed that no group adhered to the swabs in numbers significant enough to cause gross errors in estimating the proportions of each group present in the sample when plating only the first swab rinse (Fig. 2). In the remaining experiments, bacterial counts were made from only the first swab rinse.

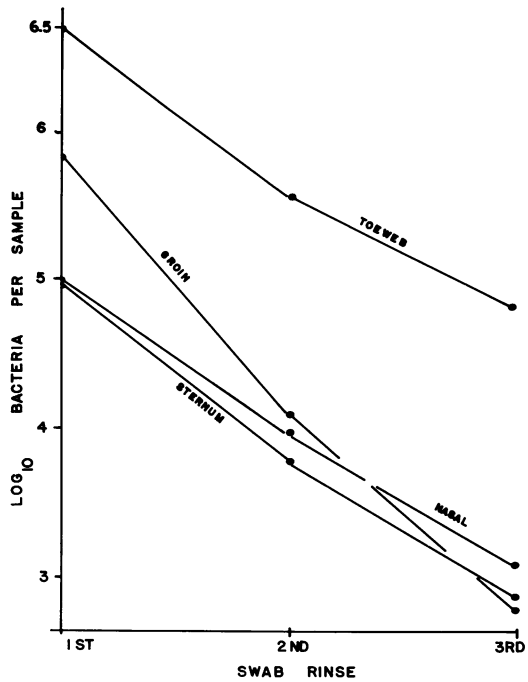

FIG. 1. *Effect of swab rinsing on elution and enumeration of total aerobic cutaneous bacteria. Counts were made with Trypticase soy agar with Tween 80.*

Enumeration of diphtheroids. The concentration of furoxone in FTO agar used to selectively isolate cutaneous lipophilic or nonlipophilic diphtheroids affected the recovery and enumeration of these bacilli from certain sites. Nasal diphtheroids were not affected by 50 μg/ml of furoxone, but diphtheroids from the groin were inhibited as the concentration of furoxone was increased (Fig. 3). Diphtheroids from the toewebs, forearms, and sternum were as resistant to furoxone as the nasal diphtheroids. The FTO agar used for diphtheroids was prepared with 10 μg/ml of furoxone in subsequent experiments.

Enumeration of lipophilic and nonlipophilic bacteria. Initially, total nonlipophilic populations were enumerated by using lipid-deficient nutrient agar without furoxone (Fig. 4a). In nasal samples of two individuals, the aerobic flora of one was dominated by diphtheroids, the other by gram-positive cocci. The toeweb sample contained more diphtheroids than cocci. In each case, the total nonlipophilic flora was similar to the numbers of cocci. Gram stains of colonies confirmed that all nonlipophilic bacteria from the nasal

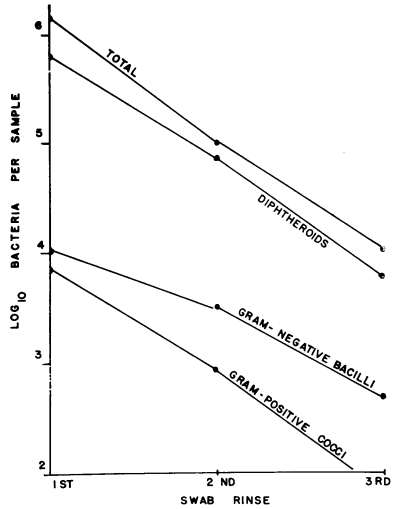

FIG. 2. *Effect of swab rinsing on elution and enumeration of different cutaneous bacteria from the nasal area. Media used included Trypticase soy agar with Tween 80 for total counts, FTO agar for diphtheroids, Herellea agar for gram-negative bacilli, and mannitol salt agar for gram-positive cocci.*

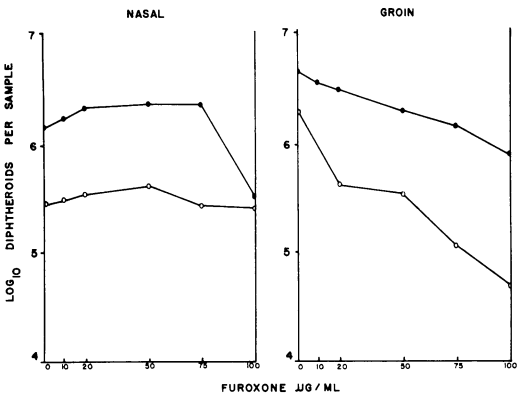

FIG. 3. *Effect of furoxone concentration in FTO agar on isolation and enumeration of cutaneous lipophilic and nonlipophilic diphtheroids. Each sample was from a different subject.*

sites were in fact cocci. Examination of nonlipophilic toeweb bacteria indicated that both cocci and diphtheroids were present. The same individuals and sites were sampled again after 6 weeks (Fig. 4b) but lipid-deficient furoxone medium was included. Nonlipophilic bacteria corresponded primarily to coccal populations. Nonlipophilic diphtheroids were either absent or represented only a small portion of the total diphtheroid flora. At 3.5 months after the first samples were taken, the same sites and subjects

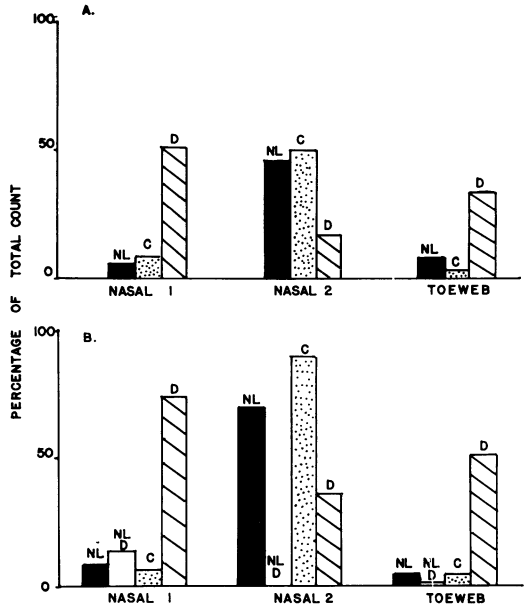

FIG. 4. *Enumeration of lipophilic and nonlipophilic cutaneous bacteria. Abbreviations: NL, total nonlipophilic bacteria counted on lipid-deficient nutrient agar; C, staphylococci or micrococci counted on mannitol salt agar; D, total lipophilic and nonlipophilic diphtheroids counted on FTO agar; NLD, nonlipophilic diphtheroids counted on lipid-deficient nutrient agar. The latter two media contained 10 µg/ml of furoxone.* (A) *Initial samples from three subjects.* (B) *Same subjects and sites sampled 6 weeks later.*

were again examined. The results were nearly identical to those illustrated in Fig. 4b except that the nonlipophilic diphtheroids of the nasal sample of the 6-week study were not present in the 3.5-month study. Repeated sampling from the scalp, groin, ear canal, and facial areas of other subjects showed that few sites contained nonlipophilic diphtheroids regardless of the total numbers of lipophilic diphtheroids or other bacterial groups.

Effect of anaerobic incubation on enumeration. Further evidence showed that the majority of the furoxone-resistant lipophilic diphtheroids were aerobic rather than facultative anaerobes (Table 1). Separate sites from three subjects sampled at three 1-month intervals showed consistently higher aerobic counts of total bacteria and diphtheroids than anaerobic counts.

Predominance of diphtheroids or cocci at certain sites. The basic aerobic and lipophilic nature of the furoxone-resistant diphtheroids led to studies of the proportions of this group present on skin compared to other bacteria. Inverse relationships in the proportions of diph-

TABLE 1. *Effect of anaerobic incubation on enumeration of cutaneous lipophilic diphtheroids*

Site[a]	Sample	Total count[c]		Diphtheroids	
		Aerobic	Anaerobic	Aerobic	Anaerobic
Nasal	1[b]	7,500,000	2,600,000	7,500,000	<50
	2	7,000,000	3,000,000	6,750,000	770,000
	3	1,250,000	545,000	630,000	<50
Groin	1	8,050,000	2,250,000	3,100,000	43,500
	2	5,750,000	1,450,000	1,100,000	320,000
Toeweb	1	4,850,000	63,000	2,350,000	<50
	2	600,000	560,000	165,000	49,000
	3	765,000	165,000	5,200	600

[a] Separate subject was used for each site.
[b] Number indicates sample taken at 1 month intervals.
[c] Total counts were made with Trypticase soy-Tween 80-agar. Diphtheroids were counted on FTO agar.

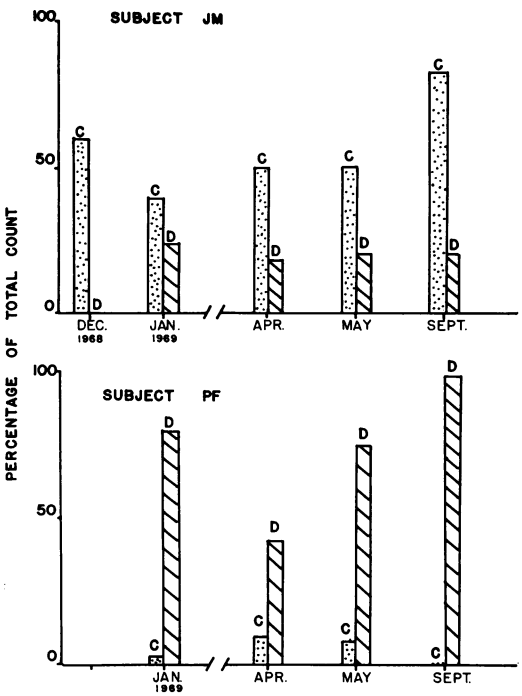

FIG. 5. *Inverse relationships in nasal diphtheroid-cocci populations of two subjects. Abbreviations: C, cocci, counted on mannitol salt agar; D, diphtheroids, counted on FTO agar.*

theroids and cocci remained constant in subjects examined for periods of 9 months (Fig. 5) One subject (J. M.) was a "low carrier" with total aerobic counts below 100,000 bacteria per sample. Subject P.F. was, by comparison, a "high carrier" with total aerobic counts rarely below 10^6 per sample. In both subjects, the cocci were primarily groups II and VI staphylococci. Diphtheroids, when present in subject J.M., were unidentified nonlipophilic strains, whereas subject P.F. contained almost exclusively group IVa corynebacteria. Inverse relationships between diphtheroids and cocci occurred over an 11-month period in six male and two female adults. Sites studied included nasal, ear canal, groin, and toeweb areas. During the 1-year study, four subjects developed non-cutaneous infections for which tetracycline, ampicillin, or penicillin were prescribed. Use of these antibiotics for 10 or 15 days was not observed to affect diphtheroid-cocci relationships. The transient occurrence of yeasts and gram-negative bacilli including *Proteus* species changes in climate, or the total number of aerobic bacteria also had no apparent affect on diphtheroid and cocci proportions. There were no streptococci or lactobacilli isolated. *Staphylococcus aureus* was isolated transiently from the groin and nose of some subjects but was not carried consistently by anyone.

DISCUSSION

Sampling skin by scrubbing an area for 1 min was reported to remove 85% of the bacteria at the site (12). In this study, enumerations based on plating the first washing of a single swab showed that between 90 and 95% of the bacteria collected on the swab were removed. This also permitted a fairly accurate means of measuring the relative proportions of different bacteria in the sample. Many skin studies measure the presence of certain bacteria by the per cent frequency of their occurrence in populations of subjects. Qualitative estimates of relative numbers of different bacteria on skin have some clinical and epidemiological value, but such data are not suitable for ecological analysis.

Lipophilic diphtheroids are the principal ba-

cilli on human skin but vary in their resistance to furoxone. A 10 μg/ml concentration of this antibiotic in media permitted a selective enumeration of this group without significant loss in their numbers. Lipophilic diphtheroids from the surface layers of the stratum corneum are primarily aerobic. Anaerobic incubation reduced or completely eradicated this group from enumerations. Isolation of diphtheroids from deeper areas such as comedones, which comprise a distinct cutaneous ecosystem, could be expected to yield facultative and anaerobic diphtheroids (M. Puhvel, *personal communication*).

Marples and Williamson (5) reported that nonlipophilic diphtheroids occurred in forehead and axillary samples with frequencies of 8–20 and 25–61%, respectively. Their subjects included groups with and without antibiotic therapy. Certain individuals, as in this study, did not contain nonlipophilic diphtheroids depending upon the site sampled. The frequency of lipophilic diphtheroids on adult skin was reported to be greater than 90% (8).

Colonization of skin by pathogens is known to occur by suppressing the normal skin flora with antibiotics or disinfectants (3, 11). Marples et al. (4) found that in a group of normal subjects not receiving antibiotics, 38% had lipophilic diphtheroids as the dominant nasal bacteria although some individuals in the group had none. Coagulase-negative cocci were found in all subjects. Marples and Williamson (5) and Smith (10) reported inverse relationships in the proportions of cutaneous diphtheroids and cocci. The former authors observed that antibiotics that reduced lipophilic diphtheroids permitted cocci to multiply. As the diphtheroids returned, the coccal density fell. Marples and Williamson felt that diphtheroids were of particular importance possibly because they control the other bacterial components of the axilla. The earlier observations by Smith (10), regarding inverse numbers of diphtheroids and cocci at nasal sites, and the data presented here indicate that dominance by one group over the other is not necessarily caused by extrinsic factors of climate or drugs nor by intrinsic factors such as age or the occurrence of other bacteria at the particular site. Although the number of subjects was small, longitudinal observations showed that the inverse relationships between cocci and diphtheroids remained stable for long periods of time. This phenomenon may have some intrinsic basis connected with the physiological activity of the skin or that of the microorganisms in question. Moisture and temperature contribute to cutaneous floral changes (2) but have not been correlated directly with coccal-diphtheroid interactions. There are many in vitro examples of cocci that inhibit diphtheroids including *Corynebacterium acnes* (7) but the opposite antagonism is not well known. Group IVa aerobic, furoxone-resistant lipophilic diphtheroids are not remarkable in their biochemical or physiological properties (9). Studies to date have not detected any specific diphtheroidal substances that inhibit groups II and VI staphylococci (*unpublished data*), yet the prospects of inhibitors in an ecosystem are often anticipated. Other forms of interactions between cocci and diphtheroids require study.

Nonlipophilic diphtheroids do not appear to be significant in the lipophilic diphtheroid-cocci relationships but can be enumerated with lipid-deficient media containing furoxone. Chemically defined media would obviously be desirable for this purpose but cutaneous diphtheroids vary considerably in their nutritional requirements (9), and a lipid-free defined medium is not presently available.

Cutaneous lipophilic diphtheroids, unfortunately ignored as insignificant commensals of man, may have an important but unidentified role in affecting colonization of skin by other bacteria.

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