Microflora Analysis of a Child with Severe Combined Immune Deficiency

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An infant with severe combined immune deficiency was delivered by Caesarean section and continuously maintained in an isolator in an effort to restrict microbial contact. However, during the past 5 years the child came in contact with at least 54 different microbial contaminants. His skin autoflora was similar to the reference group of healthy male adults in numbers of different species and the number of viable cells present per square centimeter of surface area. The subject's autoflora differed from the reference group in that significantly fewer anaerobic species were recovered from the patient's mouth and feces. The populations on the skin and in the upper respiratory tract were almost exclusively composed of staphylococci and micrococci, although several species that commonly inhabit these areas were infrequently isolated and failed to colonize. The fecal autoflora was dominated by staphylococci, bacilli, and clostridia recovered in typical numbers. *Enterobacter agglomerans*, not commonly recovered from the reference group, was a frequent inhabitant of the mouth and feces of the child. The fact that this severe combined immune-deficient patient has remained disease free in the presence of this microbial population illustrates the noninvasive nature of the bacteria present, as well as the importance of unaffected components of the child's immune defense mechanism.

This report concerns a male child suspected of having severe combined immune deficiency disease who was delivered by Caesarean section and immediately placed in axenic isolation where he has remained for 5 years (18). The diagnosis of severe combined immune deficiency was confirmed by quantitation of the child's immunoglobulins, challenge of his immune system in vivo with standard and experimental antigens and skin grafting, and in vitro testing of his lymphoid cells for reactivity to phytohemagglutinin and pokeweed mitogen. In addition, the child had a diminished number of lymphocytes, very low levels of circulating immunoglobulins G and M, and no immunoglobulin A detectable by quantitative precipitation techniques (17). Although attempts were made to restrict contamination of the subject by maintenance in a plastic isolator at all times and feeding him sterile food, Wilson et al. (21) reported recovery of a number of contaminants over the first 28 months of the subject's life. Colistin methate and gentamicin 40 were administered in the first month after delivery to successfully remove *Alcaligenes faecalis* from the autoflora (21). No other antibiotic therapy was employed.

With the indication that the subject was not germfree, a 20-month monitoring activity, starting at age 39 months, was initiated. This study

was part of a program to provide and test a mobile biological isolation system, similar to that developed for immune-suppressed cancer patients (13), which would allow the patient more freedom of movement while maintaining a controlled environment. This report describes the microbial population inhabiting the integument, oral cavity, and feces of a child completely devoid of demonstrable humoral or cell-mediated immune competence (2, 3, 18, 21).

MATERIALS AND METHODS

Sample collection. A sterile kit containing sterile snap-cap tubes, swabs, and 0.03 mM phosphate buffer was passed into the isolator before each sample collection. Samples were collected at approximately 4-week intervals from the mouth, nasal passages, feces, and body surface areas. Sample sites of the body included the forehead, ear canals, back of neck, hands (palm and between fingers), umbilicus, face (nasal folds), axilla, groin, and toe webs. On the morning of sample collection, a fecal sample was obtained, removed from the isolator as soon as possible, and placed in a reduced-oxygen atmosphere. All of the body surface areas were sampled with sterile swabs wetted in phosphate buffer. The mouth and nasal passages were sampled with dry swabs. After use, the swabs were placed in individual, labeled, snap-cap tubes and removed from the isolator in such a way as to avoid back contamination. Five milliliters of sterile Trypti-

case soy broth (Baltimore Biological Laboratory) was added to each tube. The tubes were packed in ice and transported to the Johnson Space Center laboratories for processing. All samples were analyzed quantitatively for aerobic and anaerobic bacteria, yeasts, and filamentous fungi.

Quantitation methods. All samples were held from 1 to 2 h at 4°C until processed. After serial dilution in sterile Trypticase soy broth, 0.1-ml portions were transferred to quantitation plates and spread over the surface with an alcohol-flamed glass rod.

Media used for quantitation of aerobic bacteria in the various samples were blood agar (Baltimore Biological Laboratory; Trypticase soy agar with 5% sheep blood) for all samples, chocolate bacitracin agar (Casmen agar with 5% horse blood and 300 µg of bacitracin per ml) for mouth and nasal passages, mitis salivarius agar (Difco Laboratories) for mouth and feces, and MacConkey agar (Baltimore Biological Laboratory) for the feces. The inoculated aerobic media were incubated at 35°C for 48 h. Media used for quantitation of anaerobic bacteria in the various samples were anaerobic blood agar (blood agar with vitamin K, hemin, and yeast extract) for all samples; paromomycin-vancomycin-menadione agar for the mouth, nasal passages, and feces; and Rogosa agar (Difco Laboratories) for the mouth and feces. The inoculated anaerobic media were incubated in GasPak jars (Baltimore Biological Laboratory), using GasPak H₂-CO₂ generators for the gaseous phase, at 35°C for 4 days. Portions of the Trypticase soy broth cultures were streaked to Sabouraud-dextrose agar with antibiotics, corn meal-malt extract-yeast extract agar with antibiotics, and Czapek-Dox agar for isolation of yeasts and filamentous fungi. After incubation at 20°C for 4 to 7 days, yeasts were inoculated onto Dalmau plates and appropriate biochemical media; filamentous fungi were transferred to Czapek-Dox agar, malt extract agar, and half-strength corn meal agar. Species were identified as previously outlined (19).

After 48 h of incubation, each morphologically different aerobic bacterial isolate was quantitated and picked to Trypticase soy broth, and a Gram stain was prepared. After 24 h of incubation at 35°C, all cultures were streaked to a blood agar to assure purity before

biochemicals were inoculated. After 96 h of incubation, each morphologically different anaerobic bacterial isolate was quantitated, picked to a tube of prereduced chopped meat (Virginia Polytechnic Institute), and streaked to a blood agar plate, and a Gram stain was prepared. After 48 h of aerobic incubation at 35°C, the blood agar plates were examined for growth; biochemicals were inoculated using only those isolates that were strict anaerobes. Unless noted, Virginia Polytechnic Institute anaerobic procedures were followed (7).

RESULTS AND DISCUSSION

A total of 54 different species of strains of microorganisms was identified over the 20-month sample collection period. Of these, 22 were aerobic bacteria, 10 were anaerobic bacteria, 1 was a yeast, and the remaining 21 were filamentous fungi.

The methods used were identical to those routinely used in studies of astronaut autoflora (20). Therefore it is possible to compare the results directly from a methodological point of view. The reference group was composed of healthy male adults because the subject was maintained in isolation and the possible contaminating sources were all adults.

Anaerobic bacteria. Table 1 illustrates the mean number of species of anaerobic bacteria recovered from each sample site. The most striking differences between the immune-deficient child and the reference group are the complete absence of anaerobes in the child's mouth and the comparatively low number of species present in his feces. This may very well be the result of feeding sterile food, combined with the poor survival capability of anaerobes on possible contaminating surfaces and in the air. Likewise, there were typically no anaerobes found on the child's body surfaces, a result not statistically different from that obtained within the reference group.

TABLE 1. Recovery of anaerobic bacteria from immune-deficient patient

Sample site	Patient			Reference group		
	Quantitation ^a (mean ± 1 SD)	No. of species (mean ± 1 SD)	Sample size	Quantitation ^a (mean ± 1 SD)	No. of species (mean ± 1 SD)	Sample size
Mouth	0 ^b	0 ^b	22	6.07 ± 0.70 ^b	7.52 ± 2.16 ^b	133
Nasal cavity	0.40 ± 0.66	0.09 ± 0.29	22	2.59 ± 1.73	1.04 ± 0.81	96
Ear canal	0.32 ± 0.34	0.09 ± 0.29	22	1.33 ± 1.89	0.53 ± 0.73	102
Neck	0	0	22	2.94 ± 1.28	1.26 ± 0.64	102
Hand	0	0	22	1.46 ± 1.32	0.74 ± 0.61	102
Umbilicus	0.46 ± 0.81	0.09 ± 0.29	22	1.97 ± 1.80	0.98 ± 1.03	102
Axilla	0	0	15	1.58 ± 1.13	1.00 ± 0.76	102
Groin	0.52 ± 1.17	0.27 ± 0.47	15	2.11 ± 1.86	1.01 ± 1.07	102
Toes	0	0	15	0.47 ± 1.41	0.21 ± 0.45	102
Feces	8.30 ± 0.97 ^b	3.05 ± 0.60 ^b	20	10.15 ± 0.54 ^b	9.29 ± 2.35 ^b	58

^a Expressed in log₁₀ colony-forming units per square centimeter of surface area or per gram of wet feces (± 1 standard deviation [SD] unit).

^b Significantly different at *P* < 0.05.

The quantitation of viable anaerobic bacterial cells recovered from these areas is also presented in Table 1. Although the load in the subject's feces is significantly lower than in the reference group, it is evident that even with feeding of sterile food and maintenance of the subject in an axenic environment, a fecal flora has accumulated. The nature of this microbial population will be illustrated in a later table. As might be expected, quantitations for body surface areas were quite low, although not significantly different from the reference group.

Aerobic bacteria. Table 2 shows the mean number of species of aerobic bacteria recovered from each sample site. As in the case of the anaerobes, the subject's oral flora is less complex than that typically found in the reference group. However, a typical variety of aerobes was recovered from all other sampled areas. The mean number of viable aerobic bacterial cells recovered from each of these areas is also summarized in Table 2. Except in the case of the umbilicus and the axilla, these numbers are not significantly different from those of the control group. This is even true in the mouth, where a representative quantitation was routinely observed in spite of the small number of species present.

Although 21 different species of filamentous fungi were recovered from the patient during the 20-month monitoring period, all must be considered transients on the basis of their infrequent isolation. *Candida parapsilosis* was the only yeast recovered from the patient samples and should be considered part of the fecal autoflora.

The data presented in Tables 1 and 2 illustrate that the patient, who has no B- or T-cell-mediated immunity, has not responded adversely to a bacterial population which in many ways is numerically indistinguishable from the adult

male reference group. Further, members of this population have been present for up to 5 years without a single incidence of bacterial infection in spite of the subject's lack of immune capability.

Comparison of genera and species recovered. Table 3 lists the genera isolated at least 50% of the time from the mouth of either the patient or the reference group. Although very few cohesive data exist on the composition of the oral flora during childhood (6), it is likely that, by the time the primary dentation has formed, autoflora components are essentially the same as in adults. Of course there is considerable variability depending on diet, hygiene regimen, presence of caries, etc. In one study it was shown

TABLE 3. *Microorganisms recovered from mouth of immune-deficient patient or reference group*

Genus ^a	Quantitation ^b (log ₁₀ CFU/cm ²)		Isolation frequency (%)	
	Patient	Reference group	Patient	Reference group
<i>Corynebacterium</i>	6.6	5.6	4.6 ^c	86.7
<i>Enterobacter</i>	4.2 ^d	1.9	90.9 ^d	35.7
<i>Haemophilus</i>	0 ^d	6.2	0 ^c	100.0
<i>Lactobacillus</i>	2.9	3.4	4.6	93.3
<i>Micrococcus</i>	5.2	5.3	40.9	63.3
<i>Neisseria</i>	0 ^c	6.0	0 ^c	93.3
<i>Rothia</i>	0 ^c	5.3	0 ^c	90.0
<i>Staphylococcus</i>	6.5 ^c	3.2	100.0 ^d	76.7
<i>Streptococcus</i>	4.2 ^c	7.0	9.1 ^c	100.0
<i>Bacteroides</i>	0 ^c	5.1	0 ^c	100.0
<i>Fusobacterium</i>	1.5 ^c	4.5	4.6 ^c	87.2
<i>Leptotrichia</i>	0 ^c	3.2	0 ^d	53.9
<i>Veillonella</i>	0 ^c	5.4	0 ^c	94.9

^a Only genera isolated at least 50% of the time are presented.

^b Expressed in log₁₀ colony-forming units (CFU) per square centimeter of surface area.

^c *P* < 0.01 compared with reference group.

^d *P* < 0.05 compared with reference group.

TABLE 2. *Recovery of aerobic bacteria from immune-deficient patient*

Sample site	Patient			Reference group		
	Quantitation ^a (mean ± 1 SD)	No. of species (mean ± 1 SD)	Sample size	Quantitation (mean ± 1 SD)	No. of species (mean ± 1 SD)	Sample size
Mouth	6.72 ± 0.52	3.50 ± 0.96 ^b	22	7.04 ± 0.64	11.64 ± 2.58 ^b	176
Nasal cavity	4.64 ± 0.50	3.23 ± 1.15	22	4.76 ± 0.73	4.71 ± 1.53	138
Ear canal	3.29 ± 0.57	3.45 ± 1.01	22	4.72 ± 1.13	3.64 ± 1.33	144
Neck	3.32 ± 1.00	3.45 ± 1.14	22	3.10 ± 0.81	3.80 ± 1.77	144
Hand	4.18 ± 0.66	4.23 ± 1.02	22	2.70 ± 0.77	4.31 ± 1.81	144
Umbilicus	5.67 ± 0.52 ^b	3.86 ± 0.99	22	3.12 ± 0.94 ^b	4.15 ± 1.98	100
Axilla	5.10 ± 0.93 ^b	3.47 ± 1.06	15	2.61 ± 0.85 ^b	3.23 ± 1.61	140
Groin	6.06 ± 1.07	3.40 ± 0.91	15	5.32 ± 0.74	5.38 ± 1.54	144
Toes	6.32 ± 0.62	3.93 ± 1.10	15	5.35 ± 1.03	4.43 ± 1.63	142
Feces	8.10 ± 0.55	4.40 ± 0.88	20	7.76 ± 1.33	4.97 ± 1.96	58

^a Expressed in log₁₀ colony-forming units per square centimeter of surface area or per gram of wet feces (± 1 standard deviation [SD] unit).

^b Significantly different at *P* < 0.05.

that by 12 months of age all children tested had *Streptococcus*, *Staphylococcus*, *Veillonella*, and *Neisseria* in the oral flora (10). At least half of those tested had *Actinomyces*, *Lactobacillus*, *Rothia* (*Nocardia*), and *Fusobacterium*. This observation was borne out by the reference group used in this study (Table 3) except that the genera *Corynebacterium*, *Haemophilus*, *Micrococcus*, and *Bacteroides* were also frequently found. This difference can largely be explained by differences in methodology. This is especially true in the case of *Haemophilus*, which was always isolated from reference individuals but may be easily overlooked if not actively sought out. Of the common aerobic genera, *Corynebacterium*, *Haemophilus*, *Lactobacillus*, *Neisseria*, *Rothia*, and *Streptococcus* were either missing or were only occasionally isolated from the mouth of the immune-deficient patient. Although 15 different bacterial species were recovered at least once from the subject's mouth over the 20-month test period, only *Enterobacter agglomerans* and *Staphylococcus epidermidis* were recovered frequently enough to be considered part of the autoflora (Table 4). These two species probably colonized in high numbers because of the absence of competition from the more common oral microorganisms, which either were never introduced into the isolator or were present but failed to colonize the mouth.

Although the patient's oral autoflora was reduced when compared with the reference group, the presence of even one species should be considered medically important because of his immunodeficiency. The fact that oral infections have been lacking throughout his first 5 years could indicate the importance of other factors in controlling infection. Probably the most im-

portant is the fact that he has remained in good general health and has not been subjected to frank pathogens. Likewise, species such as *E. agglomerans* and *S. epidermidis*, rather than the more typical streptococci, have been able to dominate the autoflora. This may signify the benign nature of these species. Other affecting factors could include the antagonism between the established species and newcomers (12), salivary lysozyme, and production of hydrogen peroxide (8) and bacteriocin (9) by species present, as well as diet and hygiene.

The species isolated at least 50% of the time from the patient's skin samples are shown in Table 4. As might be expected (11), strains of *Micrococcaceae* predominate in the integumentary autoflora; three strains of *Micrococcus* and three of *Staphylococcus*, as identified by the Baird-Parker procedure (1), were routinely recovered. The recovery of these microorganisms from the patient's skin compared well with the control group, as well as with other studies where *S. epidermidis* II and *Micrococcus* groups 2 and 3 were found to predominate in the skin autoflora of normal adults (16). In addition *Candida parapsilosis* and *E. agglomerans*, infrequently recovered from the normal male adult control group, were often recovered from the patient's skin. It is likely that these two species are permanent inhabitants of the lower digestive tract and occur on the skin only as secondary contaminants.

It has been suggested that in the normal cutaneous environment, the coagulase-negative staphylococci and micrococci dominate the skin surface by virtue of the bacteriocins and other inhibitors they produce (15). These factors, which traditionally have been considered minor

TABLE 4. Isolation frequency of commonly occurring microorganisms^a from immune-deficient patient

Species	Isolation frequency (% recovery)							
	Patient				Reference group			
	Mouth	Feces	Nasal cavity	Skin (total)	Mouth	Feces	Nasal cavity	Skin (total)
<i>Bacillus circulans</i>	0	70	13	27	0	0	0	0
<i>Bacillus</i> species	0	90	0	47	1	17	17	83
<i>Bacteroides clostridiiformis</i>	0	100	0	0	3	22	0	0
<i>Candida parapsilosis</i>	10	70	14	68	0	0	0	0
<i>Clostridium bifermentans</i>	0	85	0	13	0	4	0	0
<i>Clostridium subterminale</i>	0	70	0	20	0	0	0	0
<i>Enterobacter agglomerans</i>	95	100	47	80	1	0	0	17
<i>Micrococcus</i> subgroup 1	40	7	68	100	26	3	6	96
<i>Micrococcus</i> subgroup 2	7	0	7	73	1	0	3	91
<i>Micrococcus</i> subgroup 3	7	0	20	68	0	0	0	91
<i>Staphylococcus epidermidis</i> II	95	95	100	100	54	3	59	91
<i>Staphylococcus epidermidis</i> IV	27	7	20	80	6	0	19	78
<i>Staphylococcus epidermidis</i> V	59	7	27	93	3	1	19	87

^a Only strains isolated at least 50% of the time from the same area are listed.

defense mechanisms in normal individuals, may be a more important means of protection to the subject of this report. Because he has lived in an axenic environment the subject has been exposed less frequently to new microbial contaminants. Cutaneous lysozyme, free fatty acids, bacteriocins, "micrococci," and "epidermins" (15) have been shown to selectively inhibit microbial growth. Although children typically have lower concentrations of free fatty acids than do adults (16), these factors could help account for the ability of this immune-deficient patient to resist the contaminants that occasionally reached the skin.

The common genera isolated from the feces are listed in Table 5. Like the oral cavity auto-flora, the fecal populations of the child and the reference group differ in several ways. The common genera *Streptococcus*, *Escherichia*, *Bifidobacterium*, *Eubacterium*, and *Fusobacterium* were always absent from the patient's fecal samples. *Bacteroides clostridiiformis*, *Clostridium subterminale*, *C. bifermentans*, *Staphylococcus epidermidis*, *Bacillus circulans*, and a variety of other bacilli were routinely recovered. In addition, *E. agglomerans*, never found in the reference group, was always present in the patient. The yeast *Candida parapsilosis* was isolated from the patient 70% of the time.

Although recovery of *Bacteroides* is not uncommon in normal subjects (4, 5), it should be noted, in light of the subject's immunodeficient

state, that *B. clostridiiformis* has been implicated in tissue and intestinal abscesses. *C. parapsilosis* has been associated with nail infections, endocarditis, and ulcerations. Although *E. agglomerans* (*Erwinia herbicola*) has been isolated from human infections (14), its presence has not caused an infection in this severe combined immune-deficient patient.

The fact that the subject has remained disease free in the presence of a microbial population may stimulate some thought as to the relative importance of other defense factors. For example, his complement activity and phagocytic activity are within normal limits, his skin has remained intact (except for a skin graft at age 6 months [17]), frank pathogens have not been recovered from within the isolator, and microorganisms have not been isolated in unusually high numbers. Under these conditions it may be possible for the patient to remain infection free, even in the absence of specific immune activity.

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TABLE 5. Microorganisms recovered from feces of immune-deficient patient or reference group

Genus ^a	Quantitation ^b (log ₁₀ CFU/g)		Isolation frequency (%)	
	Pa-tient	Refer-ence group	Pa-tient	Refer-ence group
<i>Bacillus</i>	7.7 ^c	5.0	100.0 ^c	23.7
<i>Enterobacter</i>	6.3 ^d	4.6	100.0 ^c	7.9
<i>Staphylococcus</i>	7.1 ^c	3.2	95.0 ^c	5.3
<i>Streptococcus</i>	0 ^c	6.0	0 ^c	94.7
<i>Escherichia</i>	0 ^c	6.8	0 ^c	94.7
<i>Bacteroides</i>	7.6 ^c	10.1	100.0	100.0
<i>Bifidobacterium</i>	0 ^c	8.9	0 ^d	100.0
<i>Clostridium</i>	7.6 ^d	5.6	100.0	85.7
<i>Eubacterium</i>	0 ^c	9.7	0 ^d	71.4
<i>Fusobacterium</i>	0	8.6	0 ^d	71.4
<i>Candida</i>	1.3 ^d	2.8	70.0 ^d	12.8

^a Only genera isolated at least 50% of the time are presented.

^b Expressed in log₁₀ colony-forming units (CFU) per gram of wet feces.

^c *P* < 0.01 compared with reference group.

^d *P* < 0.05 compared with reference group.

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