Persistence of Mycobacterium bovis BCG in Soil and on Vegetables Spray-Irrigated with Sewage Effluent and Sludge

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ABSTRACT

Survival of Mycobacterium bovis BCG on garden plots spray-irrigated with sewage effluent or sludge was studied to determine persistence of mycobacteria under conditions approximating current practices. The D value (90% reduction time) in effluent-sprayed soil was 11 days, and in sludge-sprayed soil it was 8 days. On effluent-sprayed radishes, the D value was 6 days, and on sludge-sprayed radishes, 4 days; however, this difference was not significant. Isolation from lettuce was too erratic to determine a true survival rate, but the organism was isolated sporadically for up to 35 days.

Land disposal of sewage and sludge is being actively considered in the United States as a means of reducing the costs of treatment facilities, reducing discharge to water bodies, and utilizing the water and nutrient content of wastewater in an economically beneficial manner. More than 1,000 communities in the United States are using land disposal methods, and many more are considering them (19). Clear guidelines are needed when such wastes are used on food crops or on crops that may enter the human food chain. A variety of public health problems are posed because conventional treatment processes do not remove pathogens and toxic elements from sludge and effluent. Accumulations of heavy metals, ova of human parasites, and salmonellae have been observed in soils fertilized with sludge (7, 9).

This study, which was done in parallel with one examining the persistence of poliovirus on sewage-irrigated vegetables (13), was undertaken to determine the survival of mycobacteria in soil irrigated with sewage effluent and sludge, and to establish the likelihood of contamination of vegetables eaten raw. Little recent information exists on survival of mycobacteria under conditions comparable to those currently employed in the spray irrigation of fields with sewage and sludge. In 1957, Greenberg and Kupka (6) reviewed the occurrence and survival of the tubercle bacilli in wastewaters and concluded that conventional treatment processes are inadequate to reduce numbers of mycobacteria. Since that time, the tuberculosis situation in the United States has changed considerably. New reported cases have fallen from 49,000 in 1964 to 33,000 in 1975 (1, 3). At the same time, chemotherapy is allowing treatment on an outpatient basis and elimination of tuberculosis institutions. The distribution of the disease involves geographic, age, and socio-economic factors; case rates vary among the states from 2.7 to 43 per 100,000 (2). Assuming that undetected cases make the actual number higher than the annual reported figure, many urban wastes will contain a low but continuous level of Mycobacterium tuberculosis, and the majority of these will pass through conventional treatment plants and be available for application on fields in effluent and sludge.

MATERIALS AND METHODS

Vegetable plots

Vegetables were grown in plywood boxes 6 x 2 x 1 ft (1.8 x 0.6 x 0.3 m), lined with polyethylene sheeting and with bottom drains connected to a container to collect percolate. Each box was filled with 9 inches (23 cm) of fertile topsoil evenly distributed over 2 inches (5 cm) of field stone. The plots were exposed to the normal outdoor conditions of the Cincinnati area. Two weeks before the beginning of spraying, plots were seeded with Black-seeded Simpson lettuce and Early Scarlet Globe radishes. Three rows of each were sown in each plot, in rows 10 inches (25 cm) apart. Plots were cultivated by hand when necessary.

Bacterial inoculum

Mycobacterium bovis BCG cultures were provided by the U. S.-Japan Cooperative Medical Science Program — National Institute of Allergy and Infectious Diseases. Strain 1029 (Phipps) was chosen because of its growth rate on the isolation medium and its distinctive colonial morphology. Cultures were grown in 800-ml bottles of Dubos broth at 35 C for 4 weeks. Sedimented growth was harvested by pipet, pooled, and homogenized by adding 0.5% Tween-80 and mixing for 1 h on a magnetic stirrer. Activated sludge and unchlorinated secondary effluent were obtained from the pilot plant at the Environmental Protection Agency, National Environmental Research Center, Cincinnati, Ohio, or from a municipal activated sludge plant. Approximately 200 ml of the cell suspension was added to each 20-liter Millipore pressure vessel of sewage effluent or sludge to yield an estimated 10^8 colony-forming units (CFU) per ml, and the contents of the pressure vessels were mixed for an additional hour on magnetic stirrers. Pressure vessels were fitted with a nozzle that applied a gentle
spray of the inoculum to simulate existing spray irrigation equipment. The amount applied was equivalent to a 1-inch (2.5-cm) depth of liquid. Plots were sprayed four times at weekly intervals.

**Sampling**

Sampling was begun 1 day after the last spraying. Soil and vegetables were collected daily for the first week and twice weekly thereafter. About 50 g of soil was collected by taking a soil plug from a depth of 1 in. from a minimum of six points. Estimated 50-g samples of vegetables were collected. Radishes were pulled out and the clinging soil was knocked off. Lettuce was cut about an inch above soil level. After rainfalls, drainage in the collecting tanks was also sampled.

**Assay**

Five-gram portions of soil were diluted 1 to 10 in phosphate-buffered distilled water containing 0.1% Tween-80 and mixed by repeated shaking and vortexing. Whole vegetables were placed in plastic bags and weighed, and 100 ml of the same diluent was added. Bags were shaken and kneaded for 1 h to remove surface material, and the wash water was poured off for assay. Samples that could not be assayed immediately were stored at -70°C (2). The isolation medium was a modification of the selective medium of Mitchison et al. (14), utilizing the synergistic inhibitory effect of sulfadiazine and trimethoprim (5). After cooling Middlebrook 7H10 oleic acid albumin agar to 45°C, the following were added: polymyxin B, 1,000 units per ml; penicillin G, 25 units per ml; novobiocin, 15 µg per ml; amphotericin B, 100 µg per ml; sulfapyridine, 50 µg per ml; and trimethoprim lactate, 30 µg per ml. Soil suspensions and vegetable washings were decontaminated by adding aqueous Zephiran to attain a concentration of 700 µg per ml. After 60 min, decontamination was halted by diluting the samples 1:10 in buffered distilled water containing 150 µg of lecithin (brain) per ml. Plating on the isolation medium was done with a semisolid overlay (15). An equal volume of 7H10 base medium diluted to 40% of the normal concentration, melted, and held at 46°C, was mixed with each dilution to be plated (2 ml of the mixture added per plate), and plates were quickly rotated to spread the inoculum before it hardened. A minimum of five plates were used per dilution. Plates were air-incubated at 35°C for 3 days to eliminate excess moisture, then reincubated in sealed plastic bags for minimum of 35 days.

**Colony verification**

Suspect colonies were picked to Dubos oleic agar and plain 7H10 agar. An isolate was regarded as the indicator mycobacterium only if it fulfilled all the following criteria: (a) possession of typical colonial morphology on the isolation medium, (b) failure to produce significant growth in 7 days on Dubos oleic agar and 7H10 agar, (c) production of typical colonies on these media in 14-21 days, and (d) growth from Dubos plates that possessed characteristic acid-fast morphology with Ziehl-Neelsen staining. Nontypical mycobacteria were not further identified.

**RESULTS AND DISCUSSION**

No organisms similar to BCG were found in soils, effluent, or sludge before inoculation. Counts of the indicator organism in inoculated effluent and sludge are shown in Table 1. BCG was isolated from soil until day 29; thereafter the recovery methods were inadequate to suppress the high levels of soil molds and bacteria. Isolation from radishes continued until day 13, and from lettuce until day 35, at which points the crops were exhausted because of the combined effects of sampling, insects, and disease. No insecticide or fungicide sprays had been used because of concern for their effects upon the indigenous soil flora as well as the mycobacteria.

Persistence in soil, plotted on least squares curves, is shown in Fig. 1. The D value (90% reduction time) in effluent-sprayed soil is 11 days, and in sludge-sprayed soil it is 8 days. This difference is significant, but no obvious reasons can be seen for the more rapid death rate in sludge. Intuitively, the converse would have seemed more likely because of the supposed protective effect of the sludge solids. Persistence on radishes is shown in Fig. 2. The D value on effluent-sprayed radishes is 6 days, and on sludge-sprayed radishes, 4 days; however, this difference is not significant. Persistence on lettuce is shown in Fig. 3. No survival rates were estimated because of the extreme scatter of points. Rainfall was probably responsible for washing surface inoculum from the leaves and causing the rapid initial loss, but the consistent, low-level isolation for 35 days indicated that a certain number of organisms had become firmly attached.

Washout of the inoculum from the plots by rainfall did not seem to be an important factor. The total number of mycobacteria found in the percolate tanks amounted to about 1% of the total applied to the plots.

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**TABLE 1.** BCG CFU's per milliliter of effluent and sludge sprayed on vegetable plots

<table>
<thead>
<tr>
<th>Date</th>
<th>Effluent</th>
<th>Sludge</th>
</tr>
</thead>
<tbody>
<tr>
<td>June 6</td>
<td>1.8 x 10⁴</td>
<td>6.0 x 10⁴</td>
</tr>
<tr>
<td>June 13</td>
<td>8.2 x 10⁴</td>
<td>3.5 x 10⁴</td>
</tr>
<tr>
<td>June 21</td>
<td>3.9 x 10⁵</td>
<td>5.0 x 10⁵</td>
</tr>
<tr>
<td>June 27</td>
<td>4.8 x 10⁵</td>
<td>6.4 x 10⁵</td>
</tr>
</tbody>
</table>

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**Figure 1.** Recovery of BCG from effluent- and sludge-sprayed soil.
The survival times indicated should be considered closer to minimum rather than general values. Heat was undoubtedly the most important single factor in death of the mycobacteria. The period allocated for this study was later than the normal growing season in the Cincinnati area, and summer temperatures were high. Soil surface temperature as high as 45°C was observed during afternoons.

All survival studies employing mycobacteria grown in vitro should be interpreted with caution. Segal and Bloch (16, 17) demonstrated differences between the same strain of *M. tuberculosis* grown in mouse lungs and in liquid culture. Cells grown in vitro exhibited higher hydrogen transfer capacity, substrate response, and immune response, despite the fact that cells grown in vivo were more virulent for mice. Kondo and Kanai (12) determined some of the reasons for these differences. Mycobacteria grown in vitro had much higher levels of the immunologically active chloroform-soluble waxes, although organisms grown in vivo had higher levels of other fats and waxes. These findings suggest the likelihood of real differences between mycobacteria cultured for use in survival studies and those occurring in sewage as a result of active human or bovine tuberculosis. These differences may also extend to resistance to environmental stress. BCG is especially open to criticism in this respect; the strain used in this study was derived from Calmette's original 1908 isolate and has been under laboratory conditions ever since. Despite this fact, it was chosen as an indicator organism in this study because any of the more pathogenic mycobacteria would have posed an intolerable infection hazard under the outdoor gardening conditions employed.

The numbers of tubercle bacilli occurring in "typical" urban sewage cannot really be estimated because of the difficulty of isolating them from material with very high levels of other organisms. The number varies, depending on the incidence of tuberculosis. By means of animal inoculation, Jensen (10) tried to recover tubercle bacilli from sewage influents, effluents, and sludges having their origin in populations with known tuberculosis cases. Ratios of cases to total population ranged from 1:3.6 to 1:2,000. Tubercle bacilli were found in all influents, effluents, and sludges up to a ratio of 1:600 (except one effluent from an activated sludge plant where the ratio was 1:460). It took 11 1/2 to 15 months for sludge on drying beds to become free of the organisms. Using artificially inoculated sewage, Heukelekian and Albanese (8) found that effluents treated by means of chemical coagulation, intermittent sand filtration, and chlorina-

Figure 2. Recovery of BCG from effluent- and sludge-sprayed radishes.

Figure 3. Recovery of BCG from effluent- and sludge-sprayed lettuce.
tion were free of demonstrable tubercle bacilli, but there was a 13-fold increase of the organism in sludge after 6 h of settling and only an 85% reduction after digestion for 35 days. There was little, if any, decline after 25 days of drying. Weiser et al. (20) were able to isolate M. tuberculosis from sludge beds of a tuberculosis hospital, but not from treated and chlorinated effluent. Tison et al. (19) were able to isolate M. tuberculosis from the effluent samples from a sanitorium. The effluent had received full treatment, including chlorination.

Packing plant wastes are another potential source of mycobacteria. Bovine tuberculosis has been brought under nearly complete control in the United States, but occasional large outbreaks still occur (4). Although tuberculin reactors are slaughtered and the meat used only in cooked products, packing plants wastes often enter municipal treatment facilities.

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REFERENCES