Investigating the Threat of Bacteria Grown in Space

Efforts are under way to determine how reduced gravity makes some bacteria more resistant to stress and more potent as pathogens

A. Matin and S. V. Lynch

In the 1960s, President John F. Kennedy said that our thirst for "knowledge and progress" made the "exploration of space [inevitable, and] one of the great adventures of all time." Early in 2004, President George W. Bush echoed those sentiments, saying that "exploration and discovery is not an option that we choose; it is a desire written in the human heart." His vision for the National Aeronautics and Space Administration (NASA) along with recent funding decisions could lead NASA to resume sending astronauts to the moon by 2020, with plans for the moon missions becoming a springboard for exploring Mars and other planets.

Although tantalizing, space is an inhospitable and dangerous frontier for those sent to explore it. Hence, progress towards more safely navigating and perhaps colonizing space are tasks that demand that we develop knowledge on several fronts, from designing radically new means of space transport to determining how space conditions influence biological processes. Several harmful effects of space on humans are documented. During extended missions in space, for example, bones lose mass, predisposing space travelers not only to fracture their bones but also to develop renal stones from resorbed bone material.

Moreover, muscles atrophy, decreased blood production and volume damage the cardiovascular system, latent viruses (such as *Varicella zoster*, which causes shingles) tend to reactivate, the incidence of diseases such as bacterial cystitis increases, wound healing slows, pharmacologic agents act differently, and pyschological conditions such as claustrophobia and anxiety tend to be accentuated, in part because of disrupted sleep and dietary patterns. Amid these physical and psychological conditions, there is the added problem that astronauts in space are exposed to intense radiation, involving high-energy protons and nuclei of heavy elements with greater penetrating power and increased capacity to cause malignancies and other problems, than they would be on earth. Additionally, the diminished gravity of space and planets, referred to as microgravity, also poses a direct threat to human health.

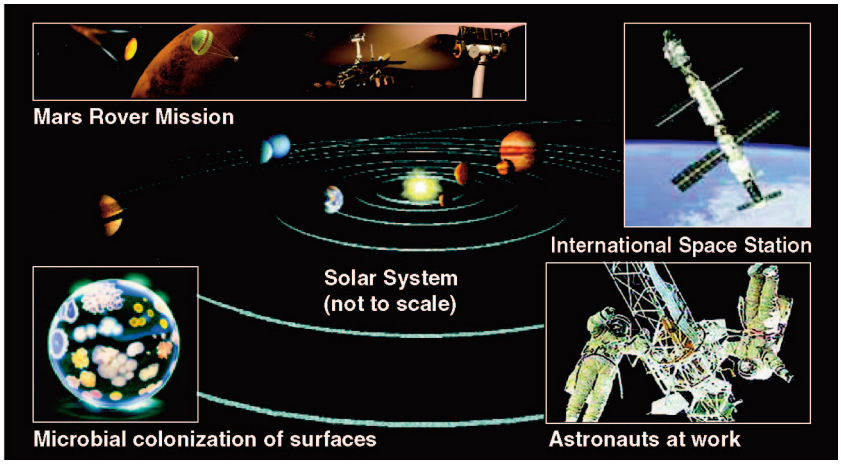
Earth-Based Systems for Studying the Biological Effects of Microgravity

Analytic equipment for studying biological processes is available on spacecraft such as the International Space Station (ISS). For example, microbes can be cultured using automated equipment on the ISS, including incubators that circulate air, control and sense temperature and humidity, and are equipped with telemetry, datafeed, video pass-through, and command capabilities. Moreover, centrifuges are installed that generate 1 × g gravity, providing the means for studying radiation effects independently of microgravity effects on biological material. The availability of radiation shields permits converse studies— of microgravity effects independent of radiation. Chemostats, equipped with hardware for processing culture samples at specified times, also are available.

Despite the availability of such instruments, however, technical difficulties and constraints on equipment accessibility and astronaut time greatly limit experimentation in space. Thus, several systems were developed to produce sim-

A. Matin is Professor and S. V. Lynch is a postdoctoral associate in the Department of Microbiology and Immunology at the Stanford University School of Medicine, Stanford, Calif.

FIGURE 1



Space, "one of the great adventures of all times."

ulated microgravity (SMG) on Earth. Because results from experiments using these devices generally can be duplicated in space, these devices have become the staples for doing microgravity research.

One particularly reliable approach for studying SMG effects on animals is the hindlimb suspension model in which animals are tethered by their tails to a guide wire, freeing their rear limbs of gravitational load. Animals that are suspended in this system become more susceptible to infection by various viruses and bacteria, according to Gerald Sonnenfeld and his collaborators at Binghamton University in Binghamton, N.Y.

Another more commonly used SMG-generating system for studying diminished gravity effects at the cellular level is the rotating cell culture system designed by scientists at Johnson Space Center in Houston, Tex. In a typical configuration, this apparatus consists of a cylindrical high-aspect-ratio vessel (HARV) that is equipped with sampling ports and a semipermeable membrane for aeration (Fig. 2). HARVs are filled to capacity with culture, leaving no headspace; aeration occurs by diffusion without bubble formation.

Two such vessels are used per experiment: one is rotated about a vertical axis (parallel to the gravitational vector) as a control, while the other rotates about a horizontal axis, providing an SMG environment. Laminar flow and the absence of bubbles greatly minimize shear in both vessels. In addition, in the vessel rotated about the horizontal axis, particles the size of bacteria and mammalian cells accelerate until they reach a constant terminal velocity. At this velocity, the pull of gravity is balanced by equal and opposite hydrodynamic forces, namely shear, centrifugal, and Coriolis forces.

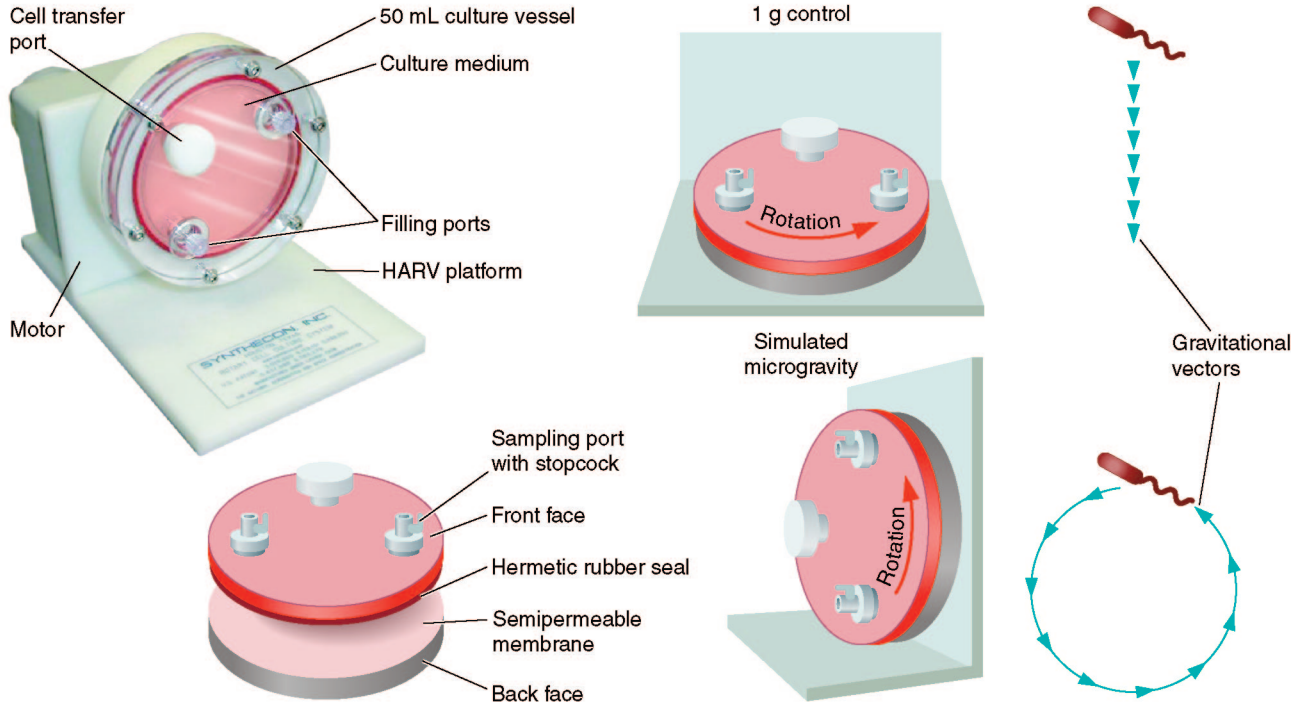
SMG Tends To Make Humans Weaker, Microbes Stronger

After realizing that astronauts show an increased susceptibility to infections and dormant viruses appear to be activated when in space, researchers launched a vigorous effort to uncover the effects of microgravity on microorganisms and also the human immune response. The latter studies provide strong evidence that diminished gravity compromises immunity. Contributing factors include detrimental shifts in the numbers and types of circulating lymphocytes, decreased production of cytokines and human leukocyte antigen (required to activate T and B cells), increased apoptosis of peripheral blood mononuclear cells, and impaired dendritic cell phagocytosis.

Meanwhile, microgravity tends to make microorganisms act more effectively as pathogens. For instance, *Salmonella enterica* serovar Typhimurium cells, grown under SMG conditions for a mere 5–10 hours, kill mice more rapidly than do such cells grown under ordinary conditions, according to Cheryl Nickerson and her colleagues at Tulane University in New Orleans, La. Moreover, the SMG-cultured bacteria colonize the spleens and livers of mice in greater numbers. These bacteria are also more resistant to a variety of stresses, such as high temperatures, that ordinarily kill such cells.

When we and others subjected *Escherichia coli* to SMG, the cells similarly showed increased resistance to the separately tested stresses of ethanol, hyperosmosis, and low pH. Bacteria in nature often persist in a low- to non-growth mode that is akin to extended stationary-phase cultures. When we kept stationary-phase cells for 24 h under SMG, they became super-resistant to the tested stresses.

FIGURE 2



The high-aspect-ratio vessel apparatus used to generate simulated microgravity on Earth for cultivating eukaryotic and bacterial cells (reproduced with permission, Synthecon Inc., Houston, Tex.). The sampling ports are on the front face of the vessel, and aeration is provided through a semipermeable membrane at the back face. Rotation of the vessel along appropriate axes provides SMG and 1 x g control conditions.

Knowing that astronauts become immunocompromised in space, these findings highlight the danger that bacteria pose to them during prolonged missions.

We also find that *E. coli* form biofilms more readily under SMG conditions than do their counterparts grown under ordinary gravity. Because of the evidence that the Mir space station was heavily colonized by biofilms, our finding could well be applicable to other bacteria. On Mir, severe biofouling damaged quartz windows and corroded various metal surfaces, contributing to a shortened useful lifetime of this station.

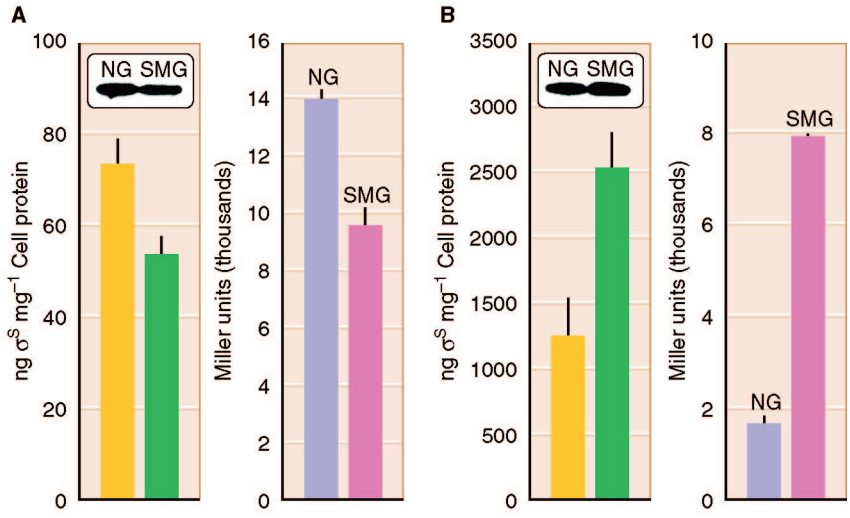
Biofilms in space pose an additional peril because the microbes within them tend to be highly resistant to antimicrobial agents, meaning they are yet another threat to the health of astronauts. On the plus side, however, biofilms are more efficient in nutrient recycling, waste disposal, and in regenerating potable water.

Bacterial Response to SMG Resembles their General Stress Response

The stresses to which bacteria become resistant in response to SMG harm them in different ways. Thus, growing in low pH acidifies the cytoplasm, high salt dehydrates cells, and ethanol damages the cell envelope. Because exposure to SMG confers resistance to these and other disparate stresses, it suggests that general protective mechanisms are at work. In particular, the response to SMG resembles the general stress response that is coordinated by a sigma factor, designated σ^s.

In 1988, we showed that when bacterial cells are treated with a small dose of hydrogen peroxide, they resist subsequent exposure to much higher doses of this otherwise toxic agent compared to untreated cells. Moreover, cells exposed to nonlethal levels of other stresses, including ethanol, heat, or starvation, also better

FIGURE 3



Immunoblots and corresponding bar graphs showing σ^s levels in exponential (A) and stationary (B) growth phases. Bar graphs on the right in the two panels represent the quantification of the sigma factor by another method and confirm the immunoblot results.

resist subsequent exposure to peroxide. This phenomenon applies to many other stresses. For example, exposure to lethal high temperatures is better resisted by cells first treated with moderate levels of other stresses, including heat, hydrogen peroxide, ethanol, acid, or starvation.

Such moderately stressed cells also show an increased capacity to cause infections. Thus, this response insulates cells not only from the stress that they experienced directly but from many other stresses while it also increases virulence. Thus, it confers enhanced comprehensive cellular resistance. Because SMG also confers comprehensive cellular resistance, SMG at first appears to be inducing this same general stress response. However, the molecular basis of the SMG effect appears to be different.

Different Roles for σ^s in SMG and Gravity-Grown Cells

The *E. coli* general stress response σ^s is considered a major determinant of cellular resistance, and there is a direct relationship between the concentration of this sigma factor and comprehensive resistance. Thus, various stresses lead to an increase in concentration of σ^s, generating a different RNA polymerase species in which the core RNA polymerase, E, combines with σ^s, forming the Eσ^s holoenzyme. Eσ^s binds to specific promoter configurations, which are upstream of genes that encode protective proteins, leading to their increased synthesis. Mutant studies confirm that σ^s is required for developing comprehensive cellular resistance.

However, although bacteria growing under SMG exhibit increased comprehensive resistance, such cells contain decreased σ^s levels (Fig. 3). Moreover, mutant strains devoid of this sigma factor retain the capacity to develop SMG-conferred comprehensive resistance. Oddly, stationary-phase SMG-grown cells contain very high levels of σ^s, and mutants missing σ^s fail to become super resistant. Thus, SMG and conventional earth-bound bacterial cell stress resistance responses differ in two respects: the former can be independent of σ^s, and its relationship to σ^s reverses in a growth-phase dependent manner.

Role of Stress Proteins

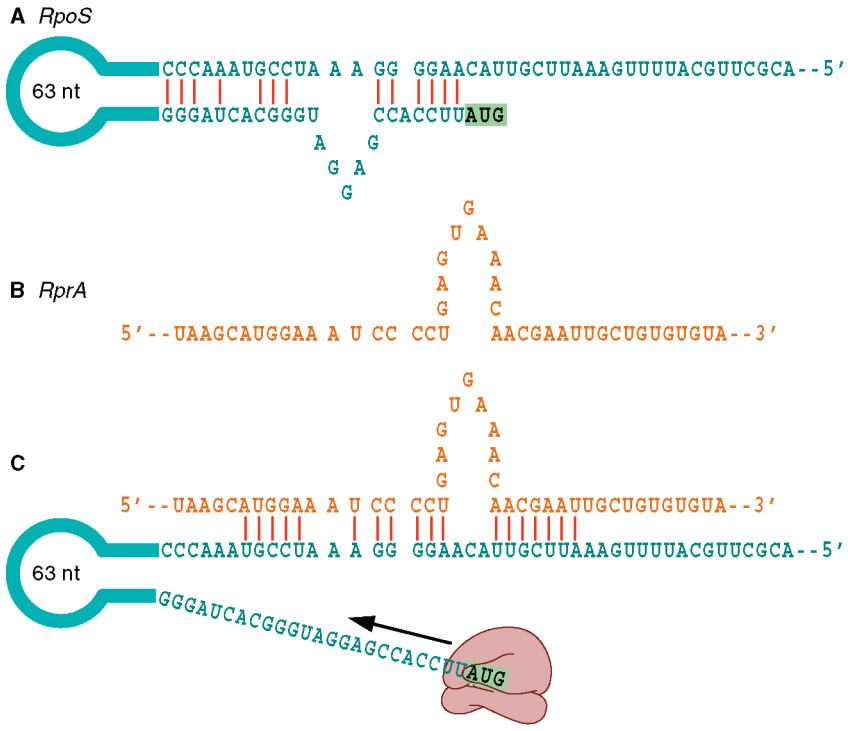
In response to stress, Earth-bound bacteria acquire comprehensive resistance by harnessing proteins that can prevent damage to and promote repair of vital cell constituents. These protective protein classes include molecular chaperones, the SOS response proteins, and PexB (Dps);
they protect or repair other cell proteins, DNA, and the consequences of oxidative stress, respectively.

How repair is brought about can be illustrated by the mode of action of the DnaK family of molecular chaperones. Apart from DnaK, this family consists of two additional proteins, DnaJ and GrpE. DnaK, when bound to ATP, has a low affinity for denatured proteins, but a high affinity for them when complexed to ADP. DnaJ initiates repairs by binding to a denatured protein and presenting it to DnaK-ATP complex. Repair proceeds, promoted by the energy released from ATP hydrolysis. A stable complex results, consisting of the substrate protein, DnaJ, and DnaK-ADP. GrpE releases the substrate protein from this complex, and the cycle continues until the damaged protein is fully repaired. The mode of action of several other classes of repair proteins, such as the SOS pro-

teins that repair DNA, is also reasonably well understood.

The SMG-conferred comprehensive resistance is, however, acquired without the induction of σ^s-regulated genes, such as the *dnaK* gene family, *groEL*, and *pexB*, and known virulence determinants that account for comprehensive cellular resistance in conventional cultures, despite the fact that the expression of over 100 genes is altered under SMG. Instead, the ferric iron uptake regulator protein, Fur, has a role in acid resistance of SMG-grown exponential-phase cells of *S. enteritica* serovar Typhimurium, and the regulatory regions of many of the SMG-induced genes appear to have Fur binding sites, according to Tulane's Nickerson and her colleagues. What mechanisms of resistance and virulence operate under SMG is under intense investigation. Many of the genes induced by SMG have no known function.

FIGURE 4



The untranslated region of the messenger RNA (*rpoS* mRNA) that encodes σ^s. Note that the sequences upstream of the translational initiation codon (ATG) of the RNA include regions of internal homologies that result in the formation of a hairpin structure. This prevents the availability of the initiation codon to the ribosomes (A). The small noncoding RNA RprA has regions of homology to the untranslated region of *rpoS* mRNA (shown in red; B). Hydrogen bonding between the homologous regions of RprA and *rpoS* mRNA opens the hairpin, permitting translation (C).

Effect of Microgravity on σ^s Regulation

The unexpected pattern of growth phase-related changes to σ^s levels under SMG prompted us to investigate the underlying regulatory mechanisms. We knew that in conventional cultures, σ^s regulation depends primarily on the efficiency with which the messenger RNA (mRNA) encoding σ^s (*rpoS* mRNA) is translated and on the subsequent stability of σ^s protein. Both are profoundly affected by growth under SMG conditions. Thus, SMG markedly increases the translational efficiency of *rpoS* mRNA regardless of growth phase. mRNAs in general contain an untranslated region that is upstream of the translation initiation codon. This codon and contiguous sequences are required for translation, and together form a translation initiation region (TIR). The *rpoS* transcript contains an unusually long untranslated region that is upstream of the translation initiation codon. Homologies in this region can result in base pairing with TIR (Fig. 4). The resulting secondary structure prevents mRNA binding to the ribosomes, decreasing translational efficiency.

Several proteins, especially the protein called Hfq, and several small noncoding RNAs (sRNAs), can modulate this process. For example, the sRNA RprA, which has regions of homology to the interfering sequence in the *rpoS* mRNA untranslated region, can, in a process evidently mediated by Hfq, disrupt the secondary structure, freeing the TIR and increasing translational efficiency. The fact that SMG increases *rpoS* mRNA translation raises the possibility that it alters the concentration of regulatory molecules such as Hfq and sRNAs, and perhaps directly reduces the tendency of *rpoS* mRNA to form secondary structures.

SMG also has a strong destabilizing effect on the σ^s protein, especially in exponential-phase cells. In 1996, we showed that in conventional cultures, σ^s stability is determined through cleavage by a specific cellular protein degrading

complex, called ClpXP in which another protein, RssB (also called SprE) was later shown to also play a role. Existence of another mechanism, independent of RssB, for σ^s cleavage regulation has very recently been hinted at by Thomas Silhavy and his collaborators at Princeton University in Princeton, N.J. In addition, ClpXP protease activity is greatly affected by the folding pattern of its substrate. SMG may alter σ^s stability by affecting any of these and perhaps other, as yet unknown, mechanisms of σ^s regulation.

Perspectives

SMG makes both planktonic and biofilm bacteria more robust and thus a more potent threat to the health of space dwellers. The increased hardiness of such bacteria appears to be mediated through a previously unrecognized mechanism that depends on σ^s but differs from the generalized stress response and appears to be also independent of other characterized bacterial stress proteins. Better understanding this mechanism should provide insights for those seeking to ensure a healthier environment for astronauts traveling in space. This understanding will also be helpful for those seeking to exploit the beneficial activities of bacteria that are indispensable for sustaining life in space. These benefits include renewal of resources such as oxygen and water, recycling wastes, and establishing sustained ecosystems on other planets.

Two of the regulatory mechanisms affected by SMG, namely the translational efficiency of *rpoS* mRNA and the stability of the σ^s protein, involve the folding patterns of macromolecules. Whether these SMG-associated phenomena are due to the direct effect of diminished gravity on RNA and protein folding should be further examined. Indeed, the implications for space exploration and residence are wide ranging.

Growth under SMG changes many bacterial responses, suggesting that they can sense gravity. Gravitropism is an area of research interest, and some researchers are studying its molecular underpinnings in plants. Given the flexibility of bacteria as experimental models, they could prove useful for studying such phenomena.

ACKNOWLEDGMENTS

Research from this laboratory was supported by a grant from NASA (#NNA04CC51G); S.V.L. was supported, in part, by a Dean's Fellowship from Stanford University School of Medicine. We thank David Ackerley, Yoram Barak, and Mimi Keyhan for helpful comments.

SUGGESTED READING

Gmunder, F. K., M. Kiess, G. Sonnefeld, J. Lee, and A. A. Cogoli. 1990. A ground-based model to study the effects of weightlessness on lymphocytes. Biol. Cell. **70:**33–38.

Gottesman, S. 2004. The small RNA regulators of *Escherichia coli*: roles and mechanisms. Annu. Rev. Microbiol. **58:**303–328.

Hammond, T. G., and J. M. Hammond. 2001. Optimized suspension culture: the rotating-wall vessel. Am. J. Physiol. Renal. Physiol. **281:**F12–25.

Kenniston, J. A., R. E. Burton, S. M. Siddiqui, T. A. Baker, and R. T. Sauer. 2004. Effects of local protein stability and the geometric position of the substrate degradation tag on the efficiency of ClpXP denaturation and degradation. J. Struct. Biol. **146:**130–140.

Lynch, S. V., E. L. Brodie, and A. Matin. 2004. Role and regulation of σ^s in general resistance conferred by low-shear simulated microgravity in *Escherichia coli*. J. Bacteriol. **186:**8207–8212.

Matin, A. 2000. Stress response in bacteria, p. 3034–3046. In G. Bitton (ed.), Encyclopedia of environmental microbiology, vol. 6. John Wiley and Sons, New York.

Nickerson, C. A., C. M. Ott, J. W. Wilson, R. Ramamurthy, and D. L. Pierson. 2004. Microbial responses to microgravity and other low-shear environments. Microbiol. Mol. Biol. Rev. **68:**345–361.

Nystrom, T. 2004. Stationary-phase physiology. Annu. Rev. Microbiol. **58:**161–181.

Peterson, C. N., N. Ruiz, and T. J. Silhavy. 2004. RpoS proteolysis is regulated by a mechanism that does not require the SprE (RssB) response regulator phosphorylation site. J. Bacteriol. **186:**7403–7410.

Stowe, R. P., C. F. Sams, and D. L. Pierson. 2003. Effects of mission duration on neuroimmune responses in astronauts. Aviat. Space. Environ. Med. **74:**1281–1284.