nuclear membrane with characteristic nucleoli and a cytoplasm free of large vacuoles. To assess neuron survival in cultures exposed to putative neurotoxic factors for 48 hours, we determined the mean number of healthy cells by scoring the number of neurons in at least 20 randomly selected fields for each of three coverslips. Ciliary ganglion neurons also were identified by immunohistochemical staining for choline acetyltransferase [D. Giulian, K. Vaca, B. Johnson, *J. Neurosci. Res.* **21**, 487 (1988)] with a 1:500 dilution of mouse 1E6 monoclonal antibody from ascites fluid.

11. Spinal cords were removed from day 15 albino rat embryos (Holtzman, Madison, WI) and dissected free of attached meninges and ganglia. After incubation with 0.08% trypsin in Ca^{+2}- and Mg^{+2}-free PBS for 10 min at 37°C, the cords were transferred to a chemically defined neuron culture medium (*10*) supplemented with 10% heat-inactivated horse serum (Gibco), and dissociated by trituration with fire-polished Pasteur pipettes. Cells were plated at a density of 500,000 cells per well of a 24-well tissue culture plate (Falcon) with each well containing a poly-L-lysine–coated 12-mm diameter glass coverslip. Twenty-four hours after plating, control or conditioned medium was added and the cultures were incubated another 3 days before fixation for 45 min with 3% formaldehyde in PBS. Neuron identification in the mixed-cell population of rat spinal cord cells was performed by indirect immunofluorescence staining with a 1:50 dilution of mouse monoclonal antibody MO3B to the 160-kD neurofilament protein followed by a 1:100 dilution of rhodamine-conjugated rabbit antibody to mouse immunoglobulin G (Dakco). Under these conditions, control cultures contained <5% NF^+ neurons and >90% glia.
12. All of the cell lines were grown as suspension cultures in RPMI 1640 medium with L-glutamine supplemented with 10% FBS at 37°C in a humidified, 95% air and 5% CO_2 atmosphere. The cell lines were free of detectable mycoplasma contamination. Continuous human T cell lines, uninfected [H9, M. Popovic, M. C. Sarngadharan, E. Read, R. C. Gallo, *Science* **224**, 497 (1984); M. Popovic, E. Read-Cannole, R. C. Gallo, *Lancet* **ii**, 1472 (1984)] or persistently infected with the $HTLV_{IIIB}$ prototype strain of HIV-1 (H9/$HTLV_{IIIB}$ NIH 1983), were obtained through the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, NIH, from R. Gallo. Two human monocyte cell lines were obtained from the American Type Culture Collection: the promonocytic cell line U937 [ATCC CRL 1593, P. Ralph, A. S. Moore, K. Nilsson, *J. Exp. Med.* **143**, 1528 (1976)] and the monocytic leukemia line THP-1 [ATCC TIB 202, S. Tsuchiya *et al.*, *Int. J. Cancer* **26**, 171 (1980)]. Both the U937 and THP-1 cell lines were infected with the $HTLV_{IIIB}$ strain of HIV-1 at a multiplicity of infection of 5×10^5 tissue culture infectivity dose of 50% ($TCID_{50}$). The cells were passaged and monitored periodically for HIV antigens by indirect immunofluorescence with pooled polyclonal antiserum from HIV-1–infected patients. At 15 to 20 days postinfection, the cells were >80% HIV-antigen positive. These cell lines, which have been in culture for more than 1 year, grow well under standard culture conditions (without the addition of cytokines), produce virus that is cytopathic in the MT-4 cell line (approximately 10^4 $TCID_{50}$ per milliliter of culture fluid), and show high titers of p24 antigen ($>1 \times 10^5$ pg/ml of culture fluid) as determined by EIA (Du Pont Biotechnology Systems). Electron microscopy of the infected THP-1 and H9 cells showed HIV-1–like retroviral particles.
13. To test the monocytoid or lymphoid cells for the production of neurotoxic factors, we plated human cells at concentrations of 10^4, 10^5, or 10^6 in 1 ml of medium (RPMI 1640 with L-glutamine and 10% FBS) in Millicell-CM chambers (0.4-μm filter pore size; Millipore), which had been placed in 12-well plates (Falcon). Each well contained about 8000 ciliary ganglia neurons growing on an 18-mm round glass coverslip coated with poly-L-lysine in 1 ml of chemically defined neuron culture medium supplemented with 10% FBS. After 48 hours of coculture, neurons were fixed and scored as described in (*10*). Culture media were collected to measure p24 antigen concentrations.
14. To prepare conditioned media, test cells were seeded at a density of 5×10^5 cells/ml and cultured for 48 hours under standard conditions (*12*). To monitor HIV-1 production, we evaluated 200-μl samples of culture media by an EIA specific for the p24 antigen of HIV. Media were centrifuged for 10 min at 300*g* and then subjected to ultracentrifugation for 60 min at 200,000*g* to remove viral particles. The media were then heat treated at 60°C for 60 min, filter-sterilized (0.2 μm; Acrodisc; Millipore), and stored at −80°C.
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20. Amino acid analysis of culture media passed through Centricon-3 filters was carried out by C. Goodman, Department of Pathology, Baylor College of Medicine, using a PicoTag System (Waters Instruments).
21. Quantitative measurements of quinolinic acid levels in culture media were carried out by S.-N. Lin, Analytical Chemistry Center, University of Texas Health Science Center at Houston, with a Finnigan MAT Incos 50 for GC-MS. As described by Heyes *et al.* (*19*), samples containing quinolinic acid were washed with an equal volume of chloroform, and the aqueous phase was passed over a small ion-exchange column (0.2 cm by 1.0 cm) containing Dowex 1-X2 (formic form), washed with 10 volumes of distilled H_2O and 10 volumes of 0.1 N formic acid, eluted with 5.0 ml of 6 N formic acid, and lyophilized with a Speed-Vac (Savant). Under these conditions, we found an ~15% loss of [^{3}H]quinolinic acid during the chloroform extraction and another 10 to 15% loss on the ion-exchange column. Lyophilized samples eluted from Dowex ion-exchange columns were derivatized with bis-trimethylsilyl trifluoroacetic acid and passed through a 60-m DB-1 fused-silica capillary column (GC oven temperature programmed from 190°C to 210°C at the rate of 1°C/min). Under these conditions, we showed good separation of quinolinic acid (2,3-pyridinedicarboxylic acid) from dipicolinic acid (2,6-pyridine-dicarboxylic acid). Selective ion monitoring by MS allowed detection of an internal standard 4-chlorobenzoic acid (mass-to-charge ratio *m/z* 213) and quinolinic acid (*m/z* 296) with detection sensitivity (after column chromatography) of <1 ng. Standards of quinolinic acid measured by GC-MS showed a linear range from 2 to 50 ng, thus providing sufficient sensitivity to detect toxic levels of quinolinic acid in conditioned media. Using large batches of culture media (10 ml), we found that the amounts of quinolinic acid secreted by various cell lines with or without HIV-1 infection did not significantly differ and were far below the toxic range for the cultured ciliary neurons.
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Poliovirus Mutants Resistant to Neutralization with Soluble Cell Receptors

GERARDO KAPLAN, DAVID PETERS, VINCENT R. RACANIELLO*

Poliovirus mutants resistant to neutralization with soluble cellular receptor were isolated. Replication of soluble receptor-resistant (*srr*) mutants was blocked by a monoclonal antibody directed against the HeLa cell receptor for poliovirus, indicating that the mutants use this receptor to enter cells. The *srr* mutants showed reduced binding to HeLa cells and cell membranes. However, the reduced binding phenotype did not have a major impact on viral replication, as judged by plaque size and one-step growth curves. These results suggest that the use of soluble receptors as antiviral agents could lead to the selection of neutralization-resistant mutants that are able to bind cell surface receptors, replicate, and cause disease.

SOLUBLE CELL RECEPTORS HAVE BEEN shown to block infection by HIV-1 (*1–5*), Epstein-Barr virus (*6*), and rhinovirus (*7*). We have shown that infection of cells with poliovirus, a small, nonenveloped animal virus, is blocked by cytoplasmic extracts of insect cells expressing cell receptors for poliovirus (*8*). As a result of these studies, it has been proposed that soluble cell receptors might be effective antiviral therapeutics. It has been suggested that viral mutants resistant to the antiviral effects of

Department of Microbiology, College of Physicians and Surgeons of Columbia University, 701 West 168th Street, New York, NY 10032.

*To whom correspondence should be addressed.

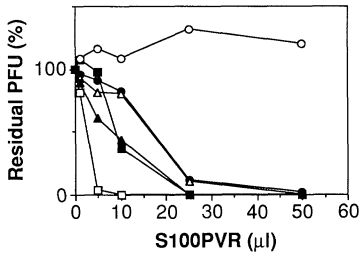

Fig. 1. Effect of S100PVR concentration on neutralization of wild-type virus and *srr* mutants. Identical amounts of purified poliovirions (10^7 PFU) were treated with increasing amounts of S100PVR (0, 1, 5, 10, 25, and 50 μl; 5 μl contained 5×10^5 insect cell equivalents) for 1 hour at 37°C; infectivity was determined by plaque assay on HeLa cells. Titers obtained from S100PVR-treated samples are expressed as a percentage of the virus titer of each sample incubated without S100PVR. Each point is the average of five independent experiments; all values were within 5% of those shown. □, wild type; ■, 111A; ○, 111C; ●, 131B; △, 141D; and ▲, 151A.

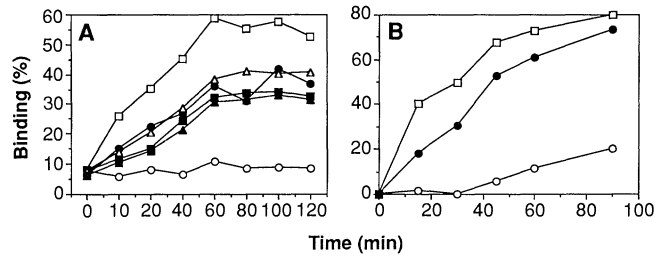

Fig. 2. Binding of *srr* mutants to membrane-anchored PVR. (**A**) Binding to HeLa cell membranes. We mixed 80,000 cpm of each [^{35}S]methionine-labeled virus preparation with 500 μl of HeLa cell membranes prepared as described (*25*) and incubated it at 25°C. Samples (50 μl) were taken at different times and membranes were pelleted at 12,000*g* for 3 min and resuspended in 0.2% SDS. The percentage of poliovirus binding to the membrane fraction was determined by scintillation counting of both supernatant and resuspended membranes. Nonspecific binding, determined in the presence of blocking anti-receptor monoclonal antibody D171, was approximately 5% of the values shown. (**B**) Binding to HeLa cell monolayers. Confluent monolayers in 10-cm plates were infected with 5×10^4 PFU of virus in 0.3 ml DMEM at 25°C. Samples (20 μl) were taken at different times after infection for titration of infectivity. In both experiments, each point is the mean of duplicate samples; all values were within 5% of those shown. Symbols are defined in Fig. 1.

soluble receptors would not arise, because mutations that abrogate binding to receptors would be lethal (7). Here we show that poliovirus mutants resistant to neutralization with soluble cellular receptor can be readily isolated, at rates similar to those observed for mutants resistant to neutralization with monoclonal antibody.

Cell receptors for poliovirus were overexpressed in insect cells by means of the baculovirus expression system (*8*). *Spodoptera frugiperda* IPLB-SF-21 cells were infected for 48 hours with wild-type baculovirus (AcNPV) or recombinant baculovirus containing the cDNA encoding the poliovirus receptor (AcPVR). Cells were lysed with 1% NP-40, nuclei were removed by centrifugation, cytoplasmic extracts were centrifuged 1 hour at 100,000*g*, and the supernatant was filtered through 0.2 μm nitrocellulose. Cytoplasmic extracts (S100) of insect cells infected with a recombinant baculovirus containing poliovirus receptor cDNA (S100PVR) but not wild-type baculovirus (S100NPV) neutralized poliovirus infectivity (*8*). Viral titers were reduced 100- and over 1000-fold by incubation with 5 or 10 μl of cytoplasmic extracts, respectively (Fig. 1). However, a fraction of the input virus consistently failed to be neutralized. For example, incubation of wild-type virus with 10 μl or more of S100PVR reduced infectivity to 2500 plaque-forming units per milliliter (PFU/ml), compared to 8×10^6 PFU/ml in the absence of extract.

To determine whether this residual infectivity was due to neutralization-resistant mutants, we neutralized a plaque-derived stock of poliovirus P1/Mahoney (designated wild type) with 10 μl of S100PVR. Virus from five plaques was then subjected to two additional rounds of neutralization with S100PVR and plaque purification. Four of the five plaques gave rise to between one in three and one in five *srr* mutants (designated 111A, 111C, 131B, 141B, 141D, and 151A). Similar results were obtained with an independent wild-type clone (*9*), indicating that the generation of *srr* mutants was not restricted to one wild-type poliovirus isolate. The frequencies with which *srr* mutants were isolated from the wild-type population were estimated at about 1 in 10^4 to 10^5 PFU, which is comparable to that reported for the isolation of mutants resistant to neutralization with monoclonal antibodies (*10, 11*). Analysis with neutralizing polyclonal antibodies identified the *srr* mutants as type-1 poliovirus (*9*).

For subsequent analyses, wild-type virus and *srr* mutants 111A, 111C, 131B, 141D, and 151A were labeled with [^{35}S]methionine and purified in 5 to 30% sucrose gradients. The *srr* mutants and wild-type virus were incubated with increasing amounts of S100PVR, and the remaining infectivity was determined (Fig. 1). The results indicate that the *srr* mutants fall into two classes: one class consisting of mutants that are relatively more resistant than wild-type virus, but could be neutralized with high concentrations of S100PVR (mutants 111A, 151A, 131B, and 141D), and the other class represented by one mutant that could not be neutralized with high concentrations of S100PVR (mutant 111C). Thus, wild-type virus was very sensitive to neutralization with S100PVR, mutant 111C was totally resistant to neutralization, and mutants 111A, 131B, 141D, and 151A were of intermediate resistance.

To determine whether the *srr* mutants and wild-type virus use the same cell surface receptor to infect HeLa cells, a protection assay was done with monoclonal antibody D171. This antibody is directed against the cell receptor for poliovirus and specifically blocks poliovirus infection (*12*). D171-treated HeLa cell monolayers were protected against infection with wild-type virus and with all *srr* mutants, but the protection was significantly higher in cells infected with mutants 111A and 111C (*9*). Thus, wild-type poliovirus and the *srr* mutants use the same PVR to infect HeLa cells.

To determine the basis for the resistance to neutralization with soluble receptor, we determined the kinetics of virus binding to membrane-bound PVR. Equal counts per minute of purified [^{35}S]methionine-labeled poliovirions were bound to HeLa cell membranes for different amounts of time. All virus preparations had specific infectivities of approximately 3.5×10^3 PFU/cpm, and the particle:plaque-forming unit ratios were between 150 to 300. HeLa cell membranes were then pelleted, and the associated radioactivity was determined by scintillation counting. Binding of wild-type virus to HeLa cell membranes saturated within 60 min at 60% of the input virus (Fig. 2A). Binding was not detected with *srr* mutant 111C, whereas mutants 111A, 131B, 141D, and 151A were all similar and bound more slowly and less efficiently than wild-type virus. Kinetics of binding of the viruses with membranes from AcPVR-infected insect cells were similar to those obtained with HeLa cell membranes, and poliovirus did not bind to membranes of AcNPV-infected insect cells (*9*).

The kinetics of *srr* mutant binding to intact HeLa cells was also examined. Equal amounts of wild-type virus or *srr* mutants 131B and 111C were adsorbed to HeLa cell monolayers at 25°C, and the binding kinetics were determined (Fig. 2B). Mutant 131B bound slightly less efficiently than wild-type virus. Mutant 111C bound very poorly, although some binding was detected. The percentage of binding of both wild-type poliovirus and *srr* mutants was higher with HeLa cell monolayers than with mem-

branes, which might account for the ability to detect low amounts of mutant 111C binding to HeLa cell monolayers. Slightly different profiles were obtained when we used membranes or whole cells. In the membrane assay, binding was detected with radiolabeled virus, whereas binding to whole cells was measured by titrating residual infectious virus in the supernatant. Furthermore, binding to HeLa cell monolayers was done with 5×10^4 PFU and 3×10^7 cells, whereas binding to membranes was done with approximately 2.8×10^7 PFU and 5×10^6 cell equivalents. Therefore, the whole cell assay was performed under conditions of receptor excess and was more sensitive than the membrane assay and able to detect low amounts of virus attachment. We found that in both assays, *srr* mutants showed reduced ability to bind to PVR.

The results of binding assays suggest that mutations responsible for the resistance of the *srr* variants to neutralization with soluble PVR also reduce the ability of these viruses to bind membrane-bound PVR. The decreased binding of the mutants to HeLa cell membranes correlated well with resistance of the *srr* mutants to neutralization with soluble PVR. For example, mutant 111C was the poorest at binding to HeLa cell membranes and was not neutralized by any concentration of S100PVR, whereas wild-type virus was able to bind most efficiently and is also best neutralized by soluble PVR. The greater sensitivity of *srr* mutant 111C to protection with D171 correlates with its high resistance to neutralization with soluble PVR, and may result from failure to compete with D171 for the receptor binding site because of its reduced affinity for the cell receptor.

When poliovirus is bound to cells at 37°C, a large fraction of the particles elute in a noninfectious form (*13, 14*). These "altered" particles cannot attach to cells, have lost capsid protein VP4, are sensitive to proteases, are hydrophobic, and sediment at 135*S* [compared to 160*S* for native particles (*15*)]. Altered particles eluted from cells are very similar to an intracellular form of the virus that predominates early after infection (*16*). We previously showed that neutralization of poliovirus by S100PVR is mediated in part by conversion of native virus to altered particles (*8*). To determine whether the *srr* mutants could be converted to altered particles, we incubated [^{35}S]methionine-labeled poliovirions with HeLa cell membranes at 37°C for 1 hour, followed by centrifugation to remove membranes (*17*). The unbound and eluted polioviruses in the supernatant were analyzed by sedimentation in a 15 to 30% sucrose gradient. When wild-type poliovirus was incubated with membranes, the bulk of the native 160*S* particles were converted to 135*S* altered particles and 80*S* empty capsids (Fig. 3). Treatment of mutants 131B (Fig. 3), 151A, and 111A (*9*) also resulted in formation of 135*S* and 80*S* particles. Mutant 111C was largely unaltered under these conditions; a trace of 135*S* particles and no 80*S* particles were observed. These results suggest that alteration is not blocked in *srr* mutants 151A, 111A, and 131B. Although it was not possible to rule out alteration defects in mutant 111C, the small amount of 135*S* particles observed suggests that alteration is inefficient because of the poor binding properties of this mutant.

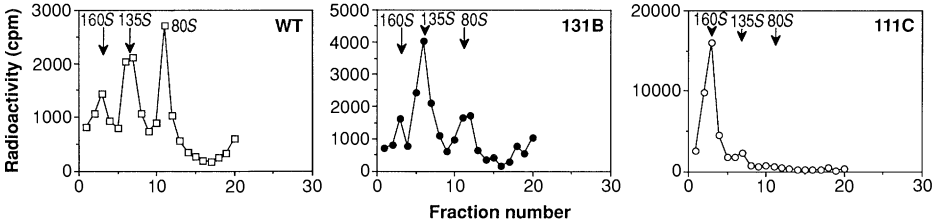

Fig. 3. Alteration of *srr* mutants by HeLa cell membranes. Purified [^{35}S]methionine-labeled poliovirions (80,000 cpm) were mixed with 500 μl of HeLa cell membranes and incubated at 37°C for 1 hour. Membranes were pelleted at 12,000*g* for 5 min and supernatants were centrifuged on 15 to 30% sucrose gradients for 2.5 hours at 40,000 rpm in an SW40 rotor at 4°C (*16*). Radioactivity of one-third of each fraction was determined by scintillation counting in 3 ml of Aquasol (NEN). Profiles of wild-type virus (top), mutant 131B (middle), and mutant 111C (bottom) are shown. Arrows indicate migration of native poliovirions (160*S*), eluted A particles (135*S*), and empty capsids (80*S*) determined by centrifugation of markers in parallel gradients.

One-step growth analyses were done to determine whether the reduced binding phenotype of the *srr* mutants affected viral replication. We found that mutant 131B replicated with similar kinetics and to similar concentrations as wild-type virus (Fig. 4). Replication of mutant 111C lagged slightly at 4 hours after infection, but reached similar final titers. Both mutants showed an initial higher background at time 0, consistent with their reduced binding and eclipse. All *srr* mutants had the same plaque size as wild-type virus when assayed on HeLa cell monolayers as described (*18*). These results show that replication of the *srr* mutants in HeLa cells is not dramatically altered, despite their reduced ability to bind cell receptors.

It is curious that the reduced ability of the *srr* mutants to bind cell receptors did not have a major effect on viral replication. In particular, binding of mutant 111C was severely reduced compared to wild-type virus, yet this mutant showed only a slight difference in replication. This observation cannot be explained by differences in the particle:plaque-forming unit ratios of the *srr* mutants, which were not significantly different from wild-type virus. Furthermore, the *srr* mutants had the same plaque size as wild-type virus. These results suggest that

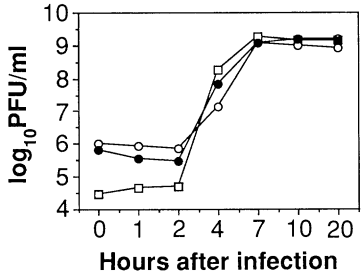

Fig. 4. Analysis of replication of *srr* mutants. Single-cycle replication of wild-type virus and mutants 111C and 131B was examined at 37°C as described (*26*). HeLa cell monolayers containing 3×10^6 cells were infected at a multiplicity of infection of 10. After adsorption at 37°C for 45 min, the inoculum was removed, monolayers were washed five times with PBS, and growth medium was added. At different times after infection, monolayers were frozen and thawed three times, medium was clarified, and total virus was determined by plaque assay in HeLa cells. Each time point is the average of duplicate samples; values did not differ by more than 0.5 $\log_{10}$. Symbols are defined in Fig. 1.

the binding assays do not measure parameters that are important for viral infection. Perhaps receptor binding is not the rate-limiting step in viral infection, and short of abolishing binding, the virus can tolerate a wide range of binding capabilities. A similar situation has been reported for coxsackieviruses B2, B4, and B6, which bind extremely poorly to HeLa cells, yet replicate as well as coxsackievirus B1, B3, and B5, which attach extremely well to these cells (*19*).

It will be important to determine the functional basis for the reduced binding of the *srr* mutants. The location of the responsible mutations, which are presumably in the viral capsid, might provide information on the mechanism by which receptor binding is altered. The mutations may occur in virion sites that directly interact with the cell receptor. Alternatively, the mutations may occur at sites that are involved in receptor-mediated conformational transitions of the virion that are believed to be essential steps in viral

replication (*16*). For example, such transitions may be required for high-affinity receptor binding required for productive infection, and mutations affecting the transitions may therefore block receptor binding. The availability of the three-dimensional structure of poliovirus (*20*) will facilitate interpretation of the mechanism of action of mutations that cause the *srr* phenotype.

It has been proposed that soluble receptors might be used as antiviral agents against HIV-1 (*1–5*), Epstein-Barr virus (*6*), and rhinovirus (*7*), and clinical trials of soluble CD4 for HIV-1 infection are currently underway. One argument for the use of receptor-based antivirals is that the virion attachment site may be a highly conserved sequence (*21*). Viruses with mutations that lead to resistance to neutralization with soluble receptors would therefore not arise, because such mutations would be lethal (*7, 22*). Here we have shown that poliovirus mutants resistant to neutralization with soluble PVR can be readily isolated. These results suggest that either mutation of the receptor binding site is not lethal, or that mutation at other sites in the virion can modulate receptor binding. Since replication of *srr* mutants in cultured cells was not significantly impaired, it is possible that these variants are as pathogenic as wild-type virus. Preliminary results with a transgenic mouse model for poliomyelitis (*23*) suggest that *srr* mutants are as neurovirulent as wild-type virus (*24*). These findings temper enthusiasm for the use of soluble receptors as antiviral compounds.

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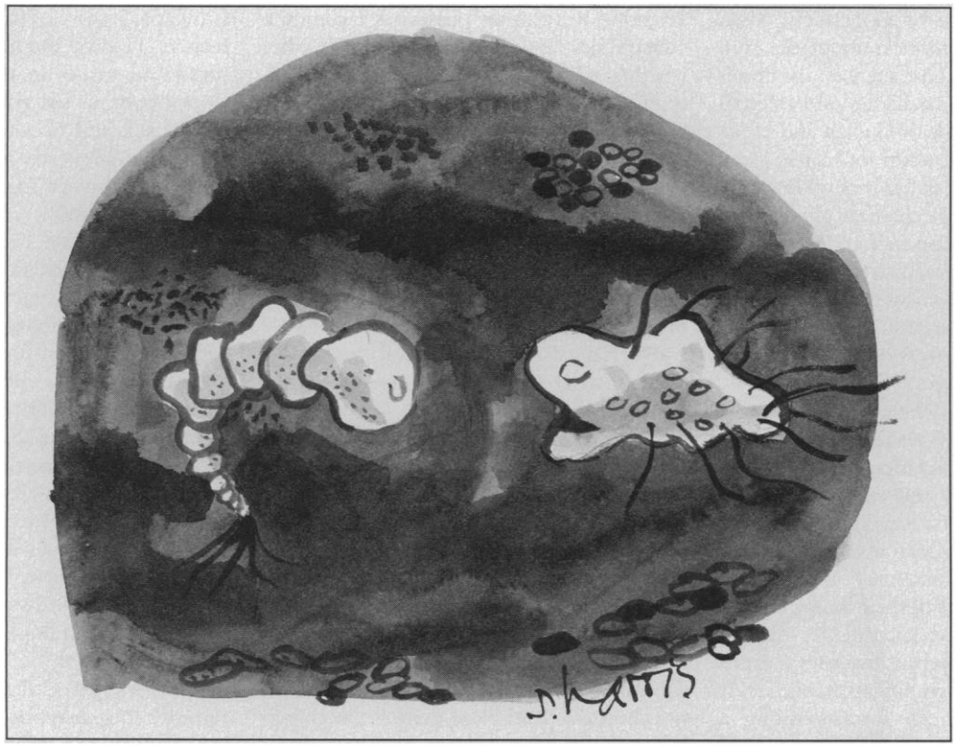

" What burns me up is I work as hard as I can, and they call me a "low-grade" infection."