Chlorovirus ATCV-1 is part of the human oropharyngeal virome and is associated with changes in cognitive functions in humans and mice

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Chlorovirus ATCV-1 is part of the human oropharyngeal virome and is associated with changes in cognitive functions in humans and mice

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chlorella virus 1 (ATCV-1) in a metagenomic analysis of DNA extracted from human oropharyngeal samples. These samples were obtained by throat swabs of adults without a psychiatric disorder or serious physical illness who were participating in a study that included measures of cog-

nitive functioning. The presence of ATCV-1 DNA was confirmed by

quantitative PCR with ATCV-1 DNA being documented in oropha-

ryngeal samples obtained from 40 (43.5%) of 92 individuals. The

presence of ATCV-1 DNA was not associated with demographic variables but was associated with a modest but statistically signif-

icant decrease in the performance of cognitive assessments of visual processing and visual motor speed. We further explored the effects of

ATCV-1 in a mouse model. The inoculation of ATCV-1 into the intes-

tnal tract of 9-11-wk-old mice resulted in a subsequent decrease in performance in several cognitive domains, including ones involving

ognition memory and sensory-motor gating. ATCV-1 exposure in

mice also resulted in the altered expression of genes within the hip-

ocampus. These genes comprised pathways related to synaptic plas-

ticity, learning, memory formation, and the immune response to

viral exposure.

chlorovirus ATCV-1 | infection | cognitive functioning | oropharyngeal virome | metagenomic sequencing

Mucosal sites of humans such as the oral pharynx and intes-

tinal tract are not sterile but contain many micro-

organisms including bacteria, viruses, and fungi. It has recently become apparent that individuals differ in the composition of the microbial flora at their mucosal sites, characterized as the “micro-
bio" comprising the “microbiome.” Studies in both humans and animal models have shown that the microbiome can affect bio-

logical functions, including cognitive performance (1, 2). Bacterial and fungal components of the oral microbiome have been the

subject of several prior studies. These studies generally rely on

PCR-based amplification and subsequent analysis of ribosomal RNA sequences conserved among most species (e.g., refs. 3-6). Analysis of viral components in the microbiome (virome) has re-

ceived less attention, largely because there are no universal target regions suitable for PCR amplification across different viral taxa. This problem can be overcome by whole genome sequencing in which viral particles are separated from the rest of the sample, se-

quenced, and then bioinformatically identified. Such analyses have identified bacteriophages as the most abundant component of the human oral microbiome (6-9). The virome can also be evaluated by

sequencing unfraccionated samples. This approach has the advan-
tage of identifying viruses that might be in low concentrations or evade detection by standard fractionation methods (10-12).

In the process of analyzing whole genome sequences obtained from unfraccionated samples of the oropharynx from healthy indi-

viduals participating in a study that involved the assessment of cognitive functioning, we unexpectedly discovered a substantial number of sequence reads very similar to virus Acanthocystis turfa-

ccea chlorella virus 1 (ATCV-1), a member of the genus Chlorovirus (family Phycodnaviridae). This family of algae-infecting viruses is common in aqueous environments but not previously thought to infect humans or animals or to inhabit human mucosal surfaces (13). Viruses that cross kingdoms are rare; however, some plant viruses can replicate in both their plant host as well as an in-

vertebrate vector. However, there is one report indicating a possible algal-infecting virus associated with humans. In this report, cervico-
vaginal secretion samples contained virus-like particles, and these samples inhibited the propagation of certain algal cultures, consist-

ent with the presence of a virus capable of infecting algae (14).

Significance

Human mucosal surfaces contain a wide range of microorganisms. The biological effects of these organisms are largely unknown. Large-scale metagenomic sequencing is emerging as a method to identify novel microbes. Unexpectedly, we identified DNA sequences homologous to virus ATCV-1, an algal virus not previously known to infect humans, in oropharyngeal samples obtained from healthy adults. The presence of ATCV-1 was asso-

ciated with a modest but measurable decrease in cognitive func-

tioning. A relationship between ATCV-1 and cognitive functioning was confirmed in a mouse model, which also indicated that ex-

posure to ATCV-1 resulted in changes in gene expression within the brain. Our study indicates that viruses in the environment not thought to infect humans can have biological effects.

Author contributions: R.H.Y., L.J.-B., M.V.P., and J.L.V.E. designed research; L.J.-B., G.K., F.D., E.S., S.S., J.R.G., I.V.A., F.L., K.L.G., O.C., B.D., and M.V.P. performed research; R.H.Y. and L.J.-B. supervised the overall performance of the analyses; D.D.D. and F.M. mapped the virus genes; G.K. performed the animal infection and behavior experiments; F.D. supervised the collection of the human samples; E.S. supervised the processing of the human samples and the measurement of the antibodies in the mouse samples; S.S. su-

pervised the experiments related to high throughput sequencing; C.C.T. and E.P. per-

formed the analysis of gene expression; J.R.G. tested for infectious virus; F.L. performed the experiments related to viral DNA detection; K.L.G. performed the antibody measurement studies; O.C. performed the experiments related to high throughput sequencing; B.D. produced the virus; M.V.P. supervised the animal infection and behavior experiments; D.D.D., C.C.T., E.P., and F.M. analyzed data; and R.H.Y., L.J.-B., D.D.D., M.V.P., and J.L.V.E. wrote the paper.

Reviewers: J.F., The Swiss Federal Institute of Technology; and A.V.K., Tulane University. The authors declare no conflict of interest.

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The surprising discovery of this apparent human–ATCV-1 association led us to conduct further investigations in humans and in mice by using high throughput sequencing methods, PCR procedures, and immunological analyses. The presence of ATCV-1 genomes in the oropharynx of many individuals was evaluated in a Baltimore, Maryland-based cohort, and correlations were discovered between the presence of ATCV-1 and cognitive performance. These results prompted us to explore the ability of ATCV-1 to infect mice under experimental conditions and to study the effect of ATCV-1 infection on cognitive performance and brain gene expression.

Results

Detection of ATCV-1 DNA in Human Pharyngeal Samples. Metagenomic sequencing was performed on DNA extracted from oropharyngeal samples obtained from 33 adult individuals without a known psychiatric disorder or physical illness. The demographic characteristics of these individuals are reported in Table S1A. The viral fraction of these metagenomic analyses revealed a wide range of viruses consistent with human and bacterial components of the oropharyngeal cavity. However, there were an unexpected significant number of sequences that resembled chlorovirus ATCV-1. Individuals with and without detectable ATCV-1 sequences in their oropharyngeal samples did not differ significantly in terms of the demographic variables of age, sex, race, educational level, level of maternal education, cigarette smoking, basal metabolic index (BMI) score, a history of travel outside of North America, or place of birth. The sequence reads mapped to many ATCV-1 sites located throughout the viral genome (Fig. 1 and Table S2).

A quantitative PCR (qPCR) procedure with a fluorescent-labeled probe (Taqman) was developed to allow throtts of more individuals to be tested for ATCV-1 DNA. The assay relied on primers directed at ATCV-1 gene z100l. The sensitivity of the assay was ~10 copies of target DNA based on standard curves generated from purified ATCV-1 DNA. The qPCR assay detected ATCV-1 DNA in all 10 individuals with at least two sequence reads homologous to ATCV-1 and in 12 of the 14 individuals who had one sequence read homologous to ATCV-1. The Taqman assay was negative when tested with either human DNA or extracts from buffer solutions. Furthermore, the assay was negative, with DNA extracted from either the ATCV-1 host Chlorella variabilis or with DNA from two other chloroviruses, PBCV-1 and CVM-1, and their hosts Chlorella variabilis and Microcystis conductrix, respectively.

The Taqman assay was used on oropharyngeal samples from 92 individuals, including the 33 individuals tested above. Overall ATCV-1-like DNA was detected in 40 (43.5%) of the 92 samples. Individuals with or without detectable ATCV-1 DNA were similar in terms of demographic variables (Table S1B). No ATCV-1 DNA was detected in blood samples obtained from the study individuals by the qPCR assay.

Because the individuals in the study cohort were also participating in a study of cognitive functioning (15), we examined the association between detection of ATCV-1 DNA and performance on a battery of cognitive tests. A significant association led us to conduct further investigations in humans—ATCV-1 infection of 10 per cell for 5 h (20) or C. heliozoae alone (control, n = 20) or C. heliozoae infected with ATCV-1 at a multiplicity of infection of 10 per cell for 5 h (C. heliozoae/ATCV-1 exposed, n = 30). The behavior tests were started 6 wk postinoculation. An open field test and dark-light box were used to evaluate the effects of viral exposure on general locomotor activity and anxiety (16, 17). No significant group differences occurred in either test. The effect of ATCV-1 exposure on learning and memory
was then tested using the Y-maze test to evaluate spatial recognition memory (17, 18). Mice exposed to ATCV-1 performed at a lower level on this test compared with control mice (Fig. 3d). ANOVA of the percentage of entries to the previously blocked arm showed a significant effect of group, F(1, 49) = 6.4, P = 0.015. This difference could not be explained by lower locomotor activity in ATCV-1–exposed mice, as both groups had similar numbers of total entrances over the observational period: 19.8 ± 1.45 for the control group and 19.5 ± 1.39 for the ATCV-1 group. The effects of ATCV-1 exposure on recognition of a novel object or a novel location were then evaluated. These tests evaluate different aspects of recognition memory in mice (19). No differences were observed in baseline exploratory activity of the objects during training between the ATCV-1–exposed and control group. In contrast, compared with control mice, ATCV-1–exposed mice spent significantly less time exploring the novel object, F(1, 48) = 62.3, P < 0.001 (Fig. 3b), or the novel location of the same object, F(1, 47) = 7.75, P = 0.008 (Fig. 3c). The passive avoidance test was used to evaluate memory to aversive stimuli in mice (20). No significant effect of virus exposure was observed in this test.

The effects of ATCV-1 exposure on the acoustic startle and its prepulse inhibition (PPI) were assessed. These are translational measures of sensorimotor gating that are impaired in patients with neurological and psychiatric illnesses (21). No group difference was found in the baseline acoustic startle response, and both groups exhibited comparable amplitudes (in nV): 193.1 ± 8.4 for control mice and 212.4 ± 8.6 for ATCV-1–exposed mice. However, ATCV-1 exposure impaired PPI in mice. Two-way repeated measures ANOVA with treatment as a between-subject factor and PPI as a within-subjects factor indicated a significant group effect, F(1, 45) = 6.9, P = 0.015, and a significant effect of type of prepulse, F(4, 234) = 98.2, P < 0.001 (Fig. 3d). No Group × Prepulse interaction was found. Post hoc test results revealed that compared with control mice the ATCV-1-exposed group had significantly reduced PPI averaged across all prepulses (P < 0.05) (Fig. 3e).

Hippocampus Gene Expression in ATCV-1–Exposed Mice. Gene expression in the hippocampus of mice was evaluated to compare ATCV-1 exposure (26 wk after ATCV-1 exposure) and control mice. The hippocampus was selected because it contains pathways essential for learning, memory, and behavior (22, 23). Exposure to ATCV-1 was associated with a significant up-regulation or down-regulation of 1,285 individual genes (Fig. S1), which could be constructed into 34 functional pathways that met the inclusion criteria (Table S3). Pathways with multiple components relevant to the response of the mice to ATCV-1 exposure and the development of cognitive changes following infection include pathways related to dopamine receptor signaling (Fig. S2), cyclin-dependent kinase 5 (CDK5) signaling (Fig. S3), antigen presentation (Fig. S4), immune cell adhesion (Figs. S5 and S6), and eukaryotic initiation factor 2 (eIF2) (Fig. S7) with color coding legend (Fig. S8).

Antibodies to ATCV-1 in Exposed Mice. Following the completion of the behavioral studies, there were a total of 47 mice from which serum could be obtained for antibody testing (~6 mo following oral inoculation. This set included 28 of the 30 mice that had been inoculated with ATCV-1–infected C. heliozoae (15 females, 13 males; 2 males died before testing) and 19 mice inoculated with C. heliozoae alone (10 males, 9 females; 1 female died before testing). We found detectable antibodies to ATCV-1 by enzyme immunoassay (ELISA) in 10 of the 28 mice exposed to ATCV-1 (5 males and 5 females) but in none of the 19 mice exposed to C. heliozoae alone (P < 0.0033, Fisher’s exact test). The presence of antibodies to ATCV-1 proteins was examined by Western blot in 12 available blood samples from mice exposed to ATCV-1 in this study. Of five tested samples that were positive by ELISA, four also reacted to multiple ATCV-1 proteins but not to C. heliozoae proteins (Fig. S9). One of the predominant proteins recognized was tentatively identified as the ATCV-1 major capsid protein. No reaction to ATCV-1 proteins occurred in Western blots with the seven samples that were seronegative with ELISAs. Sera obtained from mice exposed to C. heliozoae in the absence of ATCV-1 did not react with ATCV-1 proteins.

Discussion

Metagenomic sequencing of DNA extracted from human oropharyngeal samples identified sequences homologous to chlorovirus ATCV-1 in 14 of 33 (42.4%) individuals from a cohort of adults without a known psychiatric disorder or physical illness who were living in an urban area in the United States. DNA sequences found in study individuals mapped to many sites on the ATCV-1 genome and included many viral genes (Fig. 1). The finding of ATCV-1 sequences by metagenomic sequencing was confirmed by a sequence-specific (gene z1001) qPCR assay that detected ATCV-1 sequences in oropharyngeal samples from 40 of 92 (43.5%) individuals from the same study cohort. There were no differences between individuals, with or without ATCV-1 sequences, with respect to demographic variables, such as age, sex, race, socioeconomic status, cigarette smoking, travel history, or place of birth.

As noted in the introduction, ATCV-1 is a member of the genus Chlorovirus, family Phycodnaviridae. Viruses in the phycodnavirus family, together with those in the Poxviridae, Iridoviridae, Ascoviridae, Astroviridae, Miniviridae, and Marseilleviridae, are proposed to have a common evolutionary ancestor and are often referred to as nucleocyttoplasmic large DNA viruses (24–26). Chloroviruses have large dsDNA genomes (290–370 kb) that encode up to 410 proteins and many tRNAs (13). Chloroviruses infect certain unicellular, eukaryotic, eukaryotic chlorella-like green algae, called zoochlorellae. Zoochlorellae are associated with the protozoan Paramecium bursaria, the coelenterate Hydra viridis, the heliozoan A. turfae, and other freshwater and marine invertebrates and protozoans (27, 28). Three such zoochlorellae are Chlorella NC64A (renamed

Table 1. Association between ATCV-1 oropharyngeal DNA and performance on cognitive tests

<table>
<thead>
<tr>
<th>Cognitive Test</th>
<th>ATCV-1 DNA detected, n = 40</th>
<th>ATCV-1 DNA not detected, n = 52</th>
<th>Overall cohort, n = 93</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trails A, scaled score</td>
<td>38.2 (12.4)</td>
<td>46.7 (11.7)</td>
<td>43.0 (12.7)</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>WAIS III, Information subtest, scaled score</td>
<td>10.8 (2.7)</td>
<td>10.8 (2.6)</td>
<td>10.8 (2.6)</td>
<td>NS</td>
</tr>
<tr>
<td>RBANS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Score</td>
<td>81.3 (11.9)</td>
<td>85.4 (11.5)</td>
<td>83.6 (11.8)</td>
<td>&lt;0.014</td>
</tr>
<tr>
<td>Attention Index</td>
<td>91.4 (17.5)</td>
<td>98.5 (14.5)</td>
<td>95.4 (16.2)</td>
<td>&lt;0.011</td>
</tr>
<tr>
<td>Delayed Memory Index</td>
<td>85.2 (11.7)</td>
<td>88.3 (9.9)</td>
<td>87.0 (10.8)</td>
<td>&lt;0.039</td>
</tr>
<tr>
<td>Immediate Memory Index</td>
<td>85.8 (15.5)</td>
<td>89.3 (14.5)</td>
<td>87.8 (14.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Visuospatial/Constructional Index</td>
<td>72.6 (9.1)</td>
<td>74.4 (10.6)</td>
<td>73.6 (9.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Language Index</td>
<td>93.3 (17.0)</td>
<td>94.8 (17.0)</td>
<td>94.2 (16.9)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values listed are means (standard deviations). P values calculated by linear regression adjusted for age, sex, race, educational level, maternal education, cigarette smoking, and place of birth. NS indicates P > 0.1.
C. variabilis), Chlorella Pbi (renamed M. conductris), and C. heliozoae, the host for ATCV-1. ATCV-1 is a representative of a group of viruses that infect C. heliozoae and collectively are referred to as SAG viruses.

Genomes from 41 chloroviruses infecting these three hosts have been sequenced, assembled, and annotated (29). Collectively, the 41 viruses encode genes from 632 protein families, whereas any given virus only has 330–410 protein-encoding genes. Thus, the genomic diversity among these viruses is large. Furthermore, the viruses encode some unusual proteins that might influence brain function including potassium ion and aquaglyceroporin channels, potassium and calcium transporters, polyamine metabolic enzymes, a histone methyltransferase, and numerous sugar enzymes including several glycosyltransferases.

Chloroviruses are common in inland waters throughout the world with titers as high as thousands of plaque-forming units (PFUs) per milliliter of indigenous water, although titers are typically 1–100 PFUs/mL. The viruses cannot infect zooclorellae when they are in their symbiotic phase, and we have no evidence that the zooclorellae grow free of their hosts in indigenous waters.

To our knowledge, this is the first report of chlorovirus gene sequences in the oral pharynx of humans. There have been several recent reports of oral viromes, but ATCV-1 or other chloroviruses were not mentioned. There are two explanations for this apparent absence. First, in one report the authors specifically looked for known human viral pathogens (30), and so they could have missed the chloroviruses. Second, in three other reports, the samples were filtered through both 0.45-μm and 0.2-μm filters; the material that passed through both filters was used to extract DNA. These researchers were primarily evaluating bacteriophages (7, 8, 31). The icosahedral-shaped chloroviruses are 190 nm in diameter and have a 34-nm spike structure protruding from one unique vertex (32). Therefore, it is likely that ATCV-1 and most chloroviruses would be trapped on a 0.2-μm filter.

The fact that the individuals in our study population were participating in a project, which included measures of cognitive functioning, allowed us to examine and consider the association of detectable ATCV-1 DNA in the pharynx and performance on a range of cognitive tests. Surprisingly, the presence of ATCV-1 DNA in the oropharynx was associated with modest but statistically significant decreases in performance on tests including Trails A and RBANS Attention, both of which measure visual processing and visual motor speed.

The association between the level of ATCV-1 and decreased performance on cognitive tests was independent of demographic variables (Tables S1A and S1B). Also, there was no association between the level of oropharyngeal ATCV-1 DNA and WAIS Information, indicating that the association between ATCV-1 DNA and the other cognitive domains was not due to level of general knowledge or educational background. Studies of associations between environmental factors and cognitive performance in humans must always be interpreted with caution because it is possible that exposure to a single infectious agent might be associated with unmeasured exposures, such as other infectious agents, heavy metals, pollutants, or other environmental factors that also can be associated with alterations in cognitive functioning (33).

For this reason, we developed a mouse model for assessing behavior and cognitive functioning following ATCV-1 exposure by the oral route. As a group, mice inoculated by the oral route showed signs of impairment in new object or new location recognition memory and sensory-motor gating as measured by PPI several months after a single viral exposure (Fig. 3).

Exposure of mice to ATCV-1 also resulted in changes in gene expression within the hippocampus, the region of the brain most associated with spatial memory and navigation (22, 34, 35). Of particular interest in light of the cognitive assays were alterations in the Cdk5 pathway (Fig. S5) because this pathway is central to learning and memory formation (36). Also of note were differences in expression of genes in the dopamine pathway (Fig. S2) because perturbation of the dopamine system impairs novel object recognition and PPI in rodents (37, 38), as do alterations in the pathway of EIF2 because this factor is central to the control of long-term synaptic plasticity and memory storage (39).

Although it is difficult to directly relate the cognitive tests used in our human study with memory tests in mice, it is striking that...
exposure to ATCV-1 in both humans and mice was associated with decrements in performance on tasks calling for visual spatial abilities (Figs. 2 and 3). More detailed cognitive assessment of humans and mice exposed to ATCV-1 might better define these associations and the relationship between exposure to ATCV-1 and cognitive functioning.

There are several questions relating to ATCV-1 exposure in humans that remain to be addressed. One concerns the source of the acquisition of ATCV-1 in the virome. ATCV-1-like viruses are common in inland waters such as those around Baltimore, so exposure to these water sources would be relatively common. The factors involved in the acquisition of ATCV-1 in the oropharynx following exposure will be the subject of additional investigations. ATCV-1 is the reference genome for the SAG virus group, but we have previously sequenced 12 other SAG viruses and there are 2-3 distinct clades (29). Therefore, the exact nature of SAG viruses capable of colonizing the human oropharynx and other mucosal sites is also an important subject for future studies.

Another set of questions concerns the mechanisms by which ATCV-1 might be associated with alterations in cognitive functioning. Although the exact mechanisms of the behavioral changes in the exposed host remain unclear, the observed cognitive deficits are unlikely to be related to sickness behavior, as no overt signs of malaise were noted in exposed mice. Similar to a number of other microbial infections, we think that both direct and indirect effects of pathogens that remain to play a role (e.g., refs. 40–42). The finding of alterations in several pathways involved in antigen processing and immune cell functioning in the hippocampus of mice exposed to ATCV-1 (Table S3 and Figs. S4–S6) suggests that immune mechanisms may be involved, as have been documented in other biological systems (43). We found evidence of an immune response to ATCV-1 in about 35% of mice exposed to ATCV-1 when measured 6 mo following a single exposure. Thus, our serological and gene expression data implicate immune response to ACTV-1 as a mechanism underlying the cognitive deficits. It is conceivable that immune activation produced secretion of proinflammatory cytokines that affected neuronal functioning, leading to behavioral abnormalities. In this context, both shared and unique profiles of cytokine up-regulation have been shown for various microbial infections (Borna virus vs. Toxoplasma), and it is plausible that differential neurobehavioral outcomes of different microbial infections may be at least in part explained by unique “signatures” of cytokine expression (44).

Earlier blood samples were not obtained from this cohort of mice to avoid affecting the behavioral tests. Further studies of the kinetics of ATCV-1 infection and the immune response to infection are thus warranted. Our studies document that ATCV-1 is part of the human virome and is associated with cognitive changes in humans and experimentally infected animals. An increased understanding of the role of ATCV-1 and related viruses may lead to a new understanding of the role of the oropharyngeal virome in human health and cognition.

**Materials and Methods**

**Studies in Humans.** The study cohort consisted of 92 individuals living in the Baltimore, Maryland metropolitan area who did not have current or past psychiatric disorders and who did not have a serious medical illness that would be likely to affect cognitive performance. The overall characterization of the study population including the methods of recruitment and measures used for their characterization was previously described (45). Control individuals were enrolled after they were screened to rule out the presence of current or past psychiatric disorders with the Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition Axis I Disorders-Nonpatient Edition (46). Participants also met the following criteria: proficient in English, no history of i.v. substance abuse, absence of mental retardation, absence of HIV infection, absence of a serious medical disorder that would affect cognitive functioning, and no indication of alcohol or substance use disorder. Demographic data including age, self-reported race, level of highest educational attainment, level of maternal education, and current use of cigarettes were obtained from all participants. All of the participants provided written informed consent following explanation of the study goals and procedures. The study was approved by the Institutional Review Boards of Johns Hopkins University and Sheppard Pratt Hospital.

**Clinical samples.** Oropharyngeal samples were obtained by swabbing the back of the throat using sterile cotton swabs (BBL Culture Swabs, Becton Dickinson). On the day of collection, the swabs were transported from the collection site to the processing laboratory at room temperature and then frozen until processed further, as described below.

**Cognitive testing.** All of the participants underwent a battery of cognitive tests, as previously described (45). These included the RBANS (47), Trails A (48), and the information subtest of the WAIS III (49). Details of these tests are described in SI Materials and Methods.

**Sample Processing.** DNA was extracted from throat swabs using Qiagen’s Gentra Puregene Buccal Cell Kit. The collection brush heads from the swabs were excised and incubated at 65 °C overnight in the kit cell lysis solution. The manufacturer’s instructions were followed with some minor changes to the protocol as follows: (i) During the isopropyl alcohol precipitation, 2 μL of 5 mg/mL linear acrylamide (Life Technologies), a chemically modified alcohol, was added to the precipitant for the glucose treatment of the DNA to prevent possible contamination by reagents extracted from natural sources; (ii) following this precipitation step, incubation on ice was added for 15 min; (iii) centrifugation following the 70% (vol/vol) ethanol wash was extended to 5 min from 1 min; and (iv) final elution of DNA was reduced from 100 μL to 30 μL.

**Metagenomic Sequencing.** DNA samples from 33 individuals (two independent experiments with 17 and 16 individuals) were analyzed by metagenomic sequencing. The demographic information on these individuals is reported in Table S1A. The method used for sequencing is detailed in SI Materials and Methods.

**qPCR Analysis.** Oropharyngeal samples from a larger set of individuals (n = 92) were tested by a qPCR system. These individuals included the 33 individuals evaluated by the initial metagenomic sequencing method. The demographic information related to these individuals is presented in Table S1B. The method of sample collection and DNA extraction was identical to that used in the metagenomic sequencing. The qPCR was performed using the 5′-3′ exonuclease activity of *Thermus aquaticus* polymerase (Taqman) (50). Target primers were directed at the portion of the genome encoding ATCV-1 protein Z100L. This target region was selected because the primers did not have appreciable homology with any other viruses, bacteria, or eukaryotic organisms.

The method used for the Taqman assay was as follows: A 20-μL assay mix was made using forward primer 5′-GCA ATT CGA ATG ATA GTG CTC A-3′, reverse primer 5′- CTG GTG TGC CCT TTA ACA AA-3′, and probe 5′-FAM/AG TAA ACC CAC ACC TTT TGA TAG CCA /36-TAM5/ containing 18-μM primers and a 4-μM probe. A 20-μL reaction volume was used using 10 μL of 2× Gene Expression Master Mix (Applied Biosystems) and 1 μL of the 20-μL assay mix with the remaining volume consisting of input DNA and sterile water. The qPCR amplification profile consisted of one cycle of 10 min at 95 °C followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Reactions were performed in a Stratagene MX3005P Thermocycler (Agilent Technology). Results were quantified with a standard curve generated from the testing of 10-fold dilutions of a plasmid created to contain the target. Testing of this standard curve indicated that 10 copies of ATCV-1 DNA could be reliably detected in the qPCR reaction. A sample, which contained ≥10 copies of ATCV-1 genomic DNA, was considered to contain ATCV-1 DNA. DNA extracted from related chloroviruses PBCV-1 and CVM-1 (13, 29) and human DNA did not produce products with this assay.

**Statistical Analysis of Virome Studies.** The demographic correlates for the presence of ATCV-1 DNA were calculated by χ² analysis for categorical variables such as sex, race, cigarette smoking, history of travel outside of North America, and place of birth and by two-way analysis of variance for continuous variables such as age, level of education, BMI score, and maternal education, the latter being used as a marker of socioeconomic status (51). Individual performances on the cognitive tests described above were further compared with the performance of individuals who did or did not have detectable ATCV-1 genomes in their pharynx using linear regression models incorporating the covariates of age, sex, race, educational level, maternal education, cigarette smoking, and place of birth. Logistic regression models were used to calculate the odds ratios, which define the association between the presence of ATCV-1 DNA in the throat with low performance on
the cognitive tests as defined above, using the same covariates that were used in the linear regression models. To have adequate statistical power, analyses of cognitive functioning were only performed on the larger cohort (n = 92), on whom ATCV-1 DNA was detected by the qPCR method described above.

**Mouse Model of Infection.** Animal model studies were performed to determine the effect of ATCV-1 exposure via the oral route on cognition and behavior. The conditions of viral growth and mouse inoculation were determined by a set of preliminary experiments. All protocols were approved by the Animal Care and Use Committee at Johns Hopkins University. A total of 50 C57BL/6J male and female mice (Charles River Laboratories) were used to evaluate the effect of ATCV-1 exposure on cognition and behavior. Mice were housed five per cage (28.3 cm length × 17.4 cm width × 13 cm height) unless separated due to fighting. Animals had free access to food and water at all times. Mice were inoculated at 9–11 wk of age, as described below.

**Exposure to ATCV-1.** C. heliozoae host algae were either uninfected (C. heliozoae control) or infected with ATCV-1 at a multiplicity of infection of 10 PFU per cell for 5 h (C. heliozoae/ATCV-1) pelleted, (3,800 × g × 5 min, 4°C), and then resuspended in 0.4x PBS. Mice were gavaged with 0.2 mL of either C. heliozoae/ATCV-1 (n = 30) or C. heliozoae control preparations containing ∼4 × 10^7 cells (n = 20), with both groups equally divided between males and females.

The tests used to monitor the behavior of the mice exposed to ATCV-1, as well as the procedures used for RNA extraction, microarray analysis, data normalization and statistical analysis for microarray transcripts, pathway analysis, and measurement of antibodies to ATCV-1 and related chloroviroid viruses in mouse blood samples are described in SI Materials and Methods.

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14. Pröschold T, Darienko T, Silva PC, Reisser W, Krienitz L (2011) The systematics of C. heliozoae × C. heliozoae resuspended in 0.4 PBS. Mice were gavaged with 0.2 mL of either C. heliozoae/ATCV-1 (n = 30) or C. heliozoae control preparations containing ∼4 × 10^7 cells (n = 20), with both groups equally divided between males and females.

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Supporting Information

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SI Materials and Methods
The tests used to monitor cognitive behavior, the behavior of the mice exposed to ATCV-1, as well the procedures used for metagenomic sequencing, RNA extraction, microarray analysis, data normalization and statistical analysis for microarray transcriptomics, pathway analysis, and measurement of antibodies to ATCV-1 and related chloroviruses in mouse blood samples follow.

Cognitive Testing. All of the participants underwent a battery of cognitive tests, as previously described (1). These included the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) (2), Trail Making Test Part A (Trails A) (3), and the Information subtest of the Wechsler Adult Intelligence Scale (WAIS) III (4).

The RBANS consists of 12 subtests that are used to calculate five index scores and a total score. Test indices are Immediate Memory (comprising List Learning and Story Memory tasks), Visuospatial/Constructional (comprising Figure Copy and Line Orientation tasks), Language (comprising Picture Naming and Semantic Fluency tasks), Attention (comprising Digit Span and Coding tasks), and Delayed Memory (comprising List Recall, Story Recall, Figure Recall, and List Recognition tasks). Each index score is expressed as an age-adjusted standard score with a mean of \( \mu = 100 \) and an SD of \( \sigma = 15 \). The index scores were combined to yield an RBANS Total score, which is a measure of overall cognitive functioning. Trails A requires an individual to draw lines sequentially connecting 25 encircled numbers distributed on a sheet of paper; the score is based on the time to complete the task. Trails A is a test of visual scanning and motor speed. The Information subtest of the WAIS is a test of general knowledge, including questions about geography and literature. For the latter two tests, scores are expressed as an age-adjusted scaled score with a mean of 10 and an SD of 3. For the purposes of calculating odds ratios, a low performance on the RBANS tests was defined as less than or equal to 80 and low performance on the other tests as performance below the 25th percentile (4, 5).

Monitor the Behavior of Mice. Behavioral tests were performed on mice inoculated with Chlorella hétiozoae/ATCV-1 (exposed) and C. hétiozoae (control) between 6 and 22 wk postinoculation. The tests were performed in the following order: novelty-induced activity in open field, Y-maze, object placement, dark–light box, new object recognition or location, prepulse inhibition (PPI) of the acoustic startle, and passive avoidance.

Novelty-induced activity. Novelty-induced activity in the open field was assessed over a 30-min period using activity chambers with infrared beams (San Diego Instruments Inc.), as previously described (6).

Spatial recognition in the Y-maze. Spatial recognition memory was evaluated in a Y-maze as described by Melnikova et al. (7). In brief, one arm of the maze was blocked and a mouse was allowed to freely explore the two open arms for 5 min. After a 20-min delay, the block was removed and the mouse was allowed to freely explore all three open arms for 5 min. The percentage of time and visits into the novel (previously blocked) arm during the first 2 min of the 5-min trial was analyzed.

Dark–light box. Anxiety was evaluated using a dark–light box (Colbourn Instruments). Mice were placed in the transparent side of the box and allowed to freely move between the dark and light chambers for 5 min. The latencies to cross between chambers were automatically recorded using Graphic State v 3.03.

Novel object recognition. The novel object recognition test was used to assess recognition memory (8). Briefly, mice were habituated for 4 d to an empty mouse cage (28.3 cm length × 17.4 cm width × 13 cm height) for 10 min each day as previously described (9, 10). On day 5, two identical objects were placed on opposite ends of the empty cage, and the mouse was allowed to freely explore the objects for 10 min. After a 1-h delay, during which the mouse was held in its home cage, one of the two familiar objects was replaced with a novel one, and the mouse was allowed to freely explore the familiar and novel object for 5 min. The percent time near the novel object was calculated as the time near the novel object divided by the total time near either object.

Novel location recognition. Novel location recognition was used to assess spatial recognition memory. Briefly, mice were habituated for 4 d to an empty mouse cage for 10 min each day. On day 5, two identical objects were placed on opposite ends of the empty cage, and the mouse was allowed to freely explore the objects for 10 min. After a 1-h delay, one object was moved to a different location in the cage, and the mouse was allowed to explore for 5 min. The percent time near the object at the novel location was calculated as the time near the novel location divided by total time near novel and old location.

Sensorimotor gating. Sensorimotor gating was assessed using PPI of the acoustic startle (San Diego Instruments Inc.). Mice were acclimatized to a 70-dB background noise for 5 min. They were then given 10 presentations each of a 120-dB pulse and 0-dB pulse. This was followed by 5–6 presentations in randomized order of a 120-dB pulse, 0-dB pulse, or the following prepulses followed by the 120-dB pulse: 74, 78, 82, 86, and 90 dB. The intervals between each presentation varied from 10 to 19 s. PPI% was calculated by [(100 – (mean startle amplitude of each prepulse/mean startle amplitude of 120 dB pulse)) × 100]. Mean PPI% was calculated by averaging all PPI% values for presentations of all prepulses for each experimental group.

Passive avoidance. Associative learning and memory were evaluated using a 2-d passive avoidance test (San Diego Instruments Inc.). On day 1, mice were placed in a lit compartment with the gate to the dark compartment closed. After a 30-s delay, the gate opened, allowing the mouse to cross to the dark compartment. Once the mouse crossed over, the gate automatically closed, and after a 3-s delay, a 0.3-mA shock was administered for 3 s. Twenty-four hours later, the mouse was again placed in the lit compartment with the gate shut. After a 5-s delay, the gate opened. The trial ended either when the mouse crossed to the dark compartment or once 10 min elapsed. On each day, the latency in seconds for the mouse to cross from the light to dark compartment was automatically recorded and used in the analysis.

Statistical Analyses of Behavioral Studies. The behavioral data were analyzed with one-way analysis of variance (ANOVA) for all tests except PPI. The PPI data were analyzed using two-way repeated measures ANOVA with treatment as a between-subject factor and PPI as a within-subjects factor. We did not rely on sex or other factors as independent variables, as our analyses detected no sex-dependent effects in the tests described. If the data did not pass tests for normality or equal variance, the data were rank-transformed before further statistical analysis.

Metagenomic Sequencing. DNA samples from 33 individuals (two independent experiments with 17 and 16 individuals) were analyzed by metagenomic sequencing. The demographic information on these individuals is reported in Table SI. The method used
for the sequencing is presented as follows. A total of 75–100 ng of DNA was used for paired-end library generation using the Nugen Ultralow DR Multiplex System (NuGEN) following the manufacturer’s instructions. The libraries were purified and analyzed on the Bioanalyzer (Agilent Technologies) to confirm size and concentration. The purified libraries were sequenced using Illumina HiSeq, which generated ~200,000,000 paired-end reads of 100 nucleotides.

Sequence reads were filtered to remove low-quality sequences, resulting in a minimum length of 60 nucleotides. To evaluate putative viruses associated with the human throat sequence reads, human, bacteria, fungi, and parasite sequence reads were removed by bioinformatic filtering as follows: sequence reads with homology to human samples were removed in two stages. The first stage used the program Bowtie (bowtie-bio.sourceforge.net/index.shtml). A sliding window approach was used to align a 40-base-pair subsequence from the reads to the human genome Build 37 (www.snpedia.com/index.php/GRCh37). During each iteration of this procedure, reads mapping to the human genome were removed from the analysis and subsequences used for alignment were offset by five bases. The second filtering of human sequences used CLC Genomics Workbench Version 6 (www.clcbio.com) using a reference set of sequences based on the human genome Build 37 with the following settings: length fraction 0.4, similarity 0.4. Sequence reads that were not removed by this subtraction were filtered sequentially to remove bacterial, fungal, and protozoan sequences by matching to appropriate National Coalition Building Institute Reference Sequence (RefSeq) databases (www.ncbi.nlm.nih.gov/refseq/). The remaining sequences, which consisted of <1% of the starting sequences, were then mapped to the RefSeq complete set of viral genomes (ftp://ftp.ncbi.nlm.nih.gov/refseq/release/viral/) using CLC Genomics Workbench Version 6 with the following settings: length fraction 0.8, similarity 0.8. Sequences homologous to ATCV-1 (reference sequence NC_008724.1) by this analysis were further mapped to the ATCV-1 genome using CGView (11).

RNA Extraction. Following completion of the behavioral experiments, the mice were killed and brains were removed and placed on ice. The hippocampus was dissected, placed into RNA stabilization reagent (Qiagen), and stored at −80 °C. Total RNA was isolated from either the left or right hippocampus using miRNeasy Qiagen mini kit (cat. no. 217004) following the manufacturer’s protocol. To remove genomic DNA carryover, RNA samples were treated with DNase for 20 min at 37 °C using a TurboDNA-free kit from Ambion (cat. no. AM1907). Samples were assessed for RNA quality and concentration by TapeStation 2200 (Agilent Technologies). Based on these measurements, 24 samples were selected for analysis. These included samples from 16 mice gavaged with C. heliozoae/ATCV-1 and from eight mice gavaged with C. heliozoae alone.

Microarray Analysis. RNA transcript levels were quantified by microarray analyses. RNAs were amplified into cDNA and biotinylated by in vitro transcription with Affymetrix reagents, using the Whole Transcript Sense Target Labeling protocol as described in the Affymetrix manual (www.affymetrix.com/support/technical/product_updates/wt_1_1_assay.aff). Biotinylated cDNAs were purified, fragmented, and subsequently hybridized to Affymetrix GeneChip Mouse Gene 2.0 ST arrays.

Data Normalization and Statistical Analysis for Microarray Transcriptomics. Affymetrix CEL files, containing the raw GeneChip data, were prepared using GeneCp Command Console software. These data were extracted and normalized with Genomics Suite v6.6 (Partek Inc.) software using the Robust Multichip Analysis algorithm. One C. heliozoae inoculated control (ID no. 650) had RNA that was largely degraded and was omitted from the final analyses. To detect differentially expressed genes under various conditions, a single expression value was assigned for each transcript, including all its exons. One-way ANOVA was used to detect statistically significant changes in gene expression between samples from mice inoculated with C. heliozoae/ATCV-1 and control mice inoculated with C. heliozoae alone. Transcripts with a difference of at least 2 SDs in either direction between the ATCV-1–exposed and control mice were selected for pathway analyses (12).

Pathway Analysis. Network, function, and pathway analyses were generated using Ingenuity Pathways Analysis (Ingenuity Systems), which facilitates the interpretation of microarray data by grouping differentially expressed genes into known functional pathways. These analyses identified statistically increased representations of the differentially expressed genes in biologically relevant processes (13). Genes that showed differential expression of greater than 2 SDs between inoculated and control mice were compared with those genes that did not, using the Fisher’s exact test to identify the differentially expressed genes’ pathways for review of potential biological function. Based on its curated Knowledge Base (MAP Molecule Activity Predictor; www.ingenuity.com/products/ipa/ipa-summer-release-2014), Ingenuity Pathways Analysis further predicted whether the genes’ observed levels of altered transcription were in accordance with regulatory relationships from the literature; for example, elevated expression of gene A (a known inhibitor of gene B) is observed together with reduced expression of gene B. Due to the exploratory nature of this study, pathways with P values ≤0.05 were selected for inclusion.

Measurement of Antibodies to ATCV-1 and Related Chloroviruses in Mouse Blood Samples. Enzyme immunoassays. IgG antibodies to ATCV-1 were measured by ELISA using variations of previously described procedures. Highly purified virion stocks containing ~1011 PFU/mL were diluted 1:1,000 in 50 μL carbonate buffer and coated overnight at 4 °C on 96-well polystyrene flat bottom MaxiSorp plates (Nunc; Thermo Fisher Scientific). Plates were blocked for 1 h at 37 °C with Starting Block (Thermo Scientific). Plates were then incubated with a 1:1,000 dilution of the mouse test serum in duplicate wells, incubated for 1 h at 37 °C washed, and incubated with peroxidase-conjugated goat–anti-mouse IgG for 45 min at 37 °C (Southern Biotech). A 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonate) hydrogen peroxide solution (KPL Protein Research Products) was added for color development, and absorbance was measured at 405 nm, with a reference wavelength of 490 nm, in an automated microtiter plate reader (Molecular Devices), with the results expressed as absorbance units. A sample was considered positive for antibodies to ATCV-1 if it generated a signal in the wells coated with ATCV-1 that gave an absorbance value of at least 0.4 units.

Measurement of antibodies by Western blotting. C. heliozoae infected with ATCV-1 and uninfected C. heliozoae were added to SDS Laemmli buffer and heated to 95 °C, diluted 1:10, and loaded on a precast NuPAGE Novex 4–12% Bis-Tris gel (Life Technologies). Proteins were resolved through SureLock gel electrophoresis, and the gels were stained with SimplyBlue SafeStain (Life Technologies). Proteins were transferred to nitrocellulose using iBlot dry transfer technology (Life Technologies). Membranes were incubated overnight at 4 °C with a 1:10,000 dilution of the test sample of mouse serum, the blots were washed and incubated for 45 min with goat–anti-mouse IgG conjugated to alkaline phosphatase (Southern Biotech). Following another incubation, the blots were washed and developed with an alkaline phosphatase-based conjugation kit (BioRad Life Science). For reference, we also used a 1:500 dilution of a rabbit polyclonal antibody gener-
ated to recognize a recombinant GST-labeled major capsid protein A430L from chlorovirus PBCV-1. The same immunoblotting procedure was performed, except an anti-rabbit IgG secondary was applied.


Infected vs. Uninfected Volcano Plot

Differentially expressed genes selected based on FC > 2SD

Fig. S1. Distribution plot of genes expressed in the hippocampus of mice gavaged with ATCV-1–infected C. heliozoae (n = 16) and control mice (n = 7) gavaged with C. heliozoae alone. The dots shown in red represent transcripts with expression differing by at least 2 SDs.
Fig. S2. Differences in the statistically significant dopamine receptor signaling pathway between mice exposed orally to ATCV-1–infected *C. heliozoae* and control mice exposed orally to *C. heliozoae* alone. The numbers shown represent the linear fold change of expression between the two groups. The legend for the color coding is presented in Fig. S8.
Fig. S3. Differences in the statistically significant CDK5 signaling pathway between mice exposed orally to ATCV-1–infected C. heliozoae and control mice exposed orally to C. heliozoae alone. The numbers shown represent the linear fold change of expression between the two groups. The legend for the color coding is presented in Fig. S8.

Fig. S4. Differences in the statistically significant antigen presentation pathway between mice exposed orally to ATCV-1–infected C. heliozoae and control mice exposed orally to C. heliozoae alone. The numbers shown represent the linear fold change of expression between the two groups. The legend for the color coding is presented in Fig. S8.
Differences in the statistically significant agranulocyte cell adhesion pathway between mice exposed orally to ATCV-1–infected *C. heliozoae* and control mice exposed orally to *C. heliozoae* alone. The numbers shown represent the linear fold change of expression between the two groups. The legend for the color coding is presented in Fig. S8.
Fig. S6. Differences in the statistically significant granulocyte cell adhesion pathway between mice exposed orally to ATCV-1–infected C. heliozoae and control mice exposed orally to C. heliozoae alone. The numbers shown represent the linear fold change of expression between the two groups. The legend for the color coding is presented in Fig. S8.
Fig. S7. Differences in the statistically significant eIF2 signaling pathway between mice exposed orally to ATCV-1–infected *C. heliozoae* and control mice exposed orally to *C. heliozoae* alone. The numbers shown represent the linear fold change of expression between the two groups. The legend for the color coding is presented in Fig. S8.

**Prediction Legend**

- **more extreme**
  - more confident:
    - **more extreme**
      - **Predicted activation**
        - Leads to activation
    - **more extreme**
      - **Predicted inhibition**
        - Leads to inhibition
  - **less**
    - **more extreme**
      - **Predicted activation**
    - **more extreme**
      - **Predicted inhibition**

*Fig. S8.* The legend for the color coding in Figs. S2–S7.
Western blot assays performed with antigens derived from purified ATCV-1 (A) and C. heliozoae (Ch) prepared and reacted as described in the text. The first lane (labeled P) is from rabbit antibody prepared against the major capsid protein (A430L) of chlorovirus PBCV-1 as a reference. ATCV-1 exposed ELISA positive, reactivity of sera from mice exposed to ATCV-1 and reactive to ATCV-1 antigens by ELISA. ATCV-1 exposed ELISA negative, reactivity of sera from mice exposed to ATCV-1 and not reactive to ATCV-1 antigens by ELISA. C. heliozoae exposed ELISA negative, reactivity of serum from a mouse exposed to C. heliozoae in the absence of ATCV-1. All mice with this exposure were nonreactive by ELISA.

**Other Supporting Information Files**

[Table S1](#)  
[Table S2](#)  
[Table S3](#)