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Use of HeLa Cell Guanine Nucleotides by *Chlamydia psittaci*

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Exogenous guanine was found to be incorporated into the nucleic acids of *Chlamydia psittaci* when the parasite was grown in HeLa cells containing hypoxanthine guanine phosphoribosyltransferase (EC 2.4.2.8) activity but not when the parasite was grown in transferase-deficient HeLa cells. No evidence for a chlamydia-specific transferase activity was found in either transferase-containing or transferase-deficient infected HeLa cells. It is concluded that *C. psittaci* is incapable of metabolizing guanine, but that the parasite can use host-generated guanine nucleotides as precursors for nucleic acid synthesis.

Members of the genus *Chlamydia* are obligate intracellular parasitic bacteria which have not been propagated outside of eucaryotic host cells. Moulder (Ann. N.Y. Acad. Sci. **98**:245-247, 1962) suggested that chlamydiae are energy parasites which depend on their hosts for the energy intermediates they require for biosynthesis of macromolecules. Several lines of indirect evidence support Moulder's supposition that chlamydiae are incapable of generating useful energy. Weiss and his co-workers (19, 20, 21) determined that isolated *C. psittaci* can carry out a number of reactions in the glycolytic, tricarboxylic, and pentose phosphate pathways but that none of these reactions was associated with the net production of ATP. Gill and Stewart (5) and Moulder (12) concluded that the enhanced rate of catabolism of glucose when L cells are infected with *C. psittaci* represents a host response to infection rather than independent parasite catabolic activity. Gill and Stewart (6) found antimycin, an inhibitor of electron transport, reduced the yield of *C. psittaci* grown in L cells even though cytochromes and flavoproteins have not been detected in chlamydiae (E. Weiss and L. A. Kriesow, Bacteriol. Proc., p. 85, 1966).

Proving that chlamydiae are energy parasites requires demonstrating a direct requirement of nucleotides by chlamydiae growing in a host-free system. Since chlamydiae have not been propagated outside of host cells, Hatch (8) attempted to demonstrate indirectly that *C. psittaci* uses host-derived energy intermediates by showing that *C. psittaci* organisms multiplying within L cells incorporate exogenous uridine, guanine, and adenine labels into RNA at rates consistent with the parasites drawing exclusively on their host's ribonucleoside triphosphate pools for precursors. Although it was deemed unlikely, the possibility that chlamydiae are purine and pyrimidine nucleoside auxotrophs, rather than dependent on host ribonucleotides, could not be ruled out with certainty.

The present study was undertaken to eliminate the possibility that *C. psittaci* relies on host purine pools and to demonstrate that *C. psittaci* can and does draw on host guanine nucleotide pools for precursors for nucleic acid synthesis. The basis of the experiments was to compare the incorporation of an exogenous guanine label into *C. psittaci* nucleic acids when the parasite was grown in HeLa cells containing hypoxanthine guanine phosphoribosyltransferase ($HGPRT^+$ cells) with the incorporation of label into *C. psittaci* grown in HeLa cells deficient in transferase activity ($HGPRT^-$ cells). The transferase catalyzes the conversion of guanine plus 5-phosphorylribose-1-pyrophosphate to GMP and PP_i and is required for the incorporation of guanine into nucleic acids. It was found that *C. psittaci* can use guanine supplied in the culture medium only if the parasite is grown in a host cell capable of elevating guanine to the nucleotide level.

MATERIALS AND METHODS

Growth of organisms. Two different lines of HeLa cells were used: HeLa 229 cells, containing hypoxanthine guanine phosphoribosyltransferase activity, will be referred to as $HGPRT^+$ cells; and HeLa cell mutants, deficient in HGPRT activity, will be referred to as $HGPRT^-$ cells. The $HGPRT^-$ cells were kindly supplied by Gregory Milman of the Johns Hopkins University. Both lines of HeLa cells were grown as monolayers in Eagle minimal essential medium supplemented with nonessential amino acids (KC Biological) containing 0.1% sodium bicarbonate, 200 μg of streptomycin sulfate per ml, and 10% fetal calf serum (KC Biological). Cultures for mycoplasma were negative (Flow Laboratories, Rockville, Md.). HeLa cell numbers were determined by loosening the cell

monolayers with 0.25% trypsin (1:250, Difco) in Dulbecco phosphate-buffered saline (PBS) and counting in a hemocytometer. All experiments were done with the 6BC strain of *C. psittaci*, grown as previously described (7). HeLa cells were infected with 10 times the amount of *C. psittaci* required to infect 50% of the cells in culture, resulting in more than 95% infection of the host cells.

Transport of guanine by HGPRT$^+$ cells. Medium was decanted from monolayers of HeLa cells incubating in 25-cm^2 plastic culture flasks (4.0×10^6 cells per flask) and replaced with 1.5 ml of growth medium containing [^{3}H]guanine (1.0 μCi/ml) and an appropriate amount of carrier guanine. After 5 min of incubation, the medium was decanted and the cells were washed five times with 5 ml of PBS before being digested with 0.5 ml of 1% sodium dodecyl sulfate. The digest was dissolved in 10 ml of ACS (Amersham/Searle), and the radioactivity was determined by liquid scintillation spectometry.

Quantitation of incorporation of [^{3}H]guanine into RNA. Monolayers of uninfected and 18-h infected HeLa cells (10^6 cells per monolayer) were incubated on 5-cm culture dishes in 1.5 ml of growth medium containing 1.0 μCi of [^{3}H]guanine (10 mCi/mol) per ml. At time intervals excess guanine was removed by washing three times with 5.0 ml of PBS, the monolayers were fixed, and the soluble pools were extracted with 2.0 ml of 1.0 N formic acid. The cells were solubilized, and the ribonucleic acid (RNA) was hydrolyzed by the addition of 0.3 N KOH and incubation at 37°C for 18 h. Protein and deoxyribonucleic acid (DNA) were precipitated by neutralization of the hydrolysate with 35% perchloric acid, and the radioactivity in the supernatant was determined. Incorporation of [^{3}H]guanine into the RNA of purified *C. psittaci* was determined by measuring the KOH-soluble counts in formic acid precipitates of purified preparations of *C. psittaci*.

Assay for hypoxanthine guanine phosphoribosyltransferase activity. Uninfected and 28-h-infected HeLa cells (2×10^7 cells per assay) were suspended in 0.5 ml of PBS and disrupted in a Metler ultrasonic bath for 15 min. Nuclei were removed by centrifugation at 1,000 × *g* for 2 min, and the supernatant fluid was assayed for transferase activity. The reaction mixture (50 μl) consisted of cell extract, 5 mM $MgCl_2$, 2 mM 5-phosphorylribose-1-pyrophosphate (Sigma), and 40 μM guanine containing 2.5 μCi of [^{3}H]guanine. After incubation at 37°C for 1 h, the reaction was stopped by the addition of 100 μl of water and by heating to 90°C for 3 min. Samples (10 μl) containing carrier GMP were applied to strips of polyethyleneimine-impregnated cellulose thin-layer chromatography sheets (Brinkmann Instruments), and unreacted guanine was removed by washing the sheets two times in 500 ml of 4.0 mM tris(hydroxymethyl)-aminomethane-hydrochloride (pH 7.8). Radioactive nucleotides were separated by using the LiCl method of Randerath and Randerath (14). The area containing GMP, detected by ultraviolet absorption, was cut out, and the radioactivity associated with GMP was determined. Protein in the extract was quantitated by the method of Bradford (2).

Autoradiographic experiments. *C. psittaci*-infected cells at a density of 250,000/ml were incubated as monolayers in Leighton tubes. At 18 h postinfection, the medium was replaced with 1 ml of growth medium containing 2 μCi of [^{3}H]guanine (100 nmol/ml). The monolayers were incubated for an additional 10 h and then fixed and prepared for autoradiography. After 21 days the film was developed and the cells stained as previously described (9). In some experiments, fixed cells were treated with 250 μg of pancreatic ribonuclease (RNase), 250 μg of deoxyribonuclease (DNAse) I (both Sigma), or both for 2 h at 37°C before exposure to film.

Purification of *C. psittaci*. *C. psittaci* was harvested by the Nonidet P-40 method of Hatch (7). The crude harvest (2.5 ml) was layered over an isopycnic gradient consisting of 0.5 ml of 40%, 0.5 ml of 35%, and 1.5 ml of 30% Renografin (E. R. Squibb & Sons), and *C. psittaci* was sedimented at 134,000 × *g* for 2 h. The *C. psittaci* band, located at the 35/40% interphase, was collected, and the Renografin was removed by sedimenting the *C. psittaci* at 10,000 × *g* and washing with 50 volumes of PBS.

Isotopes. [8-^{3}H]guanine sulfate was purchased from Amersham/Searle.

RESULTS

Transport of guanine by HeLa cells. It has been reported that mutants lacking hypoxanthine guanine phosphoribosyltransferase activity also lack the ability to transport purines by a mediated process (4). Because *C. psittaci* is an intracellular parasite, it is conceivable that the failure of an exogenous guanine label to be incorporated into the RNA of *C. psittaci* growing in HGPRT$^-$ cells could be due to the failure of the label to gain entry into the host cell rather than to the inability of the parasite to elevate guanine to the nucleotide level. Figure 1 shows the rate of uptake of guanine by HeLa cells as a

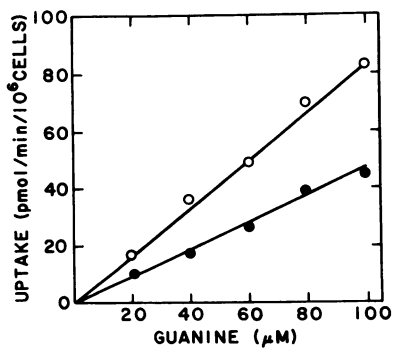

FIG. 1. *Rate of uptake of guanine by HeLa cells as a function of the concentration of guanine in the culture medium. Symbols: ○, Total uptake by HGPRT$^+$ cells; ●, total uptake by HGPRT$^-$ cells. The experiment was carried out as described in the text.*

function of the concentration of guanine in the culture medium. Uptake was non-saturable over the concentration range examined for both cell types, with the rate of uptake by the mutant HGPRT$^-$ cells being about one-half the rate by HGPRT$^+$ cells. So that rapid uptake of guanine by both HGPRT$^+$ and HGPRT$^-$ cells would occur, the concentration of guanine in the medium in experiments reported here was 100 μM.

Incorporation of [^{3}H]guanine into RNA of uninfected and infected HeLa cells. When uninfected and *C. psittaci*-infected HGPRT$^+$ cells were incubated in the presence of [^{3}H]guanine for 8 h, label was incorporated into the RNA of uninfected and infected cells at about the same rate (Fig. 2). Whether or not label was incorporated into parasite RNA in the infected HGPRT$^+$ cells was not determined in this experiment. On the other hand, the failure of label to be incorporated into the RNA of infected HGPRT$^-$ cells, despite a 3- or 4-fold increase in the mass of the parasite during the labeling period, precludes the possibility that exogenous label was incorporated to a significant degree into *C. psittaci* RNA in these cells and suggests that both HGPRT$^-$ cells and *C. psittaci* lack transferase activity.

Transferase activity in uninfected and infected HeLa cells. Hypoxanthine guanine phosphoribosyltransferase activity was measured in extracts of uninfected and *C. psittaci*-infected HGPRT$^+$ and HGPRT$^-$ cells in an attempt to detect a chlamydia-specific activity. Extracts were prepared from cells infected for 28 h, a time when inclusions containing chlamydiae were moderately large and consisted mainly of osmotically fragile, multiplying large forms of *C. psittaci* (inclusions in control samples increased twofold in size, as observed by staining and light microscopy, after 4 additional h of incubation). Transferase activity in extracts of uninfected HGPRT$^+$ cells was at least 200 times higher than in extracts of HGPRT$^-$ cells; infection with *C. psittaci* did not induce a significant increase in activity in either cell line (Table 1). Consequently, a chlamydia-specific transferase, if present at all, is present at a level significantly below that in both HGPRT$^+$ and HGPRT$^-$ cells. Also, transferase activity was not detected in lysates (prepared by ultrasonic vibration) of purified, non-multiplying, small forms of *C. psittaci*. Attempts were not made to assay for enzyme activity in purified large forms.

Autoradiographic analysis of infected HeLa cells. Infected HGPRT$^+$ and HGPRT$^-$ cells were incubated in the presence of [^{3}H]guanine and prepared for autoradiographic analysis, as described above, to detect the incorporation

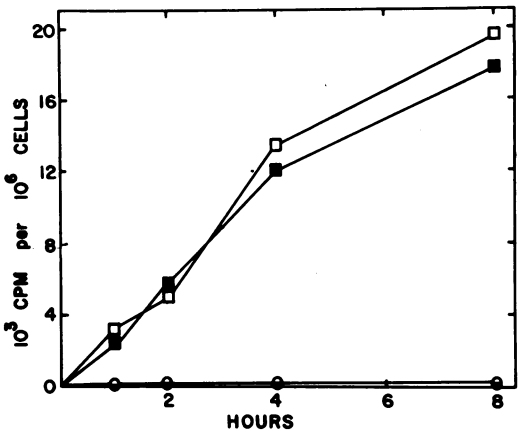

FIG. 2. *Incorporation of [^{3}H]guanine into RNA of uninfected and infected HeLa cells. Monolayers of cells were incubated in the presence of 1 μCi of [^{3}H]guanine (10 mCi/mol) per ml, and RNA was extracted as described in the text. Label was added to infected cells at 18 h postinfection. Symbols:* □, *Uninfected HGPRT$^+$;* ■, *infected HGPRT$^+$:* ○, *uninfected HGPRT$^-$;* ●, *infected HGPRT$^-$.*

TABLE 1. *Transferase activity in uninfected and C. psittaci-infected HGPRT$^+$ and HGPRT$^-$ cells*

Host-parasite system	Sp act[a]
HGPRT$^+$, uninfected	122
HGPRT$^-$, uninfected	<0.50
HGPRT$^+$, infected	110
HGPRT$^-$, infected	<0.50

[a] Picomoles of guanine converted to GMP/hour per microgram of protein.

of exogenously added guanine into parasite nucleic acids. Grains were noted over the cytoplasm and, to some extent, over the nucleus of HGPRT$^+$ cells; inclusions containing *C. psittaci* were almost completely blackened (Fig. 3A). About 50 to 75% of the grains over the inclusions were eliminated by treatment of the fixed cells with RNase before exposure to autoradiographic film, and most of the remaining grains were removed by treatment with DNase (autoradiographs not shown), indicating that the exogenous guanine label was incorporated into both RNA and DNA of the parasite. In contrast to the pattern of graining seen in HGPRT$^+$ cells, very few grains were detected over any portion of HGPRT$^-$ cells (Fig. 3B). This confirms that neither host nor parasite can use exogenous guanine when *C. psittaci* is grown in HGPRT$^-$ cells.

Quantitation of incorporation of [^{3}H]guanine into *C. psittaci* RNA. RNA was extracted from purified *C. psittaci* which had been grown in HGPRT$^+$ and HGPRT$^-$ cells incubating in

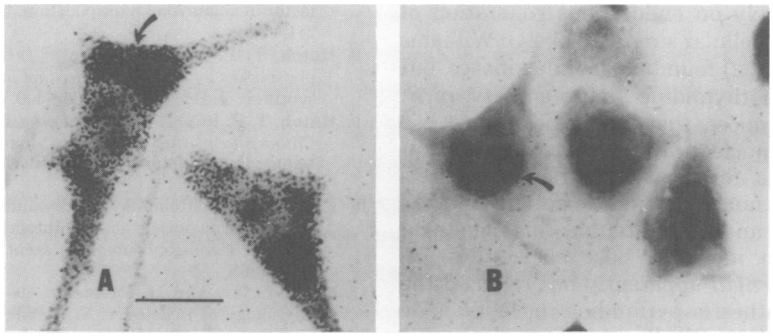

FIG. 3. *Autoradiographs of HeLa cells infected with C. psittaci. Cells were infected for 28 h and incubated in the presence of 2 μCi of [^{3}H]guanine (20 mCi/mol) per ml for 18 to 28 h postinfection. The cells were exposed to autoradiographic film for 21 days. (A) HGPRT$^+$ cells. (B) HGPRT$^-$ cells. Arrows point to inclusions containing C. psittaci. Bar, 10 μm.*

the presence of [^{3}H]guanine in order to quantitate the entry of label into parasite RNA. Although only 573 cpm were detected in the RNA extracted from 10^6 infectious units grown in HGPRT$^+$ cells, considerably less radioactivity, 10 cpm, was detected in the RNA from the same number of infectious units harvested from HGPRT$^-$ cells. The low number of counts in the RNA of HGPRT$^+$-grown *C. psittaci* is due, in part, to the low specific activity of the exogenous guanine used in the experiment. The near absence of radioactivity in the RNA of parasite grown in HGPRT$^-$ cells is consistent with the autoradiographic analysis (Fig. 3B) and the RNA extraction study on whole, infected HGPRT$^-$ cells (Fig. 2) and further confirms that *C. psittaci* is incapable of incorporating a significant amount of exogenous guanine into its RNA.

DISCUSSION

The results presented here indicate that *C. psittaci* cannot use exogenous guanine as a precursor for nucleic acid synthesis when the parasite is grown in a host cell deficient in hypoxanthine guanine phosphoribosyltransferase activity. The inability of the parasite to use exogenous guanine likely is due to its lack of transferase activity. On the other hand, guanine supplied in the culture medium is incorporated into parasite RNA and DNA if *C. psittaci* is grown in a host cell capable of elevating guanine to the nucleotide level. Therefore, it is concluded that the parasite itself cannot use guanine directly, but that it can use guanine nucleotides (most likely guanosine 5′-triphosphate) supplied by the host as precursors for nucleic acid synthesis. Although the experiments reported here do not eliminate the possibility that *C. psittaci* can synthesize guanine nucleotide de novo, they do demonstrate for the first time that an obligately intracellular parasitic bacterium can and does use host nucleotides for its own biosynthetic purposes.

It is interesting that not all host-synthesized nucleotides are used by *C. psittaci*. Tribby and Moulder (17) noted that exogenous thymidine and deoxycytidine are incorporated into host L-cell DNA, but not into chlamydial DNA. The poor incorporation of exogenous thymidine into *C. psittaci* DNA was verified by Hatch (9) in an autoradiographic study. Lin (10) demonstrated that *C. psittaci* lacks thymidine kinase activity and speculated that the parasite possesses a thymidine synthetase, thereby synthesizing thymine nucleotides from deoxyuridine 5′-monophosphate. It is possible that host ribonucleotide pools, but not host deoxyribonucleotide pools, are available to the parasite.

Pfefferkorn and Pfefferkorn (13), using *Toxoplama gondii* grown in Lesch-Nyhan and normal fibroblasts, carried out experiments similar to those reported here. Unlike *C. psittaci*, *T. gondii* can use exogenous guanine as a precursor for RNA synthesis when it is grown in hypoxanthine guanine phosphoribosyltransferase-deficient host cells.

It has been speculated, although not demonstrated, that other intracellular parasites such as *Plasmodium lopurae* and *Nosema michaelis* may draw on host-derived nucleotides in vivo, at least for the transport of nutrients into the parasite (16, 18). Host-free *Rickettsia* can generate adenosine 5′-triphosphate (ATP) using glutamate as a substrate (1). However, Winkler (24) has demonstrated that *R. prowazeki* possesses an adenosine 5′-diphosphate (ADP)-ATP transport system. Consequently, it has been suggested that these obligatory intracellular bacteria use host ADP and ATP pools for intracellular

growth and rely on endogenous generation of ATP for extracellular survival (23, 24). Williams and Peterson (22) found nucleotide kinases, but not uridine or thymidine kinase activity, in *R. typhi* and propose that these rickettsiae use host-derived nucleotides for precursors for nucleic synthesis. Similarly, Christian and Paretsky (3) found nucleotide kinase activity in *Coxiella burnetii* and speculate that this rickettsia-like organism may use host nucleotides, although *C. burnetii* apparently has retained the ability to synthesize pyrimidine nucleotides de novo as well (11). Finally, the intraperiplasmic parasite *Bdellovibrio bacteriovorus* preferentially uses host-derived thymidine monophosphate over exogenous thymidine as a precursor for DNA synthesis. Exogenous thymine and uridine are not used at all (15).

The ability of *C. psittaci* and possibly some other intracellular parasites to use host-generated nucleotides for their own biosynthetic purposes represents a logical adaptation to a nucleotide-rich environment.

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