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Adherence of *Nocardia asteroides* within the Murine Brain

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Nonlethal infection of BALB/c mice with *Nocardia asteroides* GUH-2 (GUH-2) produces a variety of neurological signs, including an L-dopa-responsive movement disorder in 10 to 15% of the infected population. To study nocardial interactions with the brain, we characterized the attachment of GUH-2 within specific regions through the use of microdissection. Following an intravenous injection of a single-cell suspension of log-phase GUH-2, viable cells were recovered from all regions of the brain, and the distribution of the nocardiae was independent of the size of the inoculum. In addition, two mutants of GUH-2 were found to possess significantly altered binding characteristics with regard to both the percentage of the inoculum bound per brain and the relative distribution of adherence to regions of the brain, when compared with the parental strain. These results indicated that GUH-2 bound throughout the murine brain and suggested that GUH-2 utilized specific receptors to facilitate this attachment.

Nocardia asteroides is a filamentous, aerobic actinomycete that is normally present in the soil (12). It is frequently recognized to cause progressive disease in compromised hosts; however, lethal infections in normal individuals with no identifiable predisposing condition have been reported often (3, 20). In humans, about 30% of the clinical cases reported in the literature involve the dissemination of *N. asteroides* from the lungs to other sites, with the brain being the most frequent secondary target (20).

A variety of neurological disorders have been reported in humans with brain lesions that were induced by various *Nocardia* species (11). These range from physical disabilities and movement disorders, such as hemiparesis (2, 7, 19, 27, 29), body tremors and parkinsonian features (14, 18, 22), seizures (13, 19), retropulsion (27), and ataxia (8, 9, 14, 27), to mental disorders, such as schizophrenia (15), manic depression (15), dyslexia (2), hallucinations (9), amnesia (7), and pruritus (24).

In mice, distinct neurological signs developed following an intravenous injection of a nonlethal dose of log-phase cells of *N. asteroides* GUH-2 (16). On the basis of the specific neurological presentation, the animals could be divided into groups which included the following: (i) mice that exhibited a levodopa (L-dopa)-responsive rhythmic vertical head shake, stooped posture, and hypoactivity (mice with parkinsonian features); (ii) mice that exhibited an L-dopa-responsive head shake and hyperactivity; (iii) mice that had a hemiparesislike syndrome with one-sided body weakness, increased muscle rigidity on the opposite side, and a deviation of the head to one side (these mice would spin when suspended by the tail); (iv) mice that were hyperactive and continually running in circles; (v) mice that had ataxic movement and sporadic convulsions or seizures; and (vi) mice that demonstrated retropulsion (16). Some of the animals exhibited mixed combinations of the above-listed features.

The interaction of a pathogen with specific sites in the host is a key component of the disease process; therefore, it is important to know the distribution and actions of the organism within the host. Previous work (5) has shown that when injected intravenously in the tail of a mouse, *N. asteroides* GUH-2 must first pass through the liver and other filtering organs, such as the spleen, before being distributed to other parts of the body. Indeed, within just a few minutes after intravenous injection, most of the nocardial cells are localized within the liver and spleen (5, 6). However, unless a very large inoculum is used, GUH-2 does not grow in either the liver or the spleen, yet it does grow within the brain. Also, this strain of *N. asteroides* does not induce abscesses in the liver or spleen in noncompromised mice (4). In humans, liver and splenic abscesses are rarely caused by *Nocardia* species, whereas about 25% of the cases reported in the literature represent brain infections (3, 20). Therefore, in both humans and mice, the brain is a site that is significant in the nocardial disease process.

Given the neurological manifestations reported in humans and mice and the predilection of the organism for the brain, the interactions of *N. asteroides* GUH-2 with the murine brain were studied further. The sequence of events which occur following nonlethal intravenous infection may involve the following steps: (i) attachment to the brain capillaries; (ii) penetration into the brain tissue; (iii) growth of nocardiae in the brain tissue; (iv) induction of neuron damage; and (v) clearance of the organism from the brain. Since it has been shown previously that these events can occur without inducing an inflammatory response (16), no microscopic evidence of a previous nocardial infection may be apparent following this infectious process (16).

The purpose of this investigation was to determine whether there are specific adherence mechanisms for the critical first step of nocardial infection of the murine brain. Through the use of microdissection, the attachment of bacteria in eight regions of the brain following lethal and nonlethal infections with *N. asteroides* GUH-2 was studied. In addition, two mutants of strain GUH-2 that had different abilities to induce neurological changes in mice were selected. Comparisons between the attachment patterns of the parent and these mutants were made to explain their different abilities to induce specific neurological signs.

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MATERIALS AND METHODS

Microorganisms. *N. asteroides* GUH-2 was isolated from the kidney of a patient with a fatal infection at Georgetown University Hospital, Washington, D.C. Its pathogenesis has been studied extensively (6). *N. asteroides* GUH-2 mutant I-38-syn (I-38-syn) is an isoniazid-resistant mutant of GUH-2 that was induced by two exposures to UV irradiation (105 s per exposure, ~1,200 ergs/mm^2 per exposure) and selected with isoniazid (100 μg/ml for primary selection and 200 μg/ml for secondary selection) (Aldrich, Milwaukee, Wis.)-containing medium. This mutant induces the L-dopa-responsive head shake in mice at a significantly higher frequency than the parent strain (i.e., 58 versus 10% at comparable nonlethal doses). Furthermore, when injected with I-38-syn at nonlethal doses, 94% of mice develop visible neurological signs, compared with 40% of mice injected with the parental strain. *N. asteroides* GUH-2 mutant NG-49 (NG-49) is a pigmentless mutant of GUH-2 that was induced by exposure to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich) as described by Delić et al. (10). It grows more slowly than parental strain GUH-2, it does not produce a melaninlike pigment when grown in brain heart infusion (BHI; Difco, Detroit, Mich.) broth, it is relatively avirulent, and it does not induce the L-dopa-responsive head shake in mice.

Frozen stock cultures of GUH-2 and NG-49 were prepared during stationary phase growth in BHI broth, while I-38-syn was prepared during stationary phase growth in MI(200), a minimal medium [7.6 mM $(NH_4)_2HPO_4$, 2.7 mM KCl, 0.81 mM $MgSO_4 \cdot 7H_2O$ (pH 7.6)] containing 0.5% glutamate and 200 μg of isoniazid per ml. These cultures were centrifuged at 55 × *g* for 5 min. Supernatants containing single-cell suspensions of coccobacillary cells were mixed 1:1 with 2% sterile skim milk in sterile, capped polystyrene tubes, and the mixtures were stored at −70°C.

Animals. Female BALB/c mice weighing 18 to 20 g were obtained from Simonsens (Gilroy, Calif.) or Bantin & Kingman (Fremont, Calif.). Following infection, the mice were kept in a climate-controlled animal room and provided food (Purina rodent chow) and water ad libitum. They were maintained by the Animal Resource Service at the University of California at Davis.

Inocula. GUH-2 and NG-49 inocula were prepared from log-phase cultures in BHI broth. Fifty milliliters of BHI broth was inoculated with 2 drops of frozen stock. Each culture was incubated at 37°C with rotational agitation (150 rpm) until the cells were in the log phase; GUH-2 cultures were incubated for 16 to 18 h, and NG-49 cultures were incubated for 40 h (as determined by comparative growth curves, NG-49 grows more slowly than parental strain GUH-2). After the incubation was completed, the cells were centrifuged at 55 × *g* for 5 min to produce single-cell suspensions. Phase-contrast microscopy of wet mounts revealed that both inocula had similar morphologies and were composed of individual branching filaments. Cell concentrations were estimated by measuring the optical density at 580 nm, and all dilutions were made in fresh BHI broth. When necessary, cells were concentrated by centrifugation (1,400 × *g* for 5 min) and resuspended in fresh BHI broth. Viable cells were quantitated by plate counts on tryptic soy agar (Difco).

The I-38-syn inoculum was prepared from log-phase cultures in MMG medium, a defined minimal medium [as described for MI(200)] containing 0.5% glutamate. The starter cultures of both *N. asteroides* GUH-2 in MMG medium and I-38-syn in MI(200) were prepared from the frozen stocks. After 4 days of incubation (37°C, 150 rpm), the cultures were centrifuged (55 × *g*, 5 min) and the supernatants were used to inoculate 50 ml of fresh MMG medium. These were incubated (37°C, 150 rpm) for 25 to 27 h (mid-log phase, as determined by growth curves). After incubation, the cells were collected on a sterile cellulose nitrate membrane (0.45-μm-pore size) in a disposable filtration unit (Nalgene). The cells were resuspended in fresh MMG medium to produce a 100-fold concentrate of the starting culture. The concentrate was centrifuged at 55 × *g* for 2 min to produce single-cell suspensions. The optical density at 580 nm was adjusted with fresh MMG medium, and dilutions were plated on tryptic soy agar for viable counts. Mutant NG-49 was not grown in MMG medium because of excessive cell clumping and the inability to obtain single-cell suspensions.

Groups of mice were injected intravenously via their tail veins with 0.1 ml of inoculum. Mice were inoculated with either a nonlethal (5 × 10^5 CFU per mouse) or a lethal (2 × 10^6 CFU per mouse) dose of *N. asteroides* GUH-2. Mutants NG-49 and I-38-syn were inoculated into mice at doses similar to the lethal dose of GUH-2 (2 × 10^6 CFU per mouse). In contrast to the parent strain, neither of these mutants kills mice at this dose. For studies of the adherence of cells grown in a chemically defined medium, the parent inoculum was 8 × 10^5 CFU per mouse.

Microdissection of the murine brain. At 3 h after injection, the mice were sacrificed by cervical dislocation. The brains were removed aseptically and kept in a sterile petri dish on ice until they were microdissected. The brains were dissected into the following eight regions: the cerebellum, substantia nigra, hippocampus, striatum, hypothalamus, cortex, pons-medulla, and midbrain, which included the thalamus. Each locale was homogenized and plated on tryptic soy agar. Plates were incubated at 37°C until colonies were large enough to count.

Perfusion of the murine brain. In separate experiments, the mice were perfused with saline to remove all residual blood from within the brain, as follows. The mice were injected intravenously with 2 × 10^6 CFU of GUH-2 grown in BHI broth. At 3 h postinjection, the mice were anesthetized in pairs by injecting 0.2 ml of a 5-mg/ml pentobarbital solution intraperitoneally. Once anesthetized, one mouse of each pair was perfused. The chest cavity was opened, the right atrium of the heart was cut, and a 25-gauge needle, which was attached to a reservoir of sterile saline, was inserted into the left ventricle. Perfusion was performed by gravity feed and monitored by observing the blanching of the extremities and liver. When perfusion was nearly completed, the other mouse was sacrificed by cervical dislocation. The brains were removed aseptically and plated on tryptic soy agar. Plates were incubated at 37°C. This procedure was repeated so that five mice were perfused and five mice were not.

Statistical analysis. Student's *t* test for the difference between two means was used to determine the significance of the values obtained (30). Comparisons between GUH-2 and mutant recoveries within each region are presented; no comparisons between regions (e.g., the cortical values were not compared with the nigral values) were performed because of difficulties in normalization.

RESULTS

Binding of *N. asteroides* GUH-2 to the brain. The averages of the total numbers of bacteria recovered from the brain and the corresponding inocula are presented in Table 1. The

TABLE 1. Percentage of inoculum recovered from the brain following intravenous injection of GUH-2 (grown in either BHI broth or MMG medium) and mutants NG-49 and I-38-syn

Inoculum (medium and organism)	No. of mice tested	Dose (CFU/mouse)	Avg recovery at 3 h (CFU/brain)	% of inoculum (10^{-2})	SEM (10^{-2})
BHI broth					
GUH-2 (nonlethal)	11	4.9×10^5	237	4.8	0.37
GUH-2 (lethal)	12	2.0×10^6	1,019	5.4	0.30
NG-49	10	2.0×10^6	79	0.39	0.05
MMG medium					
GUH-2	5	7.8×10^5	255	3.3	0.15
I-38-syn	10	2.8×10^6	386	1.3	0.10

lethal dose of 2×10^6 CFU per mouse led to a fourfold increase in the total numbers of organisms recovered from the brain, compared with the sublethal dose of 5×10^5 CFU per mouse. Statistical analysis of the total numbers of organisms recovered per dose showed that the larger inoculum did not result in a significant change in the relative percentage of cells adherent to the brain (Table 1). These results indicated that there was a linear dose-response relationship between the numbers of organisms binding to the brain and the numbers of organisms inoculated.

Effects of perfusion on nocardiae bound to the brain. To determine the effects of the numbers of organisms remaining in the residual blood in the brain, we compared counts in perfused and nonperfused mice. In this series of experiments, the brains of nonperfused mice receiving 2×10^6 CFU intravenously had an average count of 2,227 CFU, with a standard error of 335. The brains of perfused mice receiving the same inoculum at the same time had a slightly lower average count of 1,979 CFU, with a standard error of 251. The difference between the CFU in perfused and nonperfused brains was not statistically significant ($P > 0.5$). Therefore, the presence of small numbers of bacteria in the blood at 3 h after infection had no significant effect on the total CFU bound to the brain. Deterioration of tissue during perfusion prevented the use of microdissection on perfused brains. However, the lack of a significant difference between the total CFU bound to perfused and nonperfused brains suggested that the presence of blood-borne nocardiae would not significantly affect the distribution of counts within the brains.

Localization within the brain. Quantitation of the nocardiae within different regions of the brain demonstrated that the larger inoculum used for the lethal dose led to higher counts in all regions of the brain. Changes in the distribution of the organisms for both doses were determined by comparing the regional percentages of the total numbers of organisms recovered from the brain (Fig. 1). These data showed that no significant differences occurred in the nocardial distribution in the brain as a result of the different inocula (Fig. 1). Also, the higher inoculum did not significantly alter the percentage of the inoculum that was bound to the brain (Table 1).

Characterization of mutants. All prior studies with mutant I-38-syn were performed with cells grown in MMG medium. Because of our familiarity with this system, all binding studies were performed with these cells grown in MMG medium rather than BHI broth. In contrast, mutant NG-49 grew as clumps in MMG medium, and it was difficult to obtain single-cell suspensions in this medium. Therefore,

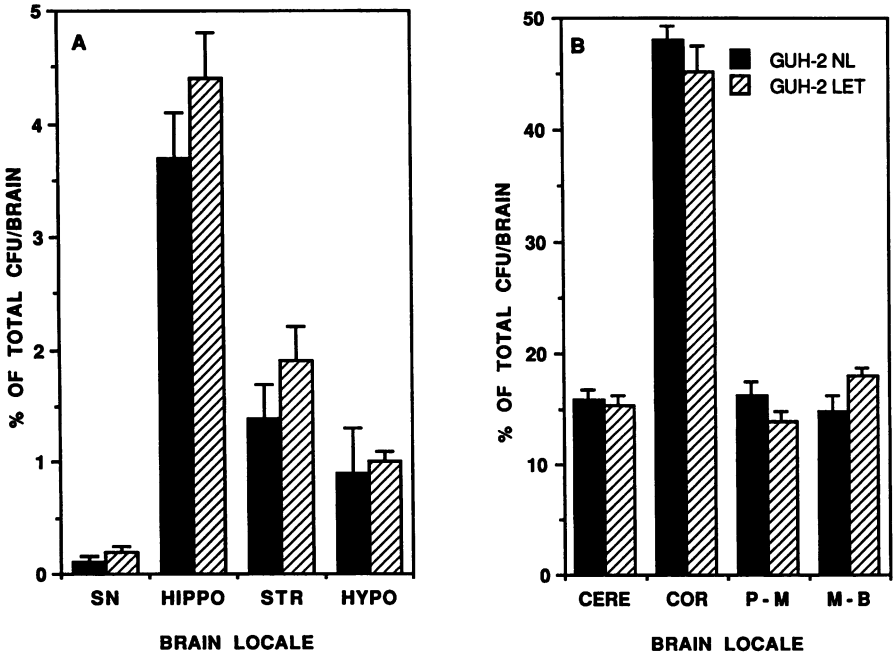

FIG. 1. Comparison of the distribution of *N. asteroides* in each brain locale of mice following an injection of either a nonlethal (GUH-2 NL) or a lethal (GUH-2 LET) dose of *N. asteroides* GUH-2. Values are presented as the mean percentages of the total numbers of cells bound to the brain ± the standard error of the mean. (A) Attachment in the substantia nigra (SN), hippocampus (HIPPO), striatum (STR), and hypothalamus (HYPO). (B) Attachment in the cerebellum (CERE), cortex (COR), pons-medulla (P-M), and midbrain (M-B). No significant differences were found ($P > 0.05$).

TABLE 2. Movement disorders induced by GUH-2 (nonlethal infection) and mutants NG-49 and I-38-syn

Organism (medium grown in)	CFU/mouse	No. of mice tested	No. (%) of mice with the following neurological sign[a]:				
			HS	sl HS	Hemiparesis		None
					With sl HS	Without HS	
GUH-2 (BHI broth)[b]	4×10^5	216	24 (11)	—[c]	9 (5)	55 (23)	128 (60)
NG-49 (BHI broth)	2×10^6	11	0 (0)	0 (0)	1 (9)	4 (36)[d]	6 (54)
I-38-syn (MMG medium)	3×10^6	36	21 (58)	1 (3)	8 (22)	4 (11)	2 (6)

[a] HS, rhythmic, rapid vertical head shake; sl HS, slight head shake.
[b] Values were reported by Kohbata and Beaman (16).
[c] —, not reported.
[d] Hemiparesis was transient; 3 weeks after injection, mice showed no neurological signs.

NG-49 was maintained in BHI broth, since uniform suspensions of single cells were easily obtained during growth in this medium.

Both mutants were shown to be less virulent for BALB/c mice than parental cells grown under identical conditions. Despite being given doses equivalent to or slightly larger than the lethal dose of the parental strain, no mice died within the first 2 weeks following infection with either of the mutant strains. In contrast, 1 of 10 mice receiving 1.7×10^6 CFU of GUH-2 died within 36 h after infection, and the remaining animals were near death at this time. Currently, no fatal infection with NG-49 has been achieved. Of 28 mice receiving 3.5×10^6 CFU of I-38-syn (a dose twofold higher than the lethal dose of GUH-2 used in this study), only 2 died, and both of these mice survived for 2 weeks postinfection. The precise 50% lethal doses of these two mutant strains were not determined; however, the 50% lethal dose of GUH-2 (grown in BHI broth) during this study was approximately 8×10^5 CFU per mouse. The neurological disorders induced by the two mutants are presented in Table 2. Of 36 mice infected with I-38-syn at inocula ranging from 1×10^6 CFU per mouse to 3.5×10^6 CFU per mouse, 21 (58%) developed a rhythmic vertical head shake. Furthermore, 34 mice (94%) inoculated with I-38-syn developed a variety of visible neurological signs. It should be noted that MMG medium-grown cells of GUH-2 did not induce more neurological signs than BHI broth-grown GUH-2 cells; therefore, the culture medium used to grow the organism was unlikely to be responsible for the greater induction of neurological disorders by I-38-syn. Of 11 mice injected with 2×10^6 CFU of mutant NG-49, 5 (45%) developed a transitory, mild hemiparesis, but no head shaking developed in any of the mice (Table 2).

Effect of mutation on adherence to the brain. Comparison of the numbers of organisms in the brains of mice 3 h after injection revealed that both NG-49 and I-38-syn had mutations that altered their attachment to the brain differently (Table 1). Strain NG-49 had a 10- to 15-fold decreased level of binding compared with the parental strain ($P < 0.001$), whereas strain I-38-syn had a 2- to 4-fold decreased level of binding compared with the control ($P < 0.001$) (Table 1).

A comparison of the distribution of the nocardiae adherent to the brain showed that the mutants displayed attachment profiles different from those of the parent. Strain NG-49 had a larger proportion of cells attached to the cerebellum and the striatum, but a lower proportion was found in the cortex (Fig. 2). A comparison between the distributions of I-38-syn and GUH-2 grown in MMG medium revealed two differences. A significantly larger proportion of the parental strain was localized in the cerebellum ($P < 0.05$) (Fig. 3); in addition, a significantly larger proportion of I-38-syn was found in the substantia nigra ($P < 0.05$).

DISCUSSION

The results of this study showed that *N. asteroides* GUH-2 could be found distributed throughout the murine brain. These observations are consistent with the variety of sites of abscess formation reported in the brains of humans (9, 14, 24, 27). In addition, they indicate that there may be an attachment receptor for nocardiae on the endothelial cells of the vasculature distributed in all regions of the brain. Although it is possible that nocardial attachment occurred by a nonspecific adherence mechanism, the independence of the distribution from the inoculum size and the fact that mutants with very different profiles of attachment were obtained suggest that there are specific binding sites.

Attachment to endothelial cells via specific receptors has been reported in other diseases. The adherence of meningitis-causing strains of *Escherichia coli* is mediated by the S fimbriae, which recognize sialic acid-containing structures (17, 21). In cerebral malaria, *Plasmodium falciparum*-infected erythrocytes express the proteins of the parasite on their surface. These bind to thrombospondin, a glycoprotein, on the surface of endothelial cells; thus, the aggregation of erythrocytes, with blockage of the blood vessels, occurs (1, 23). At present, we do not know the mechanism by which *N. asteroides* GUH-2 attaches to the murine brain; however, biochemical studies of the mutants and the parental strain should lead to a better understanding of this process.

Strain I-38-syn was shown to attach to the brain vasculature at significantly lower levels ($P < 0.001$) than GUH-2 when prepared in exactly the same manner and at the same stage of growth. Another strain, NG-49, adhered to the murine brain at significantly lower levels than either GUH-2 or I-38-syn. Furthermore, the two mutant strains displayed marked differences in distribution within regions of the brain when compared with the parent. It is possible that the decreased counts that were determined in the mutant-infected mice were due to alterations of viability within the host brain. However, preliminary data from organ distribution studies, in which the numbers of cells recovered from the lungs, spleen, kidneys, liver, blood, and brain were determined 2 h after infection, indicated that I-38-syn was not less viable than GUH-2 within the host. Therefore, it is unlikely that the numbers of I-38-syn cells that were recovered from the murine brain were a reflection of decreased viability. Regarding NG-49, no such study has been performed; however, a comparison of the glycolipid profile of NG-49 with that of GUH-2 by high-performance liquid

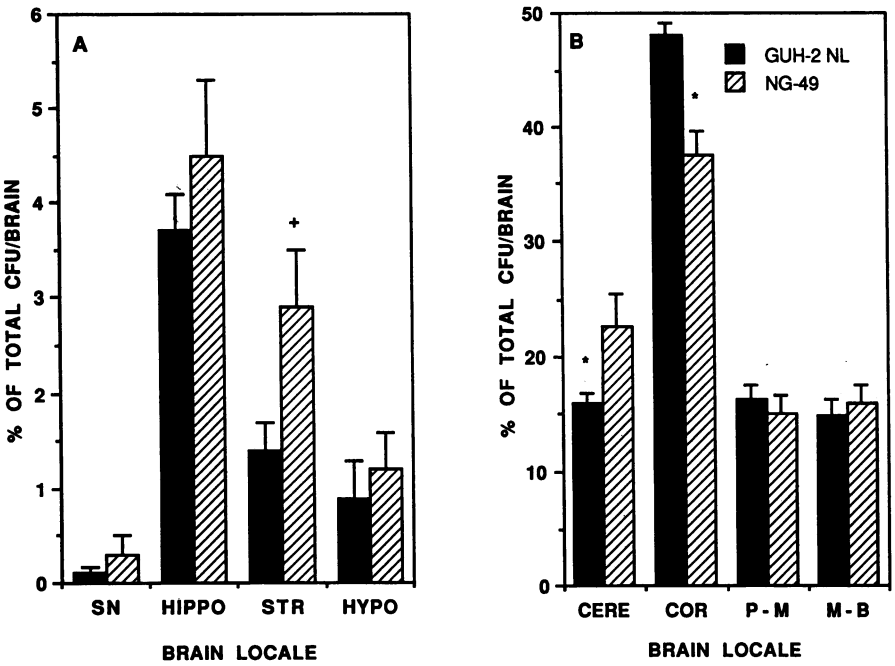

FIG. 2. Comparison of the distribution of nonlethal GUH-2 (GUH-2 NL) and NG-49 in the murine brain. Significant differences are noted with + ($P < 0.05$) or * ($P < 0.01$). Values and abbreviations are the same as those given in the legend to Fig. 1.

chromatography–mass spectrometry has revealed differences between the two organisms (as has a comparison of I-38-syn with GUH-2). This result indicates that the cell surface of NG-49 has been altered; therefore, it is possible that the decreased recovery of NG-49 from the murine brain is due to decreased adherence.

Intravenous infection of mice with a nonlethal dose of *N. asteroides* GUH-2 grown in BHI broth induces an L-dopa-responsive head shake in approximately 15% of the mice; this value is not increased by growth in MMG medium. In contrast, I-38-syn produces the L-dopa-responsive head shake at a frequency of greater than 60%, whereas NG-49 has lost this ability completely. The region of the brain for which I-38-syn exhibits a greater affinity than MMG medi-

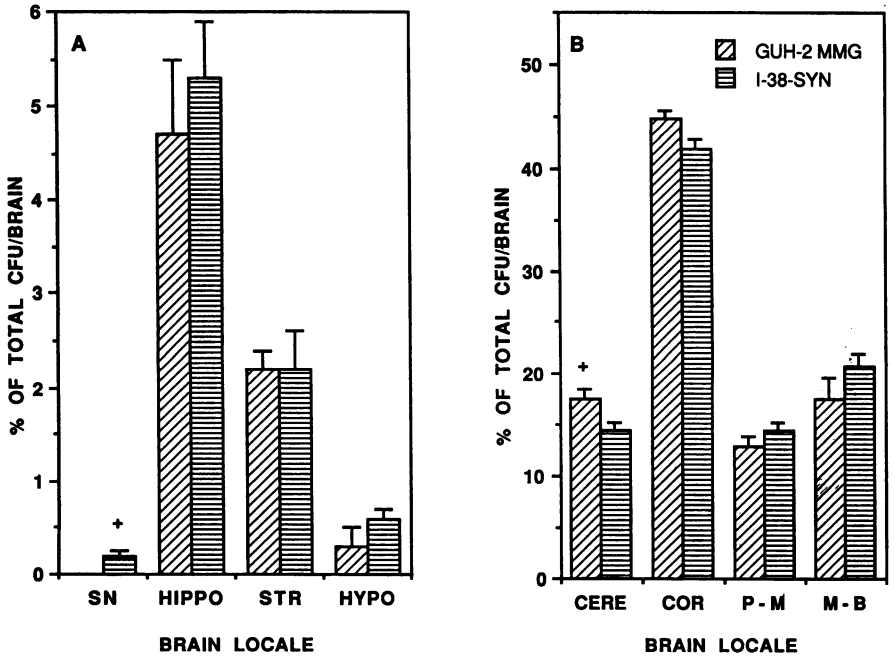

FIG. 3. Comparison of the distribution of I-38-syn and MMG medium-grown GUH-2 (GUH-2 MMG). Values, abbreviations, and levels of significance are the same as those given in the legend to Fig. 1.

um-grown GUH-2 is the substantia nigra. Perhaps the predilection of I-38-syn for the substantia nigra contributes to an increased amount of damage to the dopaminergic neurons within this region, thus producing an increased frequency of induction of the L-dopa-responsive movement disorder.

It was shown that both NG-49 and I-38-syn attach to the murine brain at lower levels than the parent; this characteristic may be one factor in the decreased virulence (based strictly on 50% lethal doses) that these mutants exhibit. Also, it was found that I-38-syn has a predilection for attachment to the substantia nigra. Attachment, however, is only the first step in the disease process. The next stage that must be addressed to better understand pathogenic mechanisms is the ability of an organism to invade and grow within each region of the brain.

Penetration of the blood-brain barrier is necessary for invasion of the brain tissue. Cells of *Treponema pallidum* and *Borrelia burgdorferi* are able to invade the brain by passing between endothelial cells through the intercellular junctions (25, 26). One possible mechanism of nocardial penetration into the brain tissue may be growth through tight junctions, as by *T. pallidum*. A second possible mechanism that the nocardiae may utilize to invade the brain may involve a process like that reported for *Rickettsia* species (28). Walker suggested that *Rickettsia prowazekii* can utilize a passive method for entering the endothelial cells of the vasculature (28). This organism attaches to endothelial cells and is then phagocytized, thus facilitating its entry into the cell. The nocardiae may utilize a similar process to penetrate through blood vessels into the brain. Therefore, attachment to and penetration into the brain by nocardiae may involve different mechanisms, which could contribute to the wide range in the ability of NG-49 and I-38-syn to induce neurological damage.

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