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Mercury Released from Dental "Silver" Fillings Provokes an Increase in Mercury- and Antibiotic-Resistant Bacteria in Oral and Intestinal Floras of Primates

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In a survey of 640 human subjects, a subgroup of 356 persons without recent exposure to antibiotics demonstrated that those with a high prevalence of Hg resistance in their intestinal floras were significantly more likely to also have resistance to two or more antibiotics. This observation led us to consider the possibility that mercury released from amalgam ("silver") dental restorations might be a selective agent for both mercury- and antibiotic-resistant bacteria in the oral and intestinal floras of primates. Resistances to mercury and to several antibiotics were examined in the oral and intestinal floras of six adult monkeys prior to the installation of amalgam fillings, during the time they were in place, and after replacement of the amalgam fillings with glass ionomer fillings (in four of the monkeys). The monkeys were fed an antibiotic-free diet, and fecal mercury concentrations were monitored. There was a statistically significant increase in the incidence of mercury-resistant bacteria during the 5 weeks following installation of the amalgam fillings and during the 5 weeks immediately following their replacement with glass ionomer fillings. These peaks in incidence of mercury-resistant bacteria correlated with peaks of Hg elimination (as high as 1 mM in the feces) immediately following amalgam placement and immediately after replacement of the amalgam fillings. Representative mercury-resistant isolates of three selected bacterial families (oral streptococci, members of the family *Enterobacteriaceae*, and enterococci) were also resistant to one or more antibiotics, including ampicillin, tetracycline, streptomycin, kanamycin, and chloramphenicol. While such mercury- and antibiotic-resistant isolates among the staphylococci, the enterococci, and members of the family *Enterobacteriaceae* have been described, this is the first report of mercury resistance in the oral streptococci. Many of the enterobacterial strains were able to transfer mercury and antibiotic resistances together to laboratory bacterial recipients, suggesting that the loci for these resistances are genetically linked. Our findings indicate that mercury released from amalgam fillings can cause an enrichment of mercury resistance plasmids in the normal bacterial floras of primates. Many of these plasmids also carry antibiotic resistance, implicating the exposure to mercury from dental amalgams in an increased incidence of multiple antibiotic resistance plasmids in the normal floras of nonmedicated subjects.

The locus for mercury resistance was the first of 12 distinct bacterial plasmid-determined metal resistance loci (including those for arsenic, antimony, boron, cadmium, chromium, cobalt, copper, nickel, silver, tellurium, and zinc) to be described. While many of the other metal resistance loci are found predominantly in bacteria from soil and industrial waste rather than in mammalian normal floras and pathogens, resistance to inorganic and organic mercury compounds is as common in the latter as in the former (36, 37).

In every case which has been examined, the biochemical mechanism of Hg resistance is the reduction of the reactive ionic form, Hg(II), to the less reactive, volatile, elemental form, Hg(0) (24). The plasmid-encoded *mer* locus consists of genes for the cytoplasmic Hg(II) reductase enzyme and a transport system (consisting of either two or three proteins) which brings Hg(II) into the cytoplasm for reduction by the Hg(II) reductase. The genes for the entire system are arranged as an operon under both the positive and negative control of the protein MerR, an exquisitely sensitive DNA-binding protein which has a 10^4-fold higher affinity for Hg than does mercaptoethanol (35). A subset of the naturally occurring loci which confer inorganic mercury resistance also carries another enzyme, the organomercurial lyase. This enzyme removes the carbon moiety from such compounds as methyl mercury and merthiolate, leaving Hg(II), which is then a substrate for the Hg(II) reductase (3, 4). Thus, strains carrying the organomercurial lyase are resistant to and biotransform both inorganic and organic mercurial compounds.

Previously, we had observed that the proportion of mercury-resistant bacteria in the gram-negative facultative bowel floras of humans varies widely from individual to individual. Some persons have floras that are less than 10% resistant, and others have floras that are more than 90% resistant (21). The incidence of Hg resistance in the gram-positive facultative oral floras of individuals has not been assessed, although Hg-resistant isolates of gram-positive bacteria have been widely found and characterized from other sources (36). The incidence of Hg(II) resistance increased in Japanese hospitals in a manner temporally related

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to their use of mercurial antiseptics (30) and in mercury-polluted soil and water (1, 17, 18). Thus, the prevalence of Hg-metabolizing bacteria varies in response to Hg in the environment. Whether this also occurs in the floras of individual humans and animals has never been explicitly examined.

Earlier investigations of humans demonstrated that toothbrushing and mastication induce increases in intraoral Hg vapor from dental "silver" fillings made of amalgam, a material which contains 50% metallic mercury (28, 39, 41, 42). The average daily absorbed does of Hg is approximately 10 μg in human subjects with amalgams (43, 44). There is now scientific concurrence that the most prevalent source of deliberate mercury exposure for the general population is from dental amalgam and that chronic inhalation or swallowing of amalgam mercury vapor is the major contributor to the total body burden of mercury (14). A recent study has demonstrated substantial Hg accumulation in the gingivae, kidneys, and intestinal contents of monkeys after installation of amalgam fillings (10, 15). The Hg concentrations in the organs and intestinal contents of these animals corresponded to concentrations routinely used for selection of Hg-resistant bacteria in rich media in the laboratory (ca. 25 to 50 μM).

The primary objective of the present work was to determine whether Hg released from amalgam fillings could select for Hg-resistant bacteria in the oral and intestinal floras of primates. Since it is well known that Hg resistance occurs on plasmids which also carry antibiotic resistance genes, additional objectives were to determine whether changes in Hg resistance in these bacterial populations correlated with changes in the incidence of antibiotic resistance and to determine whether individual Hg-resistant isolates also carried plasmids which conferred both Hg and antibiotic resistances.

MATERIALS AND METHODS

Human sample populations. From December 1977 through March 1979, 974 fecal samples from 640 hospitalized and ambulatory human donors were examined (19). The patient samples were obtained from the New England Medical Center Hospital. The ambulatory population included laboratory workers (from eight research laboratories in the greater Boston area and from the University of Georgia, Athens) and urban (Boston) and rural (Sherborn, Mass.) dwellers. Contributors answered a questionnaire which included items on sex, age, residence, employment, and history of antibiotic exposure during the previous 2 weeks (including both therapeutic and employment exposures). The subjects were predominantly adults (95.4% over age 15), and 56% were female. The mean age of the population was 42.7 years (standard deviation, 21.7 years), with a median age of 37 years. Details of sample acquisition and processing for this population have been described previously (19) and were essentially the same as described below for fecal samples from the monkeys. Antibiotic resistances assessed were those to ampicillin, streptomycin, tetracycline, kanamycin, chloramphenicol, gentamicin, and nalidixic acid.

Experimental animals. The first experiment employed two adult wild-caught male cynomolgus monkeys, monkeys 16 and 95, weighing 4.4 and 5.0 kg, respectively; the second experiment employed two adult wild-caught female rhesus monkeys, monkeys R830 and L980, weighing 7.3 and 7.6 kg, respectively; and the third experiment employed two adult wild-caught male cynomolgus monkeys, monkeys F78 and F79, weighing 8.5 and 6.5 kg, respectively.

Animal maintenance procedures. The animals were singly housed in large (48 ft^3 [1 ft = 30.48 cm]) squeeze-back cages and acclimated to their environment and diet for 2 weeks before sampling was begun. Twice daily, the animals were fed antibiotic-free Wayne 25% Primate Diet (no. 8663; Teklad/Premier Laboratory Diets, Madison, Wis.) supplemented with apples, oranges, bananas, sunflower seeds, and peanuts. Fresh water was available ad libitum. Animals maintained under such conditions do not typically engage in coprophagy, and no behavior of this type was observed in these animals. After dental surgery and sampling protocols (described below) were completed, the monkeys were anesthetized with ketamine (13 mg/kg of body weight) and then killed with an intravenous injection of sodium pentobarbital (Euthanyl; MTC Pharmaceuticals, Cambridge, Ontario, Canada) in order to obtain a complete set of samples from all organs for radiological (experiment 1) or chemical (experiments 2 and 3) quantification of Hg concentrations in tissue.

Dental surgery. Before dental surgery, each animal was made to fast for 24 h and water was withheld for 12 h. Anesthesia was induced with an intramuscular injection of a ketamine hydrochloride-xylazine mixture (Ketaset at 11 mg/kg; Austin Laboratories Canada Ltd., Joliette, Quebec, Canada; Rompun at 1.1 mg/kg; Haver/Chemagro Ltd., Etobicoke, Ontario, Canada). An endotracheal tube with an outside diameter of 5.5 mm (Portex, Inc., Wilmington, Mass.) was inserted, and unassisted general anesthesia was maintained with a Narkovet 2 anesthetic machine (North American Drager, Telford, Pa.) delivering a gas mixture of 0.6 liter of nitrous oxide per min, 0.4 liter of oxygen per min, and halothane (0.5 to 0.8%; MTC Pharmaceuticals).

The preparation and placement of dental amalgam fillings were as described by Hahn et al. (15). Each animal received 16 small, occlusal surface amalgam fillings containing ca. 93 to 100 mg of Hg(0) metal. Radioisotopic mercury metal [^{203}Hg(0)] was used only in experiment 1. Standard nonradioactive dental amalgam (Dispersalloy; Johnson & Johnson, Montreal, Quebec, Canada) filling material was used in experiments 2 and 3. In week 8 of experiments 2 and 3, amalgam fillings were replaced with glass ionomer fillings as previously described (7).

Microbiological sampling procedure. Duplicate inocula of gingival scrapings and of freshly voided fecal material were taken on sterile swabs (Culturette no. 4360210; Becton Dickinson, Cockeysville, Md.) twice weekly from each monkey. The inocula were shipped via air express to Georgia and were received there within 20 h of initial inoculation. For each swab, duplicate nonselective master plates were inoculated and then streaked in three directions for isolation of single colonies. Fecal samples were inoculated onto bile esculine azide (BEA; Difco) plates for selection of enterococci and onto MacConkey (Mac; Difco) plates for selection of members of the family *Enterobacteriaceae*. Oral samples were inoculated on mitis salivarius (MS; Difco) plates for selection of streptococci. All plates were incubated at 37°C for 24 to 48 h. Early experiments indicated that colony formation was most abundant on BEA and Mac plates when plates were incubated aerobically and on MS plates when plates were incubated in GasPak chambers.

In the first experiment (see Fig. 2), microbiological samples were taken for 10 days prior to amalgam placement and for 4 weeks with the amalgams in place. In the second experiment, microbiological samples were taken for 2 weeks

prior to amalgam placement, during 7 of the 8 weeks with the amalgams in place, and for 8 weeks after the amalgams were replaced with glass ionomer fillings. In the third experiment, microbiological samples were taken for 7 weeks prior to amalgam placement, for 8 weeks while the amalgams were in place, and for 8 weeks after the amalgams were replaced with glass ionomer fillings.

Hg quantitation. Fecal samples were placed in sealed vials, stored at −70°C, and later analyzed for total Hg concentration. In the first experiment, Hg quantitation was done radiometrically as described previously (15, 16). In the third experiment, fecal samples were analyzed for total Hg by cold vapor atomic fluorescence spectroscopy (6) by the instrumentation and analytical procedures of Brooks Rand Ltd. (Seattle, Wash.).

Determination of the percent resistant colonies. After incubation, single isolated colonies on master plates were counted and then each master plate was replica plated onto plates containing 50 (for BEA and MS plates) or 100 (for Mac plates) μM $HgCl_2$, 15 μg of tetracycline per ml, or 25 (for BEA and MS plates) or 50 (for Mac plates) μg of ampicillin per ml; a final nonselective replicate was also made to ensure that all colonies had been transferred. Secondary masters were used for BEA and Mac plates in order to prevent overinoculation of the Hg-containing plates. The colonial mass on MS agar is much firmer, and, after finding in initial experiments that the use of a secondary master resulted in underinoculation for these colonies, we used only direct replica plating for MS cultures. After 24 to 48 h of incubation, resistant colonies appearing were counted and the percent resistant colonies was determined by comparison to the original master plate and to the final nonselective replicate plate. Representative sensitive and resistant colonies from each group were purified by streaking on the same selective agar on which they had first appeared and were stored at −70°C for later studies. The effectiveness of amended agar plates in selecting for resistant isolates and in preventing growth of sensitive strains was routinely assessed by plating bona fide laboratory control strains.

Statistical analysis. Unweighted averages of all resistance determinations for a given week for a given type of bacterium in a given monkey were analyzed as a function of time pre- or postamalgam installation by analysis of factorial designs and contrast component analysis (see Table 1) (33). The percent resistance determined via duplicate platings of samples from any single animal on any given day varied by 10 to 15%. The absolute magnitude of the resistance in individual animals varied by considerably more, with the floras of some animals manifesting as high as 100% resistance (at maximum) in a given bacterial population and other animals showing only 50 to 60% resistance (at maximum) for the same bacterial population. However, the patterns of increases and decreases in resistance as a function of time after amalgam placement or replacement were the same in all six animals. The statistical significance of the differences in the average resistance during three experimental periods (preamalgam installation, weeks −7 to −1; during amalgam installation, weeks 0 to 8; and after amalgam removal, weeks 9 to 16) was assessed by computing the average and standard deviation for each period and determining whether these average values differed significantly from the average for the whole period (33).

Resistance profiles and biotypes. Mercury-resistant isolates were subcultured from −70°C storage by streaking on 50 or 100 μM $HgCl_2$ to ensure that Hg resistance had been retained. Additional resistances were determined by replica plating. Selective concentrations used were 50 or 100 μM $HgCl_2$, 25 or 50 μg of ampicillin per ml, 5 or 10 μg of erythromycin per ml, 25 μg of chloramphenicol per ml, 25 μg of kanamycin per ml, 25 μg of streptomycin per ml, and 15 μg of tetracycline per ml. Plates were scored for resistance after 24 to 48 h of incubation either aerobically or in GasPak chambers as appropriate.

Members of the family *Enterobacteriaceae* were identified with the API 20E system. Streptococci were identified with the API 20S streptococcus system. Both identification systems were from Analytab Products (Plainview, N.Y.).

Plasmid transfer and detection. Patch matings between donor strains from the family *Enterobacteriaceae* and recipient *Escherichia coli* laboratory strains were done on Luria-Bertani agar at 37°C. Strains were allowed to mate for 5 to 24 h and then were streaked on appropriate selective media; selection for each resistance marker was done independently for every strain examined. After incubation, candidate exconjugants were picked to selective masters. Replica plating was used to confirm transfer and to determine which additional resistances had been cotransferred. Recipient strains used were C600 Nal (isolated in this laboratory from the C600 *thr leu* parent by growth on 50 μg of nalidixic acid per ml), C600 Nal Rif (derived from C600 Nal by growth on 100 μg of rifampin per ml), and J53 (*pro met*; *E. coli* Genetic Stock Center). Plasmid DNA was isolated from both donor and exconjugant strains by the method of Birnboim and Doly (5).

RESULTS

Correlation between the incidence of mercury resistance and multiple antibiotic resistances in humans. Levy et al. (19) reported a surprisingly high incidence of antibiotic resistance in healthy human subjects, regardless of whether they had recently taken an antibiotic. Our analysis of the patterns of Hg resistance in the intestinal floras of these same subjects revealed strong correlations between the prevalence of Hg resistance and that of antibiotic resistance in the entire population (21).

This correlation is especially noteworthy in persons in that group known not to have recently consumed an antibiotic prior to the time of fecal flora sampling ($n = 356$; Fig. 1). In this subgroup, which had not consumed an antibiotic during the preceding 2 weeks, persons with a high prevalence of Hg resistance in their intestinal floras were more likely also to have flora strains which grew on two or more antibiotics ($P < 0.001$, chi-square). Correspondingly, persons with no detectable Hg resistance in their floras were less likely to have any strains which could grow on antibiotic-containing media ($P < 0.001$, chi-square) (Fig. 1). As this subset of the population had not recently consumed an antibiotic, another possible selective agent acting on their intestinal floras could have been Hg released from dental amalgam fillings. During the era in which these samples were obtained (1978 to 1980), adults from 18 to 75 years old had an average of 6.9 restored teeth (31), and approximately 80% of such restorations were dental amalgam (2). However, at that time it was believed that an insignificant amount of mercury vapor was released from the fillings (12, 29); therefore, we did not acquire data on the amalgam restoration status of the subjects in this survey. More recent evidence indicated that the amount of Hg escaping from amalgam fillings can be quite large (39, 41, 42) and that the majority of this Hg is eliminated via feces (16).

Response of intestinal and oral floras of primates to expo-

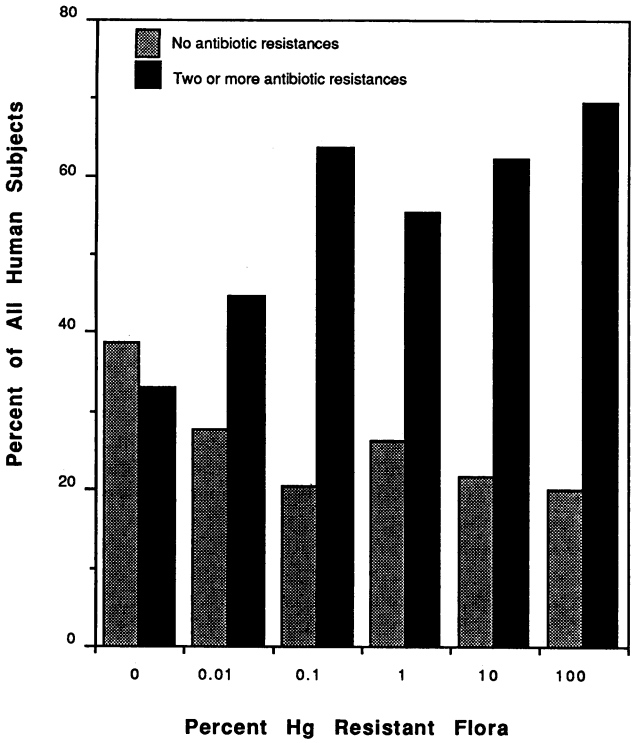

FIG. 1. Relationship between the prevalence of Hg resistance and the prevalence of antibiotic resistance in human intestinal members of the family *Enterobacteriaceae*. Antibiotic resistances assessed were those to ampicillin, tetracycline, kanamycin, streptomycin, chloramphenicol, gentamicin, and nalidixic acid (22, 40). Data are for the subset of persons known not to have been treated during the preceding 2 weeks with an antibiotic (n = 356). Hg resistance (HgR) prevalence subsets: no HgR, n = 121; 0.01% HgR, n = 58; 0.1% HgR, n = 44; 1% HgR, n = 27; 10% HgR, n = 37; 100% HgR, n = 69. The trends are significant at P = 0.001 (chi-square). The percentages of hospitalized persons in each HgR prevalence group were as follows: no HgR, 31%; 0.01% HgR, 66%; 0.1% HgR, 61%; 1% HgR, 26%; 10% HgR, 35%; 100% HgR, 65% (average percent hospitalized was 50.6% ± 11%).

sure to Hg arising from dental amalgam restorations. (i) Incidence of mercury resistance. Since the normal floras of humans and animals can vary considerably from individual to individual (11), we designed protocols in which each animal served as its own longitudinal control (Fig. 2). This allowed us to see changes in the flora occurring concomitantly with the installation or removal of amalgams in each monkey. We examined three bacterial groups which are common members of the normal floras of primates: the gram-negative facultative bacteria found in feces (members of the family *Enterobacteriaceae*), the gram-positive facultative bacteria occurring in feces (enterococci), and the facultative gram-positive bacteria which colonize tooth surfaces and gingivae, the oral streptococci. In all three experiments, resistances to mercury and to tetracycline were assessed by direct replica plating of primary streak cultures; in the third experiment, ampicillin resistance was also assessed. While there is considerable homology between the mercury resistance loci of gram-negative and gram-positive bacteria, there is presently no evidence of routine transfer of this locus between them. However, there is evidence of gene exchange among the various subgroups of streptococci. Therefore, the data for the gram-negative members of the family *Enterobacteriaceae* are considered separately from the data for the gram-positive bacteria.

The aggregate data from all six monkeys indicated that, although some Hg resistance was detectable prior to the installation of the fillings, there was a statistically significant increase (Table 1) in Hg resistance in members of the family *Enterobacteriaceae* (Fig. 3A) and in both gram-positive populations (Fig. 4A) after the installation of amalgam fillings. For example, in contrasting the average resistance in weeks −7 to −1 with that in weeks 0 to 16 (Table 1), the fact that the statistic F_1 is greater than $F_{0.05}$ indicates that the likelihood that there is no difference in the average resistances in the two time periods is less than 5% ($P < 0.05$) (i.e., the null hypothesis [H_0] is rejected). Correspondingly, the alternative (or test) hypothesis that there is a difference in resistance after amalgam placement is tenable. The variances in the data for the enterococci were sufficiently large (for all six monkeys) that pre-, during-, and postamalgam differences could not be discerned statistically for this group by itself. However, when data for both gram-positive groups were considered together (Table 1), the resistance in the time period before installation of the fillings contrasted significantly with that in the entire time period postinstallation (weeks 0 to 16) as well as with that in the period after removal (weeks 9 to 13), although it was not statistically different from that in the period immediately after installation (weeks 0 to 4). In the members of the family *Enterobacteriaceae*, Hg resistance began to rise within 2 weeks after the fillings were installed and peaked at 55% during the fifth week the fillings were in place. In the enterococci, Hg resistance peaked at 13% during the fourth week after installation, and, in the oral streptococci, the peak (23%) occurred 5 weeks after installation. These peaks in the occurrence of Hg resistance in all three bacterial populations followed by approximately 3 to 4 weeks the period of maximum throughput of Hg in the fecal material, which occurred during the first week after the amalgams were

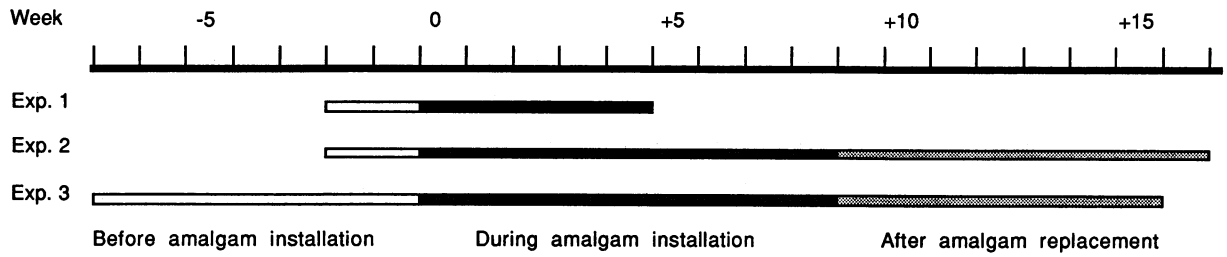

FIG. 2. Experimental sampling time lines of primates for the three amalgam installation experiments. Filling replacements with glass ionomer fillings were done only in experiments (Exp.) 2 and 3. For sampling procedure details, see Materials and Methods.

TABLE 1. Comparison of the mean percents Hg resistance at different time periods in each bacterial group

Bacterial group	Time periods contrasted (wk)	$F_{0.05}$[a]	F_1[a]	H_0[b]
Oral streptococci	T_{-7} to T_{-1} vs T_0 to T_{16}	3.96	6.12	Reject
	T_{-7} to T_{-1} vs T_0 to T_4	3.96	4.07	Reject
	T_{-7} to T_{-1} vs T_9 to T_{13}	3.96	5.14	Reject
Oral streptococci and enterococci[c]	T_{-7} to T_{-1} vs T_0 to T_{16}	3.96[d]	7.2	Reject
	T_{-7} to T_{-1} vs T_0 to T_4	3.96	1.7	Cannot reject
	T_{-7} to T_{-1} vs T_9 to T_{13}	3.96[d]	9.51	Reject
Members of the family *Enterobacteriaceae*	T_{-7} to T_{-1} vs T_0 to T_{16}	4.08	4.92	Reject
	T_{-7} to T_{-1} vs T_0 to T_4	4.08	4.43	Reject
	T_{-7} to T_{-1} vs T_9 to T_{13}	4.08	3.57	Cannot reject

[a] The statistics $F_{0.05}$ and F_1 are calculated according to reference 33 and correspond to probability estimations (see text).
[b] The null hypothesis (see text).
[c] The enterococci alone did not show statistically significant differences by weeks.
[d] Significant at the $F_{0.01}$ level.

installed (Fig. 5). Since the monkeys generated ca. 250 g of feces per week, monkey 79, for example, would have excreted approximately 72 mg of Hg during the first week after installation of the fillings, or approximately 4.8% of the mass of the Hg installed in the fillings. The postinstallation peaks were followed by a gradual decline in Hg resistance in all three bacterial groups, which also corresponded to the decline in Hg in the feces (although the latter never fell to preinstallation background levels but levelled off at ca. 2 to 5 μg/g in week 9).

When the fillings were removed (in week 8 in experiments 2 and 3), there was a much more rapid increase in Hg resistance in members of the family *Enterobacteriaceae* (peaks occurring in week 8 or 10, Fig. 3A) and in the oral streptococci (peak in week 9, Fig. 4A). For the enterococci, a marked increase in resistance began in weeks 11 and 12 and peaked roughly 5 weeks after amalgam replacement (in week 13, Fig. 4A). However, the incidence of Hg resistance in this group was nearly threefold higher than the initial peak, which occurred while the amalgams were in place. This second peak in Hg-resistant bacteria occurred shortly after the increase in fecal Hg resulting from amalgam manipulation during removal of the fillings (Fig. 5). Subsequent to this second fecal Hg peak, the incidence of Hg resistance in members of the family *Enterobacteriaceae* declined rapidly, in marked contrast to that in the gram-positive bacteria, which persisted at relatively high levels until the experiments were terminated at week 16.

The postmortem Hg contents of the kidneys of cynomolgus monkeys 16 and 95 were 3,053 ng/g (15.3 μM) and 1,518 ng/g (7.6 μM), respectively (15); those of rhesus monkeys R830 and L980 (from the second experiment) were 1,783 ng/g (8.9 μM) and 490 ng/g (2.45 μM), respectively; and those of cynomolgus monkeys F78 and F79 (from the third experiment) were 656 ng/g (3.3 μM) and 19,600 ng/g (98 μM), respectively. The basis for this 40-fold range in Hg burden in the monkeys' kidneys can be ascribed to individual differences in chewing patterns and feeding behavior. The Hg contents of the kidneys of control monkeys (similarly housed and fed but which had never had fillings installed) ranged from 40 to 90 ng/g (0.2 to 0.45 μM) for cynomolgus monkeys and from 64 to 116 ng/g (0.32 to 0.58 μM) for rhesus monkeys.

(ii) Incidence of antibiotic resistance. Because of its widespread occurrence in both gram-positive and gram-negative bacteria, tetracycline resistance was used in all three experiments as a representative antibiotic resistance for comparison with mercury resistance. The antibiotic resistance patterns of individual isolates from experiments 1 and 2 indicated that many of the members of the family *Enterobacteriaceae* were also resistant to ampicillin, so the incidence of ampicillin resistance was also assessed in experiment 3.

Tetracycline resistance occurred in all bacterial populations even prior to the installation of the fillings (Fig. 3B and Fig. 4B). This was expected since plasmids conferring resistance to antibiotics are commonly found even in the floras of wild primates (32). While tetracycline resistance persisted in all three bacterial populations (Fig. 3B and 4B), it did not fluctuate with the respective Hg resistance profile. However, in experiment 3, ampicillin resistance in the members of the family *Enterobacteriaceae* peaked when Hg resistance peaked (weeks 5 and 10) and then fell below detection after the amalgams were replaced with glass ionomer fillings. Ampicillin resistance was not observed in either gram-positive population on initial screening, although it was later observed at a low incidence in individual isolates (see below).

Resistance phenotypes and biotypes of representative Hg-resistant isolates. More than 1,300 individual strains were isolated from all six monkeys during the course of these experiments. We concentrated initially on characterizing the Hg-resistant isolates (approximately one-third of the total collected), and, while their detailed characterization is far from complete, several interesting points have emerged.

(i) Hg resistance occurs in many different biotypes in all three bacterial groups. Among the Hg-resistant strains isolated from the feces on Mac agar, the predominant biotypes were *E. coli*, *Enterobacter cloacae*, and *Citrobacter freundii*, with *Klebsiella* and *Pseudomonas* spp. being isolated less frequently. Among the streptococci isolated from the gingival samples on Hg-supplemented MS agar, the predominant biotypes were *Streptococcus constellatus*, *Enterococcus faecium*, *Streptococcus mutans*, *Enterococcus faecalis*, and *Streptococcus gemella*. The biotypes of the Hg-resistant strains isolated on BEA agar were *E. faecium*, *E. faecalis*, and others (ca. 50%) which were not identifiable with the API 20S reagents. Hg resistance occurred in both esculin-positive and esculin-negative strains, although it was more common in the latter. Hg resistance occurred in a great variety of biotypes at every stage of the experiments in all monkeys, with no particular biotype being lost or becoming solely represented during the periods of higher Hg exposure.

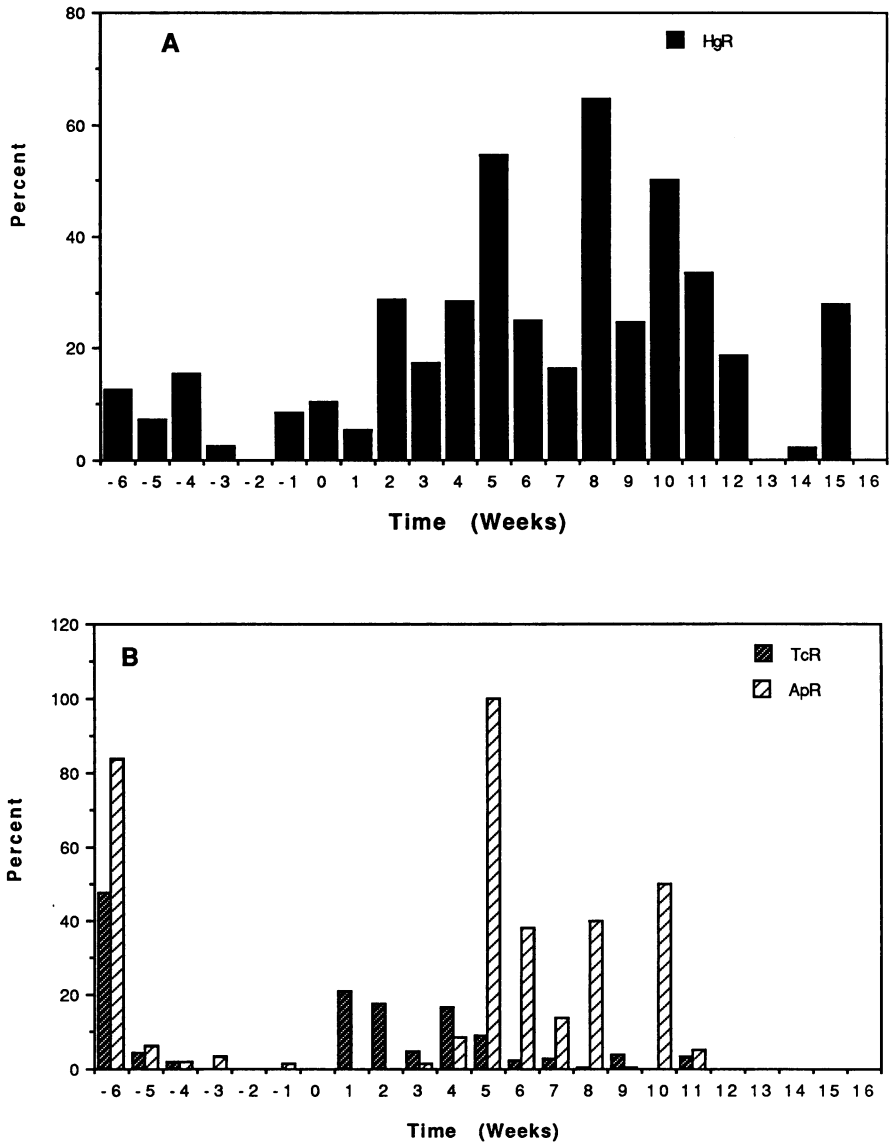

FIG. 3. Time course of the frequency of resistances in monkey fecal members of the family *Enterobacteriaceae*. (A) Occurrence of mercury resistance (HgR). Each datum point is the average percent resistance for that week for all six monkeys in three experiments; data were taken in all weeks noted in Fig. 2. (B) Frequency of antibiotic resistance. Each datum point for tetracycline resistance (TcR) is the percent resistance for that week averaged for all six monkeys in three experiments. Ampicillin resistance (ApR) was assessed only in experiment 3, and each datum point is the average percent ampicillin resistance in monkeys F78 and F79 for the indicated week.

An in-depth analysis of the population biology of the Hg resistance loci in these animals will be presented elsewhere (37a).

(ii) Most Hg-resistant strains in all genera were resistant to one or more antibiotics. In experiment 1 (monkeys 16 and 95), resistances to ampicillin, kanamycin, and streptomycin predominated in facultative gram-negative bacteria. Resistances to tetracycline, kanamycin, ampicillin, and chloramphenicol were found in *Pseudomonas* spp. In the second experiment, the enterobacterial isolates from monkey L980 were resistant to combinations of chloramphenicol, streptomycin, ampicillin, and tetracycline at various points, while those from monkey R830 had these resistances as well as resistance to kanamycin. In experiment 3, enterobacterial Hg-resistant isolates from both monkeys 78 and 79 evidenced ampicillin, kanamycin, chloramphenicol, and tetracycline resistances at various times through the course of the experiment.

Among the oral streptococci from all three experiments, 41% of the strains were resistant only to Hg. Of the remaining 59%, resistance to streptomycin was the most common, occurring in 61% of these strains. Resistances to kanamycin, erythromycin, ampicillin, and tetracycline all occurred in 5 to 15% of the strains that had any resistance in addition to Hg resistance.

Of the Hg-resistant facultative fecal isolates which were propagated on BEA agar, all were resistant to tetracycline. In addition, the enterococcal isolates in experiment 2 also had both kanamycin and streptomycin resistances at 25 and 64% in the two monkeys. In the third experiment, resistance

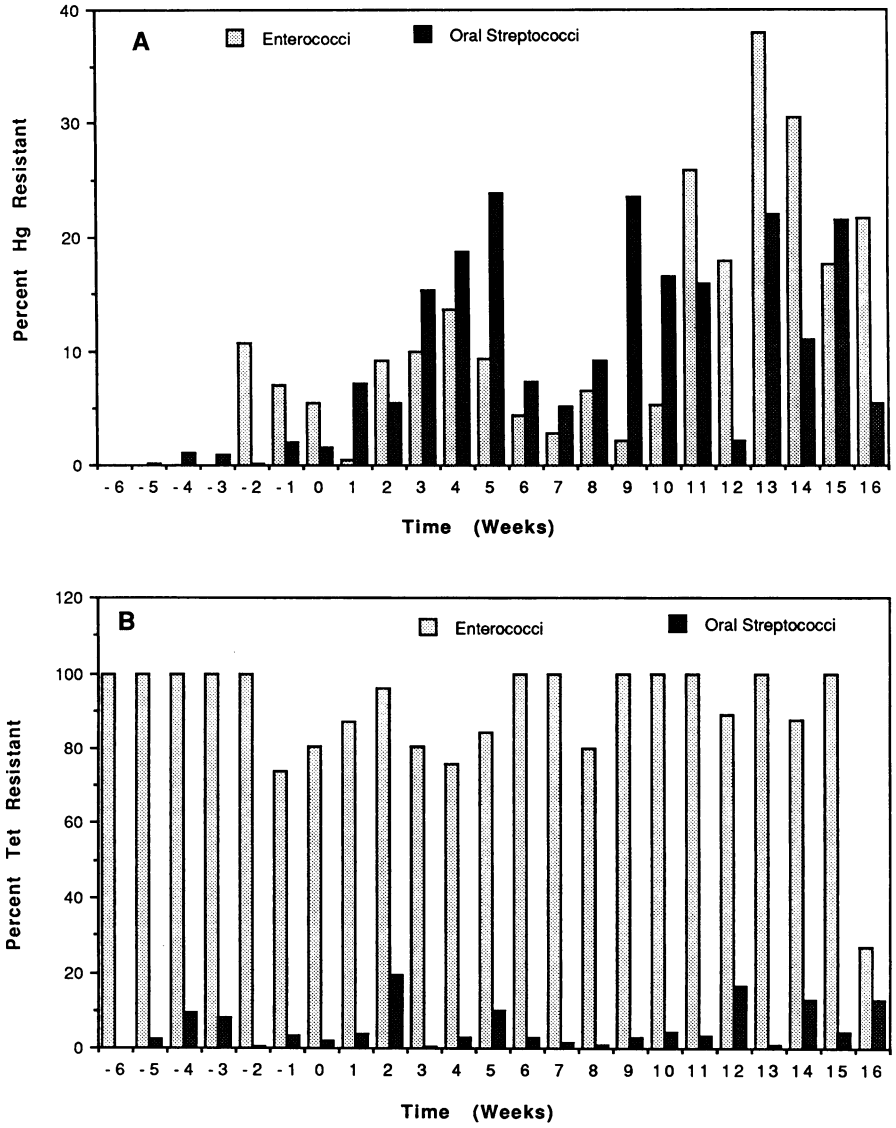

FIG. 4. Time course of the frequency of resistances in gram-positive oral and fecal bacteria from monkeys. (A) Frequency of mercury resistance in fecal enterococci and oral streptococci. (B) Frequency of tetracycline (Tet) resistance. Both data sets are averaged for all six monkeys in three experiments in all weeks noted in Fig. 2. Ampicillin resistance was assessed only in experiment 3 and was not detected in either gram-positive population.

to streptomycin or to erythromycin occurred at 13 and 15% in both monkeys.

In general, the number and kind of antibiotic resistances found in Hg-resistant isolates did not change over time; the rare preinstallation Hg-resistant isolates harbored as many different antibiotic resistances as did the more abundant postinstallation Hg-resistant strains. Thus, we wondered whether Hg resistance and antibiotic resistance were genetically linked in these strains.

(iii) Among members of the family *Enterobacteriaceae*, Hg resistance is carried on conjugative plasmids which also confer antibiotic resistance. We examined the ability of several isolates from this group to transfer their resistance markers to doubly genetically marked laboratory recipient strains. Of 12 isolates from experiment 1, each from strains having one to four resistances other than Hg resistance, two strains transferred Hg resistance and two other markers. Of 27 Hg-resistant members of the family *Enterobacteriaceae* from experiment 2, 12 transferred one or more resistance markers, and, in five of these cases, Hg resistance was also transferred. Finally, of 27 multiple resistance strains from experiment 3, three transferred resistance to streptomycin alone and one transferred resistances to Hg, ampicillin, and streptomycin. Thus, the Hg-resistant strains do carry transferable plasmids conferring antibiotic resistance. The frequency of transfer in this group of strains is as expected for strains isolated from the wild, which are typically repressed for their conjugative functions (23). In some cases, the Hg resistance locus was cotransferred, suggesting that it is genetically linked to the antibiotic resistance loci, as has often been seen (37). All exconjugants were examined for plasmid DNA, and at least one plasmid similar in size to that of the donor strain was found in each case (data not shown).

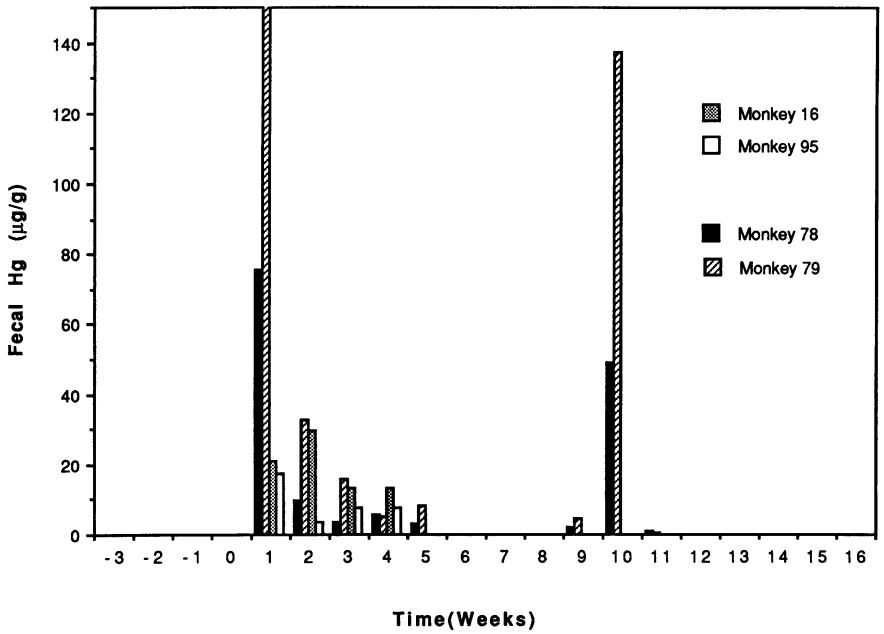

FIG. 5. Average weekly fecal Hg output of cynomolgus monkeys. Radiometric quantitation with ^{203}Hg was used for monkeys 16 and 95 (experiment 1; Fig. 2). Cold vapor atomic fluorescence spectroscopy (6) was used for monkeys 78 and 79 (experiment 3; Fig. 2). The off-scale value for monkey 79 during week 1 is 288 µg/g; 1 mM mercury is 200 µg/g. Values reported are averages for six replicates.

DISCUSSION

In the present study, the primate body tissues received extensive exposure to mercury as evidenced by both the increased fecal excretion of mercury and the increased Hg content of the kidneys following amalgam placement. The time course of occurrence of Hg resistance in all three bacterial populations studied suggests that initial exposure to high levels of mercury immediately following amalgam installation provokes an overgrowth of either rare preexisting Hg-resistant strains or of strains contaminating the food (note that fruits and vegetables are frequently contaminated with antibiotic-resistant plasmid-carrying bacteria [9]). This initial proliferation can be detected during the week after the maximum Hg throughput in the intestine, and it peaks at ca. 4 to 5 weeks after installation, at which time the Hg concentration has reached a steady state of ca. 2 to 5 µg/g (or 10 to 25 µM Hg). The diversity of the preamalgam Hg-resistant population suggests that the Hg resistance loci are widely distributed, as would be expected for a locus which is usually carried by plasmids and frequently found on transposable elements. Preliminary molecular analysis indicates that loci closely related to Tn*21*, others related to Tn*501*, and still others not closely related to either of these benchmark examples of the locus are well represented in many enterobacterial genera at all stages of the process (38). Thus, as a first approximation, it does not appear that there is a single "founder" strain in any of these populations which, under duress, becomes a monoculture.

After the first peak, Hg resistance declines in all populations; this finding is expected given that the fecal Hg concentrations fall to as little as 2% of their peak values although not to the lower levels seen prior to amalgam installation. In this circumstance, strains with Hg resistance have no selective advantage. However, when the second bolus of Hg was delivered to the system as a result of the amalgam replacement, the more rapid occurrence of peak Hg resistance suggested that the numbers of Hg-resistant bacteria had not fallen to their preinstallation levels, so the Hg-resistant bacteria were rapidly able to become dominant members of their respective populations again.

These responses of the normal floras of the monkeys to Hg released from dental amalgams suggested that this source may have provoked the surprisingly high incidence of Hg-resistant bacteria noted in humans (Fig. 1). Indeed, a recent report documents similarly high concentrations of Hg in the feces of an 11-year-old-child upon installation and subsequent removal of a single amalgam filling (20); no assessment of the normal flora was done in this experiment. Apart from placing or removing amalgam fillings, other oral factors which cause abrasion or compression of the amalgam fillings (including eating, bruxism, and having one's teeth cleaned) lead to increased Hg release from amalgam fillings (14).

With respect to antibiotic resistance, the concomitant increase in ampicillin-resistant enterobacterial strains (Fig. 3B) seen in experiment 3 and the cotransferability of Hg and antibiotic resistances in several isolates indicated that enrichment for Hg-resistant strains can result in simultaneous enrichment for genetically linked antibiotic resistance loci. An earlier assessment (13) of the antibiotic resistance profiles of the Hg-resistant human isolates described above demonstrated that many of them carried a Tn*21*-like element with an antibiotic resistance profile very similar to that of the widely found plasmid R100 (26, 34). Given the widespread use of amalgam as a dental restorative material, it is a reasonable hypothesis that in the human population described in Fig. 1, the high incidence of multiple-antibiotic-resistant strains resulted from selection by Hg released from dental amalgam fillings for strains carrying plasmids with genetically linked Hg and antibiotic resistances. This hypothesis is of more than just academic interest; the rising incidence of multiple antibiotic resistances in pathogens is a major problem in medicine (8, 25). For many years it has

been assumed that the primary source of enrichment for plasmid-carrying bacteria in the normal floras of humans is the consumption of antibiotics. However, clinicians are increasingly driven to consider the possibility that there may be factors in the environment apart from antibiotic consumption which result in an unusually high incidence of antibiotic-resistant bacteria in healthy subjects (27). The hypothesis that exposure to Hg from amalgam dental restorations maintains such strains as a higher proportion of the normal floras of humans warrants explicit testing in an appropriate human population.

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