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### Abstract

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### Keywords

non-human primates, *Actinobacillus actinomycetemcomitans*, leukotoxin, colonization, maternal antibody

### Disciplines

Dentistry



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## Intra-oral colonization of macaque monkeys by *Actinobacillus actinomycetemcomitans*

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### Abstract

*Actinobacillus actinomycetemcomitans* was acquired by captive *Macaca fascicularis* 3 to 6 months after birth, and all monkeys aged over 6 months harbored detectable levels. This microorganism was most frequently isolated from the gingival plaque of the incisor (and other) teeth compared with other oral sites. Strains were leukotoxic by bioassay and Western blot analysis. Antibodies in macaque serum contained neutralized the leukotoxin of a human *A. actinomycetemcomitans* strain. High titres of maternal neutralizing anti-leukotoxin antibodies were detected in neonates; the titre then fell rapidly so that by 6 months the antibody titer was zero. Antileukotoxin antibody production was detected after 6 months of age, rapidly reaching a high level within 2 years after birth. The presence of leukotoxic strains of *A. actinomycetemcomitans* in the gingival region did not appear to be correlated with an increase in susceptibility to periodontal disease.

### Keywords

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*Actinobacillus* spp. are isolated from the normal oral flora of many animals, including primates (11, 16). In humans, *Actinobacillus actinomycetemcomitans* is isolated from the dental plaque (14, 22) of approximately 25–30% of healthy individuals, but in those with juvenile periodontitis and severe forms of adult periodontitis the carriage rate is often much greater (14, 15, 23). The majority of patients (over 90%) with juvenile periodontitis have raised antibody titres to a potent leukotoxin produced by leukotoxic *A.*

*actinomycetemcomitans* strains (20). Production of leukotoxin may represent a virulence mechanism by destroying the host's polymorphonuclear leukocytes (PMNs) and monocytes that migrate locally in response to the microbial attack.

Non-human primates harbor leukotoxic strains of *A. actinomycetemcomitans* (16) and have detectable serum antibody titers against the leukotoxin which, *in vitro*, is lethal for PMNs or monocytes from many species. The purpose of the present investigation was to obtain more information on the association of *A. actinomycetemcomitans* with macaque monkeys. We have studied the acquisition of leukotoxic strains of *A. actinomycetemcomitans* and the dynamics of the immune response to the leukotoxin. The results may eventually be useful in

explaining why *A. actinomycetemcomitans* seems to be commensal in most primates but a potential pathogen in humans.

## Material and methods

### Animals

One group of 75 monkeys (*Macaca fascicularis*) was used in a cross-sectional study to determine the relationship between the age at which they acquire *A. actinomycetemcomitans* and the level of humoral anti-leukotoxin antibodies. The animals ranged from 2 day-old neonates to adults older than 10 years. Those less than 12 weeks old were breast-fed, the remainder were fed a variety of diets; Mazuri Primate Diet (Special Diet Services Limited, Witham, Essex, UK), a high-sucrose cariogenic sandwich diet (3) or a starch-based maintenance diet (2).

A second group of 10 neonatal monkeys was used to characterize changes in serum anti-leukotoxin antibody levels during the first 6 months after birth. These animals were breast-fed for the first 3–4 months after which they were fed the Mazuri Primate Diet. All animals had free access to drinking water.

### Sera

Blood was obtained from the femoral artery and sera were separated and frozen at  $-20^{\circ}\text{C}$ . For shipment from the U.K. to Philadelphia small volumes of sera were freeze-dried and upon arrival reconstituted with distilled water.

### Enumeration and identification of *A. actinomycetemcomitans*

Sterile scalpel blades were used to remove plaque from teeth and the mucosal surfaces were sampled with sterile cottonwool swabs. The bacteria were dispersed in 2 ml of Thioglycollate medium without dextrose or indicator, (TG medium, Difco Laboratories, West Molesey, Surrey, UK), by vigorous vortexing in the presence of sterile glass beads for 15 s. The suspensions were decimally diluted in the same medium. Duplicate, 0.1 ml aliquots of appropriate dilutions on to horse blood agar base (Oxoid, Ltd., Basingstoke, Hampshire, UK) supplemented with 5% (v/v) horse blood and menadione at  $0.5\ \mu\text{g}$  per ml and incubated anaerobically at  $37^{\circ}\text{C}$  for 5 days. *A. actinomycetemcomitans* was isolated on the medium described by Slots (12), which we (data not shown) and others (21) have found to be superior to that reported by Mandell and Socransky (9).

Colonies having the characteristic appearance of *A. actinomycetemcomitans* (12, 13) were enumerated by microscopic examination of the selective medium and usually 5 presumptive *A. actinomycetemcomitans* colonies were subcultured from each sample. These isolates were evaluated for acid production from glucose, sucrose, lactose, fructose, xylose, mannitol, mannose and dextrin, for nitrate reduction, for catalase production and for growth stimulation by X and V factors (4, 13). The number of *A. actinomycetemcomitans* was expressed as a percentage of the total colony count on the non-selective medium.

### Cross-sectional study of *A. actinomycetemcomitans*

Dental plaque was removed from the labial surfaces of the upper central incisor teeth at the gingival margin of monkeys aged from 4 weeks to over 10 years. These sites were chosen as the incisors are the first teeth to erupt (at between 3 and 4 weeks of age) permitting uniformity of sampling site on the deciduous teeth from a very young age, and from the same site on the central incisor teeth of the permanent dentition of older animals. The mucosae of the edentulous neonatal monkeys were sampled with cotton wool swabs. The proportion of *A. actinomycetemcomitans* in each sample was determined.

### **Intra-oral localization of *A. actinomycetemcomitans***

From 10 juvenile monkeys, aged 18–24 months, forming part of the group used in the cross-sectional study, plaque samples were taken from (a) the palatal groove on the first left maxillary deciduous molar tooth, (b) the gingival margin of the labial surfaces of the upper deciduous central incisor tooth (c) the dorsum of the tongue (d) the cheeks and (e) the hard palate. The mean proportion of *A. actinomycetemcomitans* at each site determined.

### **Estimation of anti-leukotoxin antibody activity in sera**

The anti-leukotoxin titers of sera were determined from 46 monkeys investigated in the cross-sectional study and in sera taken at intervals during the first 6 months after birth from the 10 neonatal monkeys.

A bioassay as described by Tsai et al. (19) was employed to ascertain whether sera neutralized the leukotoxin. Previous studies have established that toxin-neutralization in human (10) or cynomolgus monkey (17) sera was principally due to IgG antibody activity. In brief, serial two-fold dilutions of serum (25  $\mu$ l; 1:2-1:512 v/v) were preincubated (5 min, room temperature) with an LD<sub>90</sub> dose of leukotoxin (100  $\mu$ l) in 96-well round bottom microtiter plates (Linbro, Titertek, McLean, VA). The leukotoxin preparation was a sonic extract prepared from human *A. actinomycetemcomitans* strain JP2 (19). Human PMNs pre-labelled with Na<sub>2</sub><sup>51</sup>Cr O<sub>4</sub> (New England Nuclear, Boston, MA) were added (5  $\times$  10<sup>5</sup> PMNs in 75  $\mu$ l Hanks balanced salt solution containing 0.1% gelatin) to the toxin-serum mixtures and incubated at 37°C in 5% CO<sub>2</sub>-95% air for 45 min. Following centrifugation to deposit cells, the amount of label released into the ambient medium was measured by gamma spectroscopy. Controls consisted of: cells in medium alone; cells exposed to toxin in the presence of varying dilutions of a rabbit polyclonal toxin-neutralizing antiserum, or varying dilutions of a pool of normal human AB serum which had no neutralizing activity. All experiments were conducted in triplicate. Toxin inhibitory activity was expressed as the reciprocal dilution of serum required to protect 50% of the cells exposed to the LD<sub>90</sub> dose of leukotoxin.

### **Immunoblot analysis of sera for anti-leukotoxin activity**

Western blot analysis was employed to detect immunologically anti-leukotoxin antibody in sera. Leukotoxin was extracted from *A. actinomycetemcomitans* strain JP2 by osmotic shock (6), solubilized by dilution (1:1) in a solution containing 0.16 M Tris.HCl (pH 6.8), 4% sodium dodecyl sulphate and 20% glycerol and heated at 50°C for 20 min. Electrophoresis was performed as described previously (5) on 17.5% SDS-polyacrylamide gel slabs and blotted onto nitrocellulose as described by Towbin et al. (18). The blot was cut into longitudinal strips and each strip was incubated with an individual serum sample, previously diluted 1:10 in 10 mM Tris.HCl (pH 7.4) containing 0.9% NaCl, 3% bovine serum albumin and 10% normal rabbit serum, over-night at room temperature. Human juvenile periodontitis serum and a monoclonal antibody against the leukotoxin (6) were included as controls. All strips were washed and incubated with peroxidase conjugated anti-human or anti-mouse immunoglobulin (Cooper Biochemical, Malvern, PA) at 1:2000 for 2 h at room temperature, washed and developed with *o*-dianisidine (18).

### **Testing of *A. actinomycetemcomitans* isolates for leukotoxic activity**

For biological testing, *A. actinomycetemcomitans* isolates were grown for 24 h in 100 ml peptone-yeast extract broth (7) supplemented with 0.4% bicarbonate in 10% CO<sub>2</sub>-90% air at 35°C. Organisms were washed twice with PBS by centrifugation. Leukotoxic activity was estimated using human blood PMNs or HL-60 leukemia cells (pre-labelled with <sup>51</sup>Cr) as target cells (see above). Freshly harvested bacteria were added to labelled cells (5 to 200

organisms per cell) for 45 min at 37°C. Controls consisted of: cells in buffer without bacteria; cells exposed to *A. actinomycetemcomitans* human strains JP2 or Y4 (positive controls); cells incubated with heated organisms (60°C, 30 min; negative controls).

### Immunoblot analysis of extracts of *A. actinomycetemcomitans*

*A. actinomycetemcomitans* (human strain JP2) and representative isolates of this organism from the macaques were grown in broth as described above and sonicated at 4°C, cleared by centrifugation (12,000 × g) and the proteins solubilized and electrophoresed as described above. The proteins were transferred to nitrocellulose and the blot was incubated with monoclonal antibody specific for the leukotoxin of human *A. actinomycetemcomitans* (strain Y4) [6]. The blot was processed as described previously (18).

## Results

### Identification of *A. actinomycetemcomitans*

None of the isolates identified as *A. actinomycetemcomitans* produced acid from dextrin, lactose, sucrose or xylose: all produced acid from glucose, maltose and fructose; fermentation of mannitol was variable and a small number of isolates did not ferment mannose. All produced catalase and none was stimulated by either X or V factor and nitrate was reduced.

### Cross-sectional study of *A. actinomycetemcomitans* in the mouth

*A. actinomycetemcomitans* was not identified in gingival plaque or oral mucosal samples obtained from any of the 13 animals aged less than 8 weeks of age (Table 1). The organism was first detected at the gingival margin of the central incisor teeth in 2 of 4 monkeys aged between 9 and 12 weeks and in 7 of 8 monkeys aged between 3 and 6 months. All 50 monkeys over 6 months old harbored *A. actinomycetemcomitans* in plaque collected from the gingival margins.

It should be noted that there was no obvious correlation between the periodontal status of the gingival sampling sites and the presence of these organisms: *A. actinomycetemcomitans* was isolated from sites with healthy as well as inflamed gingival tissues. In no instance did the sampled areas exhibit an increase in probable pocket depth or other clinical signs of progressive, destructive periodontitis.

The median proportion of *A. actinomycetemcomitans* was 0.7% of the total cfu in animals aged from 3 to 6 months. This figure increased significantly to 2.8% in monkeys aged 6 to 12 months and to 4.4% in those animals over 1 year old.

*A. actinomycetemcomitans* constituted greater than 5% (Table 1) of the total cfu in plaque from 21 to the 62 monkeys 9 weeks of age or older.

### Intra-oral distribution

*A. actinomycetemcomitans* was not isolated from the palatal grooves of the first maxillary molar tooth of any of the 10 monkeys (age 18 to 24 months) and the frequency of isolation from various intra-oral mucosal surfaces was low (Table 2). However, as expected from the results of the cross-sectional study (above) *A. actinomycetemcomitans* were isolated from the gingival margin of the incisor teeth of each monkey.

### Leukotoxic activity of macaque *A. actinomycetemcomitans* isolates

Four strains of *A. actinomycetemcomitans* from different monkeys were tested for their ability to kill human PMNs or HL-60 tumor cell lines. All macaque isolates were cytotoxic,

causing dose-dependant destruction of target cells (Fig. 1). Biological activity was abolished by heating the organisms to 60°C for 30 min (data not shown).

### Anti-leukotoxin activity in macaque sera

In cross-sectional studies median anti-leukotoxin antibody titers were initially high (range 30–220) in monkeys aged between 0–4 weeks, then fell reaching a low point at about 26 weeks (Fig. 2). Subsequently, the level of the toxin neutralizing activity very rapidly rose until the median titer for monkeys aged over 2 years was > 512 (Fig. 2).

The anti-leukotoxin antibody profile during the first 6 months of life was investigated more closely in a longitudinal study and median titers are shown in Fig. 3. The antibody titer was initially high at birth (range 86–460) and then rapidly fell reaching a median titer of zero between 21 and 24 weeks. *A. actinomycetemcomitans* strains were not isolated from infant monkeys until they were at least 10 weeks old (data not shown).

**Immunoblot analysis of sera for anti-leukotoxin activity**—Murine monoclonal antibody to *A. actinomycetemcomitans* leukotoxin, and antibody in 2 human juvenile periodontitis sera and in both of the 2 monkey sera examined, detected the same antigenic moiety in *A. actinomycetemcomitans* strain JP2 (Fig. 4).

**Immunoblot analysis of macaque *A. actinomycetemcomitans* for leukotoxin**—Fig. 5 shows the proteins of 4 macaque strains and human JP2 strain of *A. actinomycetemcomitans* separated using SDS-polyacrylamide gel electrophoresis and probed with a monoclonal antibody raised against the JP2 leukotoxin. In strains B4 and B6 strong bands corresponding to leukotoxin are present while C2 and C7 also have a band at the same position on the gel but it is somewhat weaker. These results correlate with those obtained when these organisms were tested for biological activity (above): strains B4 and B6 were more potent in destroying target cells when compared to equivalent numbers of strains C2 and C7.

## Discussion

*Actinobacillus spp.* are members of the oral flora of many animals (11). The data presented here clearly demonstrate that *A. actinomycetemcomitans* is routinely isolated from the dental plaque of *Macaca fascicularis* and confirm previous observations that *A. actinomycetemcomitans* is a commensal of most non-human primates (16). Whether *A. actinomycetemcomitans* is a commensal of humans or an exogenous pathogen is not clear; however it is present in the dental plaque of a significant proportion of healthy individuals (14, 22).

Incisor teeth of macaque monkeys erupt when the animals are between 3 and 4 weeks of age and *A. actinomycetemcomitans* is first acquired some weeks after tooth eruption. Thus, no monkey harbored *A. actinomycetemcomitans* until at least 4–5 weeks after the eruption of the incisor teeth. Similar studies of the colonization of the teeth of macaques by *Actinomyces viscosus* and *Actinomyces naeslundii* (which are also normal members of the dental plaque flora), demonstrated a different pattern of acquisition (1). *Actinomyces* were present on the incisor teeth within 7 days of tooth eruption. The reasons for the slower colonization of the teeth by *A. actinomycetemcomitans* when compared to *Actinomyces* is not clear but may include a requirement for bacterial species with which the actinobacillus can co-aggregate. It may also be of importance that the time of actinobacillus colonization coincides with the period when the young monkeys start to reduce their intake of milk so reducing the potential influence of maternal s-IgA on colonization. Cessation of breast feeding is also associated with an increased consumption of prepared diet; in this case

usually the Mazuri diet (see Materials and Methods). Clearly significant changes to the oral environment occur simultaneously with the acquisition of *A. actinomycetemcomitans*, the importance of any one of them cannot be determined from the present study. The persistence of *A. actinomycetemcomitans* in the plaque of juveniles and adults was not related to the diet consumed (data not shown).

The role of maternal antibody in determining the resistance of the infant monkeys to colonization by *A. actinomycetemcomitans* is not clear because we do not know whether antibodies reactive with leukotoxin or other cellular components are able to interfere with the establishment of *A. actinomycetemcomitans* in neonatal monkeys. However, it is noteworthy that colonization occurred when the titer of maternal anti-leukotoxin antibody was declining or undetectable. It is also likely that maternal antibodies reactive with other actinobacillus antigens also were at a low level at the same time. As *A. actinomycetemcomitans* was harbored by each mother it is reasonable to assume that the organisms were derived from their mothers.

The present data suggest that the gingival margin is the major intra-oral habitat of *A. actinomycetemcomitans* in macaque monkeys. These findings are similar to those of Slots et al. (14) who isolated *A. actinomycetemcomitans* primarily from dental plaque in the gingival crevice region of periodontally healthy humans. The presence of *A. actinomycetemcomitans* on the dentition is not surprising as they are able to form adherant plaque *in vitro* (8). The preferential colonization of this niche by *A. actinomycetemcomitans* could be due to localization of substrates, essential for its growth, derived from the host via the gingival crevice or from other members of the gingival microflora. At present the nutritional requirements of *A. actinomycetemcomitans* growing *in vivo* are not known.

Western blot analysis demonstrated the presence of leukotoxin in representative strains of macaque *A. actinomycetemcomitans* and that macaque sera contained antibody that reacted with leukotoxin from a human *A. actinomycetemcomitans* strain. Further, monkey sera neutralized the biological activity of the leukotoxin. These observations together with the ability of macaque strains to kill human PMNs strongly suggest that isolates from monkeys produce a biologically active molecule with properties very similar to those of the leukotoxin from human strains of *A. actinomycetemcomitans*. In fact by all the criteria used in our study the macaque *A. actinomycetemcomitans* strains also were similar to those isolated from humans (13).

There are considerable data to suggest that *A. actinomycetemcomitans* may be of pathogenic significance in the development of destructive periodontitis in humans (14, 15, 23). However, this would not appear to be the case in macaques. There may be several explanations for the apparent difference in the nature and possible consequences of *A. actinomycetemcomitans* colonization in humans versus macaques, and other non-human primates. If *A. actinomycetemcomitans* leukotoxin represents one potential virulence mechanism in humans then it is of significance that monkeys begin to harbor leukotoxic strains within weeks of tooth eruption, yet appear to suffer no untoward effects. This may be a consequence of at least 2 variables. First, monkey PMNs are significantly less sensitive to *A. actinomycetemcomitans* leukotoxin when compared to human PMNs (16). Thus the level of infection and/or availability of the *A. actinomycetemcomitans* leukotoxin required to kill enough PMNs to depress antibacterial defences in the monkey gingival crevice may be higher than that needed in humans. Second, the immune response to the leukotoxin may be instrumental in protecting these animals against *A. actinomycetemcomitans*. At birth they are passively immunized by maternal antibody and by 1 year of age virtually all have actively responded to antigenic stimulation having relatively high titers of toxin-neutralizing antibodies for the rest of their lives. It is only for a short period (between 4 and 8 months of



age) that the antibody levels appear to be extremely low. The decline in maternal antibody titer to the toxin (and presumably other *A. actinomycetemcomitans* antigens) corresponds to the time when the deciduous dentition erupts and when *A. actinomycetemcomitans* is first detected in the gingival crevice in these animals. This temporary lapse in antibody activity may provide *A. actinomycetemcomitans* with the opportunity to become established in the host. However, the dynamics and the intensity of the host's immune response to the leukotoxin (as well as other potential virulence factors) may prevent the development of clinically detectable disease at colonized sites.

Little is known about the dynamics of *A. actinomycetemcomitans* infection or the immune response to this organism in humans. We believe that comparative studies of host-*A. actinomycetemcomitans* interrelationships in non-human primates may help to provide clues to better understand why *A. actinomycetemcomitans* is a commensal of both humans and non-human primates but a potential periodontopathogenic organism only in humans.

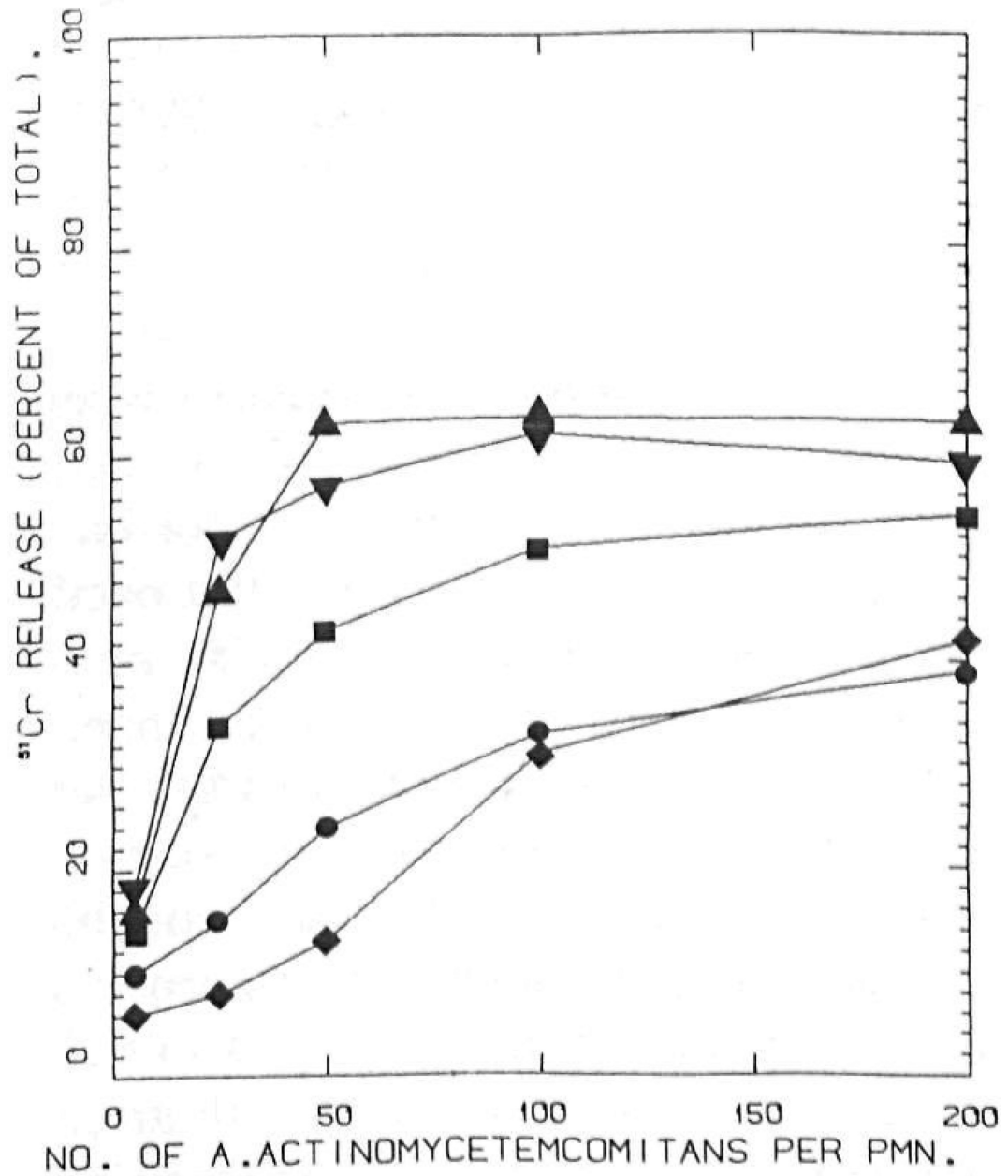
## Acknowledgments

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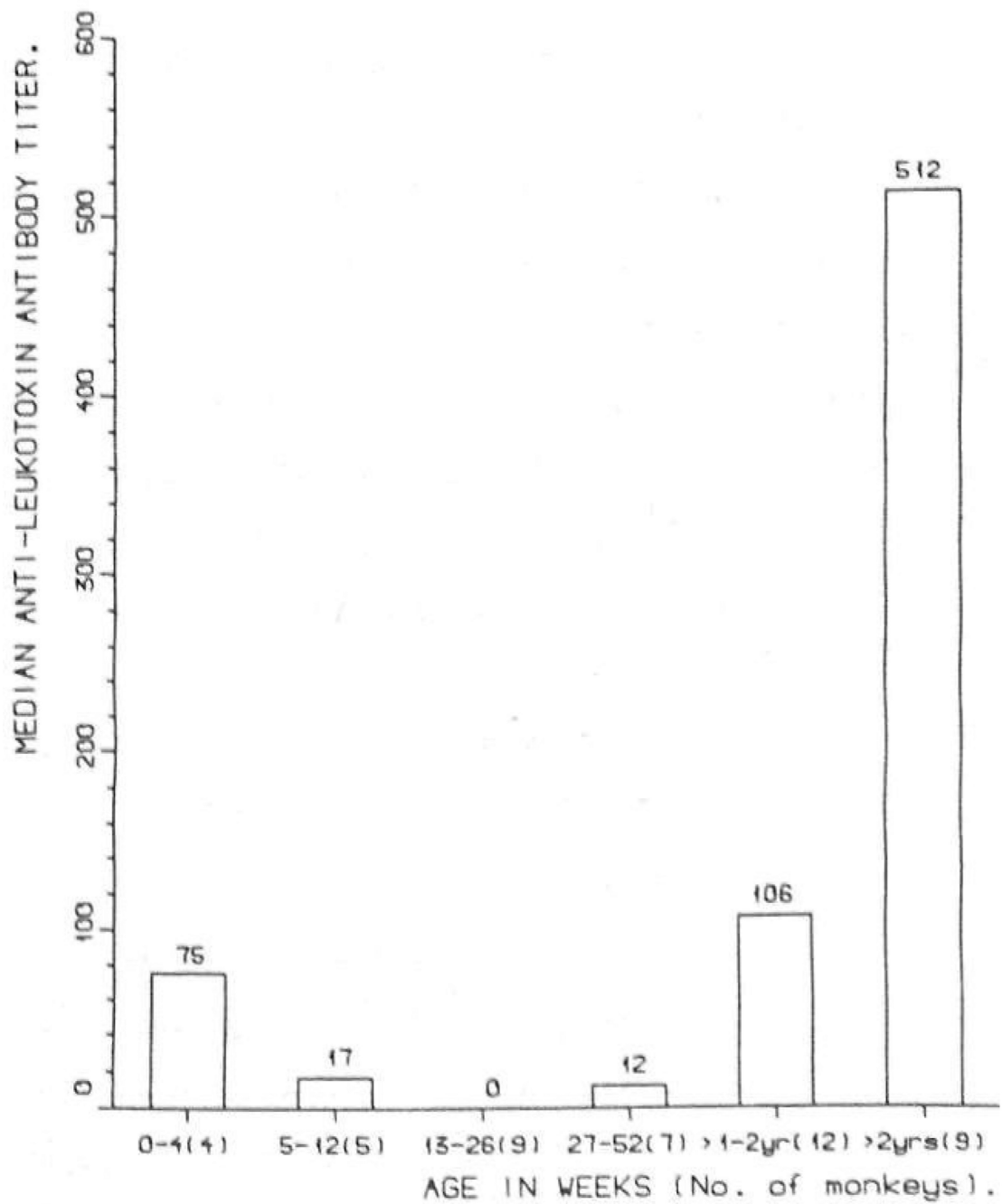
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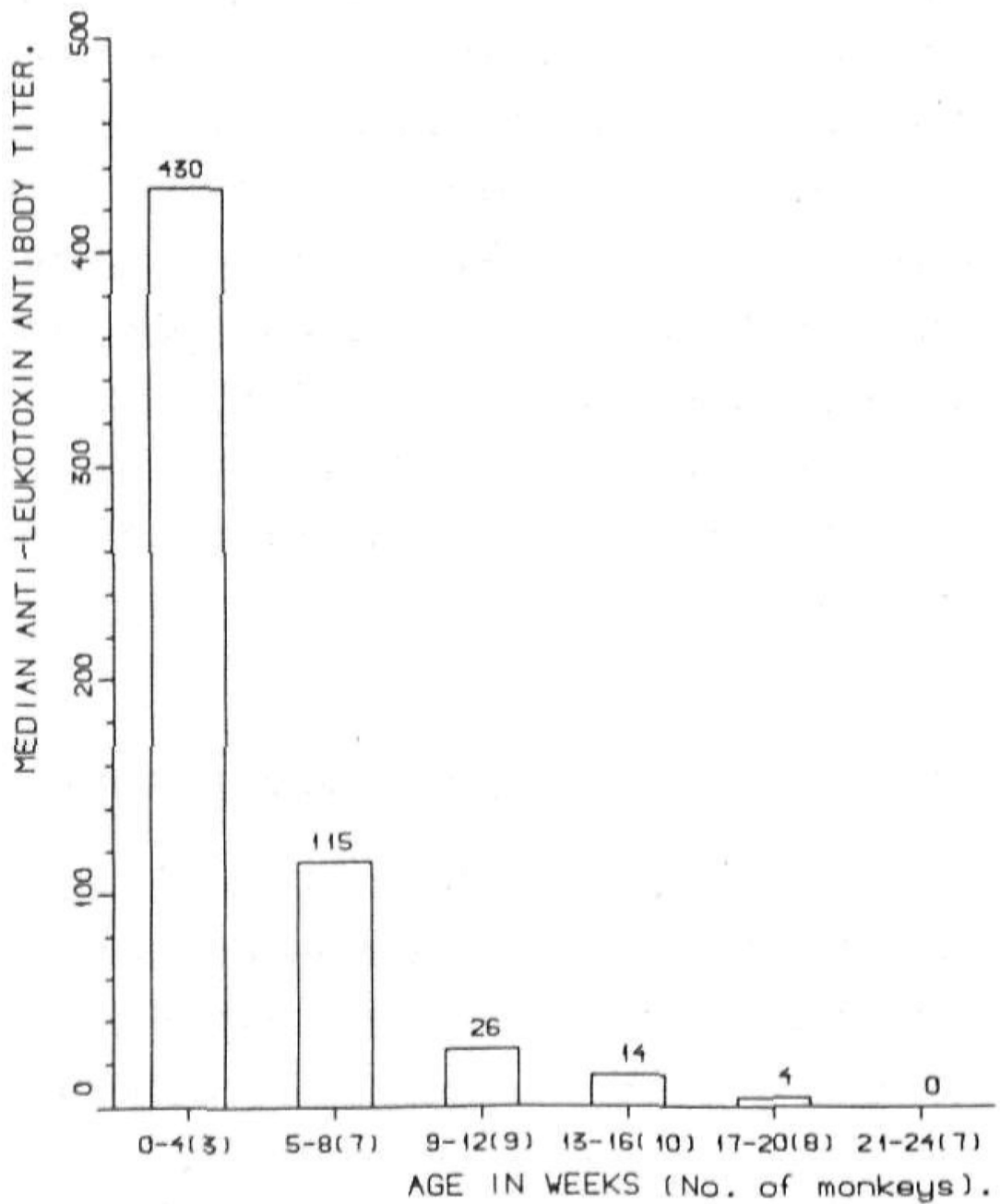
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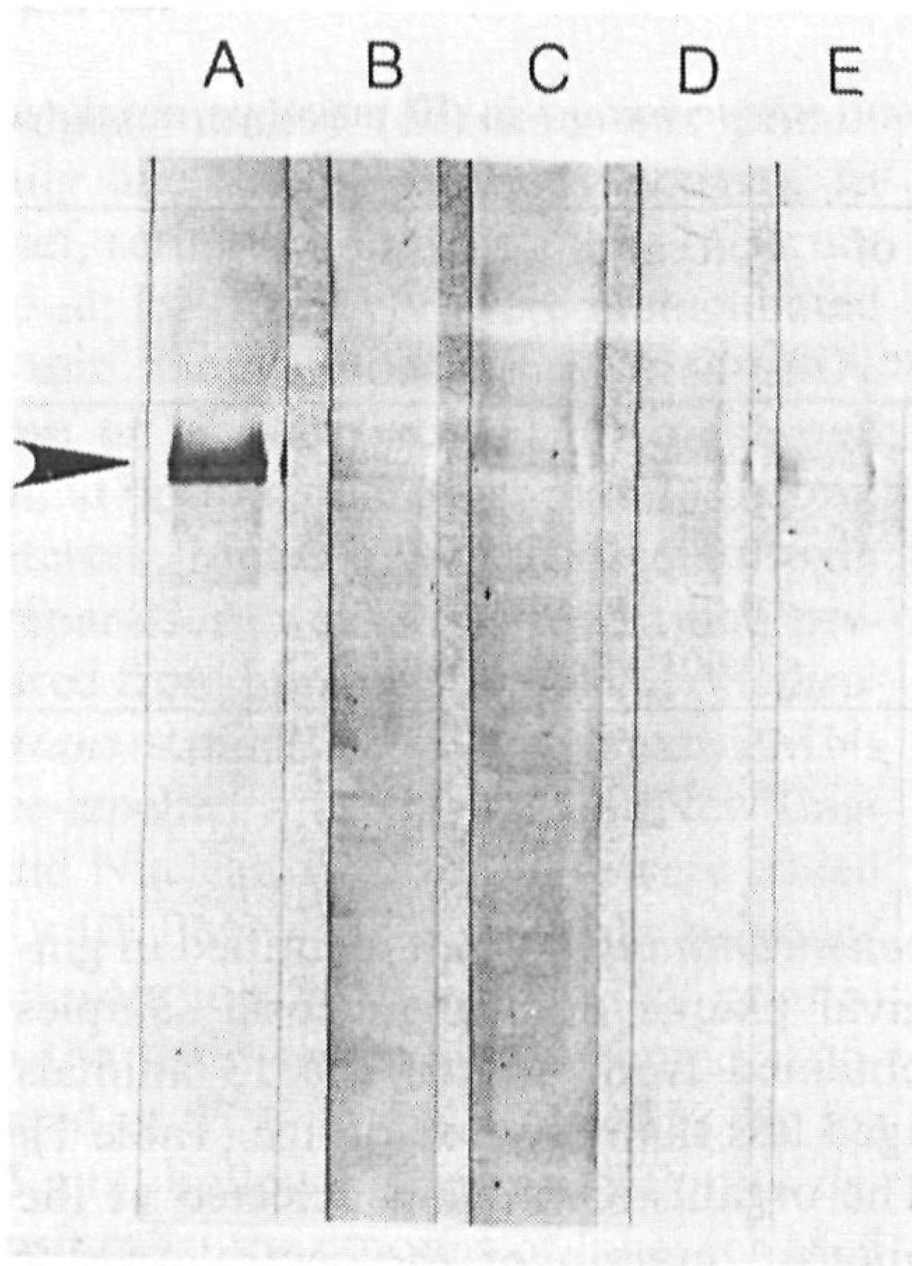
**Fig. 1.** Leukotoxic activity of *A. actinomycetemcomitans* isolated from macaques and humans. Freshly cultured organisms derived from humans (strain JP2) and from macaques (strains B6, B4, C2, and C7) were washed and incubated with  $^{51}\text{Cr}$ -labelled human PMNs (5–200 per PMN). The amount (per cent of total) of  $^{51}\text{Cr}$  released from the target cells into the medium is a reflection of the leukotoxic activity of the isolates.



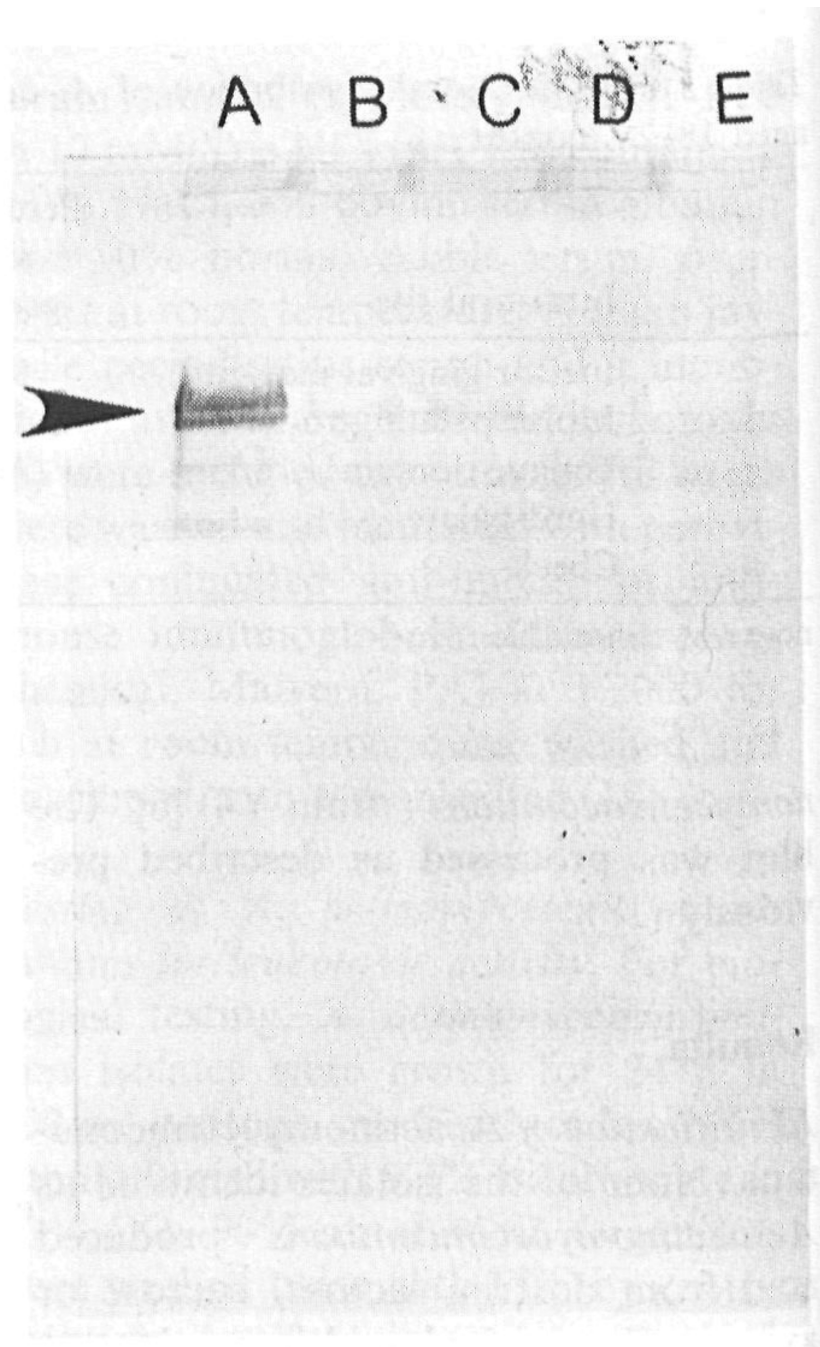
**Fig. 2.** Median anti-leukotoxin antibody titers in the sera of individual monkeys of various ages, from neonates to adults. The anti-leukotoxin titer represents the reciprocal of the dilution of serum which inhibited 50% release of  $^{51}\text{Cr}$  from prelabelled PMNs exposed to an  $\text{LD}_{90}$  dose of *A. actinomycetemcomitans* leukotoxin (see Material and methods).



**Fig. 3.** Longitudinal changes in the median anti-leukotoxin antibody titer in the serum of a group of 10 neonatal monkeys up to the age of 24 weeks. The anti-leukotoxin titer represents the reciprocal of the dilution of serum which inhibited 50% release of  $^{51}\text{Cr}$  from prelabelled PMNs exposed to an  $\text{LD}_{90}$  dose of *A. actinomycetemcomitans* leukotoxin (see Material and methods).



**Fig. 4.** Screening of cynomolgus monkey sera for anti-leukotoxin antibodies. An osmotic shock extract of *A. actinomycetemcomitans* Y4 was applied to multiple lanes of an SDS-polyacrylamide gel and the separated proteins were transferred to nitrocellulose. The filter was cut into strips and each strip was incubated with sera (1:10 dilution) from human juvenile periodontitis patient or from macaque monkeys. The strips were then incubated with peroxidase-conjugated reporter antibody (1:2000 dilution). Lane A, monoclonal antibody to leukotoxin from human *A. actinomycetemcomitans* Y4; lanes B and C, human serum; lanes D and E, monkey serum.



**Fig. 5.** Western blot analysis of cellular proteins from *A. actinomycetemcomitans* isolated from cynomolgus monkeys. The bacteria were lysed by sonication, the proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Leukotoxin-specific monoclonal antibody made against toxin from the human strain Y4 was used at a 1:1000 dilution. Peroxidase-conjugated reporter antibody (1:2000 dilution) was used to visualize the reaction. Lane A, human strain JP2; lane B, strain B4; lane C, strain B6; lane D, strain C2; lane E, strain C7. The strains examined in lanes B–E were isolated from the macaque monkeys described in Material and methods.

**Table 1**

The age-related frequency of isolation and proportion of *A. actinomycetemcomitans* in the dental plaque of macaque monkeys.

Age of monkey	No.	Frequency of	Percent of total cfu	
		isolation (%)	Median	Range
<4 weeks	7	0	< 0.001	–
4–8 weeks	6	0	< 0.001	–
9–12 weeks	4	50	< 0.01	< 0.001–0.55
3–6 months	8	87	0.7	< 0.001–7.0
6–12 months	16	100	2.8	0.09–50.0
>1 year	34	100	4.35	0.2–40.0



**Table 2**

The intra-oral distribution of *A. actinomycetemcomitans* in 10 macaque monkeys aged 18–24 months

Intra-oral site	Percent of sites	Percent of total cfu	
	positive	Median	Range
Incisor gingival margin	100	10.3	1.5–36.0
Molar palatal groove	0	< 0.001	nc
Tongue dorsum	40	< 0.002	<0.001–0.21
Hard palate	50	< 0.01	< 0.001–0.30
Cheek	40	< 0.001	<0.001–0.13

Nc = not calculable.