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Detection of *Klebsiella pneumoniae* antibodies in *Aotus l. lemurinus* (Panamanian Owl Monkey) using an enzyme linked immunosorbent assay (ELISA) test

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Summary

An enzyme linked immunosorbent assay (ELISA), was adapted to detect antibodies against *Klebsiella pneumoniae* in *Aotus l. lemurinus* monkeys. It was used to define the prevalence of infection and the immunogenicity of an $Al(OH)_3$ bacterin in a population of laboratory born *A. l. lemurinus* monkeys. This represents a preliminary step to reduce *K. pneumoniae* produced mortality. A striking finding during a cross-sectional prevalence study was that none of the babies of less than 2 months old had detectable levels of antibody. The antibody prevalence gradually increased in all other age groups reaching 87·5% in the 8–10-month-old group. These results indicate that infection with *K. pneumoniae* occurred sometime between 2 and 6 months of age, probably as a result of oral-faecal contamination and a change in the feeding and grooming behaviour. To determine whether infants had maternal antibodies or if they were asymptomatic carriers of the bacterium, a cross-sectional study was done in 15 infants less than 4 months old and their mothers. *K. pneumoniae* antibodies were detected in 11/15 mothers with serum titers ranging from 1 : 4 to >1 : 256 and the bacterium was isolated from 3 babies and one mother and her baby. Results showed that no maternal antibodies remained in babies older than 3 weeks old. A prospective study indicated a reduction in mortality from 20% for the previous 3 years to 3·7% (3/79) in $AL(OH)_3$ *K. pneumoniae bacterin vaccinated infants born during 1988–89.*

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K. pneumoniae K 65 strain bacterium has been implicated as a major cause of death since 1980 among laboratory born and captive *A. l. lemurinus* in the Gorgas Memorial laboratory (GML) monkey colony. In *Aotus sp.* the organism causes high fever, pneumonia, purulent meningitis, nephritis, septicaemia, peritonitis with mesenteric lymphonodular abscesses and Peyer's patch necrosis, together with ulceration of the caecum and small intestine as well as air-sacculitis (Snyder *et al.*, 1970; Giles, 1974; Escajadillo, 1984; Escajadillo *et al.*, 1987).

In addition, 5 out of 10 GML colony monkeys are healthy carriers of the bacterium (Escajadillo, 1984). Other investigators have isolated the organism from 17% of normal monkeys (Snyder *et al.*, 1970).

Even though parenteral bacterins for the control of bacterial enteric diseases have been regarded as of little value in protecting man and animals (WHO, 1972) attempts have been made at GML (Escajadillo, 1984) to prevent periodic outbreaks of the disease using a subcutaneous formalin-inactivated diluted *K. pneumoniae* bacterin without adjuvant.

Recently, however, *K. pneumoniae* polysaccharides and ribosomal nucleic acid immunogen vaccines have been shown to induce strong humoral immune responses in man and

mice and to protect *Saimiri sciureus* monkeys against fatal *K. pneumoniae* infection (Riottot *et al.*, 1979; 1981; Fournier *et al.*, 1981; Cooper *et al.*, 1982; Cryz *et al.*, 1984, 1985a,b; Postal *et al.*, 1988).

The intent of the present study was to adapt an ELISA test to detect antibodies against *Pasteurella multocida* in rabbits (Manning, 1984; Manning *et al.*, 1986), with modifications as described by Cryz *et al.* (1984), to determine the prevalence of anti-*K. pneumoniae* antibodies among GML laboratory born monkeys and to test the immunogenicity of a whole *K. pneumoniae* aluminium hydroxide adsorbed (Amphogel) bacterin, in an attempt to reduce the mortality among GML laboratory born and captive *A. l. lemurinus* monkeys.

Materials and methods

Animals

Monkeys One hundred and nine adult feral and infant laboratory born *A. l. lemurinus* of both sexes and weighing between 150–900 g were kept in biological pairs or family groups in wire mesh cages (112 × 96 × 120 cm) with a nest box and a PVC pipe perch. Cages were cleaned and sterilized twice monthly. Food and refuse pans were cleaned and sterilized daily. Rooms were kept at an ambient temperature of between 26° and 32 °C and 70–80% relative humidity. Ventilation consisted of 15–20 changes of air per hour. A 12 h light–dark cycle was maintained beginning with light at 0730 h.

A diet of bananas, carrots, lettuce, oranges, cooked rice, boiled potatoes, high protein monkey chow (Purina 5045®) and a vitamin and mineral supplement was offered daily late in the afternoon. Water was offered *ad libitum*.

Rabbits One adult male New Zealand rabbit was used to produce *K. pneumoniae* pre- and hyper-immune sera as described by Cryz *et al.* (1984). The animal was kept on a stainless steel cage 60 × 52 × 46 cm fed commercial rabbit chow (Purina®), and allowed drinking water *ad libitum*. Room temperature was maintained between 26–30 °C relative humidity between 70–80%, with a ventilation of 15–20 changes of air per hour and a 12 h dark–light cycle.

Mice Forty, 3-week-old male NIH standard white mice, were kept in groups of 5 in 29 × 17 × 12·5 cm stainless steel rodent cages and used as inoculated and control groups in bacterin safety tests. The animals were fed commercial mouse food (Formulab rodent chow, Purina®). Water was offered *ad libitum*. The room temperature was kept between 27° and 30 °C, relative humidity between 70–80%, with a ventilation of 15–20 changes of air per hour and a 12 h dark–light cycle.

The animals were cared and treated for as described in the *Guide for the Care and Use of Laboratory Animals* published by the National Institute of Health, Public Health Service, USA.

K. pneumoniae *bacterin and antigen production for ELISA test*

K. pneumoniae K 65 strain GML-86041 (isolated by Dr A Escajadillo and typed by Dr P B Smith at the Center for Disease Control, Atlanta; GML 1983–84 Annual Report) and a *Escherichia coli* untyped strain were inoculated in 2 ml nutrient broth vials (Difco) and incubated at 37 °C for 24 h, further seeded into 500 ml nutrient broth bottles and incubated at 37 °C for 24 h. Bacterial concentrations reached $1{\cdot}4 \times 10^8$ ml after 24 h.

K. pneumoniae K 65 strain and *E. coli* bacterium cultures were killed by adding 10% buffered formalin vol/vol to obtain a 0·5% solution. After 24 h of incubation at room temperature the cultures were tested for safety by inoculating 5 NIH standard white mice intraperitoneally (IP) per batch, and for sterility by inoculation of nutrient broth vials and further seeded on MacConkey's agar plates. A total of 3 batches of *K. pneumoniae* bacterin and antigen and one of *E. coli* antigen was prepared.

The bacterial suspensions were then centrifuged at 3000 rpm × 20 min. The pellet was resuspended in phosphate buffered saline pH 7·2 (PBS) and washed 3 times by centrifugation at 3000 rpm × 20 min. This pellet was either resuspended in PBS for bacterin production as described below or

adsorbed overnight after suspension in carbonate buffer pH 9·5 at a spectrophotometric optical density (OD) of 0·66 at 610 nm to coat Nunc-Immuno plate I® (Nunc intermed, Denmark) for ELISA antibody tests as described by Manning (1984).

K. pneumoniae or *E. coli* antigen coated immunoassay plates were washed 3 times with PBS Tween-20 (0·5 ml Tween-20 × 1000 ml PBS) pH 7·2 and either used immediately or stored at 4 °C until used.

The aluminium hydroxide $Al(OH)_3$ bacterin was prepared with a modification of the method described by Matsumoto and Yamamoto (1975). Briefly, 50 ml aluminium hydroxide gel (Amphogel) was added to 200 ml of a 0·66 OD suspension of purified bacterium in PBS 7·2. Finally, 0·2 ml of a 1 : 1000 solution of merthiolate was added as a preservative.

ELISA protocol

Optimum dilutions of all reagents were determined by checkerboard titration (Sanchez-Vizcaino & Cambra Alvarez, 1981). The 1 : 800 dilution of protein-A horseradish peroxidase conjugate was chosen as the detector reagent.

The plates were coated with 50 µl of blocking buffer (PBS with 10% fetal bovine serum, 1% bovine serum albumin) (Manning, 1984; Manning *et al.*, 1986), incubated at 37 °C for 2 h, and further washed 3 times with PBS Tween–20 pH 7·2. Four-fold serial dilutions of the test sera starting with 1 : 4 up to 1 : 256 and then 2-fold dilutions from 1 : 512 to 1 : 4096 were made in PBS Tween-20 or hydrolyzed agarose-Tris buffer pH 8·0 in order to eliminate background or naturally occurring anti-carbohydrate antibodies as described by Hamilton and Adkinson (1985). The plates were kept at 37 °C for 1 or 24 h and washed 5 times with PBS-Tween 20 pH 7·2. Protein A-conjugated horseradish peroxidase (0·1 ml original concentration, 1 mg/ml; Sigma) diluted 1 : 800 in PBS (Cryz *et al.*, 1984) was added to each well and incubated for 30 min at 37 °C. The plates were then washed 3 times with PBS-Tween 20. The substrate consisting of 0·05% O-phenylenediamine (Sigma) in a buffer composed of 0·05 M citric acid, 0·05 M sodium citrate; 0·01% hydrogen peroxide (Manning, 1984) was added before use and 0·1 ml of the mixture was added to each well. After 3–5 min, the reaction was stopped by addition of 0·1 ml of 1·6 N sulphuric acid to each well. Optical densities were read at 450 nm in a Titertek® Multiskan (Flow laboratories) immuno-ELISA reader.

A positive antibody titre was considered to be reciprocal of the last dilution of the serum in which the OD reading was greater than the negative control mean plus 2 standard deviations (2SD) (Fig. 1) (Sanchez-Vizcaino & Cambra Alvarez, 1981; Feldkamp & Smith, 1987).

The specificity of the reaction was demonstrated by including pre-immune rabbit serum, and negative antigen (PBS-Tween-20), plus pre-immune monkey serum and standard post-inoculation (PI) immune monkey serum, and by coating plates with *E. coli* purified antigen prepared as described above and reacting it against positive and negative *K. pneumoniae* control sera.

*Rabbit anti-*Klebsiella *antibodies*

A strong positive reaction was observed with the rabbit anti *Klebsiella* hyperimmune sera. With the ELISA system the end point titre was >1 : 4096 with an OD of 1·2 at 1 : 16 dilution and 450 nm and the pre-immune sera OD was less than 0·4 at 1 : 16 dilution.

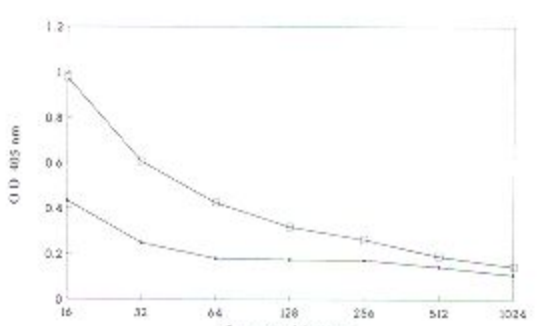

Fig. 1. Pre- and post-exposure sera mean ELISA optical density readings ± two standard deviations for adult *A. l. lemurinus* monkeys vaccinated with a 1/10 PBS diluted *K. pneumoniae* bacterin, $n = 5$. —●— = pre-mean; —□— = post-mean.

Experiment 1: feral adult vaccination experiments and ELISA standardization

Fifteen adult feral *A. l. lemurinus* were used to determine suitable conditions for the ELISA test. The monkeys were caged individually and divided into 3 groups of 5 monkeys each and a pre-immune blood serum sample was taken from the femoral vein. Five animals were each inoculated subcutaneously with 0·5 ml of a formalin PBS 1/10 diluted *K. pneumoniae* bacterin subcutaneously without adjuvant once each week for 3 consecutive weeks. Each of 5 animals in a second group was given orally 1 ml of heat inactivated formalin bacterin daily for 5 days, combined with 0·5 ml of formalin killed bacterin. The third group of 5 animals served as uninoculated controls. Serum samples were collected on days 0 and 7 and at 6 months PI in group No. 1 and at day 0 and 30 and at 6 months PI in group No.2. The control group was sampled on day 0 and at 2 months.

Experiment 2: maternal antibodies cross-sectional study

A cross-sectional study was carried out to study the prevalence of maternal anti-*K. pneumoniae* antibodies in 15 laboratory-born *A. l. lemurinus* monkeys aged 3 weeks to 4 months and their mothers. Serum samples and faecal swabs were taken from all mothers and their babies. The swabs were streaked directly onto MacConkey's agar plates and incubated for 24 h. Sera were tested for *K. pneumoniae* antibodies using ELISA.

Experiment 3: immunogenicity of a Klebsiella *$AL(OH)_3$ bacterin in laboratory-born monkeys*

Sera were collected from 79, laboratory-born (1988–89) monkeys *A. l. lemurinus* of both sexes and different ages (3 weeks to 14 months of age), weighing between 150 and 650 g and used for a prevalence study. Seventy-four monkeys were inoculated subcutaneously twice at one month intervals and 5 others only once with 0·1 ml of an $Al(OH)_3$ *K. pneumoniae* bacterin prepared as previously described. Serum samples were collected every month for two consecutive months. Seven laboratory-born (1989) monkeys between less than 1-month-old and up to 5 months of age served as non-vaccinated controls. The *K. pneumoniae* antibody prevalence for 1988 and 1989 laboratory born monkeys was calculated from their pre-inoculation serum samples (see Fig. 3).

Results

Experiment 1: feral adult A. l. lemurinus *vaccination experiments and ELISA standardization*

All of the pre-immune sera tested from 15 adult monkeys were negative for *Klebsiella* antibodies. The mean pre-inoculation OD observed for 5 monkeys as shown in Fig. 1 was slightly over 0·4 OD at 1 : 16 dilution compared to a 0·9 mean post vaccination sera OD.

The standard positive post-immune serum showed the highest titre among 15 wild monkeys tested (1 : >4096) (Kohno *et al.*, 1988; Dea & Tijssen, 1989).

In group one, 2 of 5 monkeys died of *K. pneumoniae* peritonitis 4 months PI; these monkeys received a diluted *K. pneumoniae* bacterin subcutaneously alone. The serum titres of these two monkeys were 1 : 4 and >1 : 256. The other 3 monkeys that survived had titres of 1 : 64 to <1 : 256.

In group two, one of 5 monkeys died of *K. pneumoniae* peritonitis. This monkey had a titre of 1 : 64 5 months before death. The other 4 monkeys had a titre of 1 : 128 to 1 : 256.

The monkeys from the control group remained negative for 2 consecutive months. One control animal died of *K. pneumoniae* peritonitis 4 months after the last blood sample was taken. No difference in mortality was observed between the inoculated and control groups.

Experiment 2: maternal antibodies cross-sectional study

In the 15 female *A. l. lemurinus* tested and their 3-week- to 4-month-old babies, *K. pneumoniae* serum titres varied between 1 : 4 and 1 : 256 in 11 of 15 mothers. None of the 15 babies tested had *K. pneumoniae* antibodies, although

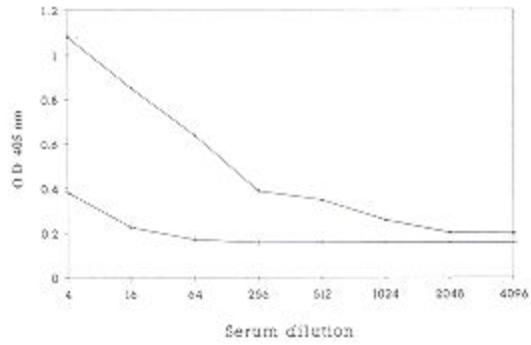

Fig. 2. Pre- and post-exposure sera mean ELISA Optical Density readings ± 2 standard deviations for laboratory-born *A. l. lemurinus* monkeys vaccinated with and $Al(OH)_3$ bacterin, $n = 10$. ——— = post-mean; ——|—— = pre-mean.

K. pneumoniae was isolated from the faeces of one mother and its baby and from 3 of 15 babies examined.

One mother that had been in captivity for more than 12 years and which had received several doses of a diluted *K. pneumoniae* bacterin without adjuvant died of an hepatic abscess caused by *K. pneumoniae*. Its serum titre was 1 : 64 and its baby remained healthy for 7 months after the mother's death.

Experiment 3: immunogenicity of Klebsiella *$Al(OH)_3$ bacterin in laboratory-born monkeys*

The mean ELISA pre-inoculation OD readings for 10 baby monkeys was less than 0·4 OD and the mean PI OD was slightly greater than one. No overlap between the plotted lines was observed (Fig. 2).

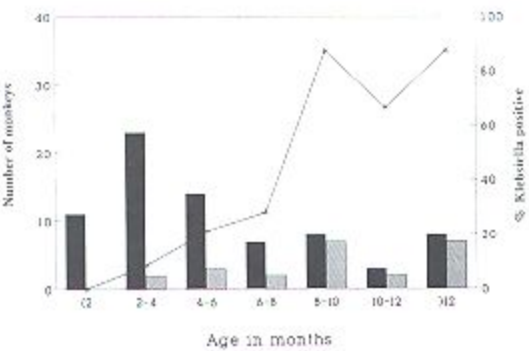

Fig. 3. Age specific *K. pneumoniae* antibody prevalence in 1988–89 laboratory-born *A. l. lemurinus*, $n = 74$. ■ = population; ▪ = antibody positive; ——•—— = prevalence.

Antibodies were not detected in 11 baby monkeys of 3 weeks to less than 2 months old (Fig. 3). The prevalence increased to 8·6% in the 2–4-month-old group, then to 21% in the 4–6-month-old and to 28% in the 6–8-month-old age groups. The prevalence reached 87·5% in the 8–10-month-old group and decreased to 66·6% in 10–12-month-old increasing again to 87·5% in >12-month-old group monkeys.

After the first dose of *K. pneumoniae* $Al(OH)_3$ vaccine 76% of 63 baby monkeys seroconverted. Their ELISA arithmetic and Geometric Mean Titre (GMT) were 1 : 1320 and 1 : 101, respectively. After the second dose of vaccine one month later, 90% of 52 vaccinated babies seroconverted. Their arithmetic and GMT titres were 1 : 1473 and 1 : 491, respectively. The pre-inoculation arithmetic and GMT titres for 21/63 babies tested was 1 : 24 and 1 : 3, respectively. Antibody titres varied between <1 : 4 and 1 : 4096 (see Table 3).

Small 0·5–1 cm granulomas with fistulous tract were formed at the site of inoculation. Histologically, there was focal necrosis and granulomatous inflammation.

Three of 7 non-vaccinated laboratory-born control monkeys died of *K. pneumoniae* peritonitis during the 1989 outbreak, at 3, 4 and 5 months of age. Four other controls died of causes other than *K. pneumoniae* during the course of the experiment and only one survived the entire course of the study.

Only 1/33 (3%) 1988 born $Al(OH)_3$ vaccinated monkeys died at 7 months of age after receiving 2 doses of bacterin (Table 1). Two of 46 (4·3%) $Al(OH)_3$ bacterin vaccinated 1989 born monkeys died of *K. pneumoniae* peritonitis during the 1989 outbreak (Table 1). The 1988 born monkey had a titre greater than 1 : 256 before dying of *K. pneumoniae* peritonitis. The other 2 monkeys born during 1989 had titres of >1 : 256 and 1 : 4096 before death.

Two 2- and 3-week-old monkeys died 3 and 5 days after receiving one dose of $Al(OH)_3$ bacterin. At necropsy there were ecchymotic haemorrhages on the surface of the lungs presumably cause of bacterial endotoxins.

Table 1. *Klebsiella pneumoniae* peritonitis survival and death rates for $AL(OH)_3$ vaccinated and non-vaccinated laboratory-born *Aotus l. lemurinus* monkeys

Year	*Born*	*Surv.*	*Surv. (rate %)*	*Died*	*Death (rate %)*	*Kleb. death*	*Kleb. vac death (rate %)*
1986	105	30	(28·5)	75	(71·4)	21	(20)
1987	66	23	(34·84)	43	(65·1)	15	(22)
1988	61	33	(54·09)	28	(45·9)	13	(21) *1/33 (3·0)
1989	63	37	(58·7)	26	(41·2)	5	(7·9) †2/46 (4·3)
Total	295	122	(41·3)	173	(58·64)	54	(18·3) 3/79 (3·7)

Kleb. = *K. pneumoniae*; Surv. = survival; vac = vaccine.
*One baby died after May 4 1989 when the babies that survived the October 1988 *K. pneumoniae* epizootic were vaccinated with an $Al(OH)_3$ bacterin.
†Two of the 5 babies that died during the 1989 epizootic were vaccinated; the others were not and died when 3, 4 and 5 months old.

Histopathological examination revealed a severe acute interstitial haemorrhagic pneumonia and a severe acute multifocal centrolobular necrotic hepatitis.

The data in Table 1 show a reduction in *K. pneumoniae* mortality rates from 20, 22 and 21% registered for non-vaccinated laboratory-born monkeys during 1988, 1987 and 1986, respectively, to 3 and 4·3% in the $Al(OH)_3$ *K. pneumoniae* vaccinated monkeys born during 1988 and 1989.

Table 2. *Klebsiella pneumoniae* peritonitis crude death rate by room in adult and 1988–89 laboratory-born *Aotus l. lemurinus* monkeys during May 18–October 30, 1989 outbreak

Room	*Adults*	*Death (rate %)*	*1988–89 Lab-born $Al(OH)_3$ vac*	*Lab-born control non-vac*
76	1/23	(4·3)	1/11	0/1
79	2/46*	(6·5)	1/7	—
85	2/52	(3·8)	0/10	0/1
81	1/48	(2·0)	0/4	—
O	4/92	(4·3)	1/33	2/4
86	2/29	(6·9)	0/8	—
84	1/42	(2·3)	0/4	1/1
Total	14/332	(4·2)	3/75‡ (4·0)	3/7§ (42·8)†

vac = vaccinated; non-vac = non-vaccinated; () = rate %; O = outside cage.
* = Index case.
† = statistically significant by Fischer's exact test.
‡Two babies less than 2 months old died after vaccination (room 76, room 86) most likely due to toxic effects from the bacterin. They were not included in the statistical calculations.
§Three of 7 unvaccinated control monkeys died of *K. pneumoniae* peritonitis. They were 5, 4 and 3 months old. Four 2 and 4 months old died of other causes and one 16 months old survived.

Table 3. Seroconversion to an $AL(OH)_3$ *Klebsiella pneumoniae* bacterin in *A. l. lemurinus* as determined by ELISA

	ELISA titre		
	Pre-inoculation	*First dose*	*Second dose*
Mean	24·6	1320	1473
GMT	3·4	101	491
Min	0	0	0
Max	512	4096	4096
CV	34·5	3·11	2·57
%	32·3	76·2	90·4
n	63	63	52

Mean = arithmetic mean titre; GMT = geometric mean titre; Min = minimum titre, 0 = < 1 : 4; Max = maximum titre; CV = coefficient of variation; % = per cent seroconversion; *n* = number of monkeys tested.
Titres are expressed as the reciprocal of the last dilution in which the OD was greater than the mean negative control sera plus 2 SD.

No significant differences in crude death rates were found using the Fisher's exact test between adult monkeys caged in different rooms during the 1989 epizootic of *K. pneumoniae*. The average crude death rate for 332 adult monkeys was 4·2% with a minimum of 2·3% for one room and a maximum of 6·5% for another (Table 2). The disease was present in all the rooms at the same point in time during the 1989 epizootic and we considered all the animals to have been at the same risk of exposure. There were not significant differences in crude death rates per room between adult monkeys inoculated with bacterin without adjuvant and the $Al(OH)_3$ vaccinated laboratory-born monkeys.

Discussion

The ELISA test used in this study as described by Manning *et al.* (1986) and Manning (1984) with modifications from Cryz *et al.* (1984), was considered to be a reliable tool in determining the antibody levels against *K. pneumoniae* in *A. l. lemurinus* monkeys.

The nonspecific background observed in some of the adult feral pre-inoculation monkey sera at a 1 : 16 dilution, was attributed to naturally occurring anti-carbohydrate antibodies that reacted with the *K. pneumoniae* antigens. However, when sera with a high background were treated with hydrolysed agarose tris buffer as described by Hamilton & Adkinson (1985) a drop in the ELISA OD readings was observed. We used PBS-Tween 20 alone as the standard dilution buffer (Cryz *et al.*, 1984).

No difference in mortality was found between vaccinated and control groups in the adult feral monkeys vaccination and ELISA standardization experiments. The monkeys from the vaccinated groups developed detectable levels of antibodies against *K. pneumoniae*, but did not survive a natural challenge.

The lack of protection observed in prior trials with the PBS diluted bacterin without adjuvant, could have been the result of a change in the molecular structure of the bacterial surface antigens during treatment with formalin or the lack of adjuvant in the bacterin preparation which may have stimulated a nonprotective antibody response.

The prevalence studies demonstrated that *A. l. lemurinus* baby monkeys between 3 weeks and 2 months of age did not have maternal antibody. These findings are different to those reported by Kohno *et al.* (1988); and Russell *et al.* (1988) in which maternal antibodies against *Campylobacter* infection were detected in *Cynomolgus* monkeys (*Macaca fascicularis*) between 2 weeks and 2 months of age.

The *A. l. lemurinus* babies in our study seroconverted when 2–4 months old, and *K. pneumoniae* was isolated from the faeces of 3/15 babies sampled when 2–3 months old, respectively. It is postulated that seroconversion

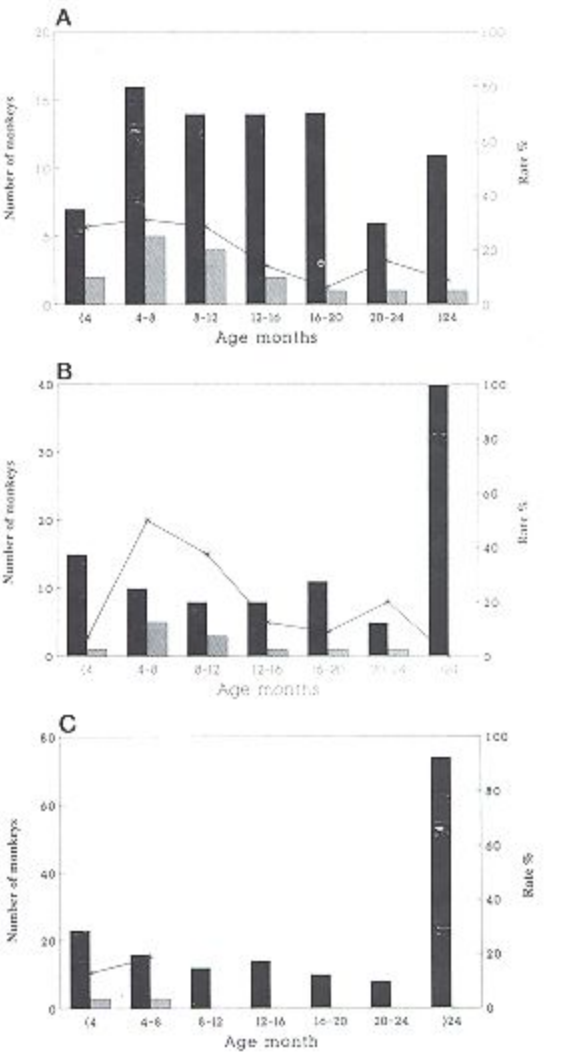

Fig. 4. Age specific *K. pneumoniae* death rate in laboratory-born *A. l. lemurinus* during: (A) October–December 1987 outbreak, $n = 84$; (B) October 1988 outbreak, $n = 109$; and (C) May–October 1989 outbreak, $n = 157$. ■ = population; ▨ = Kleb. mortality; ——●—— = % Kleb. mortality.

was related to oral-faecal contamination as a result of a change in the feeding and grooming behaviour of *A. l. lemurinus* babies, which might explain the high incidence of *K. pneumoniae* peritonitis death observed in the 4–8-month-old baby monkey groups during the 1987–88 epizootics at GML (Fig. 4 A,B). Oral-faecal contamination has been observed to occur in *Cynomolgus* baby monkeys older than 2 months (58%), and bacterial isolation from their faeces was

statistically correlated to seroconversion (Kohno *et al.*, 1988; Russel *et al.*, 1988).

As a result of these observations a vaccination protocol using an $Al(OH)_3$ *K. pneumoniae* bacterin was designed in order to control the spread of the disease in the 4–8-month-old group which was at highest risk of contracting infection with *K. pneumoniae* (Fig. 4C).

With the first dose of vaccine 76% of the babies seroconverted and when the second dose of bacterin was administered one month thereafter, 90% had seroconverted. Only 3/79 (3·7%) vaccinated monkeys died of *K. pneumoniae* peritonitis, compared to 20% for the previous 3 years (Table 1).

Perhaps the granulomas formed at the site of inoculations were the result of the $Al(OH)_3$ adjuvant incorporated in the bacterin, which stimulated a long lasting humoral and cellular immune response due to a depot effect and slow release of the antigen. This depot effect may have then contributed to the development of a stronger cellular immune response in the large intestine associated lymphoid tissue (Peyer's patches), limiting the spread of the disease. However, it could not be discounted that the granuloma was only the result of too high a concentration of adjuvant or bacteria in the bacterin preparation.

The observation that toxic effects occurred in monkeys younger than 1-month-old suggests, that endotoxins contained within the bacteria induced the stimulation of T suppressor cells (T_s cell), as has been demonstrated in mice when repeatedly inoculated with sub-immunogenic doses of polysaccharides from other gram negative bacteria (Taylor & Bright, 1989).

This T_s cell activity may have in turn caused an immunosuppression, which allowed other pathogens such as *K. pneumoniae*, *E. coli* or viruses such as hepatitis A virus (Lemon *et al.*, 1982) to evade the immune system and to induce the histopathological lesions reported herein. These pathogens could have been then the cause of the necrotic hepatitis and interstitial pneumonia found in the babies that died after vaccination.

Future challenge studies of vaccinated monkeys will be conducted in order to test the efficacy of the $Al(OH)_3$ bacterin compared to a polysaccharide version of the bacterin (Cryz *et al.*, 1985b) and to study the role of cellular immune responses to the outcome of *K. pneumoniae* peritonitis.

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