INFECTION AND IMMUNITY, Aug. 1994, p. 3146–3155
0019-9567/94/$04.00+0

Vol. 62, No. 8

Immunization with *Pseudomonas aeruginosa* Vaccines and Adjuvant Can Modulate the Type of Inflammatory Response Subsequent to Infection

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Received 29 September 1993/Returned for modification 30 March 1994/Accepted 3 May 1994

***Pseudomonas aeruginosa* is the predominant pathogen in patients with cystic fibrosis (CF). To study the possibility of preventing lung inflammation and decreasing the progression of the infection by vaccination, we have developed a rat model of chronic *P. aeruginosa* lung infection. Rats were immunized with *P. aeruginosa* whole-cell sonicates, O-polysaccharide toxin A conjugate, an alginate-toxin A conjugate, or native alginate. Control animals received sterile saline or incomplete Freund's adjuvant (IFA). The macroscopic (mean score, 2.4 versus 2.7 to 3.2) ($P < 0.05$) and microscopic (mean score, 2.0 versus 2.1 to 2.8) pathologic abnormalities were less severe in the control rats injected with sterile saline than in the immunized rats and the IFA group. The more severe lung abnormalities observed in immunized rats could be due to the result of immune complex-mediated lung tissue damage. The histopathologic results in the saline control rats were characterized by acute inflammation dominated by numerous polymorphonuclear leukocytes surrounding the alginate beads (microcolonies), as in CF patients. In contrast, the inflammatory response in the IFA group and in the immunized rats had changed from an acute-type inflammation to a chronic-type inflammation dominated by mononuclear leukocytes and scattered granulomas. Cross-reacting antibodies were induced by the two alginate vaccines, and most immunized animals developed a significant ($P < 0.001$) antibody titer elevation (in enzyme-linked immunosorbent assay) of the immunoglobulin M (IgM), IgG, and IgA classes against the homologous antigens. The bacterial clearance was significantly ($P < 0.05$) more efficient in most immunized rats than in the control rats given sterile saline. The present study shows that none of the vaccines could completely prevent chronic lung inflammation 4 weeks after challenge. However, the changed pathologic condition in immunized rats to a chronic-type inflammation might be of great benefit in future management of CF patients since the developing lung tissue damage has been shown to be caused by polymorphonuclear leukocyte-released elastase.**

During childhood, most cystic fibrosis (CF) patients have recurrent lung infections with *Staphylococcus aureus* and *Haemophilus influenzae* (31). With increasing age, chronic lung infections with mucoid *Pseudomonas aeruginosa* become more prevalent, and up to 80% of all adult CF patients become chronically infected (52). Chronic lung infection in CF patients is manifested as an endobronchiolitis (2), with the bacteria located as microcolonies without penetrating the periluminal tissue (41). At this stage *P. aeruginosa* is rarely eliminated despite a pronounced antibody response to numerous antigens (18, 40) and an abundance of polymorphonuclear leukocytes (PMNs) in the bronchial secretions (37).

Previously (34) we described a chronic lung infection induced in normal rats by intratracheal administration of mucoid *P. aeruginosa* embedded in seaweed alginate. The pathological and serological responses were similar to those observed in patients with CF (34). Alginate is a major virulence factor which provides protection from the host defense mechanisms by interfering with the clearance of *P. aeruginosa* as a result of its antiphagocytic properties (45, 52, 64). It has been shown that a subpopulation of antibodies against alginate are able to promote the uptake and killing of mucoid *P. aeruginosa* by human PMNs (1). Most commonly the initial colonizing strains in CF patients are nonmucoid, and a shift to a mucoid phenotype occurs with time (28). However, evidence that the early colonizing strains also produces small amounts of alginate has accumulated (54, 57). Furthermore, it has been found that elevated levels of opsonic anti-alginate antibodies correlate with lack of detectable *P. aeruginosa* lung infection in a group of older CF patients (58). In this study we have used native alginate and a depolymerized alginate vaccine which is covalently coupled to toxin A (10, 14). This has previously elicited antibodies which were cross-reactive with heterologous alginate (6) and which could be of importance in trying to prevent the subsequent chronic lung infection.

Lipopolysaccharide (LPS) plays an important role in the virulence of *P. aeruginosa* by activating complement and inducing production of cytokines (tumor necrosis factor, interleukin-1) (15, 19). Elevated levels of antibodies to LPS and toxin A have been found to correlate with survival from *P. aeruginosa* bacteremia (10, 60). It has been shown that in CF patients, infection-induced anti-LPS antibodies against *P. aeruginosa* possessed affinities at least 100-fold less than those induced by vaccination with an octavalent O-polysaccharide toxin A (O-PS toxin A) vaccine (5). By using a chronic lung infection model in rats (68), it has been shown that *P. aeruginosa* toxin A-positive mutant strains causes parenchymal changes and bronchial inflammation, indicating the impor-

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tance of this virulence factor. The O-PS toxin A vaccine (63), which in CF patients was able to raise high levels of antibodies to LPS and toxin A, was therefore included in the present study. In a recent study (36) we found that subcutaneous immunization with *P. aeruginosa* whole-cell sonicate on days 0, 14, and 21 elicited high immunoglobulin G (IgG) and IgA antibody responses in serum against *P. aeruginosa* antigens when measured on day 28.

In view of the above findings, the purpose of this work was to study whether antibodies induced by the vaccines *P. aeruginosa* whole-cell sonicate, O-PS toxin A conjugate, an alginate toxin A conjugate, O-PS toxin A conjugate plus alginate toxin A conjugate, or native alginate could prevent chronic lung inflammation or minimize the inflammatory response which in CF is dominated by PMNs, when given subcutaneously before challenge with *P. aeruginosa* in alginate beads in our rat model of chronic *P. aeruginosa* lung infection. Since PMN elastase plays such a crucial role in the tissue damage in lungs of CF patients (26, 31, 65, 66), prevention of PMN-dominated inflammation might be of great advantage (46, 47).

MATERIALS AND METHODS

Animals. Seven-week-old female Sprague-Dawley rats in groups of 20 to 46 weighing approximately 170 g were used (34–36).

Challenge strain. *P. aeruginosa* PAO 579, which stably maintains a mucoid phenotype and is International Antigenic Typing System (IATS) O:2/5 (kindly provided by J. R. W. Govan, Department of Bacteriology, Medical School, University of Edinburgh, Edinburgh, United Kingdom), was used (22, 27). The behavior of this strain in the chronic-infection model has been previously described (34, 55).

Immobilization of *P. aeruginosa* in spherical alginate beads. Briefly, 1 ml of the *P. aeruginosa* bacterial culture was mixed with 9 ml of seaweed alginate (guluronic acid content, 60%) and forced once with air through a cannula into a solution of 0.1 M $CaCl_2$ in 0.1 M TRIS-HCl buffer (pH 7.0) (34, 55). The suspension was adjusted to yield 10^9 CFU/ml and confirmed by colony counts.

Vaccines. We used the following control and vaccine groups. Controls consisted of (i) 0.1 ml of 0.15 M sterile NaCl (control I) and (ii) 0.1 ml of incomplete Freund's adjuvant (IFA) (Tuberculin Department, State Serum Institute, Copenhagen, Denmark) (control II). Vaccines consisted of (i) sonicated *P. aeruginosa* PAO 579, with a protein concentration of the antigen preparation of 19.7 g/liter (44) (each animal was given 0.1 ml of the vaccine mixed with IFA yielding 100 μg of protein per vaccination); (ii) *P. aeruginosa* O-PS toxin A (12, 16) (the eight *P. aeruginosa* serotypes represented in the vaccine are Fisher-Devlin immunotypes IT-1 [Habs O:6], IT-2 [Habs O:11], IT-3 [Habs O:2/5], IT-4 [Habs O:1], IT-5 [Habs O:10], IT-7 [Habs O:2/5], and Habs-3 and Habs-4 [62]) (each animal received 0.25 ml of vaccine containing 25 μg of O-PS toxin A from each serotype); (iii) depolymerized *P. aeruginosa* alginate (from strain 3064) covalently coupled to toxin A (D-ALG toxin A) (13, 14) (each animal received 0.25 ml of vaccine containing 25 μg of alginate and 77.5 μg of toxin A); (iv) 0.25 ml of O-PS toxin A plus 0.25 ml of D-ALG toxin A; and (v) purified alginates from *P. aeruginosa* 6680 and 8839 mixed with IFA (53) (each animal received 0.2 ml of vaccine containing 140 μg of alginate). Vaccines used in vaccine groups (ii) to (iv) were adsorbed to $Al(OH)_3$, whereas the vaccines in control group (ii) and vaccine groups (i) and (v) were mixed with IFA.

Immunization and challenge procedures. On days 0 and 14, each of the seven groups of rats were subcutaneously immunized with one of the above-mentioned vaccines. On day 28 all rats were intratracheally challenged with 0.1 ml of *P. aeruginosa* (10^9 CFU/ml) in alginate beads.

Blood samples. On day 0, blood was drawn from 20 randomly chosen rats, and this pool was used as reference day 0 pool in all the enzyme-linked immunosorbent assays (ELISA). On day 28, 1.5 ml of blood was drawn from the right orbital plexus from all rats as previously described (34, 35). All animals were sacrificed on day 56 (4 weeks after challenge).

Macroscopic description of the lungs. After removal, all lungs were macroscopically assigned to one of four groups, according to the severity of the infection: 1, normal; 2, swollen lungs, hyperemia, small atelectasis (1 by 1 mm); 3, adherences, small hemorrhages, small abscesses (to 1 by 2 mm), atelectasis (2 by 3 mm); 4, adherences, hemorrhages, abscesses (>1 by 2 mm), and atelectasis (>3 mm) (34). The scoring was performed in a blinded fashion to avoid bias.

Histopathologic testing. Lungs from 10 to 36 animals in each group were prepared for histologic examination. The lower section of the left lung lobe was fixed in formalin, embedded in paraffin wax, cut into 5- to 10-μm-thick sections, and stained with hematoxylin and eosin. Microscopically the sections were assigned to one of four groups according to the severity of the inflammation: I, normal; II, mild focal inflammation; III, moderate to severe focal inflammation with areas of normal lung tissue; IV, severe inflammation to necrosis and severe inflammation throughout the lung (34). The cellular changes were assigned to groups according to acute and chronic inflammation by using a scoring system based on the percentage of PMNs and mononuclear cells in the inflammatory foci: ≥90% PMNs and ≤10% mononuclear cells, 50 to 90% PMNs and 10 to 50% mononuclear cells, 10 to 50% PMNs and 50 to 90% mononuclear cells, and ≤10% PMNs and ≥90% mononuclear cells. Acute inflammation is defined as inflammatory infiltrates dominated by neutrophil granulocytes (≥90%). Chronic inflammation is defined as predominance of mononuclear cells (≥90%) (lymphocytes and plasma cells) and presence of granulomas.

Bacteriologic testing. The first 10 lungs from each group were prepared for quantitative bacteriologic examination as follows. The lungs were mixed with 3 ml of sterile phosphate-buffered saline and homogenized in a blender (34). Appropriately diluted samples were plated to determine the number of CFU. The limit of detection was <100 organisms per ml of lung homogenate.

Purification of *P. aeruginosa* LPS. LPS was purified from *P. aeruginosa* PAO 579 O:2/5 as previously described (19, 21).

ELISAs. Quantitation of anti-*P. aeruginosa* PAO 579 sonicate antibodies of the IgM, IgG, and IgA classes in serum and IgG and IgA antibodies against *P. aeruginosa* alginate (6680 + 8839) were carried out by means of ELISAs as previously reported (34). Titers, expressed as ELISA units, were obtained by dividing the mean optical density values of the samples with the mean optical density of an internal standard expressing absorbance units between 0.30 and 0.40.

For determination of IgM, IgG, and IgA antibodies against *P. aeruginosa* toxin A, *P. aeruginosa* PAO 579 LPS, and *P. aeruginosa* 3064 alginate, and IgM antibodies against *P. aeruginosa* 6680 + 8839, we used flat-bottomed 96-well microdilution plates (Microwell; NUNC, Roskilde, Denmark). Serum dilutions varied between 1:40 and 1:320, and 0.1 to 2.0% goat serum was added to eliminate nonspecific binding. For determination of IgM, IgG, and IgA specific antibodies, dilutions of peroxidase-conjugated goat anti-rat IgM, IgG, and IgA between 1:2,000 and 1:10,000 were used.

Analytic variation of ELISA. The intraplate same-day and

day-to-day variations in the ELISA results, which have not been reported before, were determined by testing in each assay 10 antibody-positive serum samples representing a wide range of antibody levels as previously described (34). By using 95% confidence limits (CL), the intraplate variation coefficient the same day was 18% (95% CL, ±25%) for the *P. aeruginosa* toxin A IgM assay, 3% (95% CL, ±4%) for the IgG assay, and 6% (95% CL, ±8%) for the IgA assay, whereas the day-to-day variation was 50% for IgM (95% CL, ±75%), 18% for IgG (95% CL, ±25%), and 11% for IgA (95% CL, ±15%).

The intraplate variation coefficient on the same day was 19% (95% CL, ±26%) for the *P. aeruginosa* PAO 579 LPS IgM assay, 12% (95% CL, ±17%) for the IgG assay, and 9% (95% CL, ±12%) for the IgA assay, whereas the day-to-day variation was 28% for IgM (95% CL, ±39%), 54% for IgG (95% CL, ±75%) and 8% for IgA (95% CL, ±11%).

The intraplate variation coefficient on the same day was 3% (95% CL, ±4%) for the *P. aeruginosa* 3064 alginate IgM assay, 4% (95% CL, ±6%) for the IgG assay, and 12% (95% CL, ±16%) for the IgA assay, whereas the day-to-day variation was 15% for IgM (95% CL, ±21%), 30% for IgG (95% CL, ±42%), and 23% for IgA (95% CL, ±32%).

The intraplate variation coefficient on the same day for the *P. aeruginosa* alginate 6680 + 8839 IgM assay was 4% (95% CL, ±6%), whereas the day-to-day variation was 14% (95% CL, ±19%).

Statistical analysis. We chose a 5% level of significance. The χ^2 test for categorical data was used. Wilcoxon's signed-rank test for pair differences and the Mann-Whitney U test for unpaired differences were used to compare continuous data.

RESULTS

Immunogenicity. The IgM, IgG, and IgA antibody responses measured on day 28 in the controls against the antigens used for the ELISA were in most cases significantly ($P < 0.01$) lower than the antibody responses obtained in immunized rats (Tables 1 to 5). The development of antibodies in the control groups was first induced after challenge with alginate beads on day 28; however, thereafter a significantly ($P < 0.05$) increased IgM, IgG, and IgA antibody response could be demonstrated against most antigens (Tables 1 to 5).

The highest titers against *P. aeruginosa* alginates 6680 + 8839 and 3064 could be demonstrated in the rats immunized with the homologous alginate ($P < 0.05$). However, a significant ($P < 0.05$) number of cross-reactive IgM, IgG, and IgA antibodies were induced by the two alginate vaccines against the heterologous alginate (Tables 1 and 4).

The PAO 579 sonicate vaccine induced significant ($P = 0.001$) IgM, IgG, and IgA antibody responses on day 28 against *P. aeruginosa* 6680 + 8839 alginate, the homologous sonicate, and LPS when compared with the responses on day 0. The rats immunized with the O-PS toxin A vaccine was expected to be able to induce cross-reactive LPS antibodies since O groups 2 and 5 are both present in the vaccine and the PAO 579 LPS used as antigen in the ELISA (43); however, only traces of cross-reacting LPS antibodies could be measured after immunization (Tables 2 and 5).

The highest anti-toxin A antibody response could be measured in animals immunized with the toxin A conjugated vaccines. A high and significant ($P = 0.001$) toxin A IgG antibody response was induced by both toxin A conjugated vaccines when compared with the other vaccine groups. Increased anti-toxin A IgG and IgA ($P < 0.05$) titers were observed in rats vaccinated with toxin A-containing vaccines after challenge when compared with the other immunization

TABLE 1. *P. aeruginosa* alginate 6680 + 8839 antibodies[a]

Control or vaccine	Antibody response (ELISA ratio [median and range])[b]								
	IgM			IgG			IgA		
	Day 0	Day 28[c]	Day 56	Day 0	Day 28[c]	Day 56	Day 0	Day 28[c]	Day 56
NaCl (control I)	0.08 (0–0.94)	0.46 (0.29–0.96)	1.39 (0.39–2.18)	0.10 (0.03–0.36)	0.18 (0.08–0.44)	3.47 (0.58–4.58)	0 (0–0.05)	0.02 (0–0.21)	0.67 (0.07–2.19)
IFA (control II)	0.08 (0–0.94)	0.58 (0–0.97)	1.34 (0–3.29)	0.10 (0.03–0.36)	0.10 (0–0.99)	0.93 (0.22–2.56)	0 (0–0.05)	0.25 (0–0.89)	0.77 (0.24–1.93)
PAO 579 sonicate	0.08 (0–0.94)	1.78* (0.70–4.77)	2.27 (0.65–4.15)	0.10 (0.03–0.36)	2.68*# (0.75–8.03)	4.93 (0.9–8.81)	0 (0–0.05)	0.81* (0.22–1.43)	1.18 (0.44–2.21)
O-PS toxin A	0.08 (0–0.94)	0.71 (0.21–1.29)	1.45 (0.17–3.00)	0.10 (0.03–0.36)	0.26 (0–0.81)	2.38 (0.14–6.71)	0 (0–0.05)	0.40 (0.05–1.46)	1.01 (0.36–1.89)
D-ALG toxin A	0.08 (0–0.94)	4.84*# (1.10–28.94)	2.52 (1.18–10.40)	0.10 (0.03–0.36)	0.72*§ (0.31–1.43)	1.07 (0.42–3.58)	0 (0–0.05)	1.07*# (0.55–1.92)	1.39 (0.55–2.07)
O-PS toxin A + D-ALG toxin A	0.08 (0–0.94)	1.27* (0.49–9.36)	1.39 (0.64–3.18)	0.10 (0.03–0.36)	0.32 (0–1.74)	0.94 (0–3.60)	0 (0–0.05)	0.74* (0.29–1.81)	1.20 (0.36–3.16)
P. aeruginosa alginate 6680 + 8839	0.08 (0–0.94)	18.24*# (1.84–29.12)	12.16 (1.84–26.21)	0.10 (0.03–0.36)	9.77*#§ (0–13.44)	11.81 (1.55–18.52)	0 (0–0.05)	2.52*# (0.31–4.62)	2.58 (1.00–4.54)

[a] Development of IgM, IgG, and IgA antibody responses (ELISA ratio) against *P. aeruginosa* 6680 + 8839 alginate after subcutaneous immunization with seven different *P. aeruginosa* vaccines on days 0 and 14 and challenge with 10^9 CFU of *P. aeruginosa* per ml embedded in alginate beads on day 28. The animals were killed on day 56.

[b] Statistical significance: *, $P < 0.05$ compared with sterile NaCl group (control I); #, $P < 0.05$ when the two (#) groups in the same column are compared; §, $P < 0.05$ when the two (§) groups in the same column are compared.

[c] Challenge took place on day 28.

TABLE 2. *P. aeruginosa* 579 sonicate antibodies[a]

Control or vaccine	Antibody response (ELISA ratio [median and range])[b]								
	IgM			IgG			IgA		
	Day 0	Day 28[c]	Day 56	Day 0	Day 28[c]	Day 56	Day 0	Day 28[c]	Day 56
NaCl (control I)	0 (0–0)	0 (0–0)	0.33 (0–1.67)	0.12 (0.06–0.35)	0.18 (0.06–0.53)	4.53 (0.53–7.65)	0 (0–0)	0 (0–0.17)	3.47 (1.90–3.97)
IFA (control II)	0 (0–0)	0 (0–1.20)	0.17 (0–2.36)	0.12 (0.06–0.35)	0.21 (0–1.46)	1.94 (0.45–4.44)	0 (0–0)	0.40 (0.02–0.95)	1.76 (0.75–4.39)
PAO 579 sonicate	0 (0–0)	4.84*# (1.62–13.40)	1.76 (0.24–5.40)	0.12 (0.06–0.35)	11.31*# (6.40–16.26)	12.09 (6.60–17.27)	0 (0–0)	8.58*# (6.53–11.14)	9.18 (5.95–11.20)
O-PS toxin A	0 (0–0)	0 (0–0.17)	0.06 (0–0.92)	0.12 (0.06–0.35)	0.63 (0–6.96)	4.17 (1.11–6.09)	0 (0–0)	0.51 (0.15–1.45)	1.94 (1.0–4.35)
D-ALG toxin A	0 (0–0)	0 (0–0.55)	0.32 (0–1.40)	0.12 (0.06–0.35)	1.36 (0.16–4.36)	4.04 (1.78–5.94)	0 (0–0)	1.22 (0.09–2.70)	4.86 (2.78–7.18)
O-PS toxin A + D-ALG toxin A	0 (0–0)	0.05 (0–0.49)	0.13 (0–0.51)	0.12 (0.06–0.35)	1.16 (0.09–2.41)	2.76 (1.91–4.67)	0 (0–0)	1.30 (0.18–4.27)	3.51 (1.14–5.77)
P. aeruginosa alginate 6680 + 8839	0 (0–0)	0.37*# (0–1.40)	0.51 (0–3.60)	0.12 (0.06–0.35)	1.44*# (0.26–4.70)	4.11 (2.37–6.15)	0 (0–0)	1.45*# (0.11–3.52)	5.26 (2.11–7.48)

[a] Development of IgM, IgG, and IgA antibody responses (ELISA ratio) against *P. aeruginosa* sonicate after subcutaneous immunization with seven different *P. aeruginosa* vaccines on days 0 and 14 and challenge with 10^9 CFU of *P. aeruginosa* per ml embedded in alginate beads on day 28. The animals were killed on day 56.
[b] Statistical significance: *, $P < 0.05$ compared with sterile NaCl group (control I); #, $P < 0.05$ when the two (#) groups in the same column are compared.
[c] Challenge took place on day 28.

TABLE 3. *P. aeruginosa* toxin A antibodies[a]

Control or vaccine	Antibody response (ELISA ratio [median and range])[b]								
	IgM			IgG			IgA		
	Day 0	Day 28[c]	Day 56	Day 0	Day 28[c]	Day 56	Day 0	Day 28[c]	Day 56
NaCl (control I)	0 (0–0.04)	0.15 (0–0.33)	0.07 (0–0.27)	0.03 (0–0.12)	0.16 (0.03–0.29)	0.13 (0.06–0.32)	0 (0–0)	0 (0–0)	0 (0–0)
IFA (control II)	0 (0–0.04)	0.02 (0–0.14)	0.02 (0–0.15)	0.03 (0–0.12)	0.19 (0.03–0.67)	0.19 (0.05–0.58)	0 (0–0)	0 (0–0.40)	0 (0–0.15)
PAO 579 sonicate	0 (0–0.04)	0 (0–0.04)	0 (0–0.08)	0.03 (0–0.12)	0.21 (0.04–0.66)	0.21 (0.04–1.78)	0 (0–0)	0 (0–0.20)	0 (0–0.50)
O-PS toxin A	0 (0–0.04)	0.07# (0–1.03)	0 (0–1.39)	0.03 (0–0.12)	2.70* (0–17.20)	3.45 (0–20.50)	0 (0–0)	0.02* (0–0.31)	0.25 (0–0.80)
D-ALG toxin A	0 (0–0.04)	0.60*# (0–4.59)	2.14 (0.23–4.50)	0.03 (0–0.12)	29.30*# (10.60–212.80)	64.40 (23.00–195.02)	0 (0–0)	0.58*# (0.27–1.27)	1.13 (0.85–1.66)
O-PS toxin A + D-ALG toxin A	0 (0–0.04)	0.05 (0–1.05)	0.62 (0–4.30)	0.03 (0–0.12)	11.60*# (0–149.60)	23.80 (0.20–135.20)	0 (0–0)	0.22*# (0–0.94)	0.63 (0.05–1.68)
P. aeruginosa alginate 6680 + 8839	0 (0–0.04)	0.09 (0–0.64)	0 (0–0.14)	0.03 (0–0.12)	0.31 (0.17–0.67)	0.33 (0.11–0.74)	0 (0–0)	0 (0–0.05)	0 (0–0.08)

[a] Development of IgM, IgG, and IgA antibody responses (ELISA ratio) against *P. aeruginosa* toxin A after subcutaneous immunization with seven different *P. aeruginosa* vaccines on days 0 and 14 and challenge with 10^9 CFU of *P. aeruginosa* per ml embedded in alginate beads on day 28. The animals were killed on day 56.
[b] Statistical significance: *, $P < 0.05$ compared with sterile NaCl group (control I); #, $P < 0.05$ when the two (#) groups in the same column are compared.
[c] Challenge took place on day 28.

TABLE 4. *P. aeruginosa* 3064 alginate antibodies[a]

Control or vaccine	Antibody response (ELISA ratio [median and range])[b]								
	IgM			IgG			IgA		
	Day 0	Day 28[c]	Day 56	Day 0	Day 28[c]	Day 56	Day 0	Day 28[c]	Day 56
NaCl (control I)	0.08 (0–0.08)	0.15 (0.08–0.31)	0.31 (0.15–0.62)	0.17 (0.07–0.33)	0.13 (0.07–0.33)	0.20 (0.13–1.00)	0 (0–0.02)	0.06 (0–0.10)	0.10 (0–0.33)
IFA (control II)	0.08 (0–0.08)	0.18 (0.05–0.47)	0.34 (0.08–1.71)	0.17 (0.07–0.33)	0.40 (0.22–0.53)	0.53 (0.33–1.78)	0 (0–0.02)	0.03 (0–0.62)	0.06 (0–0.45)
PAO 579 sonicate	0.08 (0–0.08)	0.26 (0.13–0.56)	0.31 (0.11–1.05)	0.17 (0.07–0.33)	0.32 (0.10–0.65)	0.33 (0.08–0.55)	0 (0–0.02)	0.14 (0–0.91)	0.10 (0–0.40)
O-PS toxin A	0.08 (0–0.08)	0.38 (0–1.92)	0.29 (0–1.24)	0.17 (0.07–0.33)	0.35 (0.13–1.15)	0.47 (0.15–3.70)	0 (0–0.02)	0.06 (0–0.75)	0.06 (0–0.75)
D-ALG toxin A	0.08 (0–0.08)	3.94*# (0.76–33.96)	1.64 (0.55–3.84)	0.17 (0.07–0.33)	4.86*# (3.14–9.10)	4.05 (2.61–7.30)	0 (0–0.02)	1.08*# (0.49–1.47)	0.79 (0.29–1.56)
O-PS toxin A + D-ALG toxin A	0.08 (0–0.08)	0.43* (0–2.10)	0.39 (0–0.87)	0.17 (0.07–0.33)	1.97* (0.26–3.77)	1.02 (0.18–3.16)	0 (0–0.02)	0.39* (0–0.65)	0.14 (0–0.81)
P. aeruginosa alginate 6680 + 8839	0.08 (0–0.08)	1.78*# (0.36–3.51)	1.02 (0.36–2.56)	0.17 (0.07–0.33)	2.14*# (0.38–4.14)	1.43 (0.31–4.14)	0 (0–0.02)	1.12*# (0.23–1.74)	0.79 (0.15–1.76)

[a] Development of IgM, IgG, and IgA antibody responses (ELISA ratio) against *P. aeruginosa* 3064 alginate after subcutaneous immunization with seven different *P. aeruginosa* vaccines on days 0 and 14 and challenge with 10^9 CFU of *P. aeruginosa* per ml embedded in alginate beads on day 28. The animals were killed on day 56.
[b] Statistical significance: *, $P < 0.05$ compared with sterile NaCl group (control I); #, $P < 0.05$ when the two (#) groups in the same column are compared.
[c] Challenge took place on day 28.

TABLE 5. *P. aeruginosa* 579 LPS antibodies[a]

Control or vaccine	Antibody response (ELISA ratio [median and range])[b]								
	IgM			IgG			IgA		
	Day 0	Day 28[c]	Day 56	Day 0	Day 28[c]	Day 56	Day 0	Day 28[c]	Day 56
NaCl (control I)	0.04 (0.02–0.06)	0.05 (0.02–0.33)	0.27 (0.08–0.67)	0 (0–0.04)	0 (0–0)	1.89 (0.69–3.48)	0.05 (0–0.10)	0.08 (0–0.28)	0.76 (0.30–1.28)
IFA (control II)	0.04 (0.02–0.06)	0.28 (0.03–0.62)	2.32 (0.95–22.56)	0 (0–0.04)	0.04 (0–0.30)	1.78 (0.07–13.60)	0.05 (0–0.10)	0.03 (0–0.48)	1.29 (0.19–3.13)
PAO 579 sonicate	0.04 (0.02–0.06)	3.81*# (2.03–26.19)	2.82 (1.09–4.68)	0 (0–0.04)	17.29*# (0.62–71.68)	18.29 (0.62–70.15)	0.05 (0–0.10)	2.00*# (1.08–2.77)	2.12 (0.80–3.69)
O-PS toxin A	0.04 (0.02–0.06)	0.76*# (0.24–1.83)	1.81 (0.71–3.69)	0 (0–0.04)	0.15*# (0.02–0.32)	1.63 (0–4.37)	0.05 (0–0.10)	0 (0–0.12)	0.66 (0–1.86)
D-ALG toxin A	0.04 (0.02–0.06)	0.35 (0.07–0.75)	1.09 (0.50–2.28)	0 (0–0.04)	0.04 (0–0.22)	9.97 (3.56–28.89)	0.05 (0–0.10)	0.04 (0–0.11)	0.82 (0–1.66)
O-PS toxin A + D-ALG toxin A	0.04 (0.02–0.06)	0.34* (0.11–1.19)	0.62 (0.26–2.78)	0 (0–0.04)	0.12* (0–2.05)	3.78 (1.33–16.50)	0.05 (0–0.10)	0.13*# (0.03–0.68)	0.90 (0.26–1.98)
P. aeruginosa alginate 6680 + 8839	0.04 (0.02–0.06)	0.58 (0.09–0.81)	1.48 (0.43–3.66)	0 (0–0.04)	0.27 (0.05–0.80)	12.64 (2.11–34.09)	0.05 (0–0.10)	0.24 (0–0.94)	1.35 (0.55–2.60)

[a] Development of IgM, IgG, and IgA antibody responses (ELISA ratio) against *P. aeruginosa* LPS after subcutaneous immunization with seven different *P. aeruginosa* vaccines on days 0 and 14 and challenge with 10^9 CFU of *P. aeruginosa* per ml embedded in alginate beads on day 28. The animals were killed on day 56.
[b] Statistical significance: *, $P < 0.05$ compared with sterile NaCl group (control I); #, $P < 0.05$ when the two (#) groups in the same column are compared.
[c] Challenge took place on day 28.

TABLE 6. Lethality of challenge and condition of the lungs 28 days after challenge with *P. aeruginosa* alginate beads

Control or vaccine group	% Lethality (no. killed/total no.)	Scoring of lungs according to severity of inflammation			% of lungs with *P. aeruginosa*[a] (no. infected/total no.)
		Macroscopic mean[a]	Microscopic mean	%PMN/MC/G[b]	
NaCl (control I)	5 (1/20)	2.4	2.0	≥90/≤10/−	67 (6/9)
IFA (control II)	0 (0/46)	3.0*	2.1	≤10/≥90/+	10 (1/10)*
PAO 579 sonicate	0 (0/37)	3.2*	2.7	≤10/≥90/+	40 (4/10)
O-PS toxin A	0 (0/36)	2.8	2.5	≤10/≥90/+	0 (0/10)*
D-ALG toxin A	0 (0/36)	2.7	2.8	≤10/≥90/+	0 (0/10)*
O-PS + D-ALG toxin A	3 (1/35)	2.7	2.3	≤10/≥90/+	20 (2/10)*
P. aeruginosa alginate 6680 + 8839	0 (0/37)	2.7	2.7	≤10/≥90/+	0 (0/10)*

[a] Statistical significance: *, $P < 0.05$ compared with sterile NaCl control group.
[b] PMN, neutrophil granulocytes; MC, mononuclear cells (lymphocytes and plasma cells); G, granulomas (see Materials and Methods).

groups. The antibody responses induced by the combined vaccination were lower with respect to each antigen component (toxin A IgG and IgA; $P = 0.0001$) than were the antibody responses induced by each of the vaccines when administered alone (Table 3).

Lethality. Two rats, one in the sterile saline group and one in the D-ALG + O-PS toxin A group, died of *P. aeruginosa* sepsis (blood culture positive for *P. aeruginosa* PAO 579) within the first 2 days after challenge (Table 6).

Pathologic abnormalities. Macroscopically the less affected lungs were observed among the control rats injected with sterile saline when compared with the other vaccine groups, and the differences were significant compared with results for the rats given IFA or *P. aeruginosa* sonicate ($P < 0.05$). In the sterile-saline group the pathologic changes were rather heterogeneous; some of the rats had abscesses in the lower section of the left lung, whereas others did not show any sign of inflammation. In contrast, the pathologic changes observed in the control group given IFA and the groups of rats immunized with vaccines containing *P. aeruginosa* antigens appeared much more homogeneous, with adherences, abscesses, some small hemorrhages, and smaller and larger atelectases located primarily in the lower section of the left lung (Table 6).

The most severe microscopic inflammations were observed among vaccinated rats when compared with the control groups. The histopathologic abnormalities in the rats injected with sterile saline were characterized by an acute inflammatory reaction dominated by numerous PMNs (≥90%) surrounding the alginate beads (microcolonies) (Fig. 1a). In contrast, the inflammatory response observed in the IFA group and the immunized rats had shifted from an acute-type inflammatory response to a chronic-type inflammation dominated by mononuclear cells (≥90% lymphocytes and plasma cells) and scattered granulomas (Fig. 1b) (Table 6).

Bacteriologic testing. *P. aeruginosa* could be cultured from 67% of the control rats given sterile saline; this was a significantly ($P < 0.05$) higher percentage than that for most other vaccine groups (Table 6). Three vaccines, O-PS toxin A, D-ALG toxin A, and *P. aeruginosa* alginate 6680 + 8839, were found to have completely cleared the bacteria (Table 6).

DISCUSSION

Experimental models of the chronic lung infection found in CF patients have previously been established in rats by Cash et al. (8) and others (3, 9, 17, 24, 25, 29, 33, 38, 39, 51, 55, 59, 67), who incorporated *P. aeruginosa* bacteria into agar beads, agarose beads, or alginate beads. We have recently established the alginate bead model of chronic *P. aeruginosa* lung infection in normal rats (34, 55) and athymic rats with an abundance of PMNs dominating the inflammatory reaction in both types of rats as in CF (34). The protective effect of preimmunization on the chronic *P. aeruginosa* lung infection has previously been studied by others, using different animal models. Klinger et al. (38) used a PEV-01 vaccine consisting of LPS and other cell wall antigens from 16 O groups of *P. aeruginosa* for immunizing rats before inducing the chronic lung infection. They found significantly milder lung abnormalities but no decrease of the *P. aeruginosa* count in the lungs 8 to 10 days after challenge. Similar results as regards protection were obtained by Gilleland et al. (24, 25), using outer membrane protein F from *P. aeruginosa* for immunization prior to challenge of rats with agar beads containing *P. aeruginosa*. Pennington et al. (56) found that in guinea pigs intratracheally challenged with *P. aeruginosa*-containing agar beads, immunization with a *P. aeruginosa* LPS vaccine resulted in smaller numbers of viable *P. aeruginosa* bacteria and reduced histopathologic abnormalities of the lungs.

In the present study all rats either were prevaccinated or received a control injection. All vaccines containing *P. aeruginosa* antigens were able to induce high antibody responses against their homologous antigens in ELISAs (Tables 1 to 5). In general the macroscopic and microscopic abnormalities were more pronounced in immunized rats and in the IFA group compared with those in the controls given sterile saline. This severity could be due to hypersensitivity reactions, e.g., immune complex-mediated lung tissue damage as in CF (32). When these results were compared with previous findings in nonvaccinated rats, the only group which persistently showed similar histopathologic findings was the control group receiving sterile saline. In these rats 4 weeks after challenge, PMNs surrounded the alginate beads containing the bacteria as in CF. With regard to changing the inflammatory response, the effect caused by the *P. aeruginosa*-containing vaccines was equal to that in rats injected with IFA, since the inflammatory response in these rats also changed to a chronic-type inflammation with mononuclear leukocytes and eradication of most of the bacteria (Table 6). The vaccine-induced antibodies therefore seem to play a minor role in prevention of the inflammation. This shift from an acute to a chronic inflammation is potentially very important since the tissue damage in CF has been shown by many authors to be caused by PMN elastase (26, 31, 46, 47, 65, 66).

There is a considerable body of evidence that adjuvants can influence the antibody isotype and cell-mediated immunity in rodents (4). All the *P. aeruginosa* vaccines in the present study were adsorbed to either $Al(OH)_3$ or IFA. These two adjuvants both enhance the IgG1 antibody response and induce Th1

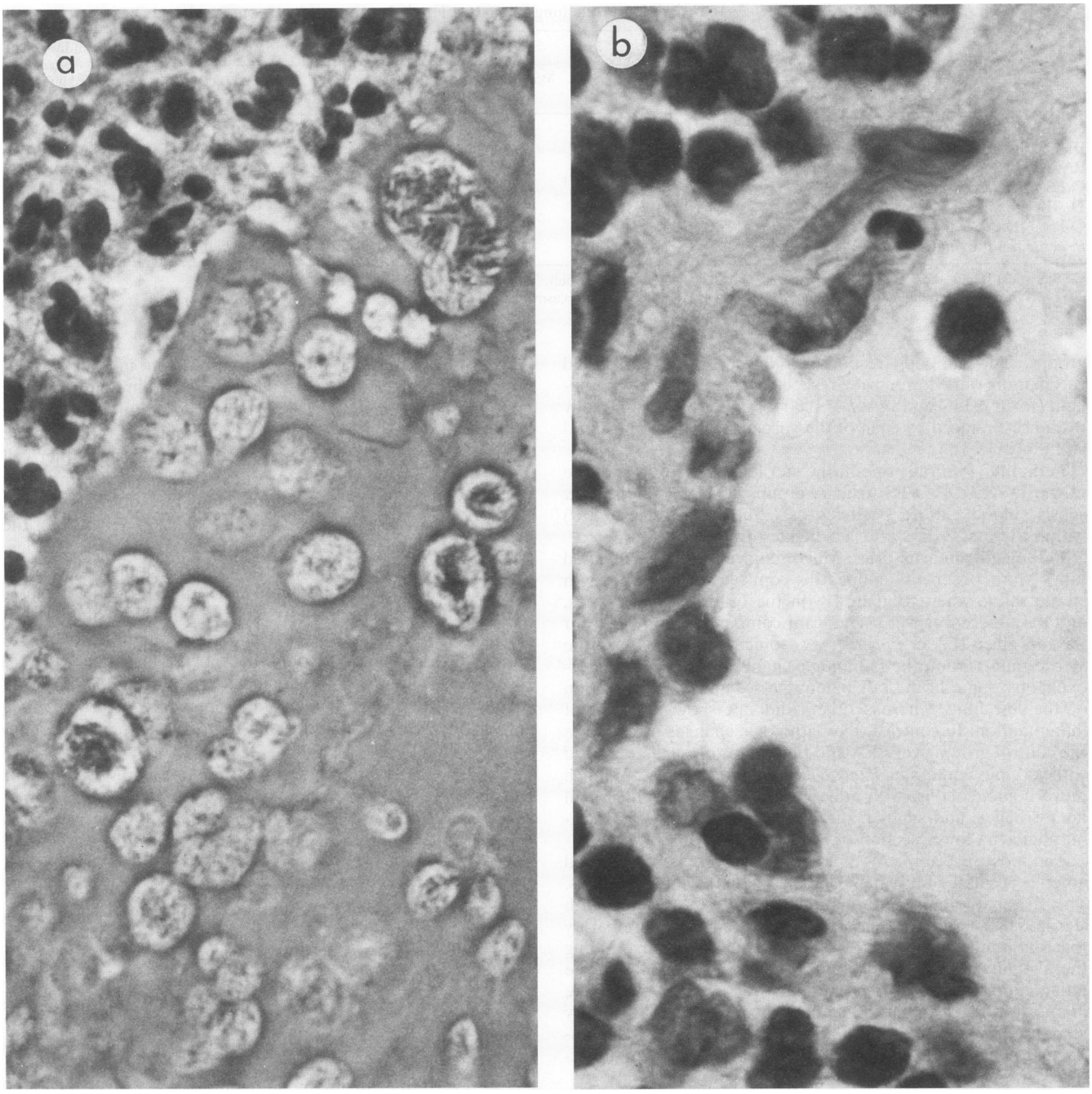

FIG. 1. (a) Photomicrograph of a section from the lower portion of the left lung in a normal rat injected with sterile saline (group 1) 28 days after challenge with 10^9 CFU of *P. aeruginosa*-containing alginate beads per ml. Note the dense accumulation of PMNs surrounding the alginate bead with *P. aeruginosa* bacteria located as microcolonies within the bead as in CF patients. Stain, hematoxylin and eosin. Magnification, ×1,000. (b) Photomicrograph of a section from the lower portion of the left lung in a normal rat immunized with D-ALG toxin A conjugate (group 5) 28 days after challenge with 10^9 CFU of *P. aeruginosa*-containing alginate beads per ml. Note the alginate bead surrounded by mononuclear leukocytes, different from the situation observed in CF patients. Stain, hematoxylin and eosin. Magnification, ×1,000.

lymphocytes, and they should therefore induce similar pathologic and serologic effects in the vaccine groups (4).

The adjuvant effect caused by IFA is in accordance with previous findings by Roberts et al. (61), who found that injection with complete Freund's adjuvant (CFA) reduced the severity of an acute pneumonia caused by *Streptococcus pneumoniae* in rat lungs when compared with that in rats not receiving CFA. Buhles et al. (7) found that immune stimulation of granulocytopenic mice with CFA partially protected them against the lethal consequences of subsequent infection with *Staphylococcus aureus*. The tendencies in our experiment with IFA and the two experiments with CFA are similar. However, CFA includes mycobacteria in the oil and therefore possesses even stronger immunomodulatory traits than IFA

since it also leads to the appearance of IgG2 antibodies and to a delayed hypersensitivity reaction (4).

The slight increase in IgM, IgG, and IgA antibodies to, e.g., alginate (Table 1) in the control animals injected with NaCl or especially IFA correlates with the older age of the animals, which is known to lead to higher titers of "natural antibodies" (30). Since IFA stimulates Th1 lymphocytes (4) to produce the cytokines interleukin-2, gamma interferon, and tumor necrosis factor beta (48, 49), this may explain the developing antibody level seen in these rats.

The pathologic changes observed in the D-ALG toxin A- and *P. aeruginosa* alginate-immunized animals were similar in degree; the total clearance of the bacteria in the two groups was also similar. However, the doses and composition of the two alginates used for immunization were rather different. Rats immunized with depolymerized alginate received two 25-μg doses, whereas rats immunized with purified alginate received 140 μg per dose. Although we did not investigate the opsonic capacity of sera in this study, our findings do not seem to be in agreement with those of Pier et al. (59), who found that mice and rats immunized with 1 to 10 μg of MEP (alginate), respectively, elicited opsonizing antibodies, which reduced the chronic lung infection and induced complete bacterial clearance compared with the results in nonimmune controls. However, doses of ≥40 μg per mouse and 100 μg per rat induced only nonopsonic antibodies, resulting in more severe lung damage and more animals with detectable *P. aeruginosa* in the lungs, when compared with results for animals immunized with low doses. Furthermore, it was found that in mice, a higher-molecular-weight preparation of MEP elicited opsonic killing antibodies over a wide dose range (1 to 400 μg) (23). We have no obvious explanation for the differences between our results and those of Pier et al.

Woods and Bryan (67) immunized rats twice with 100 μg of alginate and showed that animals inoculated with a nonmucoid strain cleared the bacteria whereas the rats inoculated with a mucoid strain remained infected on day 30. These findings are also different from ours, since we found that rats immunized with either 140 μg of purified alginate or 25 μg of depolymerized alginate were able to clear the mucoid challenge strain. The severity of the pathologic changes observed in our rats immunized with alginate could be due to hypersensitivity reactions, e.g., immune complexes formed during the chronic lung infection, as suggested by Woods and Bryan (67).

LPS was included in two of the vaccines used in the study, the O-PS toxin A conjugate and the *P. aeruginosa* sonicate. The number of cross-reactive IgM, IgG, and IgA antibodies elicited by the toxin A conjugate against PAO 579 LPS was small. Considering the O antigens involved (43), this is surprising since two of the eight serotypes (immunotypes IT-3 and IT-7) represented in the vaccine have a similar O group 2/5 to that of the antigen used in the ELISA, and we would therefore expect a certain cross-reactivity. The low level of cross-reacting antibodies could be due to differences in the typing antisera and typing methods, which may lead to apparently similar Habs O-groups with antigenically different LPS types (21). The O-PS toxin A conjugate vaccine has previously been used (63) in a clinical study of noncolonized CF patients. It is thought-provoking that the antibody responses are low in rats whereas the vaccine induces high and functional antibody levels when used in humans (60). When rats were immunized with a combination of the two toxin A conjugate vaccines (group 6), diminished immune responses were recorded against all antigens used. Since both vaccines have previously evoked high titers of IgG antibodies against alginate, LPS, and toxin A in healthy volunteers (11, 12), an immunologic interference may have occurred in the rats, resulting in an "antagonistic" antibody response. The reason for this is unclear but may be related to immunosuppressive effects of some *P. aeruginosa* LPSs (20).

Toxin A is known to be an important virulence factor, and when it is secreted by *P. aeruginosa*, it has been shown to be associated with severe bronchial inflammation and parenchymal changes (68). Anti-toxin A antibodies could be demonstrated in all rats immunized with toxin A conjugated vaccines. However, toxin A did not significantly improve the lung abnormalities when compared with the sterile saline controls.

The protective capability of the LPS vaccine (O-PS toxin A) used in the present study is not completely in accordance with the findings of Pennington et al. (56). We found that rats immunized with LPS-containing *P. aeruginosa* sonicate had more severe lung damage and reduced bacterial elimination but had higher antibody titers directed against most of the antigens used in the ELISAs. The severe abnormalities and reduced clearance could be due to hypersensitivity reactions, e.g., immune complexes (32, 67), of which there was no evidence in the study performed by Pennington et al. (56).

The more severe macroscopic abnormalities observed among our immunized rats fit well with the observations of Langford and Hiller (42), who found that vaccination of noninfected CF patients with a polyvalent *P. aeruginosa* vaccine induced more rapid deterioration than that found in nonvaccinated controls. The accelerated course of the disease in vaccinated patients may also be explained by hypersensitivity reactions, e.g., immune complex-mediated lung tissue damage, which occurs during chronic lung infection in CF (32, 50, 52).

In conclusion, this study shows that none of the vaccines used could completely prevent chronic lung inflammation 4 weeks after challenge with *P. aeruginosa*-containing alginate beads. In all immunized rats and the IFA control group, we succeeded in changing the inflammatory response from an acute-type inflammation dominated by PMN leukocytes as in CF patients to a chronic-type inflammation dominated by mononuclear leukocytes. The altered abnormalities in immunized rats and the improved bacterial clearance might be of great advantage in future management of CF patients, since the ongoing lung tissue damage has been shown to be caused by elastase (26, 31, 46, 47, 65, 66) secreted by PMNs, which dominate the chronic *P. aeruginosa* lung infection in CF patients.

ACKNOWLEDGMENTS

The expert technical assistance of Ellen Frederiksen and Jette Pedersen is greatly appreciated.

This study was supported by grants from the King Christian IX and Queen Louise Jubilee Foundation; Danish Hospital Foundation for Medical Research of Copenhagen; The Faroe Islands and Greenland grants 54/91, 47/92 and 48/93; Director Jacob Madsen and Olga Madsen's Foundation grants 2029/1992 and 2272/1993; Ville Heises Foundation grant 5/92; the King Christian X Foundation; and Queen Louise Research Foundation grant 8/92.

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