Eur Respir J 1999; 13: 565–570
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European Respiratory Journal
ISSN 0903-1936

Reduction in the adherence of *Pseudomonas aeruginosa* to native cystic fibrosis epithelium with anti-asialoGM1 antibody and neuraminidase inhibition

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Reduction in the adherence of Pseudomonas aeruginosa *to native cystic fibrosis epithelium with anti-asialoGM1 antibody and neuraminidase inhibition. J. Davies, A. Dewar, A. Bush, T. Pitt, D. Gruenert, D.M. Geddes, E.W.F.W. Alton. ©ERS Journals Ltd 1999.*

ABSTRACT: The high incidence of colonization of the cystic fibrosis (CF) airway with *Pseudomonas aeruginosa* has been attributed to several mechanisms including increased numbers of asialoglycolipid receptors, which may be further increased by exposure to the bacterial exoproduct, neuraminidase. This study examined whether the adherence of *P. aeruginosa* to fresh CF respiratory epithelial cells can be reduced *in vitro* by anti-asialoGM1 (anti-aGM1) antibody, neuraminidase inhibition, or the use of asialoGM1 tetrasaccharide as a competitive inhibitor.

CF nasal epithelial cells were incubated with a nonmucoid strain of *P. aeruginosa*, in the presence or absence of a polyclonal anti-aGM1 antibody, the neuraminidase inhibitor 2,3-dehydro-2-deoxy-*N*-acetyl-neuraminic acid (DANA), or the tetrasaccharide moiety of aGM1. Adherence of bacteria to the apical surface of ciliated epithelial cells was quantified using scanning electron microscopy. Incubation of the cells with bacteria in the presence of either anti-aGM1 antibody or DANA significantly reduced bacterial adherence by 51(7)%, ($p<0.01$), and 34(9)%, ($p<0.01$), respectively. In contrast, no significant effect on *P. aeruginosa* binding was seen in the presence of aGM1 tetrasaccharide.

The data are consistent with previous studies on cultured cells, and suggest that the *in vivo* effects of such interventions should be explored as potential mechanisms to reduce *Pseudomonas aeruginosa* colonization in cystic fibrosis.

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Keywords: AsialoGM1, cystic fibrosis, neuraminidase, *Pseudomonas aeruginosa*

Received: June 18 1998
Accepted after revision November 8 1998

Supported by the Cystic Fibrosis Research Trust, the Association Française contre la Lutte Mucoviscidose (ARTEMIS project), the Medical Research Council, and a Wellcome Trust Senior Clinical Fellowship (E.W.F.W. Alton).

The characteristic pulmonary manifestations of cystic fibrosis (CF) are chronic bacterial infection and inflammation progressing to bronchiectasis and death from respiratory failure in >90% of patients [1]. The underlying defect relates to a mutation in the CF transmembrane conductance regulator (CFTR) protein, resulting in a reduction in the normal cyclic adenosine monophosphate (cAMP)-mediated chloride permeability of epithelial cells [2]. However, the relationship between this and the clinical features of the disease remains incompletely defined. Infection arises early, with a characteristically narrow range of bacterial pathogens, typically *Staphylococcus aureus* and *Haemophilus influenzae* in infancy and early childhood, and *Pseudomonas aeruginosa* later in the course of the disease. *P. aeruginosa* is thought to be responsible for the majority of pulmonary damage seen in CF. Acquisition is related to a decline in lung function [3], and, once present in the lower respiratory tract, it is rarely eradicated, despite an intense host inflammatory response. Rather, chronic colonization occurs in 80% of CF patients by the time of adolescence [4], despite it remaining a relatively rare pathogen in other forms of obstructive lung disease. This has led to several hypotheses regarding the predisposition of the CF airway to this organism.

Adherence to cell surfaces, thought to be the initiating step in many bacterial infections, has been shown in the majority of studies on *P. aeruginosa*, to be significantly greater to cells of CF origin than to non-CF cells [5–9]. A prior study confirmed that this was related to the absence of normal CFTR, since *in vitro* liposome-mediated *CFTR* gene transfer that corrects the chloride transport defect also significantly reduces *P. aeruginosa* adherence to fresh respiratory epithelial cells [9]. Pilin, the major adhesin for *P. aeruginosa*, adheres to cell surfaces *via* the Ga1NAc β 1–4 Gal moiety of certain asialylated glycolipids [6] including asialoGM1, which has been detected in increased amounts on the surface of CF respiratory epithelial cells [7]. In addition to the baseline increase in aGM1 levels on CF cells, the *P. aeruginosa* exoproduct, neuraminidase, may further increase the availability of such receptors by cleaving terminal sialic acid residues from cell surface gangliosides. Increased *P. aeruginosa* adherence has been reported after exposure of cultured epithelial cells to bacterial exoproducts or purified neuraminidase [5, 10].

Two recent reports have suggested mechanisms other than the increased availability of receptors to explain the high rate of *P. aeruginosa* colonization in CF. SMITH *et al.* [11] described the inhibition of naturally occurring epithelial

antimicrobial peptides by the high salt concentration found in CF airway surface fluid (ASF), and demonstrated restoration of bacterial killing ability after adenovirus-mediated *CFTR* gene transfer [11]. The human β-*defensin 1* gene has now been cloned [12]. However, other workers have failed to demonstrate an increase in ASF salt content in CF [13], and the clinical significance of this mechanism therefore remains to be determined. PIER *et al.* [14] described defective ingestion and elimination of *P. aeruginosa* by immortalized CF cells of the ΔF508 genotype [14], and postulated a role in normal pulmonary defence of ingestion and clearing of bacteria. It remains uncertain to what extent each of these mechanisms, or others as yet undescribed, contribute to the high colonization rate of the CF airway with *P. aeruginosa*.

As *P. aeruginosa* plays such a major role in the aetiology of CF lung damage, and cell adherence is thought to be an initial step in establishing the majority of bacterial infections [15], mechanisms to reduce adherence may be of therapeutic benefit. The potential of several such mechanisms was tested: an antibody to the aGM1 receptor, a neuraminidase-inhibitor, and exogenous aGM1 tetrasaccharide, (containing the Ga1NAc β 1–4 Gal receptor) to block adherence of piliated *P. aeruginosa* to freshly-obtained, non-cultured CF epithelial cells.

Materials and methods

All chemicals were obtained from Sigma, Poole, Dorset, UK, unless otherwise stated, and were of AnalaR grade or the best available.

Epithelial cells

Cell samples were examined from a total of 20 adult patients attending the CF clinic. All patients were chronically colonized with *P. aeruginosa*, although none was acutely unwell at the time of sampling. Seven patients were homozygous for the ΔF508 mutation, five were compound heterozygotes with two identified mutations (three ΔF508/G542X, one ΔF508/R117H, and one ΔF508/R347P), four were compound heterozygotes with ΔF508 and an unknown mutation, and four patients had not been genotyped. Nasal epithelial cells were obtained from the inferior surface of the inferior turbinate by brushing with a 3-mm cytology brush (Diagmed, Thirsk, UK) without the use of topical anaesthesia. Cells combined from both nostrils were suspended in 1 mL of Modified Eagle′s Medium (MEM; GIBCO, Paisley UK), and washed 2–3 times in cold phosphate-buffered saline (PBS) to remove mucus. Cell number was estimated from a 20 μL aliquot placed on a haemocytometer; typically 4×10^5–10×10^5 cells were obtained.

CFNPE9o cells [16] were cultured for 48 h to 30% confluence in MEM containing 10% foetal calf serum, 1% penicillin/streptomycin, 1% L-glutamine, and 1% nonessential amino-acids. Monolayers were trypsinized for 5–10 min with 20 mL trypsin/ethylenediaminetetraacetic acid, following which 2 mL foetal calf serum was added, and cells were washed in PBS. Aliquots containing approximately 2.5×10^5 cells were used for experiments.

Bacterial strains and culture

Two laboratory reference strains of *P. aeruginosa*, international antigenic typing scheme (IATS) serotype 0:1 (NCTC 11440, ATCC 3348) [17] and *P. aeruginosa* strain K (ATCC 25102) [18], were used. These are nonmucoid strains, chosen since they are believed to be representative of the initial colonizing phenotype in CF subjects [19], and piliated so as to allow assessment of pilin-mediated adherence mechanisms. The bacteria were maintained at 4°C on soft agar slants, plated onto blood agar, and subcultured in tryptone soya broth (TSB) (Unipath Ltd, Basingstoke, UK). Broths were either cultured for 36–48 h, and then applied to cells whilst in the culture broth, or were cultured overnight, pelleted by centrifugation ($5{,}000 \times g$, 10 min), and resuspended in PBS. In resuspended samples, bacterial concentration was estimated by spectrophotometry and colony counts, as described previously [9].

Confirmation of bacterial piliation status

The presence of pili was examined by negative staining using transmission electron microscopy. Bacteria were removed from agar plates, diluted in distilled water and a 10 μL drop placed on a formvar/carbon-coated grid (Agar Scientific Ltd, Stanstead, UK) for 5 min. The suspension was then removed with filter paper, the grids allowed to dry, stained with 0.65% sodium phosphotungstate for 1 min, and examined under the transmission electron microscope for the presence of pili.

Incubation conditions

Cells from each patient were divided into aliquots and either incubated with bacteria alone or with one of the following treatments:

Anti-asialoGM1 antibody. Polyclonal rabbit antibodies to aGM1 (Wako Chemicals, Neuss, Germany) and to mouse serum (Sigma) were incubated with the cells for 1 h at 37°C, at 1:10 dilutions in 1% bovine serum albumin (BSA), based on previous published data [6]. Cells were washed twice by centrifugation in 1 mL PBS, prior to incubation with 200 μL bacterial broth at 37°C for 3 h.

*2,3-dehydro-2-deoxy-*N*-acetyl-neuraminic acid.* DANA (Boehringer Mannheim, Mannheim, Germany), a broad-spectrum neuraminidase inhibitor, was used at a final concentration of 50 μM, based on previous published data related to other neuraminidases [20, 21]. The DANA was added to cell samples at the same time as application of 200 μL bacterial broth, and incubated for 3 h at 37°C. Control samples were incubated with broth alone.

AsialoGM1 tetrasaccharide. The tetrasaccharide of aGM1 (Biocarb, Lund, Sweden) was used at a final concentration of 250 μM in PBS, based on previous experimental data [7]. Primary nasal epithelial cells or CFNPE9o cells were incubated at 37°C with 50 μL washed bacteria (approximately 2.5×10^8 organisms) in the presence or absence of the tetrasaccharide.

Processing of samples

Samples were agitated gently, layered onto a 50% Percoll gradient (Pharmacia Biotech, St Albans, UK), and centrifuged ($20{,}000 \times g$) for 30 min at 4°C as previously described [9]. Nonadherent bacteria and red blood cells formed bands near the base of the centrifuge tube, leaving the epithelial cells with adherent bacteria at the Percoll/PBS interface. These were gently removed and cytospun for 2 min at $20 \times g$ onto a thermanox coverslip (Emitech, Ashford, Kent, UK). Cells were fixed for at least 1 h with 2.5% cacodylate-buffered glutaraldehyde, washed in sodium cacodylate buffer, and dehydrated with serial concentrations of ethanol followed by 50% and 100% hexamethyldisilazane (TAAB, Aldermaston, UK). The coverslips were mounted onto aluminium stubs, coated with gold, and viewed using a field emission scanning electron microscope (400 S; Hitachi, Mountain View, CA, USA). In each of the described studies, samples were coded to ensure that the investigator remained blinded as to the treatment received. Bacteria were quantitated on the naturally exposed (apical) surface of each ciliated cell within a field of view, when adherence was clearly seen to be directly to the cell surface. Quantitation was not performed for nonciliated or squamous cells due to difficulties in differentiating such cells with certainty from mucus, and an inability to identify the apical and basolateral surfaces. The mean (SEM) number of cells counted per sample was 43(2).

Statistics

Adherence values are expressed as a percentage of the mean for each strain of bacteria to allow grouping of data from both strains. Effects of interventions are described as mean (SEM) percentage reduction from baseline on paired samples. Statistical significance was determined using the Wilcoxon signed-rank test. The null hypothesis was rejected at $p<0.05$.

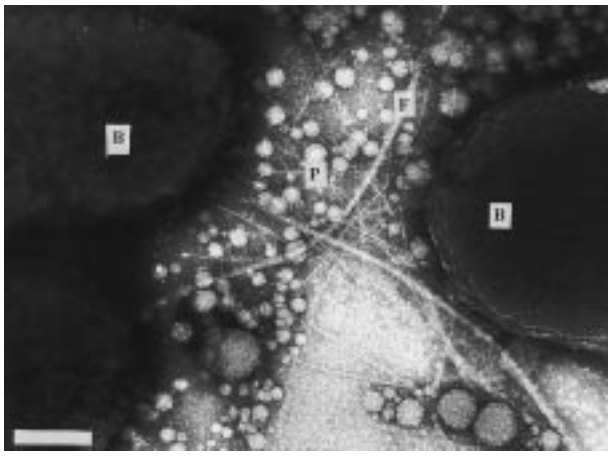

Fig. 1. – Transmission electron micrograph (TEM) of a standard culture of *Pseudomonas aeruginosa* negatively stained with 0.65% sodium phosphotungstate. Bacteria (B) possess both pili (P) and flagellae (F). Bacteria cultured directly from frozen were nonpiliated upon TEM examination, and demonstrated significantly lower levels of adherence than those cultured under standard conditions. Internal scale bar = 200 nm.

Ethical approval

The study was approved by the Ethics Committee of the Royal Brompton National Heart and Lung Hospital and informed consent was obtained from each participant prior to nasal brushing.

Results

Presence of bacterial pili

The presence of bacterial pili was found in early experiments to depend crucially on culture conditions. Bacteria (serotype 0:1) cultured overnight in TSB from frozen stock were nonpiliated and adhered at levels of <50% of those seen previously with this strain ($p<0.005$, data not shown), whereas storage at 4°C and subculture consistently yielded piliated organisms (fig. 1). Culture conditions did not have an effect on the expression of flagellae. Only nonfrozen, piliated cultures were used for adherence experiments.

Bacterial adherence

Anti-asialoGM1 antibody. Of 12 samples pre-incubated with anti-aGM1 antibody, the mean reduction in bacterial adherence compared with baseline was 51(7)% ($p<0.01$; fig. 2). Inhibition was variable, ranging 2–82% of baseline. For six samples incubated with control anti-mouse antibody, no change from baseline was seen (data not shown).

2,3-dehydro-2-deoxy-N-*acetyl-neuraminic acid.* Incubation of cells from 12 patients with bacterial broth containing 50 mM DANA resulted in a significant reduction in adherence compared with broth alone (34(9)%, $p<0.01$; fig. 3).

There was again a wide range of inhibition (18% increase–73% decrease). The two samples demonstrating the highest baseline values (173 and 192% of the strain mean) also demonstrated the greatest degree of inhibition (73 and 66%, respectively) after application of DANA.

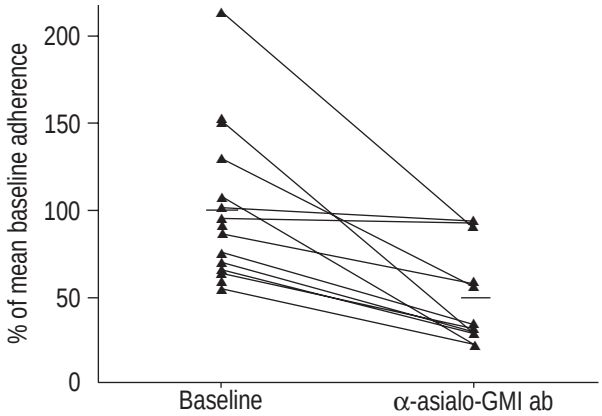

Fig. 2. – Significant ($p<0.01$) reduction in numbers of bacteria adhering to the apical surface of ciliated cystic fibrosis nasal epithelial cells after pre-incubation with anti-asialoGM1 antibody (n=12 paired samples, horizontal lines indicate mean values). Results are expressed as percentage of the mean baseline adherence, with a mean reduction of 51 (7)%.

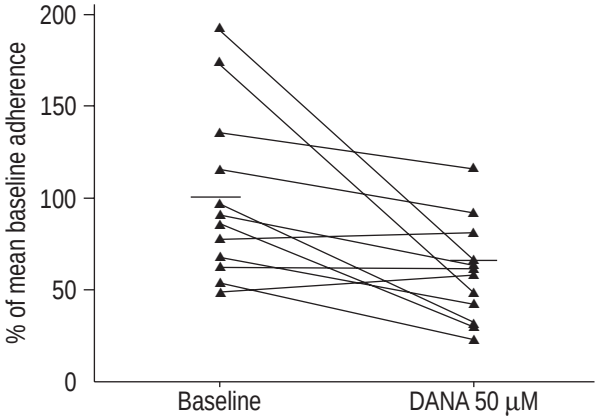

Fig. 3. – Reduction in bacterial adherence (mean 35 (9)%) when nasal epithelial cells in bacterial culture broth were exposed to 50 μm 2,3-dehydro-2-deoxy-*N*-acetyl-neuraminic acid (DANA) (n=12 paired samples, horizontal line indicate mean values, $p<0.01$).

AsialoGM1 tetrasaccharide. There was no significant effect of aGM1 tetrasaccharide (250 μM) on bacterial adherence to eight paired samples of nasal epithelial cells (fig. 4). Owing to the wide variability seen, three paired samples of CFNPE9o cells were studied to remove any possible effect of mucus. Again, no effect was seen (fig. 4).

Discussion

This study demonstrates that the increased binding of piliated, nonmucoid *P. aeruginosa* to fresh, noncultured CF respiratory cells can be significantly reduced *in vitro* either by blocking the cell-surface receptor, aGM1, or by inhibition of the bacterial exoproduct, neuraminidase. It was not possible, however, to reduce adherence by applying exogenous aGM1 tetrasaccharide.

Various mechanisms have been proposed to account for the high *P. aeruginosa* colonization rates in CF. Several previous studies have demonstrated increased adherence of *P. aeruginosa* to CF epithelial cells [5–9]. Pilin is the major *P. aeruginosa* adhesin and studies using isogenic nonpiliated mutants have demonstrated up to 50% less adherence [22, 23]. Both pili and exo-enzyme-S [24] adhere to the cell surface *via* a disaccharide, GalNAc β 1–4 Gal, which is present on certain asialylated glycolipids, but not their sialic acid containing counterparts. Thus, the finding of increased levels of aGM1 on cultured CF cells [7] provides a possible explanation for increased adherence. A prior study has shown by liposome-mediated *CFTR* gene transfer that the observed increase in adherence is related to the absence of normal CFTR [9]. The link between this and the enzymatic abnormalities leading to defective sialylation is not yet completely understood as data from published studies conflict [10–12].

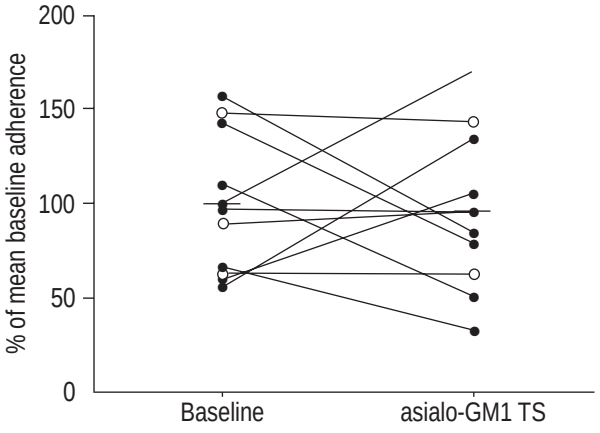

Fig. 4. – Nasal epithelial cells (●, n=8 paired samples) and CFNPEo- cells (○, n=3) incubated with *Pseudomonas aeruginosa* in the presence or absence of asialoGM1 tetrasaccharide (250 μM). No significant reduction was seen, with a highly variable effect on binding to the nasal cells. Horizontal lines indicate mean values.

As well as the increased numbers of asialoglycolipids occurring on CF cells, DE BENTZMAN *et al.* [25] have demonstrated increased asialo sites on damaged and repairing epithelium. As the majority of CF patients will have experienced lower respiratory infections with both viruses and other bacterial pathogens before acquiring *P. aeruginosa*, the expression of receptors on repairing epithelium may play an important contributory role in encouraging adherence of this organism. The blocking of such receptors would be expected to reduce adherence of the bacteria, and could be of benefit in inhibiting the initiation of infection. However, recent work has demonstrated the production of the pro-inflammatory cytokine interleukin IL-8 by cultured respiratory epithelial cells, upon binding either of *P. aeruginosa*, purified pilin, or anti-aGM1 antibody [26]. As excess inflammation is characteristic of the CF airway, and a pro-inflammatory intervention would clearly be undesirable, further study is required to assess the response of fresh, noncultured cells in this respect.

The removal of sialic acid from cell surface gangliosides could further encourage bacterial binding to newly exposed asialo sites. Neuraminidase, which cleaves terminally-positioned sialic acid residues, is produced by many bacteria and viruses, and is recognized as pathogenic in certain of these [27]. It may be relevant that the gene encoding neuraminidase expression, *nanA*, is upregulated under conditions of hyperosmolarity [10]. Although data confirming the role of the enzyme *in vivo* are lacking, CF airway fluid [28, 29] may encourage *nanA* expression. Thus, there may be a potential role for neuraminidase inhibitors, which have been shown to be of benefit in limiting the pathogenicity of influenza virus [30], in inhibiting colonization of the CF airway.

The demonstration that a disaccharide portion of aGM1 is a receptor for *P. aeruginosa* adherence raises the possibility of using this sugar as a competitive inhibitor to binding. This concept has been developed for certain other infectious agents, and has been applied with success in animal models of *Escherichia coli*-induced diarrhoea [31]. IMUNDO *et al.* [7] demonstrated a dose-dependent reduction in adherence of nonmucoid *P. aeruginosa* to small numbers of immortalized CF bronchial cells with aGM1 tetrasaccharide [7], although other work has shown that the lipid portion of the ganglioside may be necessary for binding [32]. The potential for lipid moieties to incorporate into cell membranes and create neoreceptors may limit the clinical feasibility of such an approach.

The effect of each of the above interventions on the adherence of nonmucoid, piliated *P. aeruginosa* to fresh, noncultured CF respiratory epithelial cells was assessed. This confirmed that at least 50% of the adherence of

piliated *P. aeruginosa* to CF cells is mediated through aGM1 receptors, and that inhibition of adherence can be achieved by blocking such sites with antibody. A significant reduction in binding was seen with the sialic acid analogue DANA, a highly-specific neuraminidase inhibitor. It is interesting to note that the greatest degree of inhibition by DANA was seen on those samples with the highest levels of baseline adherence, suggesting that this high baseline may have been as a result of neuraminidase-induced exposure of increased asialo receptors. The question of whether or not this enzyme is present and contributes to disease pathogenesis in the pseudomonas-colonized CF airway should now be assessed as, were this the case, neuraminidase inhibitors, such as those currently effective in clinical influenza trials, could be of therapeutic value.

In contrast to a previous report describing inhibition of pseudomonas binding to immortalized CF bronchial cells with aGM1 tetrasaccharide, and despite using the maximum concentration reported in that study [7], no consistent inhibitory effect of the sugar on binding to fresh non-cultured cells was found in the present study. In fact, in some samples, the presence of tetrasaccharide appeared to increase the numbers of adherent bacteria. It has been previously suggested that whole aGM1 could, by incorporating into the extracellular matrix, create neoreceptors. This raises the possibility that the tetrasaccharide could similarly bind to the cell and increase adherence in a variable fashion. Furthermore, although the GalNAc β 1–4 Gal disaccharide is known to be the binding portion of the aGM1 molecule, other authors have reported a lack of binding in the absence of the ceramide/long chain fatty acid portion of the glycolipid [32]. The reason for this is unclear. However, these results and the present data suggest that attempts to decrease adherence *in vivo* with this approach may be complex and will require further study.

In view of the clinical significance of *Pseudomonas aeruginosa* in the pathogenesis of cystic fibrosis lung disease, mechanisms whereby the initial colonization could be prevented or reduced may play an important role. The aetiology of lung infection in cystic fibrosis is complex, and the relative contribution of mechanisms including impaired mucociliary clearance, increased bacterial receptors, and defects in bacterial clearance both by internalization and defensin-like action, remain to be elucidated. The demonstration of two mechanisms which reduced bacterial adherence *in vitro* suggests that these should be further investigated with regard to the potential therapeutic use of such approaches to reduce or delay pulmonary colonization in cystic fibrosis.

Acknowledgements. The authors would like to thank the volunteers for their help in obtaining the samples, and R. Bethell for helpful discussion.

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