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Oral non-typable *Haemophilus influenzae* enhances physiological mechanism of airways protection

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Summary

Oral immunotherapy with inactivated non-typeable *Haemophilus influenzae* (NTHi) prevents exacerbations of chronic obstructive pulmonary disease, but the mechanism is unclear. The aim of this study was to determine the mechanism of protection. This was a placebo *versus* active prospective study over 3 months in 64 smokers. The active treatment was three courses of oral NTHi given at monthly intervals, followed by measurement of bacteriological and immunological parameters. The results can be summarized: (i) NTHi-specific T cells increased in the placebo treatment group over time ($P < 0{\cdot}05$); (ii) the T cell response in the oral NTHi group started earlier than that in the placebo group ($P < 0{\cdot}05$); and (iii) serum NTHi-specific immunoglobulin (Ig)G had significantly greater variation in the placebo group ($P < 0{\cdot}0001$). The increase in antibody in placebos over time correlated with exposure to live *H. influenzae* ($P < 0{\cdot}05$) determined from culture of gargles; (iv) reduction in saliva lysozyme over time ($P < 0{\cdot}05$) was detected only in the oral NTHi treatment group. These data are consistent with T cell priming of gut lymphoid tissue by aspiration of bronchus content into the gut, with oral immunotherapy augmenting this process leading to enhanced bronchus protection. The evidence for protection was a stable IgG antibody level through the study in the oral NTHi treatment group, contrasting with an increase in antibody correlating with exposure of the airways to *H. influenzae* in the placebo group. Saliva lysozyme was a useful biomarker of mucosal inflammation, falling after oral NTHi consistent with a reduction in the level of intralumenal inflammation.

Keywords: bronchitis, chronic obstructive pulmonary disease, pulmonary emphysema

Introduction

Smoking and inhaled environmental toxins damage the airways, with about 20–25% developing significant chronic obstructive pulmonary disease (COPD) [1]. Those with frequent exacerbations have more rapid loss of lung function [1]. The cause of exacerbations remains controversial, although some estimate that 30–50% are due to bacterial infection [2–6]. The most frequently isolated bacteria from sputum in subjects with smoking-related lung disease are non-typeable *Haemophilus influenzae* (NTHi) [2–6], and when present this organism usually predominates [6–9]. Studies in rodent models have shown that T lymphocytes derived from the gut-associated lymphoid tissue (GALT) mediate protection within the airways [10–12]. Following intestinal immunization enhanced recruitment and activation of neutrophils occurs [12–14], increasing phagocytic capacity which in turn reduces the intrabronchial bacterial load. Intercurrent virus infection can uncouple this control process, causing an inappropriate and excessive influx of neutrophils detected clinically as purulent sputum [4,15]. To test these concepts in man and to identify a new therapeutic approach, an inactivated preparation of NTHi was given orally to subjects with recurrent episodes of acute bronchitis. Those taking NTHi had a reduction in the frequency of culture positive sputa [16] with, in one study, a reduction of colonizing density of three logs [17] and a reduction in frequency and severity of acute episodes [16–20], including those with severe chronic obstructive pulmonary disease [21]. This study aimed to examine both humoral and cellular response to oral NTHi immunization in an attempt to understand more clearly the mechanism of this protection.

Materials and methods

Subjects and experimental design

This was a single-site, placebo-controlled prospective study of smokers, to determine the mechanism of action of an orally administered preparation of inactivated non-typeable *H. influenzae*. Sixty-four subjects with a smoking history of at least 10 cigarettes per day for a minimum of 2 years were recruited, and randomized into active and placebo groups. The active group consisted of 32 subjects, 14 male and 18 female (mean age and range: 40 years; 18–64 years). The placebo group consisted of 32 subjects, 18 male and 14 female (mean age and range: 43 years; 20–26 years). The study protocol was reviewed and accepted by the ethics committee of the University of Newcastle and all subjects gave written informed consent. A sample size of 25 per group was calculated to allow detection of differences between treatment and placebo groups of 0·6 of a standard deviation for continuous measures (change in all immune markers) with 5% significance and 80% power. Assuming a 20% dropout, 32 were required per group. Sixty-four subjects were recruited and 60 completed the study. Active tablets contained 45 mg of lyophilized, formalin-killed NTHi (NTHi-164) and were enteric-coated for protection against stomach acid. The dosing schedule was: two tablets per day for 3 consecutive days with the course repeated at day 28 and day 56. Subjects attended seven visits at 2-week intervals between mid-July and mid-December.

Bacteriology

Gargles were collected for the detection and quantitation of *H. influenzae*. Identity was determined by X and V factor requirement tests. Quantitation was by serial dilution and plating on chocolate agar.

Anti-NTHi antibody

Haemophilus influenzae-specific immunoglobulin (Ig)G and IgA were measured by enzyme-linked immunosorbent assay (ELISA) where plates were coated with a polyvalent NTHi sonicate antigen preparation. The concentration of NTHi-specific IgG in ELISA units (EU)/ml in the samples was calculated from a standard curve prepared using an in-house pooled standard serum, and anti-human IgG horseradish peroxidase conjugate [Chemicon (Millipore), Billerica, MA, USA] to detect bound IgG, and anti-human IgA horseradish peroxidase conjugate (Chemicon) to detect bound IgA. The within-assay variation was < 5% and the between-assay variation was < 10%.

Lymphocyte stimulation

Blood leucocytes were cultured with an NTHi-164 sonicate antigen preparation at 1 or 10 μg/ml or with phytohaemagglutinin (PHA) as a positive control. On day 5 cultures were pulsed with [^{3}H]-thymidine and harvested onto glass fibre filters for determination of radioactivity in a beta counter. The mean ± standard error of the mean (s.e.m.) counts per minute were calculated for each triplicate set of wells. The stimulation index (SI) was calculated for each test stimulation: SI = [cpm (antigen-stimulated cultures)]/cpm unstimulated.

Lysozyme, lactoferrin quantitation, interferon (IFN)-γ, nitric oxide (NO)

Lysozyme and lactoferrin in saliva samples were measured by ELISA, where plates were coated with rabbit anti-human lysozyme capture antibody (Nordic Immunological Laboratories, Tilbery, the Netherlands) or goat anti-human lactoferrin capture antibody (Nordic), and bound lysozyme or lactoferrin was detected using peroxidase-conjugated rabbit anti-human lysozyme antibody (Nordic) or peroxidase-conjugated goat anti-human lactoferrin antibody (Nordic). The concentration of lysozyme or lactoferrin was calculated from standard curves prepared with standard human lysozyme (MP Biomedicals, Seven Hills, NSW, Australia) or standard human lactoferrin (Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia). IFN-γ was measured by ELISA. The concentration of IFN-γ was calculated from a standard curve prepared with standard recombinant human IFN-γ. Monocolonal anti-human IFN-γ capture antibody, monoclonal anti-human IFN-γ biotinylated detecting antibody (Becton Dickinson, North Ryde, NSW, Australia) and streptavidin–horseradish peroxidise conjugate (Chemicon) were used to measure the bound antibody. NO was measured by adding equal volumes of 1% sulphanilamide (p-Aminobenzene sulphonamide; Sigma) and 0·1% N-1-naphthyl ethylenediamine-dihydrochloride (NEDD), incubating for 5 min at room temperature and determining colour intensity at 560 nm immediately on a Bio-Rad 680 plate reader (Bio-Rad Australia, Gladesville, NSW, Australia). The concentration of NO in saliva samples was calculated from a standard curve prepared with a $NaNO_2$ standard.

Statistical analysis

An independent-sample *t*-test was used to compare any differences between groups at the $P < 0{\cdot}05$ level. In addition, at each time-point for each treatment group, a paired *t*-test gave an indicative comparison of the parameter value with the baseline value, for each group individually and for all groups combined. Where more sensitive statistical testing seemed appropriate, as in analysis of *in vitro* lymphocyte proliferation, a repeated-measures model was fitted to the data, using SAS PROC MIXED.

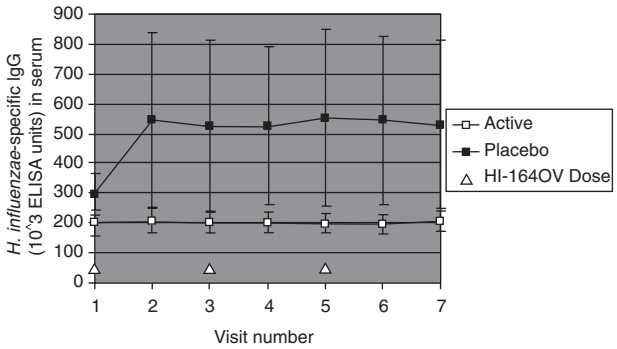

Fig. 1. Serum non-typeable *Haemophilus influenzae* (NTHi)-specific immunoglobulin (Ig)G. The mean serum NTHi-specific IgG for active (□) and placebo (■) treatments groups is shown. Error bars are standard error of the mean.

Results

Description of study subjects

Two subjects from each group did not attend beyond visits 2 (one subject) or 4 (three subjects) and were excluded from analysis comparing values at visits 2–7 with baseline value at visit 1. They were also excluded from microbiological analysis. One subject (placebo group) was excluded from IgA antibody analysis due to IgA deficiency. There were adverse events identified in 31 subjects in each treatment group. Eight severe adverse events were reported, three in the active and five in the placebo group. Only four adverse events (three in the active group and one in the placebo group) of gastrointestinal upset were considered possibly related to the study drug.

Bacteriology

There were 31 *H. influenzae* positive gargles in 13 placebo subjects and 29 *H. influenzae* positive gargles in 14 active subjects, thought to demonstrate similar environmental exposure to *H. influenzae* in the two treatment groups. The mean level of *H. influenzae* in the positive gargles was similar between groups and was $1{\cdot}8 \pm 0{\cdot}4 \times 10^5$ in the placebo group and $1{\cdot}5 \pm 0{\cdot}3 \times 10^5$ in the active group, and was not significantly different between the two treatment groups at any point.

NTHi-specific antibody (Fig. 1)

Serum NTHi-specific IgG levels are shown in Fig. 1. Serum NTHi-specific IgG over time showed different patterns in the placebo and test treatment groups. Levels increased in the placebo group to twice those in the active group, but the dramatic difference was with respect to the variations in levels within groups, with the ratio of standard errors having a point estimate and 95% confidence interval of 1·88 (1·71, 2·08 $P < 0{\cdot}0001$). The serum NTHi-specific IgA profile showed no significant difference at any time-point, but the level was maintained in the active group where it fell slightly in the placebo group. The NTHi-specific IgG and IgA profile in saliva showed no significant differences either between treatments or in change over time.

Relationship between *H. influenzae* carriage and serum NTHi-specific IgG

As it was considered probable that the increases in NTHi-specific IgG levels in the placebo group were due to NTHi reaching the lower airways (and being prevented from reaching the lower airways in the active treatment group) the association between change in serum NTHi-specific IgG from baseline (visit 1) and *H. influenzae* carriage was investigated (Fig. 2). The median change in serum IgG from baseline at each visit in subjects who had low *H. influenzae* exposure (*H. influenzae* detected in nil or one gargle) and those who had high *H. influenzae* exposure (detected in two to six gargles) was plotted for placebo and active subjects (Fig. 2 below). The median was used due to high variability in the placebo group; however, plots using the mean appear to be similar (data not shown). Subjects in the placebo group in which *H. influenzae* was detected in two to six gargle samples had a higher level of median change in serum IgG and a greater change from baseline than those placebo subjects in which *H. influenzae* was detected in nil or one gargle; this difference was statistically significant at visits 6 ($P = 0{\cdot}005$) and 7 ($P = 0{\cdot}015$). In contrast, there was a decrease in the median change in IgG in the active group for subjects in whom *H influenzae* was detected in two to six gargle samples compared to those in whom *H. influenzae* was detected in nil or one sample. This was statistically

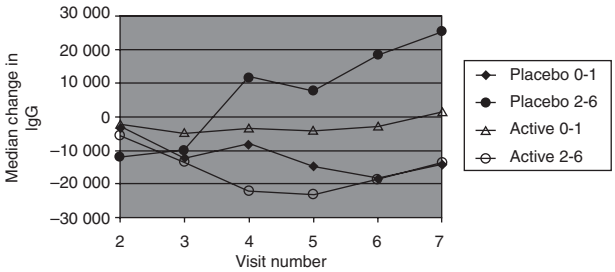

Fig. 2. Median change in serum immunoglobulin (Ig)G (EU/ml) for placebo subjects with *Haemophilus influenzae* detected in nil to one or two to six gargles. Relationship between *H. influenzae* carriage and serum non-typeable *H. influenzae* (NTHi)-specific IgG. The change (visit 7 minus visit 1) in median serum NTHi-specific IgG is plotted for active (□) or placebo (■) treatment groups for subjects with *H. influenzae* detected in nil or one gargle sample, or those with *H. influenzae* detected in two to six gargle samples. In the placebo group there were 23 subjects with nil to one and seven subjects with two to six positive gargles. In the HI-164-OV group there were 24 subjects with nil to one and six subjects with two to six positive gargles.

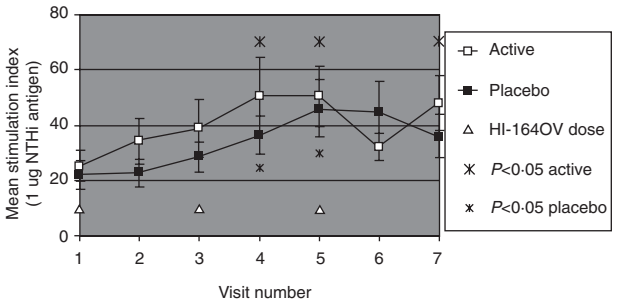

Fig. 3. Blood leucocyte stimulation *in vitro* with non-typeable *Haemophilus influenzae* (NTHi) antigen. The mean stimulation index for peripheral blood leucocytes in response to *in vitro* stimulation with 1 μg NTHi antigen/ml for placebo (■) and active (□) treatment groups is shown for each visit. The stimulation index (SI) for the active treatment group is significantly different ($P < 0{\cdot}05$) at visits 4, 5 and 7 to that at baseline (visit 1). The SI for the placebo treatment group is significantly different ($P < 0{\cdot}05$) at visits 4 and 5 to that at baseline (visit 1).

significant at visits 5 and 6 ($P = 0{\cdot}023$ and $P = 0{\cdot}026$, respectively). For subjects with *H. influenzae* detected in two to six gargles placebo subjects had a significantly higher median change in IgG level from baseline at visits 6 and 7 than HI-164OV subjects ($P = 0{\cdot}022$ and $P = 0{\cdot}032$, respectively). For subjects with *H. influenzae* detected at nil to one visits placebo subjects had a significantly lower median change in IgG from baseline than HI-164OV subjects. This was statistically significant at visits 5, 6 and 7 ($P = 0{\cdot}014$, $P = 0{\cdot}020$, $P = 0{\cdot}013$, respectively), suggesting that exposure to live NTHi in the placebo group leads to induction of NTHi-specific IgG antibody due to NTHi entering the lower airways, whereas such lower airway exposure appears to be prevented in the HI-164OV group.

Lymphocyte stimulation

Blood leucocyte stimulation with NTHi antigen at 1 μg/ml *in vitro* is shown in Fig. 3. The change in SI from baseline (visit 1) was significant for both active and control groups by visit 4 (for both groups $P < 0{\cdot}05$). The difference from baseline at the last visit (visit 7) was significant for the active group, but not for the placebo group. The SI data using a higher concentration of antigen (10 μg/ml) repeat this pattern, but with a greater response. When the 1 μg/ml and 10 μg/ml data were combined in a repeated-measures model to assess 'shape' differences, a cubic relationship with time was found to fit the data well, with the active and placebo groups having significantly different shape profiles ($P < 0{\cdot}05$). In simple terms, this means that the active group has a significantly earlier proliferation response than the placebo group. The response in the active group is also characterized by a transient dip following the third course of NTHi tablets. The increase in *H. influenzae*-specific T cell response in the placebo group did not correlate with exposure to NTHi in the airways as measured by NTHi-positive gargles, which differs from the correlation noted for the IgG antibody response in this treatment group. PHA SIs for the oral NTHi group were significantly higher than baseline for visits 3, 4 and 5, but had fallen by visit 6. The response of the placebo group was lower and only significantly greater than baseline at visit 6. By visit 4 the NTHi dosed group had a significantly higher response than the placebo group. The NTHi group showed no 'dip' following the third course of oral NTHi (Fig. 4).

Parameters of inflammation

Analysis of lysozyme and lactoferrin data used a mixed model approach with two periods – an early period corresponding to visits up to and including visit 3 (i.e. completion of dosing), and a second period corresponding to visits 4–7 (period following completion of dosing). Lysozyme (Fig. 5) showed a significant decrease from the beginning of the trial to the last visit in the active treatment group ($P = 0{\cdot}05$), while

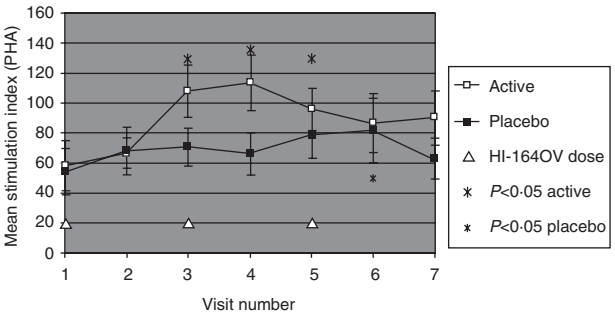

Fig. 4. Blood leucocyte stimulation *in vitro* with phytohaemagglutinin (PHA). The mean stimulation index for peripheral blood leucocytes in response to *in vitro* stimulation with PHA for placebo (■) and active (□) treatment groups is shown for each visit. The stimulation index (SI) for the active treatment group is significantly different ($P < 0{\cdot}05$) at visits 3 and 4 to that at baseline (visit 1). The SI for the placebo treatment group is significantly different ($P < 0{\cdot}05$) at visit 6 to that at baseline (visit 1).

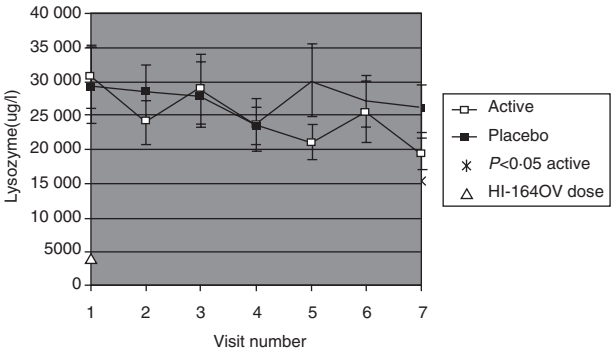

Fig. 5. Saliva lysozyme. The saliva lysozyme concentrations for placebo (■) and active (□) treatment groups are shown for each visit.

the placebo group showed no such reduction. Lactoferrin showed a similar profile over the seven visits (not shown), although this change was short of statistical significance. There were no significant changes in NO or IFN-γ levels in saliva between groups.

Discussion

Protection against acute bronchitis (defined as an increase in volume and purulence of sputum) following oral immunotherapy with NTHi has been demonstrated in smokers with both early airways disease [18] and those with severe COPD [21]. The current study of mechanisms of protection included established smokers to ensure a mucosal immune status similar to that of those with smoking-related airways disease, as smoking alters the mucosal response to oral antigen [22,23]. In this placebo-controlled study, several observations were made relevant to mechanisms of action of oral NTHi immunotherapy.

First, during the winter–spring period of the study, a significant increase occurred in antigen-induced T cell proliferation in the placebo group, reflecting exposure to NTHi antigen due to either inhalation of bacteria into terminal airways (where antigen can stimulate a systemic immune response) or aspiration of NTHi from the airways into the gut with uptake into Peyer's patches. Direct systemic stimulation of T cells is unlikely, as there is a dissociation of response profiles between the IgG antibody and T cell responses in the placebo group, and there was no correlation between T cell responders and exposure to inhaled NTHi (data not shown), as occurred for circulating IgG antibody (Fig. 2). Finally, in those immunized orally with NTHi, a similarly shaped response curve to that in the placebo group was detected, with no detectable increase in IgG antibody. These observations are most consistent with the seasonal increase in antigen-reactive T cells originating from the gut-associated lymphoid tissue (GALT), following delivery of aspirated NTHi antigen to Peyer's patches in the upper small bowel. Approximately 10^9–10^{11} live bacteria are swallowed each day in subjects with COPD [17]. Despite the stomach being a hostile environment for NTHi antigen (unpublished data), it appears that sufficient antigen enters the gut to stimulate a mucosal T cell response.

Secondly, there was a difference in the pattern of antigen-specific T cell stimulation between the placebo and active groups with an earlier, higher and more sustained increase in stimulation index in the active group, and a 'dip' following the third course of NTHi. The post-immunization 'dip' has also been noted following oral immunization of subjects with bronchiectasis [23] and attributed to 'trapping' of antigen-specific T cells within the GALT [24]. The absence of a similar 'dip' in proliferative response to the polyclonal T cell mitogen PHA is consistent with the more selective reduction of NTHi-specific T cells 'trapped' temporarily within the regional (mesenteric) lymph nodes. The transient enhanced response to PHA, following oral NTHi, is a commonly noted non-specific mitogenic effect of many complex antigenic preparations. Its presence is consistent with an additional level of immunization in the active group. These dynamics and effector T cell function at the level of the bronchus have been demonstrated in a rodent model using thoracic duct T cells from immunized mice transferred into naive recipients [10–12]. The absence of a mucosal IqA antibody response reflects the down-regulation of mucosal humoral immunity in smokers [22]. The pivotal role for gut-derived T helper type 17 (Th17) cells in mediating protection in the lung against bacterial infection would suggest that biomarkers related to Th17 cells and their secretion products would be appropriate to trace antigen-specific T cells and their activity in the human bronchus with more sensitivity and specificity [25].

Thirdly, a marked difference in pattern of IgG antibody in serum in the two groups was noted over the duration of the study. Organisms inhaled into the lung induce a brisk systemic antibody response [26]. In the active group the level of antibody was stable, less than half the level measured in placebos, and with error bars less than 20% of those not taking oral NTHi. The irregular and greater secretion of serum IgG antibody in the placebo group may reflect a variable presence of NTHi in less protected terminal airways. This suggestion is supported by the significant correlation between the frequency of detection of NTHi in gargles and the level of circulating IgG antibody in the placebo group. The significant negative correlation in the active group between more frequent isolation of NTHi in oral washings and serum IgG antibody may reflect a boosting of mucosal immunity by inhaled NTHi, a phenomenon that has been demonstrated in animal models [[27] and M. Dunkley, unpublished observations].

Lysozyme is an anti-bacterial factor in respiratory tract secretions that is elevated in COPD, especially those prone to recurrent exacerbations [28]. Demonstration that a significant reduction occurred only in the active group identifies lysozyme as a candidate biomarker for mucosal inflammation in the airways. The capacity of oral NTHi immunotherapy to reduce airways inflammation in smokers would be valuable, as progression of airways disease occurs in smokers with increased levels of CRP [29]. Currently available biomarkers, however, are imprecise and primarily of epidemiological value [30]. More formal monitoring of airways function over time, following oral NTHi with salivary lysozyme as a biomarker, is required to determine whether long-term control of airways inflammation can alter the noted decline in respiratory function in COPD irrespective of whether or not subjects continue to smoke. Recent claims that progressive inflammation and lung destruction correlate with anti-elastin autoimmunity [31] are challenged by the current findings suggesting that oral NTHi drives protective immunity and reduction of a biomarker of lumenal inflammation. Demonstration of

phenotypic changes in dendritic cells isolated from lungs of patients with emphysema, characterized by a capacity to induce Th1 and Th7 responses in CD4 T cells irrespective of whether or not the donor of the dendritic cells continued to smoke [32], is consistent with an appropriate micro-environment for colonizing bacteria to drive a damaging (hypersensitivity) response, unless controlled by immigrant gut-derived T cells.

Taken together, these results are consistent with the idea that oral NTHi enhances mucosal protection, limiting the amount of inhaled NTHi accessing the terminal airways in a smoking population. The significant reduction in level of lysozyme as a marker of lumenal inflammation in orally immunized subjects is of interest, as this biomarker has been noted to correlate with frequency of exacerbations in COPD [28]. Further studies using additional markers of airways colonization and inflammation are needed to substantiate mechanisms of protection induced by oral immunization, as well as consolidating the data supporting the idea of a central role for aspiration of bronchus content in activating the common mucosal system.

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Disclosure

Professor Clancy is a Director and Shareholder in Hunter Immunology Ltd, and Dr Dunkley is Chief Scientific Officer of that company.

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