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Editorial

New Editorial Board

The Journal came of age with the 1st issue in 1946. The main aim in commencing the Journal was the dissemination of all knowledge thought to be of interest and use to the members of the Association. This is still valid today. Right from its inception, the Journal has been peer-reviewed. Since 1956 members of the profession, as Journal representatives or as Editorial Board members, have assisted the Journal's Editor. Over the years, past Editors have selected these members for their specialist knowledge and/or their geographical laboratory location.

I took over as Editor from Marie Gillies in 1994 and selected as Editorial Board members, senior specialist medical laboratory scientists from throughout the country. This has continued up to the present date, except that from April 1999 these medical laboratory scientists have been the Special Interest Group conveners.

The main reason for our Journal, as do most international peer-reviewed journals, to have an Editorial Board is to call upon its members to act as referees, or suggest suitable referees, for submitted articles. Sadly, over the years our Journal does not attract many original articles. Lately the number of submitted articles average two to three per year.

Nothing in life is static, least of all the make-up of the Editorial Board, and I have decided it is time for change. For the last four years there has been a steady stream of members of our profession undertaking the Institute's Fellow qualification. This is the highest professional qualification set by the NZIMLS and a very high standard is set. Apart from candidates doing Fellowship by thesis, set at Masters by thesis standard, part 2 of Fellowship entails the writing of a treatise. Again, standards for the treatise has been set at a high level, as past and present candidates can testify to. This has resulted in a series of treatises published in the Journal that, in my opinion, are of a very high standard.

I have decided to utilise the special skills Fellows have in writing scientific papers/critical reviews by asking them to join the Editorial Board. As mentioned, we do not attract many article submissions, thus I have expanded the Editorial Board members roles. The following is what I have asked them to undertake:

- Act as referees, or nominate specialists, for submitted articles.
- To review submitted books from medical publishers (the reviewer gets to keep the book for their work place), or to suggest suitable reviewers.
- To suggest ways to improve the Journal.
- To suggest specialists the Editor can approach to write critical Review or Viewpoint Articles.
- To occasionally write Editorials on appropriate topics by invitation of the Editor
- To contribute to the Journal by submitting a Review, Viewpoint, or Original Article once during their three year term.
- To encourage members of the profession (they do not have to be members of the Institute) to contribute to the Journal. These are med lab scientists or QTAs from their work place, or from interesting presentations by these at SIG and other meetings.

As can be seen above, one of the requirements is for Board members to submit at least one original or critical review article during their term. Having produced excellent published treatises, I am sure that the readers can expect further excellent material from them in future editions of the Journal. These Fellow Board members will have an initial three years term, after this either being replaced by another Fellow, or asked to stay for a further period.

So, what will be the make-up of the new Editorial Board? Firstly, it will not be entirely new as I will be retaining Gordon Purdie, biostatistician from the Wellington School of Medicine, as Statistical Adviser. He will separately review any submitted articles containing substantive statistical analysis, or which in the view of the Editor or Editorial Board members needs statistical input. Also, since 1995 the co-Editors of the *Australian Journal of Medical Science* have been members of our Journal's Editorial Board under a reciprocal arrangement. This will continue as will my membership of their Editorial Board.

Four Fellows have accepted my invitation to join the Editorial Board. They are:

- Vanessa Thomson, FNZIMLS, Hawkes Bay
- Gloria Evans, MMLSc, FNZIMLS, Christchurch.
- Jenny Bennett, FNZIMLS, ESR, Porirua
- Jackie Wright, FNZIMLS, Nelson
- Ann Thornton, FNZIMLS, Wellington

Actually Ann Thornton is a present Board member, but she will now take on the additional role as Deputy Editor of the Journal. She will learn the ropes and assist me in the production of future issues. If for any reasons I am unable to produce an issue, she will have the experience to continue the Journal, which is now in its 57th annual volume. I welcome her wise council and look forward to working with her. This is made easier as she is only a few floors down from me.

Negotiations are continuing with a few other Fellows and I anticipate that the Board's list will increase next year. Look out for a more in-depth profile of the new Board members in the Journal next year. I anticipate that with these changes the Journal will continue its high profile and strengthen over the years. I wish to thank the immediate past Board members for their help and advise over the years.

I hope that members of the profession, and you don't have to be a NZIMLS member to publish in the Journal, will support the Journal by submitting original articles, research letters, and review articles. The Editor and Board members are more than willing to offer practical advise and assistance and certainly the new Board members, as well as the rest of the Board, have the experience to assist you if contacted.

Rob Siebers, MIBiol, FNZIC, FNZIMLS.
Editor.

Is latent iron deficiency as benign as once thought?

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Abstract

The aim of this review is to explore the area of latent iron deficiency that increases the haemoglobin concentration with iron supplements.

A literature search was conducted using Entrez-PubMed. The search terms used were "latent iron deficiency", "iron and performance" and "iron depletion without anaemia". The bibliographies of the retrieved articles, textbooks and the Internet yielded further references.

The iron deficient stage where the haemoglobin is in the normal reference range but increase with iron supplements represents a relative iron deficient anaemia. i.e. the haemoglobin is lower in comparison to the individual's personal 'norm'. It has been proposed by investigators to call this Stage IIIa iron deficient anaemia and classical iron deficient anaemia Stage IIIb.

As well as a trial dose of iron Stage IIIa may possibly be detected by increased zinc protoporphyrin, serum transferrin receptor and serum transferrin receptor : ferritin Index. Computer algorithms may be useful to detect variations from an individual's personal 'norm'.

The term functional anaemia has sometimes been used to describe Stage IIIa. The functional consequence most apparent is the impairment of physical exercise. The effect of Stage IIIa on cognitive and psychomotor development of infants is less convincing. Some studies show that the detrimental effect of iron deficient anaemia is irreversible.

Stage IIIa makes physiological sense. Treatment with supplements is recommended to improve and prevent functional consequences of iron deficiency. Further studies are recommended to investigate Stage IIIa specifically to reveal the prevalence and repercussions of this relative / functional anaemia.

Key words: iron deficiency, anaemia, latent

Introduction

Iron deficiency affects more people than any other condition, constituting a public health problem of pandemic proportions. The World Health Organization (WHO), estimates 66 - 80% of the world's population may be iron deficient and over 30% are anaemic. This equates to 4 - 5 billion people and 2 billion people respectively (1). Nine out of ten anaemia sufferers live in developing countries. Iron deficiency without anaemia, known as latent iron deficiency (LID), is quite common even in affluent groups (2) and in prosperous countries (3).

The aim of this paper is to explore the area of latent iron deficiency (LID) that increases haemoglobin concentration when supplemented. This investigation takes the form of a literature review looking at the classification of iron deficiency and measurable physical performance and mental developmental consequences related to the target group. Current laboratory analysis to detect and manage the study group is discussed.

Method

Entrez-PubMed was used to perform a literature search. Articles were sought using the terms: "latent iron deficiency", "iron and performance", "iron depletion without anaemia" that were published from 1966 to 31 December 2000. The search was limited to English language and human studies only and used the Boolean search method.

The search yielded 646 titles and abstracts, which were scanned; 366 had relevant articles. 87 were deemed relevant to review and were retrieved and examined fully. The bibliographies from the reviewed articles provided 42 additional articles that were obtained and reviewed fully. Of the 126 articles reviewed, 60 were excluded, as they either did not yield relevant data or additional information. Also examined were five recent textbooks, two of which were used, and the Internet site of the World Health Organization (WHO).

Results and discussion

Classic categorisation of iron deficiency

Iron metabolism is dynamic. Traditionally iron deficiency has been categorised into three overlapping stages (4-6). Various names have been used to indicate each iron deficient category (Table 1).

Table 1. Classic categorisation of iron deficiency

Stage	Pseudonyms used for stage
Stage I -	Pre latent iron deficiiency (Pre LID) Early negative iron balance Storage iron depletion Latent iron deficiency (LID)
Stage II	Tissue depletion of iron without anaemia Iron deficient erythropoiesis
Stage III	Iron deficient anaemia (IDA) increases with supplementation

Classically, normal has been defined as "not iron deficient", with ferritin and haemoglobin (Hb) in the normal reference ranges for age and gender. Whether this is optimal depends on the extent to which iron replenishment is considered desirable or if the Hb is truly maximal for that individual. The two major options for defining optimal iron nutrition are the correction of the more severe anaemic stage (IDA), or the correction of the milder iron deficiency (ID). Many favour the correction of IDA because they say there is a lack of substantial evidence that latent iron deficiency is associated with any overt functional problems (7). This view is in accord with the suggestion that it does not matter clinically, except in haemochromatosis, whether the iron stores are full or only half full (8).

The other option proposes there is a stage where iron status may be regarded as adequate when the IDA is corrected, but optimal when ID is corrected (7,9). It has been suggested that iron status be called "maximal iron repletion" when the ferritin is higher than the lower end of the normal range and Hb does not increase with supplementation (9). Others agree that optimal iron nutrition should include a small iron reserve (7).

The issue also depends on the definition of iron deficient anaemia. Stage III, the anaemic stage of iron deficiency, is defined by haemoglobin concentration cut-off values. A person is described as anaemic if their Hb falls below the normal range for their age and gender. However, what is often overlooked is that by using Hb reference ranges to differentiate normal and LID from the anaemic state it may not be adequate for some individuals whose Hb, though in the normal range, increases when supplemented with iron (9,10).

It is instructive to examine the methodology for the determination of the Hb reference range. The World Health Organization criteria have cut off values of 120 g/L for adult females, 130 g/L for adult males, and 110 g/L for pregnant females (12). It has been proposed that 117 g/L for females and 132 g/L for males may be more reasonable due to statistical problems caused by the overlapping of the normal and abnormal ranges. It is rationalised that because the cut-off point is usually assigned on the basis of a 95% normal range that 2.5% of the "normal" population lie below the cut-off value. This phenomenon, they say, tends to exaggerate an otherwise low prevalence. The use of mixed distribution analysis or the response to a test dose of iron is said to be more accurate (13).

Others agree that it is difficult to establish the lower limit of the reference range of Hb concentration, especially in adult females. However, their emphasis is opposite to the previous argument in that some persons have Hb in the normal reference range, and yet respond to a test dose of iron, demonstrating that there was a deficit that required treatment (3,10,14). In other words their iron deficiency anaemia is hidden - latent. It is this group that is the subject of this review.

The concept that haemoglobin in the context of latent iron deficiency may be abnormal for that individual does not appear to be a new one. Studies from as far back as 1883 have been cited in the literature (15). Beutler and colleagues (15) have been credited with the recognition of latent iron deficiency's (16).

The observations of iron therapy by Beutler and colleagues on a group of non-anaemic, iron deficient women displaying non-specific symptoms of iron deficiency, showed that relief of symptoms was higher in the iron-treated group than the placebo group. There was also an increase in the average Hb level in the iron deficient group given iron. It was concluded that although the Hb value was originally in the normal range, this improvement showed that it was "frequently below their 'norm'". There was no significant change in Hb in the iron-replete group when supplemented, which implies that Hb reaches a maximum set point for an individual when iron is replete. No significant increase in Hb was noted during placebo administration, which implies that the increase in Hb observed was due to iron supplementation (15). Similar results were reported in another study although in this case there was no specific effect on the symptoms of iron deficiency (17).

The concept that people have personal 'norms', i.e. a personal set point, is further suggested by studies looking at the reasons for the differences between male and female Hb levels. In females, the mean haemoglobin concentration remains lower than in males, even when females receive long-term iron medication. This difference is probably hormonal in origin and related to the stimulating effect of testosterone compounds on erythropoiesis. There seems to be a correlation between muscular development and hemoglobin concentration(3). The latter may also be a reason for intra-sex differences in Hb, as well as between males and females.

Iron and performance

What matters of course is whether there are any measurable consequences for a person whose Hb is below their personal 'norm' if the Hb is in the normal reference range. This review will concentrate on the physical endurance and cognitive performance.

Optimal physical performance is the central goal for the elite athlete. For this reason much of the literature involving iron deficiency, particularly LID and physical performance has investigated athletes, and has been the subject of several recent reviews (10,18-26). Two extensive reviews stand out as they have included many of the papers identified in the literature search for this article (22,23). These reviews both concluded that low iron alone in the absence of anaemia is not associated with reduced endurance performance.

Put another way, two things are clear. Firstly, even very mild anaemia caused by iron deficiency impairs maximal exercise capacity. A decrease in performance is proportional to loss of Hb concentration (10). For example a 20% decrease in treadmill endurance is reported in female subjects with Hb in levels of 110 to 119 g/L compared to females with a Hb greater than 130 g/L. i.e. a reduction in Hb of only 10-20 g/L (26). Secondly, no significant increase in work capacity has usually been noted in supplementation studies that have not shown an increase in Hb even though there were significant rises in ferritin (22). The target group for this review however is the group that increase their Hb within the normal reference range when given iron supplementation. No studies were found that directly investigated this group. Indirect evidence sometimes is noted as a confounding factor in studies that are endeavouring to isolate the effect of iron deficiency without anaemia on physical performance. This is because the group's performance also increases with increases in Hb as measured by maximal consumption of oxygen (VO_2max - an indicator of aerobic work capacity) (22).

Isolating the target group

Females have frequently been the subjects of studies as they have a high prevalence of LID (18). Most studies have used 120 g/L as the lower cut-off value for Hb reference range for women. When Hb is in the reference range conclusions differ regarding the response to iron supplementation and performance and often depend on the pre-therapy Hb concentration. Studies in females reporting increases in Hb with supplementation seem to have pre-therapy Hb in the low normal range (27), Hb 121±7g/L (28), 128±8g/L (29), 131±7g/L (30) and 122 ±4g/L (31). Studies that did not report Hb increase with supplementation had pre Hb values of 144±3g/L (32), 138±11g/L (31), and 147±7g/L (27). What can be said is that it is unlikely that there is any beneficial effect of iron supplementation in female athletes with baseline Hb levels of 138g/L or higher (27).

Does increasing haemoglobin in the normal reference range enhance performance?

Haematological manipulation, commonly known as blood doping, has helped elucidate the value of increasing Hb even within the normal range with a view to enhancing optimal physical performance. In theory aerobic endurance athletic activities are limited by oxygen delivery to the working muscle therefore, the greater the oxygen delivery capacity the greater the endurance. Increasing the Hb is one way to achieve this. It is estimated that 500mL of whole blood can add about 100mL of O_2 carrying capacity(25).

Experiments of blood loss and the subsequent enhancement of Hb by the reinfusion of red cells show an increase in the maximal aerobic power and physical performance. In this case it is of no importance if the subjects baseline Hb is 130 g/L or 170 g/L (21). The increase in maximal aerobic power per gram increase of Hb is similar to a studied Hb level of approximately 210 g/L (33). There is a linear relationship between Hb and work performance (34). In other words, the Hb effect is continuous, not threshold bound (35). The conventional use of cut-off values to identify anaemia assumes a threshold effect of Hb on

functional outcomes and therefore results in misclassification. The women in a recent study were not anaemic on the basis of Hb greater than a 120g/L cut-off value (35). Multiple regression analysis showed energetic efficiency was improved with increases in Hb. This data indicates that the cut-off value for functional anaemia may be much higher than 120 g/L for pre-menopausal women when their oxygen delivery system is stressed such as in the 15 km time trial of the experiment. The authors suggest that females with Hb concentrations as high as 132 g/L may still be functionally anaemic because their Hb concentrations are insufficient for optimal physical performance (35). A similar functional anaemia was found in a follow-up experiment (36).

The practical consequences of iron deficiency and performance can also be seen in the lives of manual workers. The linear effect of Hb and work performance has been demonstrated in practical situations. The results of decreased productivity from IDA are often reflected as economic loss. For instance, the take home pay packet of rubber tappers in one study was proportional to Hb concentration (37). Supplementation reduced the morbidity of infectious disease and so there was less worker absenteeism (37). It has been has been shown that with supplementation, workers improve productivity. The collection of more tealeaves per hour in one study (38) and the weeding of a greater area in another (37) demonstrated this. Once again the evidence for the target group of LID subjects, who's Hb improves with supplementation, is indirect because the studies used anaemic subjects. However, with the linear nature of Hb and work performance it is postulated that similar improvements in productivity would be noted for this reviewed target group.

Another observation on the effect of supplementation on IDA in workers on a mixed coffee and sugar plantation in Guatemala, as reported by their foreman, was that they were more "more willing, intelligent and effective" after supplementation. Whereas before the same individuals were considered to be "poor workers, lazy and stupid."(37).

Studies of work and performance of manual workers in developing countries also show the laboratory testing of workers using the Harvard Step Test to measure physical performance (5). The kind of short, near maximal effort required for some tasks, such as using an axe, hoe, or machete was severely impaired by IDA in Guatemalan agricultural workers (37). A review of these studies concluded that "even when individuals with reduced ability to perform maximal exercise, as judged by heart rate data, manage to compensate in productivity by working harder, they are under more physiological stress than the other subjects" (5).

A study in China of female cotton mill workers showed that production efficiency was demonstrated to parallel Hb concentration. When these women were supplemented with iron they were able to do work with lower energy expenditure (39). The same sort of energy efficiency and greater endurance with supplementation as has been described above in athletes with LID. Therefore, it could be postulated that manual workers with LID would find the same improvement.

Cognitive and psychomotor development

IDA alters both cognitive and psychomotor development in infants. Most studies were on the vulnerable 9-24 month age group. Various studies on the effect of iron on the mental and motor development in infants have been reviewed extensively (40-43). Most show no significant correlation between iron deficiency without anaemia, and poor performance on cognitive and psychomotor tests observed.

Pertinent to this review is the group of infants who responded to a therapeutic trial of iron with a significant rise in Hb (>10g/L) (20). Even though they had haemoglobin concentration below their personal 'norms', they were not below the standard reference range. This relative anaemia showed no tendency to lower developmental test scores (44).

One study of 9 to 12 month old infants used erythrocyte protoporphyrin to identify the stage of iron deficient erythropoiesis without anaemia. It showed that iron deficiency alone (ferritin <12 ng/L, Hb >110 g/L) did not affect developmental tests, but that they were affected by iron deficient erythropoiesis as detected by increased red cell protoporphyrin (45).

The main concern for infants is that when ID progresses to IDA, the adverse influences in the performance of developmental tests appear and persist in some studies, despite the normalising of Hb by iron therapy (46,47) even when it is extended to six months (48). However, other studies of 12 - 18 month infants have shown reversal of the effects of IDA, provided the anaemia is of short duration (49,50). It is suggested therefore, that timing, severity, and chronicity of the IDA may have an effect on later cognitive performance even when supplementation fixes the deficiency (51).

If it is true that the effects of IDA are irreversible and IDA is not prevented, it may affect the educational achievements and economic productivity of large numbers of people. The most vulnerable groups are already the poorest and least educated (1). So, although iron deficiency may be only one part of a complex problem with regard to development, it may be that if iron deficient anaemia is not prevented at an early age it may limit a person's potential, and contribute to continued poverty. With such uncertainty as to the reversibility of the cognitive and psychomotor developmental problems caused by IDA in infants, most investigators suggest erring on the side of caution and treating the LID stage.

There are very few studies of older children and adults concerning cognition and psychomotor development. However, one study of 9-11 year old children revealed an association between iron status and measures of intelligence (52). A recent study of non-anaemic, iron deficient adolescent girls showed that iron supplementation improved verbal learning and memory. Also noteworthy is the decrease in Hb in the placebo group. Over 6% of girls in this study developed IDA (53).

A longitudinal study of New Zealand adolescent girls showed that those who were anaemic at 11 years old were most likely to be anaemic at 21 years old (54). This suggests that anaemia and all its consequences may persist through teenage years. A further concern is that pregnant women, who are iron deficient at the beginning of their pregnancy, may not be able to respond to the demands of pregnancy if they have insufficient iron for erythropoiesis. This may affect the health of mother and baby (55).

Other consequences of iron deficiency are beyond the scope of this review. These include effects on immunity (56,57), thermogenesis (58-60), iron's role in cellular and neural processes (61,62), and non-haematological and other consequences (63).

Proposed expansion of the ID categories

Recently, expansion of the ID categories has been proposed in an effort to try to relate the laboratory findings with the clinical realities. A new stage, termed Stage IIIa, has been proposed (22). This will identify the ID category where the Hb is in the normal reference range but increases with iron supplementation. Stage IIIb would then describe patients with low iron and Hb below the normal reference range (Table 2).

Table 2. The expanded classification of the stages of iron deficiency

	Pre latent iron deficiency (Pre LID)
Stage I -	Early negative iron balance Storage iron depletion Latent iron deficiency (LID)
Stage II	Tissue depletion of iron without anaemia Iron deficient erythropoiesis Iron deficiency erythropoiesis with Hb in the reference range which increases with supplementation
Stage IIIa	Relative / functional IDA
Stage IIIb	Iron deficient anaemia (IDA) Manifest Iron deficiency

Stage IIIa has sometimes been termed relative anaemia. Hb levels are 'relatively anaemic' compared to the patient's 'norm' (set point), even when they are in the normal reference range (21,22). The term functional anaemia has also been used, as it describes the same stage from a metabolic perspective (35,36). A key problem is that these patients' iron category can be confused with pre-latent, LID, or even normal, and possibly not treated if Hb reference range cut-off values are the only criteria used to assess whether they are anaemic. It is important to remember that laboratory data only provides a snap shot of this dynamic process.

Stage IIIa may represent:

1. Reducing iron and Hb levels where the Hb has fallen below that person's set point. Thus, developing classic iron deficiency anaemia, but Hb has not yet fallen below the normal reference range, for example, negative iron balance caused by gastrointestinal bleeding.
2. Improving iron status and Hb levels that are not yet optimal, for example, infants going from milk, to meat containing diets
3. Sub-optimal group where iron input and excretion have formed a sub-optimal equilibrium, for example, pre-menopausal women.

Studies pointing to a sub-optimal equilibrium show that Hb and ferritin can be increased with supplementation, but therapy must be continued to maintain the raised optimal Hb (64). There are many pre menopausal women (1/3 of healthy women in one study (65)) that have low iron stores but apparently just enough to maintain adequate erythropoiesis (66). It is probable, when the Hb is in the normal range, that there is insufficient absorption of iron from a modern diet to cause the accumulation of iron stores to an adequate level (65). It was demonstrated that iron dosage of 27mg daily corrected both IDA and storage iron depletion, whereas only 9 mg daily did not correct the iron stores (67). The main concern for this group is that any increased demand for iron, for example pregnancy or increased blood loss, will cause the quick development of frank anaemia (3,65). The precarious nature of a significant minority of women with LID has been demonstrated in the placebo groups of supplementation experiments. They have been shown to developed frank anaemia within two years if untreated (68). This group also showed impaired adaptation to physical exercise (35).

Identifying iron deficiency

Identifying iron deficiency from the earliest stages of iron depletion through to IDA involves a multi-disciplinary approach, i.e. chemistry, haematology and clinical tests are involved in testing for iron in the human body. There is no single "best marker". Each test has merit depending on the stage of the iron depletion being examined (7,8). These are presented in Table 3. Tests have varying sensitivity, specificity and confounding conditions. The most appropriate approach to testing depends on the anticipated severity of the lack of iron, the age and sex of the target populations, the potential for associated disease, and the feasibility of various sampling and availability of economic and laboratory resources, to name just a few of the variables (7).

Note that increased iron absorption occurs even before a decrease in ferritin, or an increase in serum transferrin receptor is detected. This is observed in ferrokinetic studies using $^{59}Fe^{2+}$. This absorption shows an indiscriminate increase in the ability to absorb metals, which may include harmful cadmium and lead in a diet poor in iron (23).

Bone Marrow examination appears to be the only method that is both sensitive and specific for each stage of iron metabolism. The

Table 3: Tests for iron deficiency and the stages detected. (Modified from Herbert V, *J Am Diet Assoc* 1992;92:1502-9. Ref.69).

	Normal	Stage I Pre-LID	Stage II LID	Stage IIIa Functional/ Relative IDA	Stage IIIb IDA\
Iron absorption of $^{59}Fe^{2+}$	N	sl▲	sl▲	▲	▲
Response of ferritin to trial of iron	-	▲	▲	▲	▲
Reticuloendothelial marrow iron	2 - 3+	1+	0 - 1+	0	0
Serum Ferritin	N	sl,	▼	▼	▼
Transferrin Iron Binding Capacity	N	N	▲	▲	▲
Plasma Iron	N	N	sl,	▼	▼
Transferrin Saturation	N	N	▼	▼	▼
Sideroblasts in bone marrow	N	N	N	▼	▼
Serum transferrin receptor	N	N	N	▲	▲
Zinc protoporphyrin	N	N	N	▲	▲
Response of Haemoglobin to trial of iron	-	-	-	▲	▲
Haemoglobin/haematocrit	N	N	N	N	▼
MCV /MCH	N	N	N	N	▼
Erythrocyte morphology	N	N	N	N	microcytic hypochromic

staining of bone marrow for haemosiderin has come to be known as the "gold standard" for the establishment of iron depletion (3). The third stage, iron deficient anaemia, can not by definition be diagnosed from the bone marrow alone, but by the haemoglobin concentration being below normal for the age and gender of the patient. This examination of iron stores, although extremely sensitive to all the stages of iron deficiency, is invasive, costly, and cannot be performed in large epidemiological studies (8). A sensitive, yet innocuous substitute for bone marrow examination has been sought for many years (15). The results of several methods, other than bone marrow examination, can render iron depletion probable with the limitations of each method (3).

Giving a trial dose of therapeutic iron and looking for a significant increase in Hb has traditionally identified ID erythropoiesis. An increase in Hb of 10g/L or more proves the anaemia was due, at least in part, to ID (20). Supplementation studies identify not just those that are anaemic by definition of Hb below the normal reference range, but also those that may be relatively anaemic. A recent review criticised iron supplementation protocols saying that protocol differences had contributed to variable increases in Hb. The authors recommended 100mg daily of ferrous (Fe^{++}) iron taken on an empty stomach for three months of more (23).

There are more tests in the battery available to identify and categorise ID but have we come far since 1965 when Fielding et al. wrote, *"The significance of iron depletion without anaemia will remain unknown until its presence can be recognised without a therapeutic trial of iron"* (65).

Computer algorithms may assist the detection of this stage if a person's Hb was seen to drop significantly, even if it was still in the normal range. This could also be useful for other parameters that are normally stable in health. In an ideal world a person's set point could be determined in health and any significant variation from this value could be investigated. Trends in ill health can also be monitored. This may assist a clinician to make an early response to relative iron deficiency.

Other laboratory tests for predicting the people with this relative / functional Stage IIIa anaemia may be zinc protoporphyrin (ZPP), if no confounding factors are present (8), or more recently by serum transferrin receptor, sTfR, and the sTfR:ferritin ratio (7). This is a significant advance for those individuals whose optimal performance is vital, such as athletes and manual workers, as well as those groups at risk, such as young children, adolescents, and premenopausal and pregnant females.

A recent review suggested the most effective combination of laboratory tests was Hb, sTfR and serum ferritin (7). Normal Hb shows not anaemic (i.e. No IDA), normal sTfR shows an adequate tissue iron supply, and a normal serum ferritin shows the presence of storage iron.

Conclusions

A new classification of iron deficiency with functional consequences has been proposed. This new category has been called Stage IIIa IDA, with classical IDA being call Stage IIIb. Stage IIIa consists of persons that have reduced iron stores, but Hb levels still in the normal range, which increase with iron supplementation. Stage IIIa represents a functional anaemia and not a latent iron deficiency as was once thought. The Hb is reduced relative to the person's set point, i.e. they are relatively anaemic for their personal norm. Stage IIIa can be confused with pre-latent LID or even normal if using classic criteria of iron values and Hb reference cut-off values, thus it may go untreated.

The use of an algorithm to monitor intra-patient variation is recommended. This looks for trends departing from the person's set point, which act as warning signals for the early detection of iron deficiency. The inclusion of methods, such as ZPP, sTfR, or sTfR/ferritin index, to identify adequate tissue iron supply, may help identify those people with iron deficient erythropoiesis. This may help predict those individuals who may benefit from iron supplementation, while preventing harmful supplementation to people with such conditions as hereditary haemachromatosis or thalassaemia.

The functional consequences of Stage IIIa appears to be most apparent in athletes and perhaps manual workers, where maximal oxygen supply is necessary for maximal performance. In females it is unlikely that iron supplementation will improve Hb when the Hb is over 138g/L. For levels in the normal range but less than 138g/L, a test dose or other relevant iron studies may be required to elucidate if there will be an improvement in Hb for that individual.

Cognitive consequences do not show detrimental results for those whose haemoglobins are in the normal reference range. It is, however, recommended to supplement at risk groups to prevent anaemia, and possible irreversible consequences. It is suggested that Stage IIIa is closer to the frank anaemic stage and thus represents those most at risk. Further studies are recommended to investigate the Stage IIIa group to discover the prevalence of this condition, and the functional consequences specific to this group.

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Antibiotic treatment of Psuedomonas aeruginosa in cystic fibrosis: teaching an old model new tricks?

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Abstract

Pseudomonas aeruginosa is a major prognostic determinant in cystic fibrosis (CF). It is usually acquired during late childhood and once established within the CF lungs, *P. aeruginosa* is rarely eradicated. Chronic infection is marked by mucoid strains and deteriorating lung function. Within the CF lung *P. aeruginosa* adopts several survival strategies, including production of both exotoxins and exopolysaccharide as well as biofilm formation, which protect against host immunity and antibiotics. These virulence factors are coordinated by quorum-sensing systems that become activated when a critical bacterial density is reached.

Tissue injury results from complex interactions between bacterial products and host immunity. Failure to eliminate even susceptible *P. aeruginosa* strains results from several factors. Altered pharmacokinetics, poor endobronchial space penetration and inactivation by sputum means little antibiotic reaches infection sites. Not only does *P. aeruginosa* have the genetic capacity to express a broad range of resistance mechanisms, its slow growth within biofilms makes it less susceptible to cell-cycle dependent antibiotics. Furthermore, exposure to aminoglycosides selects for antibiotic-resistant populations that either have decreased uptake or actively extrude the drug. Nevertheless, despite lacking consistent bactericidal activity, antibiotics do benefit CF patients by reducing bacterial load and inhibiting virulence factors.

Treatment strategies include eradication of early infecting non-mucoid strains, antibiotic susceptibility testing that more accurately reflects the CF lung microenvironment thereby allowing more rational antibiotic choices, inhaled antibiotics to overcome limitations of poor penetration into the lung and macrolides, which possess antibacterial and anti-inflammatory properties. By further understanding *P. aeruginosa* pathogenetic mechanisms, future research will identify new targets for novel interventions.

Key words: Cystic fibrosis, *Pseudomonas aeruginosa*, inhaled tobramycin, macrolides

Paediatric Respiratory Cystic fibrosis (CF) is the most common lethal inherited disorder of Caucasian populations. It is an autosomal recessive condition with an incidence of approximately one in 2500 live births and its prevalence is estimated at 60,000 recognised cases world-wide (1). Multiple systems are affected and these may include the respiratory, gastrointestinal, liver, skin and genitourinary systems. Ultimately, 90-95% will die from respiratory failure secondary to chronic bacterial infection and its accompanying inflammation.

CF results from a single gene mutation on the long arm of chromosome 7 encoding a 1480 amino acid protein, the cystic fibrosis transmembrane conductance regulator (CFTR) (2). This protein is an epithelial cell membrane-bound cyclic adenosine monophosphate (c-AMP)-regulated chloride channel that also helps regulate the transport of other electrolytes across epithelial cell membranes (3). More than 1000 mutations have been described, but the most common mutation accounting for approximately 70% of all CFTR alleles leads to a deletion of phenylalanine at position 508 and impaired intracellular processing of the protein (4).

Life expectancy of CF patients has increased dramatically in the last five decades from a median of two-years to survival estimates exceeding 45-years of age (5). Enhanced survival is attributed to early and aggressive antibiotic therapy, improved airway clearance techniques, optimising nutrition, and specialist centre care (6). Nevertheless, recurrent lower respiratory tract infections remain a significant clinical problem and once *Pseudomonas aeruginosa* is established within the airways it is only rarely eradicated by the host immune system or by antibiotic therapy (7,8). Chronic infection by *P. aeruginosa* is associated with accelerated deterioration of lung function, increased hospitalisation and an overall 11-year reduction in life expectancy (9-12).

The finding of mucoid strains (Figure 1) in the sputum of patients with CF usually marks the establishment of chronic *P. aeruginosa* infection (9). Although antipseudomonal antibiotics from this point lack any consistent bactericidal effect, they still lead to transient improvements in lung function and overall well-being (13). Subinhibitory antibiotic concentrations, for example, may alter microbial function and even modify host immune responses. Such information is now beginning to influence the management of lung disease in CF patients.

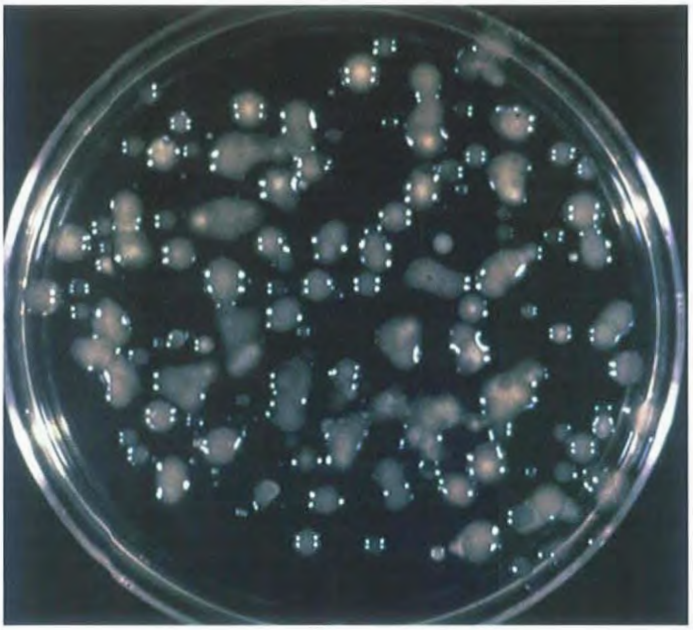
Bacteriology of CF lung disease

Bacterial infection of the lower airways of patients with CF may occur within the first few weeks of birth, is frequently focal and accompanied by massive neutrophil-infiltration (14-16). During the first three-months of life more than one-third of infants with CF identified by a newborn screening programme were found by bronchial lavage to have large numbers of bacteria in their lower respiratory tract, many of whom remained asymptomatic. *Staphylococcus aureus* was the predominant organism detected within this young age group. Cross-sectional and longitudinal studies, some employing bronchial lavage in non-expectorating children, have shown that early in life bacteria such as *S. aureus* and *Haemophilus influenzae* predominate, while in older children and adults *P. aeruginosa* is the most important pathogen (17-21). The United States CF Foundation Patient Registry is the largest database of its kind. While there are limitations to the data it provides because of variations in specimen collection and processing, it nevertheless gives an important overview of the respiratory pathogens detected in CF patients (22). The data for 2001 (Figure 2) confirm that while *S. aureus* prevails during childhood, *P. aeruginosa* is detected early, becoming predominant during late childhood and adolescence such that, by 18 years of age, 80% are infected by this organism.

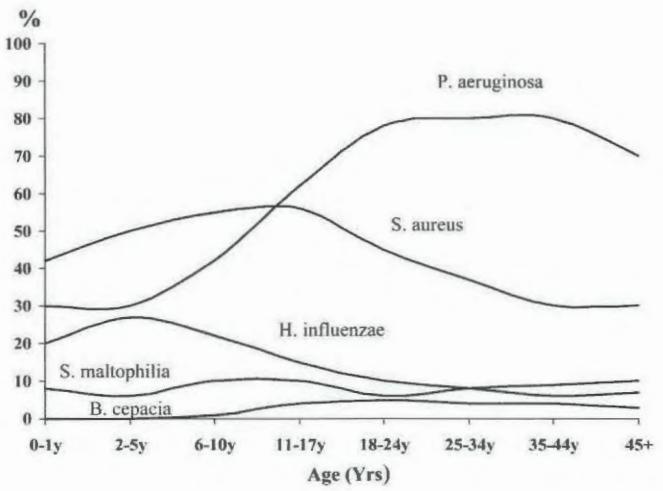
Pseudomonas aeruginosa

P. aeruginosa is a motile gram negative rod, which thrives in moist environments. It is extremely versatile and can grow in many habitats, including soil, surface waters and plants. In hospitals,

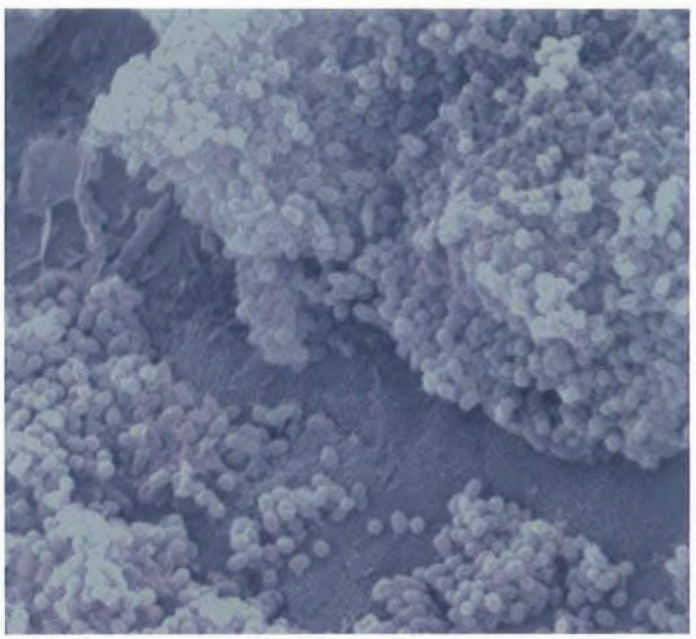
P. aeruginosa can be found in sinks, respirators, humidifiers and on the hands of staff (23). In healthy individuals, mucociliary clearance and innate immunological defence mechanisms rapidly clear bacteria



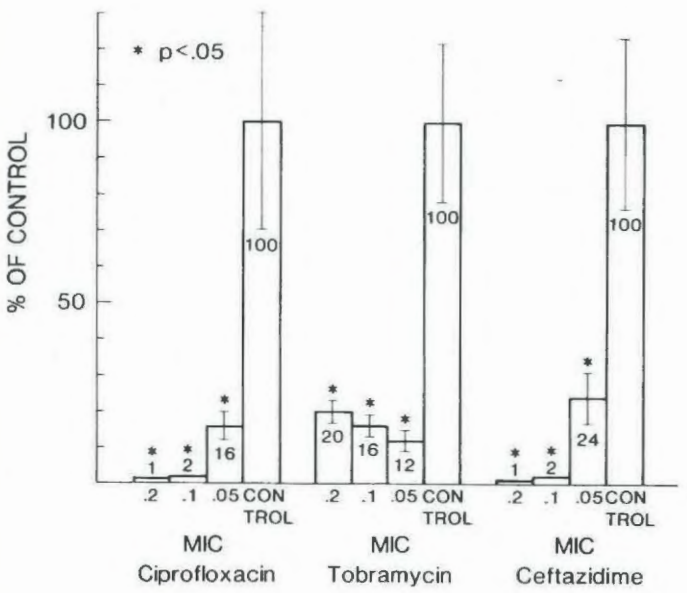
1. Over-production of alginate, a polysaccharide polymer, produces the typical "wet" appearing mucoid colonies of *P. aeruginosa* found in the sputum of a CF patient.



2. Age-specific prevalence of respiratory pathogens in CF patients: CFF National Patient Registry data 2001 (21).



3. Scanning electron micrograph depicting a mature biofilm (courtesy of Dr Chris Sissons, Wellington School of Medicine & Health Sciences).



5. In-vitro dose-responses of *P. aeruginosa* elastase for subinhibitory antibiotic concentrations.

entering the lungs. Why bacteria, and in particular P. aeruginosa, are able to colonise and infect the lower airways of patients with CF is unknown. The pathogenesis is complex and likely to be multi-factorial. Postulated mechanisms include one or more of the following: (i) abnormal airway surface electrolyte composition leading to defective mucociliary clearance and/or impaired function of antimicrobial peptides, (ii) increased availability of bacterial receptors on epithelial cell surfaces secondary to defective CFTR function, but especially in regenerating cells following damage from prior viral and bacterial infection or from aspirated gastric acid, and (iii) altered CFTR function preventing the internalisation and destruction of *P. aeruginosa* (24). However, data supporting these hypotheses are conflicting and none have been accepted as a single unifying explanation for CF lung infection.

Patients with CF may acquire *P. aeruginosa* early in life (11,14,20,21). Longitudinal studies show that once infection is established most harbour the same isolate for many years (25,26). The initial source is unknown, but acquisition is accelerated by continuous anti-staphylococcal prophylaxis from a young age (27,28) It appears that most acquire their own unique strains from the environment and, except after prolonged contact within families, are unlikely to cross-infect one another (29). Nonetheless, transmissible strains of *P. aeruginosa* have infrequently emerged and spread rapidly amongst patients attending the same clinic (30,31).

Survival strategies by *P. aeruginosa*

Early colonisation and infection by *P. aeruginosa* can be intermittent and transient, involving the same or unrelated strains (21,32). During the early stages of infection, strains resemble those from the environment, being motile, relatively susceptible to antibiotics, possessing smooth lipopolysaccharide (LPS) with side-chains and a colonial non-mucoid appearance. Within months to years, the isolated strains lose their flagella, have decreased pyocin production, show increasing resistance to all antibiotics, they develop rough LPS by losing side-chains and become mucoid from over-production of a complex exopolysaccharide heteropolymer known as alginate (33). Once a particular clone has infected the lung, DNA macrorestriction may reveal shifts in the macrorestriction fragment patterns, identifying subclonal variation, which can result from sequence alterations in restriction recognition sites, genomic rearrangements and incorporation of extrachromosomal DNA from bacteriophages (34). These hypermutable strains are unique to CF patients and allows them to promptly adapt to their environment by not only switching genes on and off, but also by an increased frequency of mutation events (35).

P. aeruginosa has many virulence factors (Table 1) that permit its establishment within the lungs (3,24,36,37). It expresses adhesins, such as pili, that allow the organism to attach to damaged mucosal epithelial surfaces and non-pilus adhesins that bind to the altered CF mucins. Once attached, *P. aeruginosa* must resist the host immune system. Several toxins are produced, for example proteases such as elastase that impair phagocytosis by cleaving immunoglobulins, hydrolysing complement and inactivating pro-inflammatory cytokines. It also has a contact-dependent type III secretion system that directly injects proteins, such as exoenzyme S and exoenzyme U, into host cells. These proteins are toxic to neutrophils and macrophages respectively, further protecting the bacteria from host defences. The most characteristic feature of persistent infection by *P. aeruginosa* is the production of mucoid alginate and the formation of microcolonies. A mutation triggers conversion to the mucoid phenotype, which is positively selected for under the environmental stresses of nutritional limitation and hypoxia found within a CF bronchiolar mucous plug. The excessive alginate facilitates biofilm development and protects the organism from the host immune response and antibiotics. Biofilms, as seen in Figure 3, are heterogenous microcolonies of bacterial cells enclosed in a self-produced extracellular matrix that allows adherence to surfaces, provides protection against phagocytosis and interferes with antibiotic activity. They exhibit a developmental sequence and have a complex architecture, characterised in mature biofilms by "mushrooms" of cells enmeshed in exopolysaccharide and separated by water filled channels that convey nutrients to the thick biofilm and remove their waste products (38).

The co-ordinated expression of the regulatory and structural genes responsible for these survival strategies adopted by communities of *P. aeruginosa* is part of a general bacterial function called quorum sensing. This is directed by small, diffusible molecules, homoserine lactones (HSL), called quorum sensors (39). *P. aeruginosa* has two complete quorum sensing systems (*las* and *rhl*) that it utilises to regulate more than 600 growth-dependent genes in a density-dependent manner. Figure 4 shows that under conditions of low bacterial density, bacterial expression of quorum sensors is negligible and planktonic growth ensues. If, as occurs within mucous plugs of the CF airways, numbers of *P. aeruginosa* increase greatly, the secreted quorum sensors achieve a critical density. The small signalling molecules (3-oxo-C12-HSL and C4-HSL) then diffuse back into the bacterial cells and bind to and activate their global transcriptional quorum sensing regulators (LasR and RhlR) to direct the expression of several factors that facilitate bacterial persistence within the lung and promote biofilm formation (40). These virulence factors include the proteases, elastase and alkaline protease, exotoxin A, the haemolysin phospolipase C, iron-scavenging proteins and both catalase and superoxide dismutase, which protect against host phagocytes. Some postulate that bacteria employ quorum sensing for regulation of virulence to ensure that immune responses are activated only after a sufficient number of pathogens are present to overwhelm host defences. The role quorum sensing plays in regulating the expression of several *P. aeruginosa* virulence factors makes it an attractive target for antimicrobial therapy.

Lung injury

Lung tissue injury results from a complex interaction between bacteria and the host immune system (Table 1). *P. aeruginosa* secretes several exoproducts. Some, such as the proteases elastase and alkaline protease degrade tissue proteins, others like exotoxin A and exoenzyme S are directly cytotoxic, while the haemolysin phospholipase C interferes with surfactant. In the rat lung model of chronic *P. aeruginosa* infection, isogenic mutants singularly deficient in elastase, exotoxin A or exoenzyme S are less virulent than their parent strains (41). Installation of these purified products into the lungs of experimental animals produces similar histological injury to those seen with infected rats. Some exoproducts may play a more important role in certain infections. *P. aeruginosa* strains from the sputum of patients with respiratory infection yield greater quantities of exoenzyme S and elastase than isolates from burns, blood, urine or wounds (42). Cross-sectional studies of ill, hospitalised CF patients report that *P. aeruginosa* exoenzyme mRNA transcripts are increased in sputum and bronchial lavage fluid, while their sputum isolates also yield greater quantities of exoproducts, compared with the mRNA transcripts and exoproduct expression found in the respiratory secretions of stable patients regularly attending a CF clinic (43-45). A six-year longitudinal study of a single patient found increased exoenzyme S expression by sputum isolates before each exacerbation, followed by a transient increase in serum anti-exoenzyme S antibodies (46). These laboratory studies and clinical observations suggest a direct role for *P. aeruginosa* exoproducts in CF lung injury.

Table 1. *P. aeruginosa* cell-associated and soluble virulence factors.

Virulence factor	Examples	Functions
Motility	unipolar flagellum	motility and adherence
Adhesins	type 4 pili	bind to epithelial cells twitching motility - biofilm formation bind to mucins
	neuraminidase	cleaves epithelial cell surface glycoprotein sialic acid residues to promote pilin-mediated adherence
	non-pilus adhesins	bind to mucins
xcp secretion system	exoproducts	secretes toxins and enzymes outside cell
	pilin protein	aids pilus synthesis
Type III secretion system		injects toxins directly into eukaryotic cells
	exoenzyme S	ADP-ribosylates GTP-binding proteins upregulates proinflamamtory cytokines/chemokines
	exoenzyme U	alters cell cytoskeleton impairing neutrophil migration toxic to macrophages
Exoproducts	elastase	degrades elastin, immunoglobulins, complement
	exotoxin A	ADP-ribosylates elongation factor-2, stops host cell protein synthesis
	phospholipase C	? destroys surfactant
	rhamnolipid	? destroys surfactant
	pyoverdine	iron-scavenging siderophore, ciliotoxic
Phase variation	lipopolysaccharide	short O-antigen chains - resist defensins
Exopolysaccharide	alginate	prevents opsonophagocytosis
		? aids biofilm formation
		? adhesin for mucins
		highly antigenic, forms tissue-damaging immune complexes
Biofilm formation	microcolonies	adhesion to inert and living surfaces
		blocks osonophagocytosis
		altered microbial physiology reduces susceptibility to many antibiotics
Quorum sensing - autoinducers	3-oxo-C12-HSL	externalised by MexAB-OprM efflux pumps
		regulates lasB, lasA, aprA, toxA, xcp,LasI, rsaL, rhlR and rhlI genes and promotes biofilm formation
	C4-HSL	immunomodulator - eg. induces IL-8, IL-12, Cox-2
		diffuses rapidly across cell membranes
		regulates rhlAB, lasB, aprA, xcp, RpoS and pyocyanin genes
Antibiotic resistance	permeability barrier	ß-lactams, fluoroquinolones, aminoglycosides
	efflux pumps	ß-lactams, aminoglycosides
	inactivation	fluoroquinolones, polymyxins
	changes to target	ß-lactams, fluoroquinolones, aminoglycosides
	biofilm formation	

Table 2. Antibiotic susceptibility (mg/L) of *P. aeruginosa* as planktonic (MIC) * and biofilm (MBEC) † populations [modified after ref. 76]

Antibiotic	Planktonic MIC *	Biofilm MBEC †
Amikacin	2	16
Gentamicin	2	128
Tobramycin	0.5	2
Aztreonam	2	> 1024
Ceftazidime	1	> 1024
Imipenem	1	> 1024
Piperacillin	2	> 1024
Ciprofloxacin	0.25	4

* minimum inhibitory concentration , † minimal biofilm eradication concentration

Large numbers of bacteria within the CF lung stimulate a marked influx of activated neutrophils into the lower airways and the increased expression of pro-inflammatory cytokines, interleukin (IL)-1, IL-6, IL-8 and tumour necrosis factor-ß (16,47). However, the inflammatory response is usually ineffective and *P. aeruginosa* is not cleared from the lungs. Instead neutrophil products, such as neutrophil elastase, other proteases, cytokines and oxidants result in further lung injury by overwhelming local anti-protease defences and damaging structural proteins (48). Of interest, *P. aeruginosa* itself may directly contribute to lung inflammation and injury by modulating the immune response. Exoenzyme S is a T-cell mitogen and induces transcriptional expression of several pro-inflammatory cytokines and chemokines before stimulating apoptosis (49). Quorum sensors are also immunomodulatory when produced in large quantities by *P. aeruginosa* biofilms. They activate transcription factor nuclear factor (NF)_B, inducing IL-8 and other chemotactic factors, which stimulate the migration of several inflammatory cell types into the lungs (50). In addition, quorum sensors induce other inflammatory mediators such as prostaglandin E2, which is synergistic with IL-8 as a neutrophil chemoattractant and stimulates both mucous secretion and oedema (51). Meanwhile, neutrophil elastase is also a potent stimulant for mucous secretion and IL-8 (52). These complex host-pathogen interactions establish a vicious cycle of infection, inflammation and tissue destruction.

Antibiotic-host-*P. aeruginosa* interactions

Although antibiotics have been associated with improved survival (53), it was only recently that a measurable, but short-term direct benefit, was demonstrated for individual patients (13,54,55). Once *P. aeruginosa* forms biofilms and adopts a mucoid phenotype, there is only as much as a 2 log10-fold reduction in bacterial numbers with antibiotics and it is rarely eradicated, even when using drug combinations with confirmed potency *in-vitro*. Moreover in older CF patients with chronic *P. aeruginosa* infection, improvements in well-being and lung function are independent of whether the antibiotics are active against their own strain in-vitro (56). Successful clinical responses are also unaccompanied by improvements in lower airway inflammatory markers (55).

The failure to eradicate even susceptible strains of *P. aeruginosa* results from a combination of factors. Clinical breakpoints defining resistance are determined by antibiotic concentrations safely achieved in serum. However, the bacteria are located within the lumen of the lower airways in association with mucus and injured epithelial cells (57). To reach the site of infection antibiotics must breach damaged bronchial walls and tenacious respiratory secretions, into which ß-lactam and aminoglycoside antibiotics penetrate poorly (58). Furthermore, ß-lactam and aminoglycoside pharmacokinetics are altered in CF patients secondary to an increased extracellular volume associated with malnutrition and elevated renal clearance leading to reduced peak serum levels and half-lives (59). Within mucous plugs, antibiotic activity of aminoglycosides, for example, may be reduced by as much as 95% by (i) binding to negatively charged glycoproteins and DNA, (ii) competition with calcium and magnesium ions for LPS binding sites and (iii) inactivation from low endobronchial pH or high osmolality (60).

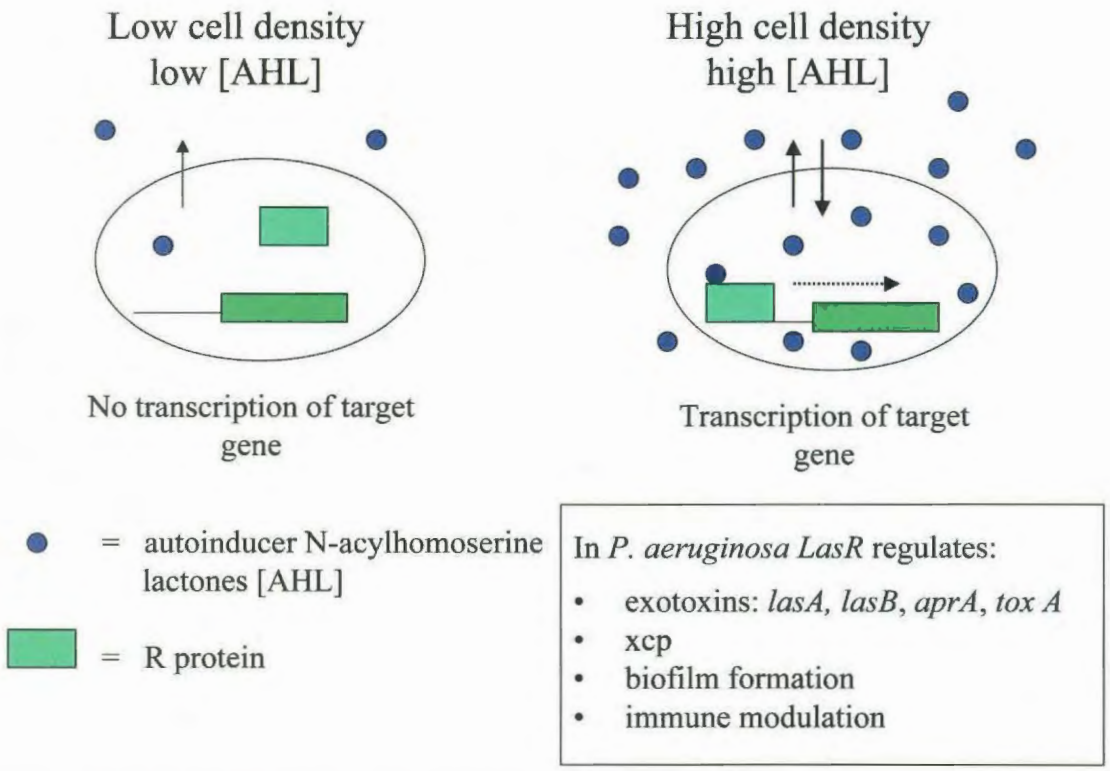
The poor permeability of its cell walls means that *P. aeruginosa* is inherently resistant to many antibiotics. It has the genetic capacity to express a broad range of resistance mechanisms that develop following mutation in chromosomal genes or following the acquisition of additional genes from plasmids, transposons and bacteriophages (61). These include changes in the outer membrane proteins and LPS, which further decrease antibiotic cell wall permeability, whilst multidrug efflux systems actively pump out antibiotics that have successfully penetrated the cell. Class and antibiotic specific enzymes are concentrated in the periplasmic space of the bacterial cell wall where they inactivate antibiotics approaching the cytoplasmic membrane. Finally, mutational changes in target enzymes allow maintenance of their cellular function while resisting selective inhibition by antibiotic classes such as the fluoroquinolones and polymyxins.

P. aeruginosa also has other strategies to resist antibiotic activities. The anionic polysaccharide layer of alginate surrounding *P. aeruginosa* binds cationic antibiotics, such as aminoglycosides, restricting their diffusion (62). Within biofilms lowered oxygen tension and restricted iron availability encourages anaerobic growth by *P. aeruginosa* and slows cell division. These changes in microbial physiology allow fully susceptible strains in the free-living planktonic phase to become resistant to cell-cycle dependent antibiotics, such as ß-lactams, and also to aminoglycosides, which rely upon oxygen-dependent uptake mechanisms (63). Exposure to aminoglycosides can also select for drug-resistant subpopulations. These include slow growing, small colony variants that are deficient in the energy-dependent uptake of aminoglycosides and result in treatment failure (64). Of interest, a single genetic locus is associated with both the ability to form biofilms and to develop antibiotic resistance by forming these small, energy deficient colony variants (65). A second subpopulation within the CF lungs survives the early, concentration-dependent killing of aminoglycosides and fluoroquinolones by inducing the rapid expression of an active, but reversible, efflux system (MeXY-OprM) allowing the organism to become transiently refractory to the bactericidal actions of the antibiotic (66,67). This is termed adaptive resistance and it disappears when the bacteria are no longer exposed to the drug.

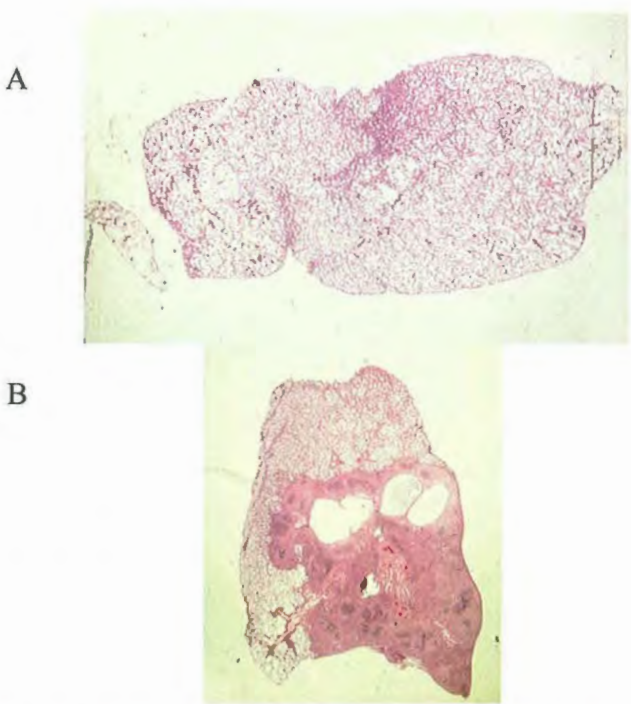
Traditionally, the therapeutic benefits of antibiotics come from killing bacteria and sterilising the infection site (68). Nevertheless, clinical improvement in CF following antibiotics is often associated with only an overall small decrease in sputum colony counts (13,54). The benefit from anti-pseudomonal antibiotic therapy may be partly explained by the reduction of exoproducts achieved with subinhibitory concentrations of antibiotics. Figures 5 and 6 show that such effects have been observed *in-vitro*, in animal models and during pulmonary exacerbations where increased concentrations of *P. aeruginosa* virulence factors, such as elastase and exoenzyme S, are reduced with antibiotic therapy despite their failure to reduce the numbers of bacteria present (45,69,70). The decrease in bacterial load and virulence factors, including quorum sensor molecules, may decrease the inflammatory stimulus and slow damage to the walls of the lower airways. However, subinhibitory antibiotic concentrations may also increase mutation rates, resulting in the mucoid phenotype, and induce adaptive resistance to aminoglycosides and fluoroquinolones such that the ultimate effect is unclear. Attempts to achieve bactericidal antibiotic concentrations within the airways should therefore continue to be a major therapeutic aim. Anti-pseudomonal antibiotics may also help protect against the consequences of an exaggerated inflammatory response. Aminoglycosides can block myeloperoxidase release from activated neutrophils preventing the conversion of chloride into toxic hypochlorous acid in the presence of hydrogen peroxide, while ß-lactams are also powerful hypochlorite scavengers (71).

Antibiotic treatment strategies

There is some evidence that prevention of chronic *P. aeruginosa* infection has important benefits for CF patients. They have fewer symptoms, less rapid decline in lung function and longer survival (9-12). Unfortunately the origins of *P. aeruginosa* isolates among CF patients remain obscure, but as most have unique isolates that initial resemble those from environmental sources, even rigorously enforced



4. Quorum sensing in *P. aeruginosa* involves two regulatory components, the autoinducer molecule (AI; eg. 3-oxo-C12-HSL and C4-HSL) produced by the autoinducer synthase gene (eg. LasI and RhlI) and the autoinducer-dependent transcriptional activator protein (R-protein; eg. LasR and RhlR). Accumulation of AI is cell density dependent and when a threshold level is reached AI binds to and activates the R-protein, which then induces gene expression. Quorum sensing regulates the expression of several exoproducts and anti-oxidising molecules, type II secretion and biofilm formation. In addition quorum sensor molecules can induce eukaryotic cells to express pro-inflammatory cytokines and chemokines by activating the transcription factor NF_B [modified after ref. 40].



6. Haematoxylin-and-eosin stained whole lung sections from rats infected with *P. aeruginosa* embedded agar beads and treated for 10-days by subinhibitory doses of (A) ciprofloxacin compared with (B) untreated controls. Bacterial numbers in lung homogenates from each of the study groups were comparable. However, isolates from ciprofloxacin treated rats had significantly less exoenzyme S and elastase expression and their lungs were significantly less damaged than the untreated controls (69).

Table 3. Effects of macrolides upon *P. aeruginosa* virulence and host immune factors

P. aeruginosa	virulence factors	reduced expression
	motility	flagella
	adherence	pili
	exoproducts	elastase and alkaline protease
		phospholipase C
		exotoxin A
	exopolysaccharide	alginate
	microcolonies	biofilm formation
	quorum sensing autoinducer molecules	3-oxo-C12-HSL
		C4-HSL
Host	innate immunity	reduced expression/function
	chemokines	Il-8
	cytokines	IL-1, IL-6, TNF_
	neutrophils	chemotaxis, superoxide production, accelerated apoptosis
	mucociliary clearance	sputum viscosity

infection control measures are unlikely to delay the onset of infection. Since early *P. aeruginosa* acquisition has been associated with prolonged anti-staphylococcal prophylaxis such measures are probably best avoided (27,28). Early treatment following the first isolation of *P. aeruginosa* may provide an opportunity to eradicate the organism or at least postpone the establishment of chronic infection. Shortly after acquisition patients with CF still have a low bacterial burden, the strains are non-mucoid, are not yet forming biofilms and are more susceptible to antibiotic therapy (16,21,33). There are accumulating data to support the early aggressive therapy of initial *P. aeruginosa* infection. Several of these studies have however been limited by their observational design, use of historical controls, failure to collect lower airway samples, small subject numbers or restricted period of follow-up (11,28,72-74). All used different antimicrobial regimens including various combinations of ciprofloxacin and inhaled or intravenous anti-pseudomonal antibiotics that ranged in duration from two-weeks to one year. The most recent was a randomised, double-blind, placebo controlled trial of phenol-free tobramycin solution for inhalation (TOBI) given 300mg twice daily for 28-days in children aged six months to six years who underwent bronchoalveolar lavage before and at the end of treatment (74). This study was abandoned after the enrolment of only 21 subjects as both mucoid and non-mucoid strains of *P. aeruginosa* were no longer detected on day 28 amongst any of the eight children who received TOBI compared with its persistence in 12 of the 13 placebo recipients. Nevertheless, compelling evidence of a clinical benefit from early intervention is lacking (11,74) and the risk of selecting increasing antibiotic resistant strains or promoting the emergence of new pathogens should also be considered (75). Further carefully controlled longitudinal studies evaluating both the microbiological and clinical outcomes of early intervention are needed.

Once *P. aeruginosa* is established antibiotic choices become more complicated. Conventional susceptibility testing of *P. aeruginosa* to individual antibiotics is conducted under *in-vitro* conditions mimicking those in serum, where organisms from sputum exhibit planktonic growth in broth or on agar. This however is unlikely to represent the situation within mucous plugs where *P. aeruginosa* is growing either very slowly or is in a stationary phase. Table 2 shows that compared to broth cultures, as much as 1,000 fold greater concentrations of antibiotics are required to inhibit *P. aeruginosa* grown as a biofilm *in-vitro* (76). Furthermore, since patients now live longer they are receiving more courses of antibiotics. This leads to increasing rates of antibiotic resistance where more than 50% of isolates are non-susceptible to at least one antipseudomonal antibiotic and 10-20% are resistant to almost all commonly used agents (31). From single sputum cultures, there may be clonally related isolates with multiple morphotypes and significantly different antibiotic susceptibility profiles (77,78). Testing individual morphotypes is labour-intensive, but is more accurate than mixed-morphotype testing for detecting antibiotic resistance (79). When multi-resistant strains of *P. aeruginosa* are identified, synergistic testing *in-vitro* either on single colonies, in biofilms or directly from respiratory secretions may identify which combinations of two or three antibiotics are active, but not antagonistic against these pan-resistant strains (80-82). A Canadian multi-centre study is presently being conducted to help determine whether synergistic bactericidal testing will better predict responses to therapy than conventional susceptibility tests

Currently, most CF physicians use a ß-lactam and an aminoglycoside in combination when treating an acute pulmonary exacerbation. Compared with monotherapy, antibiotic combinations bring about a greater reduction of bacterial numbers and produce a longer clinical remission (83). This effect has been attributed to decreasing the development of antibiotic resistance, but the data are unconvincing (83,84) Observations that clinical responses to ceftazidime and tobramycin occur independently of individual antibiotic susceptibility test results have added to the controversy (56). None of these studies have however utilised synergistic testing. It is possible that the superior record for combination therapy results from its greater bactericidal activity *in-vitro* and to higher subinhibitory concentration *in-vivo*. In the rat lung model, for example, less lung injury from *P. aeruginosa* was observed at antibiotic tissue concentrations of 1/5 versus 1/20" the minimum inhibitory concentration (MIC) (69,70). Therefore, if possible, when treating CF patients for acute exacerbation of their *P. aeruginosa* lung disease the most effective antibiotics as determined by current susceptibility testing should be administered. Optimal timing and delivery of antibiotics are also important. Although aminoglycosides are most commonly prescribed three-times a day, once-daily dosing in non-CF children (85) and adults (86) has proven to be safe and effective. Once-daily tobramycin therapy is an attractive prospect for CF patients (87). The high peak levels achieved enhance bactericidal activity and the prolonged dosing interval not only reduces the risk of toxicity, but it could also help overcome adaptive resistance (66). Currently the role of extended interval parenteral tobramycin administration is being evaluated by a British multi-centre trial (TOPIC).

In chronically infected patients, lung function returns to pre-treatment levels within weeks of intensive treatment (13,55). CF clinics, particularly those in Europe, have therefore adopted regular elective three-monthly intravenous antibiotic treatments to help reduce this decline in lung function. Subsequently, the five-year survival of patients with chronic *P. aeruginosa* infection increased from 54% in 1971-75 to a 10-year survival of 90% during 1976-85 (5,53). However, these data relied upon historical controls and remain controversial because of the disruption to patient's lives, the greater risk of antibiotic hypersensitivity and resistance, increased cost and the failure to show improved outcomes over treatments determined by symptomatic state (88). The addition of regular inhaled antibiotics has recently provided an alternative form of maintenance therapy that is gaining greater acceptance (54).

Inhaled tobramycin

Aerosolisation attempts to overcome the limitations of poor penetration into endobronchial spaces by intravenously administered antibiotics. This approach produces very high lung concentrations and low serum levels of antibiotics, but it is very cumbersome, taking more than 15-minutes to administer by jet-nebuliser (89) with only 1-10% of the drug reaching the distal airways (89,90). Moreover, deposition within the lungs is determined by lung function where those with the worst percentage of predicted forced expiratory volume in one second ($\%FEV_1$) are more likely to have the antibiotic deposited in the proximal airways, distant from the sites of infection in peripheral airways and poorly ventilated lung regions (91). Early efficacy and safety studies used various antibiotics and delivery systems and were small and underpowered. Only the recent trials with TOBI provide strong evidence-based medicine (54). A 300mg dose of TOBI provides tobramycin levels above 1,200 _g/g of sputum, easily exceeding the 10-to-25 fold of MIC values required to kill *P. aeruginosa* in sputum (92,93). Despite these high antibiotic concentrations *P. aeruginosa* is not eliminated in those with chronic infections, possibly because of biofilm formation and poor penetration into mucous plugs within peripheral airways. Also, with regular daily administration almost 75% of sputum isolates became less susceptible to tobramycin (94). To address concerns over increasing antibiotic resistance and of toxicity developing from drug accumulation in the kidneys and inner ear, TOBI is still administered twice-daily, but now on alternate 28-day cycles.

Results from a randomised double-blind, placebo-controlled trial involving 520 chronically infected patients aged six-years and above who received 300mg of TOBI twice daily during alternate 28-day cycles found that after 24-weeks treated patients had improved lung function, accelerated weight gain and reduced hospitalisation (54). At 20-weeks an average 10% increase in $\%FEV_1$ was seen in those receiving TOBI, compared with a 2% decline for those taking placebo. Improvements in lung function were maintained despite there being a decreased effect upon bacterial density after the first two cycles of therapy. This could not be explained by the 7% increase in patients harbouring isolates with tobramycin MIC values ≥ 8mg/L and, instead, was attributed to the subinhibitory effects of the antibiotic upon bacterial virulence factors (45). Furthermore, treatment with TOBI was not associated with increased isolation of *Burkholderia cepacia complex, Stenotrophomonas maltophilia, Achromobacter xylosoxidans* or other multi-resistant organisms (95). Additional subanalyses of the placebo group showed that the 139 patients who received intravenous tobramycin for acute symptomatic exacerbations had a similar net fall in $\%FEV_1$ (1.4%) as the 123 patients without such episodes (2.1%). This implies that treatment of acute exacerbations alone does not prevent the progressive deterioration in lung function seen with chronic *P. aeruginosa* infection (96).

Follow-up open-label studies have recently reported sustained improved lung function. For example in adolescents where there was a highly significant initial treatment effect, after two-years of TOBI their $\%FEV_1$ remained 14% above the initial baseline measurement (97). Improvements in lung function in this subgroup were correlated with *P. aeruginosa* density, further emphasising the additional benefit of reducing bacterial burden in the airways. In contrast, those with an original delay in initiating TOBI had only 2% improvement over baseline, suggesting an irreversible component to their decline in lung function.

Despite problems arising over the efficiency of drug delivery systems that can adversely affect drug costs and patient compliance, these data provide strong evidence to support the routine use of inhaled TOBI as maintenance therapy of chronic *P. aeruginosa* infection. Unfortunately, outside of North America TOBI is viewed as being too expensive for routine clinical use. Instead less intensively investigated antibiotics, colistin and intravenous tobramycin preparations are administered, often with substantial variations in dosing and delivery devices (98). How well conventional doses and preparations of tobramycin compare with TOBI is unknown. Future studies need to evaluate more simple and efficient delivery systems that will improve compliance and enable lower drug doses of TOBI and other inhaled antibiotics to be used (99). The role of inhaled once-daily or extended interval aminoglycoside administration should also be evaluated, both for its convenience and potential effects upon adaptive resistance.

Macrolide antibiotics

A chance observation that erythromycin dramatically improved the outcome of middle-aged Japanese men suffering from diffuse panbronchiolitis has stimulated considerable interest within the CF community (100). This rare respiratory condition is characterised in its later stages by bronchiectasis and infection with mucoid strains of *P. aeruginosa* that are difficult to eradicate. However, long-term administration of erythromycin has improved 10-year survival from 12% to 90%, although the reasons for this beneficial effect remain uncertain.

Macrolides inhibit bacterial protein synthesis by binding to the 50S ribosome subunit. They have no intrinsic bactericidal activity against *P. aeruginosa* by conventional susceptibility testing, but as shown in Table 3, they are capable of *in-vitro* suppression of several virulence factors and possess anti-inflammatory properties (101,102). Subinhibitory concentrations reduce adherence to epithelial cells and suppress the elaboration of exoproducts such as elastase, phospholipase C and exotoxin A, and inhibit flagellin synthesis. Macrolides also disrupt biofilm formation by blocking quorum sensor molecules and inhibiting alginate expression. Recently, it was observed that *P. aeruginosa* strains growing in biofilms were susceptible to the bactericidal action of azithromycin at concentrations 1/64th MIC and under such restricted growth conditions, genes controlling the quorum sensing system were responsible for this increased susceptibility (103,104). Finally, macrolides paired with conventional anti-pseudomonal antibiotics *in-vitro* have a modest synergistic effect against multi-resistant strains of *P. aeruginosa* (105).

Macrolide anti-inflammatory properties appear to be mediated by inhibiting neutrophil chemotaxis, reducing neutrophil elastase, suppressing the oxidant burst and accelerating neutrophil apoptosis. This is likely to be secondary to their direct suppressive effects upon IL-8 and other pro-inflammatory cytokine gene expression by inhibiting activator protein-1 binding sites and the transcription factor NF_B (102). Other properties include reduced sputum viscosity, possibly by their suppressive cytokine effects, which inhibit the mucus

secretagogue neutrophil elastase and promote neutrophil apoptosis instead of disintegration and release of high mol-wt DNA. Upregulation of the multidrug-resistant-associated protein, a drug efflux pump that may also increase extracellular chloride transport, has also been implicated. Nevertheless, it is tempting to speculate that many of the diverse antimicrobial and anti-inflammatory functions exhibited by macrolides are attributed to their inhibition of the bacterial quorum sensing system.

Despite the *in-vitro* evidence of potential benefits from macrolides in CF patients there are few published clinical studies (106). Most have been open, uncontrolled and of limited duration. Azithromycin has been the preferred macrolide. It is well tolerated, has a long half-life (40-hours) that allows convenient once or alternate-daily dosing and, as it does not interfere with cytochrome P_{450} mediated metabolism, it has a lower risk of drug interactions.

A randomised, placebo-controlled trial of daily 250mg azithromycin for three-months in 59 adults with moderate-to severe CF lung disease showed a significant improvement in lung function. Overall, $\%FEV_1$ increased by 3% at the end of the study compared with an almost 1% decline of $\%FEV_1$ in those receiving placebo (107). The treatment group had significantly fewer intravenous antibiotic courses and significant improvements in quality of life scores. Overall, 50 patients had mucoid strains of *P. aeruginosa* and 25 had *S. aureus* in their sputum, suggesting that some clinical benefit may have been from the anti-staphylococcal effects of azithromycin.

A second trial used a cross-over design, involving 41 children aged 8-18 years with moderate-severe disease (108). On at least three occasions in the preceding year 21 had *P. aeruginosa* and 12 had *S. aureus* detected in their respiratory secretions. All except three were receiving inhaled anti-pseudomonal antibiotics, while 25 were also taking oral anti-staphylococcal prophylaxis. After the six-month treatment period there was an overall improvement in $\%FEV_1$ of 5.4% from a mean pre-treatment FEV_1 baseline of 60%. However, the response was not uniform. While 13 of the 41 patients had a clinically significant improvement in their $\%FEV_1$ of at least 13%, five others experienced deterioration in lung function of 13% or more. A sub-analysis found that those receiving the mucolytic agent, recombinant human (rh) DNase, had a 3.6% fall in $\%FEV_1$, while those without this agent showed 11.5% improvement when taking azithromycin. It is uncertain whether patients receiving rhDNase were those with the worst initial lung function and possibly the least likely to benefit from treatment.

The results of a large, North American multicentre study evaluating alternate-day azithromycin for six-months in CF patients chronically infected with *P. aeruginosa* are awaited. Important questions that need to be addressed include whether with azithromycin's prolonged half-life there is potential for accumulation and chronic toxicity. Macrolides inhibit rhDNase *in-vitro* (109) and the poorer outcome in children receiving both rhDNase and azithromycin suggests that this may be a clinically significant interaction. As with all antibiotics there is also a risk of emerging bacterial resistance, particularly with *S. aureus* and non-tuberculous mycobacteria that may co-infect the CF airways. Although macrolides might have a potential role in CF, their onset of action is delayed as long as three-to-four months, clinical improvement appears to be modest and extended studies are needed to determine whether any benefit is sustained.

Future research goals

- Identify the source and means of *P. aeruginosa* acquisition, including reinfection.
- Gain further understanding of *P. aeruginosa* pathogenetic determinants, which will help identify novel therapy that targets virulence factors and antibiotic resistance mechanisms, especially early in infection and before the development of irreversible lung injury.
- Explore future roles for combined antimicrobial and anti-inflammatory therapies.
- Establish simple, non-invasive and reliable means of detecting early *P. aeruginosa* acquisition and infection.
- Demonstrate a clinical benefit from the early eradication of P. aeruginosa, which may include serial assessments by high resolution computed tomograms of the chest, infant pulmonary function testing and measures of airway inflammatory responses.
- Identify the least intensive treatment able to achieve sustained P. aeruginosa eradication.
- Determine the most reliable and clinically relevant means of identifying *P. aeruginosa* isolate's susceptibility to antibiotics *in-vivo*.
- Resolve the optimal dosing frequency in CF patients of intravenous and inhaled aminoglycosides.
- Develop improved and more patient acceptable delivery systems for inhaled drugs, especially for infants and younger children.
- Decide upon the role of macrolides in the treatment of CF lung disease.
- Re-examine the role of inhaled antibiotics for acute exacerbation of CF lung disease.
- Encourage research in young CF patients, the group most likely to benefit from early intervention. Ironically it will take longer to demonstrate improvements in these patients because of their overall mild lung disease.

Conclusions

A better understanding and an expanding knowledge base of the mechanisms responsible for CF lung disease will lead to further improvements in clinical management. Until recently antibiotic treatments have been determined by a model of acute infection that relies upon eradicating relatively small numbers of rapidly dividing bacteria by cell-cycle dependent antibiotics. While this model accurately predicts therapeutic responses of acute bacterial sepsis and pneumonia, it has not proven consistently reliable in chronic *P. aeruginosa* CF lung infections. Determination of MICs in culture conditions mimicking those of serum may not precisely represent those of mucoid *P. aeruginosa* strains that have become established in the CF lung and where large concentrations of slowly growing bacteria form microcolonies within highly proteinaceous mucous plugs of inflamed peripheral airways. Treatment models for CF lung disease now have to consider the effects of altered microbial physiology and the airway microenvironment upon antibiotic susceptibility and therapeutic outcome. Meanwhile, it is too soon to determine whether in the last decade new antipseudomonal antibiotics such as TOBI, have along with rhDNase, the anti-inflammatory agent ibuprofen and new airway clearance techniques have contributed to improved survival in younger CF patients (110). Nevertheless, before too long patients with CF may each day be taking a chloride transport agonist, a mucolytic agent, and one or more antibiotic and anti-inflammatory agents to help preserve lung function.

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The hygiene hypothesis: the good, the bad, and the evil - a low-down on dirt

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Abstract

Asthma and atopic diseases are on the rise throughout the world, especially in developed countries, where children from affluent households live in cleaner, relatively aseptic environments, as part of the so-called Western style of life.

Many hypotheses have been proposed to explain the increases in atopic diseases and in particular asthma. The most widely discussed - and the most controversial - is the so-called "hygiene hypothesis". The hygiene hypothesis states that the western lifestyle has caused a decrease in the incidence of infections early in life, but these infections may have a protective effect on the subsequent development of atopic diseases.

This hypothesis proposes that infections early in life help to mature the immune system to a Th1 biased state and not a Th2 state, the latter predisposing the child to atopic disease. One substance proposed to shift this immune system balance to the Th1 state, hence altering the outcome of atopy, is bacterial endotoxin.

While many epidemiological studies support the hygiene hypothesis in the development of atopic diseases, including asthma, the evidence remains inconclusive. It is possible that other environmental exposures in early childhood also alter the predisposition towards asthma. Both the affirmative and negative views on the hygiene hypothesis are reviewed in this paper.

Key words: Asthma, atopy, endotoxin, hygiene hypothesis, immune system, TH1, TH2

Introduction

Over recent decades many countries, including the United Kingdom and New Zealand, have reported increases in the prevalence of asthma symptoms (1-4) and hay fever (3,5). Recently, the World Health Organisation estimated that over 40% of the global population are atopic. Asthma is estimated to affect between 100 and 150 million people worldwide, occurring in around 1-15% in the paediatric population, this placing an enormous strain on health resources in many countries. It is a major cause of hospitalisations in children in the western world (6).

Although the cause of this epidemic remains unclear, one proposed hypothesis for these increases is that broad changes in hygiene along with a decrease in infections during early childhood may be partly responsible. This hypothesis was first developed in 1976 when Gerrard and co-workers described the virtual absence of allergy and asthma among members of the Metis Indians of Northern Saskatchewan in Canada, in comparison to non Metis Indian members of the community (7). They reported that the Metis community, due to helminth infestations, had higher mean serum IgE levels than the "white community (7)". This initial finding led onto the suggestion that "atopic diseases are in part the price paid by some members of the white community for their relative freedom from infectious and parasitic diseases (7)."

With increases in allergy and asthma prevalence around the world (6), Gerrard's findings were recognised by Strachan in 1989. Strachan offered a parsimonious and coherent explanation for his own studies, which included a number of important agents relating to hygiene, such as, declining family size, improved household amenities, and higher standards of personal cleanliness all which appeared to reduce opportunities for cross-infection in young families. He suggested that the increase in the clinical expression of atopic disease could possibly be due to these more hygienic conditions (8).

Endotoxin

A further development of the "hygiene hypothesis" has proposed that the increases in allergic disease prevalence could be due to a reduced exposure to microbes. More recently endotoxin levels have been used as a marker of microbial exposure. Endotoxin is a term often used loosely to describe a group of lipopolysaccharides, found on the cell surface of Gram negative bacteria and fungi (9).

Endotoxins have been considered detrimental to health with house dust, which contains endotoxins, being shown to be associated with asthma severity. Endotoxin in particular has the ability to exacerbate existing allergy and asthma symptoms in atopic individuals. Inhalation of high levels of endotoxin has been associated with respiratory symptoms, particularly in occupational settings. For more than a century, cotton dust exposure in cotton mills caused cyclical asthma symptoms that were most severe on Mondays, after a day or two off from work, with improvement during the work week. This "Monday Asthma" phenomenon was ultimately found to result, not from exposure to cotton dust itself, but from endotoxin in the cotton dust (10).

How endotoxin exposure can induce asthma symptoms has been clarified through airway challenge studies with inhaled endotoxin. High doses of endotoxin induce an immediate and prolonged bronchoconstrictor response in most people. Lung segmental challenges with endotoxin have recently revealed that the inflammatory response is characterised by an early phase of neutrophilic inflammation and cytokine/chemokine release, followed by a phase 24-48 hours later with additional monocyte, macrophage and lymphocyte infiltration (10).

In the late 1990s the possibility of a beneficial role of endotoxin in promoting the Th1 phenotype and therefore protecting from atopic disease was reviewed (9). Studies initially in farming communities have suggested that endotoxin exposure at higher levels is associated with less atopic sensitisation (11,12). Exposure to endotoxin during the first year of life may protect against asthma by promoting an enhanced Th1 response and tolerance to allergens. Endotoxin binding to receptors on macrophages and other cells generates IL-12, which inhibits IgE production. It also generates cytokines like IL-1, TNF-alpha, and IL-8,

which cause inflammation. The main difference seems to be that endotoxin exposure recruits neutrophils, whereas allergen exposure recruits eosinophils, and the details of the tissue injury from these granulocytes differ (13).

Despite over 30 years of research, with a particularly strong focus over the last decade, a burden of proof of a cause and effect relationship between endotoxin exposure within organic dusts and respiratory disease remains elusive (9).

The affirmative view

Many published articles provide convincing data in support of the hygiene hypothesis. Specific areas that have been researched focus on; childhood infections, the impacts of having older siblings, decreasing family size, tuberculosis, hepatitis A, toxoplasma, antibiotics, crèches, and rural lifestyles.

Farming studies

Riedler and colleagues conducted a cross-sectional survey of rural areas of Austria, Germany, and Switzerland (14). With a 75% response rate, 2,618 parents of 6 to 13 year-old-children, completed a standardised questionnaire on asthma, hay fever and atopic eczema symptoms. Nine hundred and one of these children had blood taken for specific IgE antibodies to common allergen, however complete IgE data was only available from 812 children, 319 farmer's children and 493 children from urban areas. They concluded that children from farming backgrounds had a lower prevalence of asthma, as defined by doctor diagnosed, recurrent asthmatic, obstructive, or spastic bronchitis, and hay fever, as doctor diagnosed, than children from non-farming environments, odds ratio (OR; 95% CI) was 0.30 (0.15-0.61) compared to 0.43 (0.24-0.77). Children living on farms were also less likely to be atopic than non-farming children (OR=0.61; 95%CI: 0.41-0.92). The frequency of eczema did not differ between groups.

There were several limitations in this study. Firstly, children whose parents agreed to their child having a blood test had slightly more allergic symptoms than those whose parents refused consent, which possibly led to a higher number of atopic children and therefore a sample bias. Secondly, exposure to environmental farming factors was not only restricted to children who lived on farms. Many of the urban children had exposure to most of these environmental factors in the first year of life. The assessment exposures were retrospectively assessed for the first year of life, which may also have resulted in recall bias. Finally, in the study an objective marker was measured for hay fever, being allergic sensitisation to pollen, however no objective marker was used for asthma.

The level of endotoxin found in dust samples from bedding was inversely related to the occurrence of hay fever, atopic asthma, and atopic sensitisation among children in rural areas of Germany, Austria, and Switzerland according to a cross-sectional study carried out by Braun-Fahrlander and colleagues (15). In this study, complete data on hay fever and asthma were available for 812 children aged 6 to 13. Dust samples from the mattresses of those living in farming households contained an average of 37.8 endotoxin units (EU)/mg of endotoxin versus 22.8 EU/mg for samples from the mattresses of children from non-farming households. Endotoxin exposure was also much greater among children living on farms when the exposure was expressed as units per square metre of mattress surface area. However, it was the level of endotoxin, and not residence on a farm, that appeared to be protective. When the analysis was confined to the 493 children who did not live on farms, a negative correlation between endotoxin exposure and atopic conditions was still seen. Endotoxin exposure appeared to protect against atopic diseases and atopic sensitisation.

Von Mutius and colleagues also reported on the possible protective effects of endotoxin exposure (16). Identifying 84 farming and non-farming families in rural areas of Southern Germany and Switzerland, they concluded that the level of environmental exposure to endotoxin and other possible bacterial cell wall components is an important protective determinant for the development of childhood atopic diseases. Endotoxin concentrations were highest in stables of farming families. But they were also higher in dust from kitchen floors (143 EU/mg versus 39 EU/mg, $P<0.001$) and children's mattresses (49,479 EU/m^2 versus 9,383 EU/m^2, $P<0.001$) as compared to children from non-farming families.

Von Ehrenstein and colleagues also carried out a farming study to investigate whether traditional lifestyles were associated with a reduced risk of atopy (11). They conducted a cross-sectional survey in two Bavarian districts in Germany among children 5-7 years of age, concluding that these farmers' children had a lower prevalence of hay fever, asthma, and wheeze than their peers not living in an agricultural environment. The reduction in risk was stronger for children whose families were running the farm full-time compared to a part-time basis. Among farmers' children increasing exposure to livestock was related to a decreasing prevalence of atopic diseases.

Wickens and colleagues carried out a study looking into farm residence, exposures, and the risk of allergic diseases in Dannevirke children in New Zealand (17). Unfortunately, the study only had a 60% response rate so selection bias could not be ruled out. However, it was found that despite finding a protective effect of early-life animal exposures, they also found a greater prevalence of hay fever, allergic rhinitis, asthma, wheeze, eczema, dermatitis, but no more positive skin prick tests (SPT) to inhaled allergens, in farming children. In contrast to this they found farm abode during the first year of life was not associated with an increased risk of developing SPT positivity or any allergic disease. Endotoxin levels in this study were lower in farming houses than in the town houses. The practice of removing all farming clothes and boots and washing before entering the farm-home may be effective in preventing high indoor endotoxin levels. The levels found in these dwellings showed an association with an increased risk of disease development, but no increase in SPT positivity, which is consistent with the role of endotoxin in exacerbating symptoms.

These findings contrast with the European studies described above. Explanations for this may be differing farming styles between New Zealand and Europe. Countries in Europe tend to have low humidity and small farm holdings in comparison to New Zealand. New Zealand also has different distributions of animal exposures, with children living in the townships having high exposures to animals. The study population also seemed to have a different distribution of socio-economic status than found in the European studies. A limitation of the New Zealand study may have been the lack of a proper comparison group since the town children in this study may have had similar exposures as the rural population. Nevertheless, these findings are relevant to the New Zealand population and from this study it appears that people who live on farms in New Zealand have a greater prevalence of allergic disease than those children who are not living on farms.

Sibling effect

Ball and colleagues recently carried out a study involving 1035 children followed since birth as part of the Tucson Children's Respiratory Study (18). Asthma in this study was defined as one or more episodes of asthma diagnosed by a physician between 6 and 13 years of age, while the prevalence of frequent wheezing was taken as more than three wheezing episodes during the previous year. It was found that having one or more older siblings protected against the

development of asthma, relative risk (RR) was 0.8 (95% CI: 0.7-1.0), as did attendance at day care during the first six months of life (RR = 0.4; 95% CI: 0.2-1.0). However, it was also found that children who had more exposure to other children either at home or day care centres were more likely to have frequent wheezing at two years of age, than children with little or no exposure (RR = 1.4; 95% CI: 1.1-1.8). But they were also less likely to have frequent wheezing after six years of age (RR = 0.8; 95% CI: 0.6-1.0).

Potential limitations of this study were to do with interpretation of available data regarding day care attendance. Subsequent development of asthma was further complicated by the existence of multiple causes of wheezing during childhood. Wheezing in pre-school children is primarily associated with infections, whereas in school-age children it is primarily associated with atopy (19, 20).

Another study, looking into the association between the "sibling effect" and the development of atopic disease, in particular allergic rhinitis, was conducted by Marshall and colleagues (21). Questionnaires were sent out to 26,100 households selected at random from postcodes in the United Kingdom. They reported that people who had three or more siblings were more likely to have perennial rhinitis than those with no siblings (OR = 1.21; 95% CI: 1.02-1.44). However, there was no significant statistical effect for those people who had one or two siblings.

The main difficulty with this sort of study is that the participants label themselves as having hay fever or not. Previous studies have shown that 46% of people who admit to suffering from hay fever have never had their condition diagnosed by a doctor (22). Without having SPTs or IgE levels monitored it is difficult to define the outcome variable of this study. It is also difficult to determine the exposure, which was the number of siblings, however no information was taken on the age of the siblings, which birth order the participant was, or how long they had lived together.

The opposing view

Overall, variations in the early life environment may affect the development of childhood asthma, but are these factors sufficient to cause asthma by themselves? Platts-Mills and colleagues reason that many changes in lifestyle, later in childhood, including diet, immunisation, increasing obesity, and the associated decline in physical activity, could all have contributed to the increase in childhood asthma (23).

In order to consider the relevance of the hygiene hypothesis Platts-Mills and colleagues suggest that it is important to consider when major changes in public health occurred (24). They take New York City as a model and point out that eradication of malaria occurred in 1910, chlorinated water supplies about 1920, universal wearing of shoes before 1900, minimal contact with farm animals, and the cure of major infectious diseases occurred before 1946. They state that the major increases in asthma have occurred between 1970 and 2000 and argue that the major changes in "cleanliness" occurred long before the increases in asthma.

While early exposure to infectious burden may affect the Th1/Th2 balance in the developing immune system, it is also necessary to look at other environmental risk factors for the development of childhood asthma, which may also affect immune system development. These risk factors include the protective effect of early exposures to farm animals, via endotoxins (11), to household pets, via immune tolerance (25), and the increased risk associated with antibiotic usage (26). Other early childhood exposures also increase the risk for the development of asthma, such as household polyvinychloride exposure and environmental tobacco smoke. Recently, it has also been shown that childhood exposure to chlorinated swimming pools is strongly associated with asthma prevalence (27).

Martinez and colleagues studied the relationship between childhood respiratory disorders, their prognosis and the development of asthma, as part of the Tucson Children's Respiratory Study (28). Children, who suffered wheezing before three years of age were divided into two groups, persistent wheezers and transient wheezers. Differences were observed between these two groups in the response at the time of the first infective wheezing episode, which occurred at around one year of age and was due to Respiratory Synctical Virus (RSV) in two-thirds of the episodes. Persistent wheezers showed an increase in serum IgE, and eosinophils counts were maintained, which was not seen in transient wheezers. It has been suggested that persistent wheezers will therefore be over represented in studies of children hospitalised with RSV infection, and this may explain why such studies show a strong association with subsequent asthma (29). This also supports the idea that asthma prevalence has increased because of increased persistence of wheeze rather than increased incidence.

The Tucson cohort was further investigated by Stein and colleagues (30), who reported that RSV lower respiratory tract illnesses were associated with increased wheeze (odds ratio (OR) of 3.2 (95% CI: 2.0-5.0) and an increased risk of frequent wheeze (OR = 4.3; 95% CI: 2.2-8.7) by six years of age. Risk decreased markedly with age and was not significant at 13 years old. Similar increased risk was observed with other pathogens and indeed with lower respiratory tract infection. No associations were found between infection by pathogens and the subsequent development of atopy.

After conducting a study in England/Wales and New Zealand, Wickens and colleagues also point out the importance of other asthma risk factors, which may have changed over time (31). The time frame that was assessed was from 1961-1991 and the relationship studied was between declining family size and the increase in asthma and hay fever prevalence. Finding a progressive reduction in family size during this period, it was estimated that a 1-5% relative increase in asthma in New Zealand and the United Kingdom and a 4% increase in allergic rhinitis in the United Kingdom might have occurred as a result of this decline in family size. Although strong associations were found between family size and asthma (OR = 0.56) for children with 3 or more siblings compared to only one child, the increases that may have occurred are very minor in comparison to the increases in asthma prevalence, estimated at 30-372%. One limitation of this study was that it was difficult to estimate the relative size of the increase in asthma prevalence between 1961 and 1991.

Barker has suggested another theory, when he explored the idea that the 20th century epidemic of coronary heart disease in western countries may have originated in foetal life (32). Paradoxically, the epidemic coincided with improved standards of living and nutrition, yet in Britain its greatest impact was in the most deprived areas. Barker observed that early in the 20th century these areas had the highest rates of neonatal mortality and by inference the highest rates of low birth weight. He postulated that impaired fetal growth might have predisposed the survivors to heart disease in later life. Now this theory has been applied to the development of asthma.

This theory is supported in a study carried out by Brooks and colleagues (33). They carried out a cross sectional study using the 1988 United States National Maternal-Infant Survey combined with a 1991 longitudinal follow-up survey. Eight thousand and seventy one infants were weighed and their caregivers completed questionnaires at birth and three years later. The prevalence of asthma varied by birth weight category; 6.7% in children 2500g or more at birth, 10.9% in children 1500 to 2499g at birth, and 21.9% in children less than 1500 g at birth (very low birth weight). The sole outcome measure in this study

was physician-diagnosed asthma in the first 3 years of life. Children were categorised as having asthma if there was a positive response to the question in the longitudinal follow-up survey: "ever been told by a doctor, nurse, or other health care provider that [child's name] has asthma? (33)."

There were some limitations to this study. Firstly the ability to accurately classify children with a history of bronchopulmonary dysplasia was limited. The purpose was to identify the contribution of low birth weight to asthma prevalence, regardless if any comorbid conditions existed. In early childhood however, significant respiratory disease related to prematurity may obscure the diagnosis of asthma. A second limitation is the potential for selection bias. Identifying asthmatic children on the basis of parental reports alone leaves room for error. Young children with asthma may have been misclassified as normal when their symptoms were mild and less persistent. The likelihood is greater, therefore, that these prevalence values are underestimates of true disease burden.

Summary

Overall, there appears to be plentiful and supportive epidemiological evidence for the role of early exposures to non-respiratory infections as a protective factor against the development of childhood asthma and atopic diseases. Farming studies have given us convincing evidence that, whatever the agent the timing of exposure is what appears to be important, and that the first year of life is crucial. As we have seen in the studies mentioned above, endotoxin levels have been demonstrated as being greater in farming communities than in urban communities (except for New Zealand) with these higher levels of endotoxin being related to lower levels of atopic diseases. However, bearing in mind the clear pro-inflammatory potential of endotoxin, such a response might represent an excessive or premature reaction.

Endotoxins are derived predominantly from Gram negative bacteria, however it has been shown that dust and air-borne bacterial flora in schools and day care centres are dominated by Gram positive bacilli and actinomycetes (34). The cell walls of these organisms are composed of a different polymerised structure comprising peptidoglycans. Peptidogylcans are able to interact with the immune system in a very similar way to endotoxin and may exert a larger effect on atopy and asthma than endotoxin (35). Both endotoxins and peptidoglycans are principal molecules that are recognised by the immune system. Entirely independent of the IgE mechanisms, both molecules may induce inflammatory responses in the airway leading to asthma, or make asthma symptoms worse, both in those who are allergic, and those who are not.

Could it be another of the risk factors or a combination of risk factors associated with rural living that helps prevent atopic development in children? Differences in living conditions of farming families compared to the life-styles of urban families could explain the differences in the development of allergic diseases and asthma.

Studying the indoor environment has a number of problems, since the home environment is comprised of a complicated milieu of allergens, fungi, bacteria, pollutants such as nitrogen dioxide from gas appliances, and toxins. It is possible that endotoxin is just a convenient measurable marker of these environmental exposures and it may well act as a marker for other substances.

It is likely that hygiene, as measured by endotoxin levels, is only one of several independent environmental risk factors in the development of childhood asthma. Postnatal environmental risk factors are themselves only part of a greater scheme that also includes foetal development and genetic predisposition. It appears that interactions between factors such as human development, genetics, and the environment, are the most important factors in the development of atopic childhood asthma.

Acknowledgements

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Letter to the Editor

Comparison of two kits for detection of extractable nuclear antigens

Dear Editor

Re: Comparison of the RELISA ENA multi-parameter antibody screening test kit and INNO-LIA ANA Update kit for detection of antibodies to extractable nuclear antigens - Tong-Kyung (Sophia) Shinn, Laboratory Services, Wellington Hospital - Published in the *New Zealand Journal of Medical Laboratory Science*, Volume 57, Number 2, August 2003.

Med-Bio Limited supplied this kit to the Immunology Laboratory at Wellington Hospital free of charge on 23 August 2001. The kit batch number was 01030817 and the expiration date was 30 September 2001. Our records show that at 13 June 2002 the work with this kit had not been completed. (We suspect that it was still unused, but have nothing factual to support that). At this stage (13/6/02) the kit would have been almost 9 months past its expiration date.

We are concerned about this Scientific Letter because the author did not specify that her work was carried out using expired reagents. As we should all be aware, immunoassay reagents, by their very nature, will deteriorate over time. The rate of deterioration is dependent on many factors. Because of this, all manufacturers specify expiration dates for their products. Manufacturers, including Innogenetics, state very clearly that reagents should not be used passed their expiration date. (Product Insert, page 10, Remarks and Precautions). IANZ, who carry out accreditation for New Zealand laboratories, also will not allow laboratories to use reagents that are past the manufacturers expiration dates. When expired reagents are used, there is an increasing risk of lowered assay sensitivity and specificity from that determined by the manufacturer. This may well result in incorrect or misleading results. Immunoassay reagents, such as the the Innogenetics InnoLIA ANA Update kit, may experience deterioration in the following reagents over time:

- Deterioration in the antigens that have been immobilised onto the nylon membrane. The epitopes of the antigens may become damaged over time resulting in less binding of the corresponding antibody, or possibly no binding at all.
- Deterioration of the alkaline phosphatase labelled goat x human conjugate. This could be a similar deterioration in the ability of the antibody to bind to its epitope or a loss in enzyme activity resulting in low or no signal, even when specific antigen/antibody binding has occurred.
- Deterioration in the cut off control used to compare positive reactions against.

These are the most likely problems that we may see when these reagents age. It is possible that other kit components, that are designed to work together, could also deteriorate over time and result in both lowered sensitivity and specificity for the assay.

Because the author used at least one set of reagents (INNO-LIA ANA Update kit) that had long expired in her comparison, I believe that she had a responsibility to state this clearly in this Scientific Letter. This information would then allow readers of the Research Letter to interpret her results with this knowledge. Med-Bio Limited, as Innogenetics representative in New Zealand, is concerned that other potential users of this product will be unfairly influenced by the information contained in this Research Letter. It is possible that her results do not reflect the true performance of this product. I would request that this letter be referred to the author for her comment. I would like her to submit a correction in the next issue of the journal that notifies readers of the true nature of her comparison and the possible impact that this may have had on her results.

Warren Dellow, Managing Director, Med-Bio Ltd., Christchurch

Author's response

Dear Editor

I acknowledge the comments made by Warren Dellow in regard to my recently published Research Letter (1). I should have mentioned that the INNO-LIA ANA Update kit supplied by Med-Bio was outside the manufacturer's expiration date. For this I apologise to Med-Bio, the Editor of the *New Zealand Journal of Medical Laboratory Science*, and to the readers. However, the control bands on the test strips (apart from one mentioned in the Table), when used for testing according to the manufacturer's instructions, turned brown, as required for the validity of the test.

Reference

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Sophia Shinn, Biological Investigations Group, Wellington School of Medicine & Health Sciences, PO Box 7343, Wellington South, Wellington

Editor's comment

The above comments from Warren Dellow and the response from Sophia Shinn bring up an important point for potential contributors to the Journal. As stated in the Instructions to Authors, contributors are responsible for the scientific content of their submitted articles. It is not possible for the Editor, Editorial Board members or referees to second-guess what the authors may have omitted, intentionally or otherwise. They are totally reliant on authors to honestly and fully present any data or information that is relevant to the submitted study.

In no way do I suggest that the author intentionally set out to mislead. Most likely her omission was due to her inexperience in writing scientific papers. The above is a salutary lesson for all aspiring authors.

Rob Siebers, Editor, Wellington School of Medicine of Medicine & Health Sciences, Wellington

Book Reviews

Blood Cells. A Practical Guide. (3rd Edition 2003) by Barbara Bain. Published by Blackwell Publishing (UK).
Ordering information: Blackwell Publishing Asia, PO Box 378, Carlton South, Victoria 3053, Australia. Cost (GST incl.): A$240.90

Barbara Bain has written many creditable haematology textbooks. This one is no exception. It contains all the essential basic information of the previous two editions such as methods of collection, blood film preparation, performing blood counts, morphology of blood cells in health and disease as it guides the reader to perform important supplementary tests to assist in establishing a diagnosis.

The book serves as an atlas of haematology as well as a comprehensive practical bench manual. It is highly visual and contains over 350 colour illustrations. The concise updated boxed tables and charts that are a feature of Bain's books are annotated with reference to relevant texts. The references at the end of each chapter have increased from earlier editions by approximately 30 - 90 per chapter.

The emphasis throughout the book (as in the earlier editions) is on microscopy and automated blood counts but has been greatly expanded to accommodate recent advances in automated technology and the wider range of currently available automated instruments performing 5—7 part differential counts. The text outlines details of the operating principles of each instrument and alerts the reader to inherent inaccuracies relative to the technology employed that may occur under certain conditions e.g. the presence of abnormal cells, pathological samples and thalassaemias.

Chapter 4 is devoted entirely to the detection of erroneous blood counts. Advanced instruments currently available for full blood counts and the counting of reticulocytes are listed alongside the underlying technologies employed and highlighted in boxed tables. Chapter 4 on "Normal Ranges" has been updated and includes re-formatted layout of normal range tables, which are easy to follow. The author succinctly explains the use of normal ranges and the concept underlying the derivation of reference ranges.

Chapter 7 on " Important Supplementary Tests" has been expanded to include the practical relevance of immunophenotyping, cytogenetic analysis and molecular genetic analysis. The importance in particular of immunophenotyping in the diagnosis of leukaemia and lymphoma is emphasised. There are concise easy to follow reference tables illustrating typical immunophenotypic findings in acute myeloid leukaemia, acute lymphoblastic leukaemia, chronic lymphatic leukaemia and non Hodgkin's lymphoma of B lineage as well as typical immunophenotypic findings in chronic T- cell lineage and natural killer (NK)- lineage lymphoproliferative disorders. The purpose and clinical relevance of laboratory tests are clearly stated with excellent guidelines as to supplementary tests and expected results that should be performed to assist in diagnosis.

Practical useful additions to Chapter 8 " Disorders of Red cells and Platelets "and Chapter 9 " Disorders of White Cells " include: details on red cell membrane disorders together with an excellent diagram illustrating the structure of the red cell membrane; referral to a website updated annually on drugs recognised as causing thrombocytopenia; clear tables demonstrating the French-American-British (FAB) classification of leukaemias and the World Health Organisation (WHO) classification of leukaemias These tables can be easily accessed for quick reference and enable comparisons to be made between the classifications. To date the most generally accepted is the FAB classification but indications are that the WHO classification will be increasingly adopted.

An additional feature of this edition is the inclusion of a self-assessment at the end of each chapter. A questionnaire entitled "Test Your Knowledge" provides the reader with both multiple choice and extended matching questions. Some of the questions, particularly in the specialist areas, are challenging. Readers would be well advised to return to the text of the chapter to consolidate knowledge before taking the easy way out and referring to the answers provided.

This book is a useful diagnostic guide for Haematologists and Medical Laboratory Scientists providing a practical, concise and logical approach to the wide range of blood disorders. The excellent photomicrographs of blood cell morphology enhance its value as an essential practical bench reference manual and teaching tool for medical students and for students of medical laboratory science. This is a book that justifies the investment and should be readily available in all laboratories where haematology is practised. There should be no hesitation in updating Vol 2 to Vol 3.

In Bain's preface she states, *" My overriding purpose has been to show that microscopy not only provides the essential basis of our haematological practice but can also lead to the excitement of discovery. If I succeed in sending the reader back to the microscope with renewed interest and enthusiasm I shall be well satisfied"* The reviewer was well satisfied and believes Barbara Bain has achieved her purpose.

Reviewed by Marilyn Eales, PPTC, Wellington

Leukeamia Diagnosis (3rd edition 2003) by Barbara Bain. Published by Blackwell Publishing (UK).
Ordering information: Blackwell Publishing Asia, PO Box 378, Carlton South, Victoria 3053, Australia. Cost (GST incl.): A$220.00.

Barbara Bain is the author of a successful range of books on haematological morphology and diagnosis. Leukaemia Diagnosis in its previous editions has been well received and found a place in many haematology laboratories. The third edition comes after a relatively short time span from the second edition. The necessity for this is due to the rapid progress in flow cytometric and, particularly, molecular adjuncts to the diagnosis of the acute leukaemias. This is reflected in the new edition being 50 pages longer than the old one and its incorporation of the old FAB classification with the new WHO and MIC-M classifications.

As in all of Barbara Bain's books the sequence and layout of the chapters is highly logical. There are high quality illustrations, tables and diagrams. To my eye the tinctorial quality of the illustrations is much better than in the second edition where many of the blood films had an unpleasant yellow tinge to them. There are five chapters covering acute leukaemia (two), myelodysplastic syndromes, chronic myeloid leukaemias and chronic lymphoid leukaemias.

Many laboratories around the world are in a quandary as to how best to report their acute leukaemias given, in particular, the arrival of the new WHO classification system. During this period of transition many of them have chosen to incorporate both classifications into their diagnostic reports. The new edition of Leukaemia Diagnosis will be helpful for reporting in this manner. It also contains very up-to-date

synopsis of the described molecular defects in the acute leukaemias that are useful for both a diagnostic and prognostic viewpoint. For those laboratories where leukaemia diagnosis is a significant part of their workload they will also supplement this book with the 2001 edition of the WHO book on leukaemia diagnosis which also includes the classification for lymphoma, Hodgkin's disease and the myeloproliferative disorders. The Bain book being the more recent of the two, albeit by only two years, contains significantly more material on the molecular defects in acute leukaemia.

I would recommend this book to haematology units and the third edition incorporates significant new material over and above the previous second edition

Reviewed by Associate Professor John Carter, Haematology Laboratory, Wellington Hospital

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E Coli: Shiga Toxin Methods and Protocols. Edited by Dana Philpott and Frank Ebel. Published by Humana Press, New York, 2003.
Ordering information: Blackwell Publishing Asia, PO Box 378, Carlton South, Victoria 3053, Australia. Cost (GST incl.): A$316.80.

The list of contributors of this book, part of the Methods in Molecular Medicine series, reads like a "Who's who" of the shiga toxin-producing Escherichia coli (STEC) world. As the title indicates, the book is full of detailed methods and protocols for working with these very interesting organisms, but it is much more than that. It is also contains a great deal of background information, so that the reader is fully informed of every aspect of STEC, from clinical significance and epidemiology to cellular biology. Mohamed Karmali, who in 1983 published the first paper associating a link between infection with cytotoxin-producing Escherichia coli (E coli) and the development of haemolytic uraemic syndrome, is the author of the first chapter. This deals with the medical significance of Shiga toxin-producing E coli infections.

Subsequent chapters detail methods of detection of STEC in humans, in animals and in food samples. Each chapter begins with an informative introduction, followed by detailed protocols of test methods, including lists of materials. They are well written and easy to follow, although there are some typographical errors and occasional indications that English is not the first language of many of the authors. A Notes section at the end of each chapter provides an insight into potential problems that may be encountered, which is very useful for workers trying out a new technique for the first time. There is welcome advice on details such the carcinogenicity of some reagents.

The use of PCR for detecting STEC is described in detail. This technique is becoming used more frequently by diagnostic laboratories and is ideally suited for the examination of complex material such as faeces and foodstuffs for the presence of STEC, because it is very sensitive and specific. Primer sequences are given, although first-time users might find details for obtaining custom primers useful.

The mid-section of the book deals with techniques used by reference laboratories. The chapter on molecular typing protocols gives three methods including the 'gold standard' pulsed-field gel electrophoresis (PFGE). I felt this section would benefit from an amplified discussion on the relevance of the results obtained. Tenover's Principles are referenced, but given their importance to the interpretation of PFGE patterns, they should have formed part of the chapter. In addition, the clonal nature of E coli O157 isolates means that the interpretation of PFGE patterns of epidemiologically unlinked strains should be undertaken with caution, a point which is not made in this chapter.

The rest of the book contains material of use to university and research laboratories, including cellular biological tests and the use of animal models of infection. Although these techniques are very specialised and unlikely to be used by diagnostic or reference laboratories, the introduction to each chapter contains useful background information. For example, the introduction to the chapter on detection and characterisation of EHEC-haemolysin notes that EHEC O103:H2 overproduces E-hly to the extent that an (-haemolysin-like phenotype occurs on washed blood agar plates. This information is of interest to diagnostic laboratories that used washed sheep blood agar (EHEC agar) to identify non-O157 STEC.

The editors have endeavoured to produce a useful book for the clinical microbiologist as well as the cellular microbiologist, and I feel they have succeeded. This book will be a welcome addition to the bookshelf in the Enteric Reference Laboratory, and diagnostic, research and university laboratories will also find it a valuable resource.

Reviewed by Jenny Bennett FNZIMLS, Enteric Reference Laboratory, ESR, Kenepuru Science Centre, Porirua

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Manual of Diagnostic Antibodies for Immunohistochemistry (2nd Edition) by Anthony S-Y Leong, Kumarasen Cooper, F Joel W-M Leong. Published by Greenwich Medical Medica, 2003.
Ordering information: Blackwell Publishing Asia, PO Box 378, Carlton South, Victoria 3053, Australia. Cost (GST incl.):

This book is the 2nd edition of the Manual of Diagnostic Antibodies for Immunohistochemistry co-authored by leading pathologists in the field of diagnostic immunohistochemistry. The increasing role of immunohistochemistry in the diagnostic laboratory, together with the ever-increasing number of antibodies available and evolving detection techniques has led to the publication of a 2nd edition of this manual.

The manual is divided into 3 sections. The first section features a broad list of 181 antibodies. These include the more common and widely used antibodies, to those that have a more specialised use in a diagnostic laboratory. For each antibody there is a dedicated section that includes information on the source of the antibody and/or its clone, fixation and preparation, comprehensive detail on the background of the antibody, applications, comments and a list of references. For effortless use the antibodies are listed alphabetically, with an additional feature of a blank page titled "notes" before each letter of the alphabet.

Section 2 provides a broad set of appendices that contain concise information for simple reference. Appendix one details antibody panels for specific diagnostic situations that are particularly useful when a differential diagnosis is required. This information is in a tabulated form that is easy to read and provides rapid information. Appendix two provides similar information for lymphoid neoplasms, while appendix three supplies an inventory of each antibody with a brief description of its applications. The third section is a practical list with details of supplier's names and their websites.

Overall this book would be a valuable addition to any pathologists, medical laboratory scientists or students library, useful in any diagnostic or research laboratory. The value of this manual extends from details of individual antibodies to assistance in differential diagnoses. The information contained in the manual is current and well formatted.

Reviewed by Ann Thornton, FNZIMLS, Pathology Department, Wellington School of Medicine and Health Sciences, Wellington

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Haematology

Special Interest Group

HSIG Journal based learning - Questionaire

"The diagnosis and management of hereditary spherocytosis"
Paula H. B. Bolton-Maggs FRCPath, FRCP, FRCPCH

Bailliere's Clinical Haematology Vol 13, No 3, pp 327-342, 2000

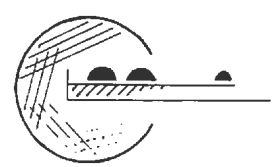
1. When was hereditary spherocytosis (HS) first described?
2. Genetic defects in what 5 membrane proteins result in HS?
3. What is the main anion-exchange protein?
4. HS due to a mutation in what membrane protein results in frequent acanthocytes?
5. What is protein 4.2 also known as?
6. What HS defect is most frequently found in the Japanese?
7. What condition masks HS film appearances and reduces haemolysis?
8. What automated parameters are the most helpful indicators of HS?
9. What increases the sensitivity of the osmotic fragility test?
10. Explain MCF
11. What are the disadvantages of the osmotic fragility test?
12. What co-existing conditions with HS result in a normal osmotic fragility?
13. What dye is utilised in flow cytometry for HS, and why?
14. What is meant by a "private" mutation?
15. Aplastic crises in HS are chiefly caused by what?
16. How does splenectomy benefit the HS patient?
17. What is the main risk of splenectomy?
18. HS is always caused by a dominant gene-True or False
19. In general the clinical severity of HS parallels the measured quantitative defect in spectrin- True or False
20. HS has not been described in blacks- True or False
21. Neonatal jaundice is a common feature of HS- True or False
22. 50% of cases of HS will have a family history- True or False
23. Splenic rupture is fairly common in HS- True or False

For a copy of this journal article, contact Jacquie Case at Haematology Dept., Middlemore Hospital, Otahuhu, Auckland. Ph 09 2760044, extn 8515 or e-mail
jcase@middlemore.co.nz

Answers on page.....

Microbiology

Special Interest Group



I Saw a Ship a Sailing

C Nicol[1], J Hewitt[2]. Enteric Reference Laboratory[1] (ERL) and Norovirus Laboratory[2] (NVL), Institute of Environmental Science and Research Ltd, Porirua

An increased number of cruise ships have been visiting New Zealand waters over recent months, one in particular provided specimens to ESR and eventually received much attention from the New Zealand public health services.

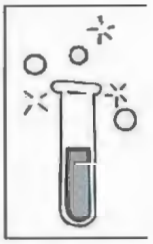
The cruise liner has a capacity for 1,804 passengers and a crew of 735. It is operated out of the United States of America and makes annual South Pacific cruises. This account concerns 5 voyages (284-288).

Voyage 284 began in Honolulu on 19 December 2002 and arrived in Tauranga, NZ on 4 January 2003. Six out of 7 faecal specimens referred to NVL for norovirus (NV, formerly Norwalk-like Virus) tested positive.

Passengers from voyage 285 embarked in Auckland on 5 January and sailed 6 January. A total of twelve faecal isolates were referred to ERL and were subsequently confirmed as S. Typhimurium phage type 160. This phage type has spread rapidly throughout New Zealand since 1998 and is rarely isolated from human cases in other parts of the world.

NV was also confirmed in specimens from voyages 286, 287 and 288 that took place between 19 January and March 2003. All noroviruses were sequenced and compared to others in a NV database. NV genotype GI/3 was identified from voyage 284 and GII/1,4,8 identified from voyage 286. The NVs from voyages 287 and 288 were also identified as GII/1,4,8, however, they were different from the NV from voyage 286 but identical to each other. Sequence analysis is a valuable tool in outbreak investigation of NV.


NZIMLS BIOCHEMISTRY SPECIAL INTEREST GROUP SEMINAR

CONCURRENTLY

OCCUPATIONAL HEALTH AND SAFETY SPECIAL INTEREST GROUP SEMINAR




Where: Heritage Auckland, 35 Hobson Street, Auckland.
BSIG: Robert Laidlaw Room 1 & 2. OHSSIG: John Healey Room
When: Saturday 3rd April 2004
Registration : 9.00 - 10.00am, Morning tea and coffee on arrival.

Proffered papers for both seminars: 10.00am to 5.00pm

Costs:
Registration: Members NZIMLS: $75.00,
Non members NZIMLS: $125.00
Student registration available on request.
Includes morning and afternoon tea, lunch and access to papers at both seminars.
Seminar Dinner: $55.00 inclusive of GST

Accommodation: Heritage Hotel, 35 Hobson Street, Auckland.
Single Room: $152 inclusive of GST.
Twin Share and Breakfast options available on request.

This meeting will host the Biochemistry SIG seminar and the newly resurrected Occupational, Health and Safety SIG seminar. Branko Vidakovic from LabPLUS, Auckland Hospital has agreed to act as Chairperson of the OHSSIG. The registration fee allows delegates to listen to papers presented at both seminars.

BSIG and OHSSIG Seminars
Proffered Papers

Heritage Auckland, 35 Hobson Street, Auckland.
Saturday 3rd April, 2004

Presenter:

First Name: _____
Surname: _____
Laboratory / Organisation_____
Address: _____
_____
Contact Phone Number: _____Fax_____
Mobile: _____Email Address: _____

Title of Presentation:

_____
_____
_____
_____

Biochemistry SIG Seminar ☐ Occupational, Health & Safety SIG Seminar ☐

Abstract Provided Yes ☐ No ☐ Oral presentation ☐ Poster ☐

Equipment Required:

Overhead projector ☐

Slide Projector ☐

Data show (Powerpoint) laptop provided ☐

Other_____

Please return this form to : Ross Hewett, LabPLUS, P.O.Box 110031, Auckland Hospital, Auckland. Tel. 09 3074949 ext 5466, Mob 021 439 388, Fax (09) 307 4939. Email rossh@adhb.govt.nz

NZIMLS President's address

Chateau on the Park, Christchurch 1st August 2003

Dear Members, Associate and Life members of the NZIMLS.

As part of the annual President's address it is customary to recall the activities of the NZIMLS over the past year. The Council of the NZIMLS operates the affairs of the profession under various portfolios which include:

- Finance
- Education - Fellowship, BMLSc. programmes, QTA & QPT
- Professional Affairs - CPD, SIGs
- Promotion/Publicity - Website, Journal, Conference, Newsletter
- Rules and Membership
- Executive Office

Finances

The NZIMLS continues to maintain a buoyant financial position thanks to strong performances of past SIG activities and the Annual Scientific Meetings. The annual report details the costs of running the NZIMLS and it should be heartening for the membership to see the current financial status of the Institute's accounts. Perhaps of greatest concern in the accounts is the cost of publishing the NZIMLS Journal. Reduced income streams from advertising have lead to a larger than expected deficit for the journal for the 2002-03 year. This has been identified as an area requiring close scrutiny for the upcoming year and Council is looking into the options available to contain the costs.

One notable, but yet to be realised financial success in the past year has been the agreement negotiated between the AIMS and the NZIMLS over the profit/loss sharing of future South Pacific Congresses. It has long been the wish of the NZIMLS to reach some agreement with AIMS over this matter, which was raised following the last SPC hosted in Christchurch in 2000. This meeting ran at a significant financial cost to the Institute after a poor turn out by the Australians. The new agreement means that from this year, there will be a financial return to the NZIMLS based on the number of NZ delegates to the Gold Coast Conference.

Education

The Fellowship convener and his band continue to process the large number of candidates currently working towards completion of their Fellowship Treatises. The profession continues to be represented on the various Boards' of Study and Management programmes of the Universities providing MLS training in NZ. The Institute's QTA examinations continue to be well supported and in 2003 the new QTA equivalent qualification for phlebotomy, the Qualified Phlebotomy Technician examination is to be run. Interest in this new qualification is high with over 100 applications to sit the examination in 2003.

Professional Affairs

Much of the collective effort of the Institute over recent years has been directed toward the Competency and Professional Development programme and the interest in the upcoming HPCA Bill. The Institute looks well positioned with its CPD programme to meet the much talked-about requirements of the new Bill. It is therefore with cautious optimism that I am able to report that the Institute looks set for a bright future as the provider of a programme for all practising Scientists in NZ. If this is to be the case, then the prophecies and the work of previous members of the NZIMLS Council with the MOLS programme and more latterly with the earlier versions of the current CPD programme will finally galvanise into reality, to become an integral part of MLS practice in the years ahead.

The SIG's of the NZIMLS continue with their active programmes of promotion of the profession through their seminars, workshops etc. Last year the NZIMLS welcomed the NZ Association of Phlebotomist's as a new SIG, the result of growing interest in the pre-analytical phases associated with laboratory endeavour. The SIG has got off to a fast start and organised itself in a relatively short-while with their first seminar to be held here in Christchurch this weekend. As a result of the interest in this new SIG, the Institute welcomes the many new members who makeup the group calling themselves the NZ Association of Phlebotomists.

Promotion and publicity

Over the last year the NZIMLS Website has undergone a makeover to provide further functionality. New to the site are features such as the MLS forum, the smiling faces of the EO and Council, and soon to be the SIG convenors. The site now includes a section whereby employers looking for staff and persons looking for work, can advertise their interests. The site also allows online CPD enrolments; CPD points claims for activities and downloadable certificates showing accumulated CPD points.

The Institute's Journal continues to provide a very high standard of scientific publications thanks to the efforts of the Editor and those providing articles for publication. Council is currently investigating providing full text articles published in the journal as downloadable .pdf files on the website to complement the full text publication.

In 2004 the Institute's Annual Scientific meeting and AGM will be held in Hamilton and an enthusiastic band of locals are already well underway with plans for the meeting. In 2007 the SPC will again return to New Zealand with plans already in place to stage the meeting in Auckland.

Last year the Institute moved toward distributing the newsletter via email. This has been well received and has reduced significantly the past costs associated with the previous method of postal distribution.

Rules & membership

In recent times the Institute has undertaken a review of the Mission Statement and is looking toward producing a modified version of the Code of Ethics once the details and the future impact of the HPCA Bill has been identified. The Institute has enjoyed a jump in membership over last year an encouraging sign for the future of the Institute.

Executive office

The NZIMLS continues to maintain a contract with E.events to provide office facilities and Executive Officer duties. Council is currently looking into the possible impact upon the EO of the provision of the CPD programme to an expanded market over the next years. This looks set to increase the workload of the EO with the possible need for additional personnel to meet the demands of the programme.

In closing the Institute looks to be entering a period of growth in the numbers of persons using the services provided by the NZIMLS. The future role of the NZIMLS as a well-established provider of Continuing Education and associated programmes for practitioners of MLS in New Zealand looks assured. It is therefore with an element of cautious confidence I can state that Council looks forward to a period of increased activity and growth over the next few years.

Chris Kendrick
NZIMLS President, 2003

NEW ZEALAND INSTITUTE OF MEDICAL LABORATORY SCIENCE (INC)

MINUTES OF THE 59th ANNUAL GENERAL MEETING HELD AT CHATEAU ON THE PARK, CHRISTCHURCH ON FRIDAY 1ST AUGUST 2003 AT 7.30AM

CHAIRMAN
The President (Mr C Kendrick) presided over the attendance of approximately 37 members.

APOLOGIES
Motion:
Moved C Kendrick, seconded T Rollinson
That the apology from S Gainsford be received.

PROXIES
Motion:
Moved J Deans, seconded A Thornton
That the list of four proxies as read by the Secretary be received.

MINUTES
Motion:
Moved C Kendrick, seconded R Siebers
That the Minutes of the 58th Annual General Meeting held on 9th August 2002 be taken as read and accepted as a true and correct record with the time of the meeting to read 7.30am not 7.30pm.
Carried

BUSINESS ARISING
Nil.

REMITS
Motion:
Moved J Deans, seconded T Rollinson
That Policy Decision Number 4 be reaffirmed
Policy Decision No 4 (1991): That the Code of Ethics as circulated to all members be adopted by the New Zealand Institute of Medical Laboratory Science (Inc).

Motion:
Moved J Deans, seconded W Dellow
That Policy Decision Number 6 be reaffirmed.
Policy Decision No 6 (1979): That the Council must be informed in advance of national workshops, seminars or similar gatherings which are being conducted under the aegis of the NZIMLS.

PRESIDENT'S REPORT
Motion:
Moved C Kendrick, seconded W Dellow
That the President's Report be received.
Carried

ANNUAL REPORT
Motion:
Moved R Allen, seconded T Rollinson
That the Annual Report be received and adopted.
Carried

FINANCIAL REPORT
Motion:
Moved R Siebers, seconded K Taylor
That the Financial Report be received and adopted.
Carried

ELECTION OF OFFICERS
The following members of Council were elected unopposed:

President	C Kendrick
Vice President	T Rollinson
Secretary/Treasurer	J Deans
Region 1 Representative	R Hewett
Region 2 Representative	R Allen
Region 4 Representative	K Taylor
Region 5 Representative	A Buchanan

The results of the elections for:

Region 3 Representative	A Thornton	26
	S Khull	16

Motion:
Moved, C Kendrick, seconded T Rollinson
That the Election of Officers be approved.
Carried.

AWARDS
The award winners were announced and the awards where possible were presented by the President:

Qualified Technical Assistant Awards

Clinical Biochemistry	Dianne Gillard, Medlab Tauranga
Medical Cytology	Jacqueline Lee, Southern Community Laboratories
Haematology	Louise Fuhrer, Medlab Gisborne
Histology Susie Warwick,	Pathlab Waikato
Immunology	Sara Thomas, Medlab South
Microbiology	John Britto, Diagnostic Medlab
Transfusion Science	Christy Steer, NZBS Waikato
Transfusion Science Blood Products	Blair Keats, NZBS Waikato
Mortuary Hygiene & Technique	Diane Woodford, Dunedin Hospital
Virology	Marita Smit. Canterbury Health Laboratories

HONORARIA
Motion:
Moved R Siebers, seconded R Hewett
That no honoraria be paid.
Carried

AUDITOR
Motion:
Moved J Deans, seconded T Rollinson
That Hilson, Fagerlund and Keyse be appointed as the Institute's auditors.
Carried

GENERAL BUSINESS
Harold Neal, Chairman, NZ Medical Laboratory Technologists Board reported that the HPCA bill in its 2nd reading in Parliament. There will be even stronger links with the NZIMLS and MLTB with registration and the CPD programme.

VENUE FOR THE YEAR 2004 ANNUAL GENERAL MEETING
Robin Allen advised the meeting that the NZIMLS 2004 conference will be held in Hamilton from 24th August to 28th August 2004 and the AGM of the Institute will be held in association with the meeting. The ASM is to be held at the Quality Hotel, Hamilton with a theme of 'Windows in Waikato'.

VENUE FOR THE YEAR 2005 ANNUAL GENERAL MEETING
No venue was confirmed. Council will follow-up.

Meeting closed at 8.00am

Answers to HSIG questionnaire

1. 1871.
2. Spectrin, ankyrin, band 3, protein 4.1, protein 4.2.
3. Band 3.
4. Beta spectrin.
5. Pallidin.
6. Pallidin (protein 4.2) gene defects.
7. Obstructive jaundice.
8. MCHC & RDW together.
9. Performing test after 24hours incubation of sample at 37°C.
10. MCF = mean corpuscular fragility (the conc. of saline causing 50% lysis).
11. Time consuming to perform, does not differentiate between causes of spherocytosis (immune vs non-immune), 10-20% of HS cases have normal fragility results.
12. Iron deficiency, obstructive jaundice, recovery phase from an aplastic crisis (increased reticulocytes).
13. Eosin-5-maleimide, it binds to band 3 protein on the red cell membrane.
14. They are specific to one family.
15. Parvovirus infection.
16. Reduces haemolysis, prolongs rbc life span (though not necessarily to normal), anaemia & gallstones are much reduced in severe HS, and abolished in milder cases.
17. Risk of severe life threatening sepsis from encapsulated organisms, especially
streptococcus pneumoniae.
18. False.
19. True.
20. True.
21. True.
22. False (it's 75%).
23. False.

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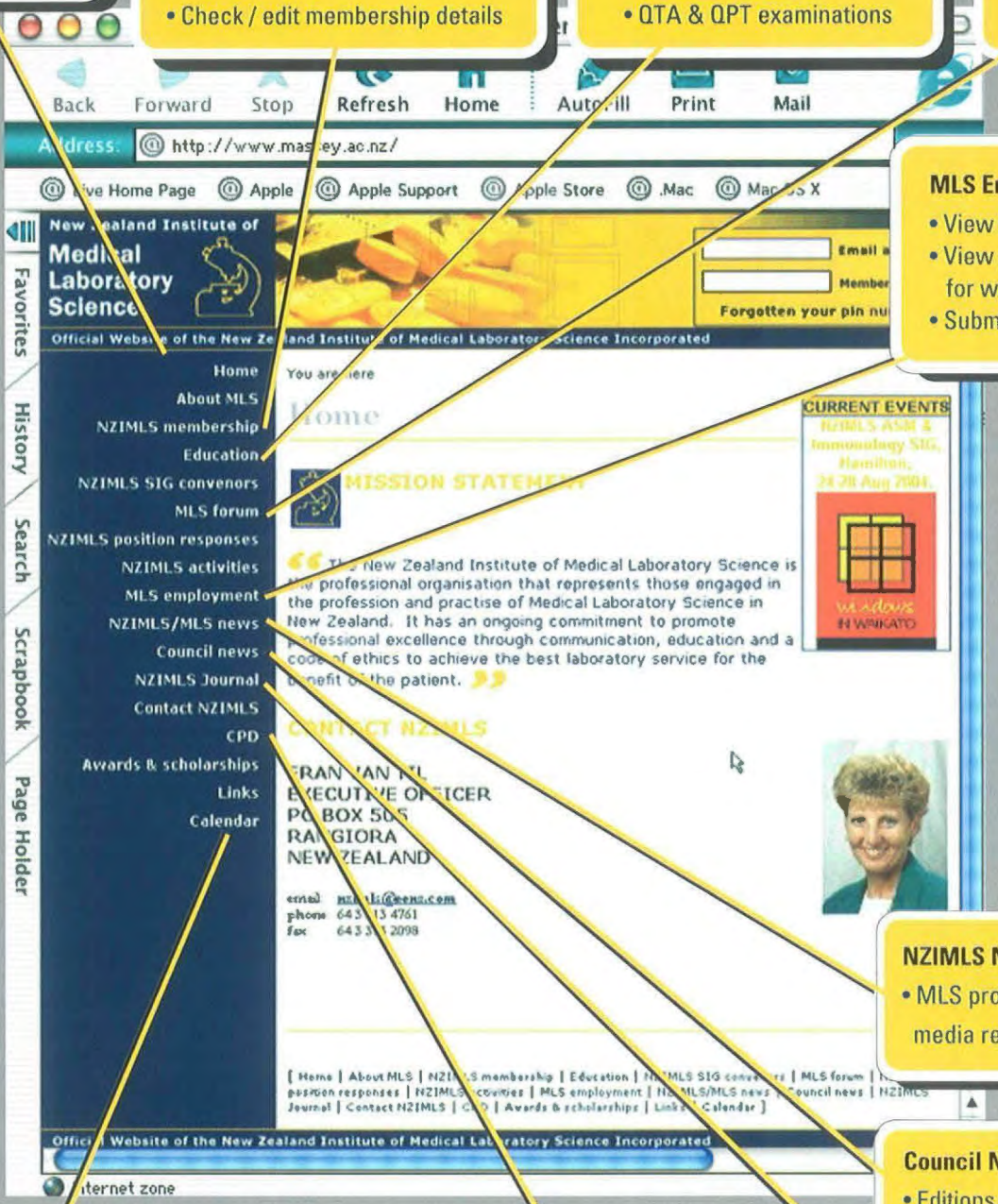

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- View vacancies
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- Submit an advert

NZIMLS News
- MLS profession related media releases etc.

Council News
- Editions of the newsletter

NZIMLS Journal
- Editor
- Instructions to authors
- Editorial board
- Journals

Calendar
- MLS releated events - 2003/04

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