Growth faltering in Gambian children under 15 months of age is associated with a chronic enteropathy leading to a breakdown of gut barrier function, and systemic inflammation

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Summary

Background: Gambian children have severe growth faltering in the first two years of life. Poor growth is strongly associated with an enteropathy, which exhibits abnormal lactulose and mannitol (L:M) permeability of the gastrointestinal tract. This thesis aimed to examine the following hypotheses relating to the enteropathy:

1. Impaired gut barrier function is related to poor growth in Gambian children (up to and beyond 2 years of age).
2. Impaired gut barrier function is persistent and associated with systemic inflammation.

The final aim was to characterise the enteropathy and provide insights into aetiology.

Methods: Three groups of subjects were recruited.

1. Keneba longitudinal cohort, was designed to assess the impact of impaired gut barrier function (as assessed by the L:M permeability and plasma endotoxin levels) systemic inflammation (measured by acute phase reactants and plasma immunoglobulin levels), upon growth. Seventy-two children were included in this group and were recruited from 2 months of age and followed until they were 15 months of age.

2. Cross-sectional permeability survey. One hundred and sixty-two residents from the three MRC study villages, between 2-60+ years of age, were recruited. The natural history of enteropathy (measured by L:M permeability) and growth was examined in this group.

3. Hospital ward and clinic based patients with more severe malnutrition underwent endoscopic small bowel biopsy to investigate features and mechanisms of enteropathy. Forty children 6 months to 3 years old were studied.
Children in the Keneba cohort were studied approximately every 2 months and changes in height and weight were recorded. At these same clinic visits L:M permeability (each sugar measured by automated enzymatic assay), plasma acute phase reactants (C-reactive protein measured by ELISA), plasma immunoglobulin subtypes (measured by automated turbidometry, Cobas Bio) and plasma endotoxin and endotoxin antibody were measured (by ELISA). Stool was collected for the identification of parasites and viruses that could cause enteropathy and also measured for faecal neopterin concentration (a marker of cell mediated inflammation, also measured by ELISA). The effect of enteropathy (measured by L:M permeability, or faecal neopterin) upon age corrected height and weight gain was studied by linear regression. Likewise the effect of enteropathy upon plasma endotoxin and plasma markers of inflammation was studied within each individual by linear regression. The overlapping effect of the different variables upon growth was studied using partial correlation modelling.

Features of the enteropathy and possible mechanisms were studied using standard linear morphometry and peroxidase immunohistochemistry with standard methods for quantifying positively staining cell types including the use of no primary or cytokeratin control slides to correct for non-specific background staining.

Results: L:M permeability was negatively associated with age corrected (height and weight) growth rates \( P < 0.003, r = -0.46 \). Endotoxin core IgG was positively related to percentage recovery of lactulose \( P < 0.003, r = +0.36 \) and all plasma immunoglobulin subclasses \( P \leq 0.0002, r \geq 0.47 \). Both plasma endotoxin antibody and immunoglobulin levels were related to age corrected height and weight growth rates \( P < 0.0001, r \geq -0.63 \) and \( P \leq 0.006, r > 0.33 \) respectively. Using partial correlation models, plasma endotoxin and plasma IgG levels overlapped in their growth limiting effects by 98%, suggesting a common mechanism of action. Small
bowel morphometry showed that even children without severe malnutrition (weight z-score >-4, n = 15) showed some mild villous shortening crypt hypertrophy and increase in IEL numbers (mean 40.5, > 40 per 100 epithelial cells is consistent with coeliac disease). Immunohistochemistry allowed quantification of the density of inflammatory cell numbers: CD3 cells was nearly 4 times that of UK controls; CD4 and CD8 were twice UK control numbers; many of these cells were expressing CD25 indicating activation (approximately 10 times higher than that observed in UK children); crypt epithelial cells were all HLA-DR positive and had a high percentage of γδ + IEL (mean 39.9 per 100 epithelial cells, normal < 10%). Together with high numbers of TNF-α and IFN-γ positive cells these findings were consistent with a cell mediated inflammatory reaction of the small bowel mucosa, that was being attenuated by equally high numbers of anti-inflammatory IL-10 and TGF-β producing cells. There was evidence of decompensation in the anti-inflammatory response in the more malnourished children, with TGF-β numbers falling faster than pro-inflammatory TNF-α numbers (P = 0.03, r =0.48).

Conclusions:

1. The data demonstrate that impaired gut barrier function is related to poor growth in Gambian children and, despite considerable improvement, persists up until 10 years of age.

2. The data suggests the translocation of endotoxin across the gut, provides a potential mechanism for systemic inflammation.

3. The enteropathy appears to be due to a cell mediated inflammatory reaction, which may be associated with a loss of oral tolerance in situations of severe malnutrition.
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doing.
“Daddy, is it a good idea to put poo in the fridge?”

Alice Campbell age 3 years
Growth faltering in Gambian children under 15 months of age is associated with a chronic enteropathy leading to a breakdown of gut barrier function and systemic inflammation.

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## Abbreviations

<table>
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<tr>
<td>95% Confidence interval</td>
<td>95% CI</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>r</td>
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<tr>
<td>Cow’s milk protein intolerance</td>
<td>CMPI</td>
</tr>
<tr>
<td>Deoxyribonucleic acid</td>
<td>DNA</td>
</tr>
<tr>
<td>Degree centigrade</td>
<td>°C</td>
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<tr>
<td>Enzyme linked immunosorbant assay</td>
<td>ELISA</td>
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<tr>
<td>Erythrocyte glutathione reductase coefficient</td>
<td>EGRAC</td>
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<tr>
<td>Gross national product</td>
<td>GNP</td>
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<tr>
<td>Gram</td>
<td>g</td>
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<tr>
<td>Haemoglobin</td>
<td>Hb</td>
</tr>
<tr>
<td>Hour</td>
<td>h</td>
</tr>
<tr>
<td>Intraepithelial lymphocyte</td>
<td>IEL</td>
</tr>
<tr>
<td>Interferon gamma</td>
<td>IFN-γ</td>
</tr>
<tr>
<td>Interleukin</td>
<td>IL</td>
</tr>
<tr>
<td>International Units</td>
<td>IU</td>
</tr>
<tr>
<td>Kilogram</td>
<td>Kg</td>
</tr>
<tr>
<td>Lactulose-mannitol ratio</td>
<td>L:M</td>
</tr>
<tr>
<td>Litre</td>
<td>L</td>
</tr>
<tr>
<td>Microgram</td>
<td>μg</td>
</tr>
<tr>
<td>Milligram</td>
<td>mg</td>
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<tr>
<td>Millilitre</td>
<td>ml</td>
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<tr>
<td>Molar</td>
<td>M</td>
</tr>
<tr>
<td>Nicotinamide adenine dinucleotide phosphate</td>
<td>NAD</td>
</tr>
<tr>
<td>Nicotinamide adenine dinucleotide phosphate reduced</td>
<td>NADPH</td>
</tr>
<tr>
<td>Number</td>
<td>n</td>
</tr>
<tr>
<td>Optical Density</td>
<td>OD</td>
</tr>
<tr>
<td>Phosphate buffered saline</td>
<td>PBS</td>
</tr>
<tr>
<td>Phosphoglucoisomerase</td>
<td>PGI</td>
</tr>
<tr>
<td>Ribonucleic acid</td>
<td>RNA</td>
</tr>
<tr>
<td>Revolutions per minute</td>
<td>rpm</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>SD</td>
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<tr>
<td>Standard error of the mean</td>
<td>SEM</td>
</tr>
<tr>
<td>Thrive index</td>
<td>TI</td>
</tr>
<tr>
<td>Term</td>
<td>Abbreviation</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Transforming growth factor beta</td>
<td>TGF-β</td>
</tr>
<tr>
<td>Triethanolamine</td>
<td>TE</td>
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<tr>
<td>Tumour necrosis factor alpha</td>
<td>TNF-α</td>
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Chapter 1

Thesis Introduction, Background And Literature Review

1.1 The Gambia: Geography and basic demography

The Gambia is a small country situated 13°30’N, enveloped on three sides by Senegal on the Atlantic coast of West Africa. It has a population of 1.1 million inhabitants, 25% of whom live in the coastal conurbation surrounding the capital of Banjul. The Gambian interior is dominated by arid savannah and has a mean altitude of only 20 metres above sea level. The country is divided in to north and south banks by The River Gambia which runs in a east to west direction.

According to 1993 data The Gambia is a relatively wealthy African country with a gross national product (GNP) of 350 US $ per capita, derived mainly from tourism and export of cash crops such as ground nuts. However, under 5 mortality rates are the eighth highest in Africa and eleventh in the world (UNICEF, 1996). The 1994 infant mortality rate is 129 per thousand live births the ninth highest in Africa and considerably higher than the United Kingdom (6 per thousand live births).

1.1.2 Keneba
Since 1974 The Dunn Nutrition Unit has run a field station in an isolated part of the Gambian interior on the outskirts of a village called Keneba. Prior to the presence of the Dunn Nutrition Unit, Keneba was run by Sir Ian McGregor. During this time Keneba served as a centre for malaria research and community nutritional surveys. Keneba is situated in the province of West Kiang and like the country as a whole suffers from low rainfall, in the region of 400-1000 mm per year falling between the months of July-October. West Kiang has proven to be a good location to study community dietary practices and morbidity because it remains relatively isolated.

Keneba lies in a delta of land made by The River Gambia and one of the larger tributaries, the Bintang Bolon. West Kiang is cut off from the main communication route, the south bank road by the southerly situated Bintang Bolon and so historically this part of the country has been slow to develop. Until the mid to early 1980's Keneba was without a road and could only be reached through narrow bush tracks, it now has a laterite road, which is passable at all times of the year. Keneba is still without telephones or news papers so inhabitants rely upon the Gambian and world service radio to catch news headlines.

The Keneba field station comprises of a laboratory, clinic, calorimeter, power station providing 24 hour electricity, and various residential buildings for the scientific and clinical staff. Outside the compound there is a building dedicated to nutritional rehabilitation of children with malnutrition.

The station provides medical services to Keneba and the two adjacent villages of Manduar and Kantong Kunda (that will feature in chapter 3.4 of this thesis) as well as the research work. The combined population of the three villages is in the region of two thousand five hundred residents. All three villages are of the Mandinka tribe, with less than 50 inhabitants of Fula extraction who run the village shops.
The unit has collected detailed demographic information of these three communities that can date back over 40 years.

1.1.3 The Diet and Nutritional Status of Keneba citizens

The life style of the Mandinka people is subsistence farming of sorghum, maize, rice, millet and groundnuts (McCrae and Paul, 1996a). These staple crops only grow in the brief rainy season and many families can not grow enough to last them through the whole year. During this time of the year the bush produces many edible wild leaves and locust beans which supplement the diet. Latterly, with the provision of more wells, it has been possible to grow some market vegetables such as tomatoes, chilli peppers, sweet potatoes, onions and cucumbers. The main protein sources from the diet are groundnuts and fish (dried and fresh). A typical meal prepared in Keneba would be a “sauce” of green leaves containing groundnut paste on top of a bowl of rice (or steamed millet).

Children breast feed throughout the first two years of life, exclusively through the first 3-4 months and then going on to weaning foods and finally to adult foods in late infancy (Prentice, 1991). Cow’s milk is rarely taken in the diet, except by the more wealthy families (McCrae and Paul, 1996b). Breast feeding continues throughout this time and weaning and adult foods supplement breast milk.

The months of June and July are always associated with a shortage of foods, heavy manual labour (mainly for the women) and a rising disease burden from malaria and diarrhoeal diseases. During these months most people in the community lose weight and the prevalence of severe malnutrition rises.

The next section will outline the relevant background literature on growth faltering, dietary interventions and intestinal disease in the tropics.
1.2 Literature Review

1.2.1 Use of growth standards and growth faltering in the tropics

This thesis aims to examine the impact of defective gut barrier function upon growth in Gambian children. Essential to this aim is the definition of normal growth, against which the growth of Gambian children can be compared. The debate on what constitutes normal growth has focused on several issues (WHO, 1994): is maximum growth desirable?; is it realistic to expect children from very different environments to grow equally as well?; and following on from this is it appropriate to use an international growth standard to define normal growth, and if so which standard should be used?

The World Health Organisation adopted The National Centre for Health Statistics (NCHS) standards as the international standards against which most comparisons of growth were made (WHO 1983). These standards were based on a very large cohort of healthy North American boys and girls. From this data separate growth curves were drawn for weight-for-age, length-for-age and weight-for-length, for males and females. It is felt by some, to be inappropriate to make judgements about growth performance in different geographically and ethnically diverse communities using growth standards derived from affluent North American citizens, from which some of the measurements were made over sixty years ago (Waterlow, 1991). Goldstein and Tanner (Goldstein and Tanner, 1980) and Van Loon argue that selection of malnourished children by anthropometric variables can only be done successfully by local references, which “...represent acceptable growth in a given environment.” (Van Loon and Vulylsteke, 1986).
The contrary opinion is advocated by Habicht and Martorell (Martorell et al., 1975) who have shown that in pre-school children of well to do families in different ethnic groups (in affluent and deprived North America, UK, Australia, rural Colombia, India, Thailand, Nigeria and Guatemala) the growth potential is similar to the NCHS standards. The effect of socioeconomic status on growth is far greater than the genetic influence of ethnic group in preschool children. Children from different racial groups thus show similar potential for growth in the first 6 months of life, but in developing countries (ie Guatemala, Malawi, Mexico, Kenya (Martorell, 1994) and Indonesia (Chwang et al., 1988)) severe growth faltering occurs from 3-4 months of age. Environmental constraints that accompany poverty are also of greater importance than genetic differences in the growth of children of school age (Graitcer and Gentry, 1981). Martorell has shown that the height of a school aged child is positively related to its’ parents, demonstrating that genetic influences in this age group are of some importance. However these are of relatively minor importance compared to the greater influence of the child’s environment (Martorell, 1985). It appears that much of the difference in height between ethnic groups becomes manifest at puberty, when genetic factors predominate. Before puberty dietary and environmental factors are more important (Waterlow, 1991).

Gopalan (Gopalan, 1989) argues that if a standard is to represent a target, it has to be drawn from a healthy population. This undermines the validity of the view that local standards are needed in the tropics, where the level of nutrition is suboptimal and the disease burden higher. It could therefore be said that a local growth reference, in deprived areas of the tropics, reflects growth under prevailing conditions of abnormal health rather than the true, genetic potential for growth. Further evidence that international standards help define normal and abnormal growth in developing countries comes from a study on 800 preschool children in Papua New Guinea which
related signs of protein energy malnutrition, intestinal parasite load and splenomegally to anthropometric scores on the NCHS standards (Kow et al., 1991). The results show a clear relationship between disease and growth, which becomes most evident below -2 standard deviations weight for age of the NCHS standards. Because growth represents changes not only in weight but also in length and body composition, the concepts of wasting and stunting were introduced by Waterlow and Rutishauser (Waterlow and Rutishauser, 1974) to describe different types of abnormal growth. Stunting is said to be present when a child is less than 90% of the median height (equals a z-score of -2) of the NCHS reference height for age and implies that a child’s genetic potential is probably not being reached (WHO, 1985). Wasting is present when the child is less than 90% of the median NCHS weight for height standard (also equals a z-score of -2). A further definition, which usually encompasses to some degree both of the former concepts, is “underweight”. This is defined as less than 80% weight for age. For the purpose of this thesis I will be using the term “growth faltering” or “growth failure” to refer to a child following a growth curve of less than 2 standard deviations below the UK 1990 reference (Cole, 1991) without showing any evidence of catch-up growth in weight or height, or a growth velocity which causes a downward crossing of these centiles. In each case I will specify the aspect of growth failure that I am referring to. The UK 1990 reference was chosen partly for convenience (integrates easily with the chosen computer software) but also as it has some advantages over the NCHS standards, ie longitudinal in design, making it more compatible with a longitudinal cohort study (Cole, 1994).

Figure 1.1 illustrates the typical pattern of growth faltering (compared to the UK 1990 references) seen in The Gambia and all over the developing world. Children are born small for gestational age but show catchup growth in the first 2-3 months. There after there is a progressive decline in growth velocity.
See table 2.1.4 for comparisons of different growth standards often used:

Having defined the means of comparing growth between Gambian children and an accepted “norm”, the next section will discuss some of the dietary factors associated with growth faltering.
Fig 1.1

Age related changes in Weight z-score in Keneba Children
(n=71)

*SD (z) score

Age weeks

*UK 1990 Reference
1.2.2. Macronutrient deficiencies

In West African countries such as The Gambia the major dietary source of nutrients in the first two years of life is breast milk. At the end of the second year of life the majority of children are still provided with 36% of the dietary energy, 60% of the dietary fat and 21% of the protein from breast milk (Prentice, 1992a).

Mothers begin introducing weaning foods at about 3-4 months of age (McCrae and Paul, 1996b). The weaning foods are called "monos" and are consumed by all members of the community, but for the weanling infant they are watered down to make them more easy for the child to consume (Hudson et al., 1980). The effect of diluting the mono is to lower the energy content drastically. The typical weaning food has a total of 380 kcal per kg compared to breast milk has an available energy content of 780 kcals per kg. The result is the weanling infant replaces a nutritionally complete, sterile food, for one that is both inadequate in energy density and often contaminated (Rowland and Barrell, 1978). More energy dense adult foods are not introduced until after 6 months of age (Prentice, 1991), and even then in small quantities (McCrae and Paul, 1996b).

The volumes of breast milk required to meet infant energy requirements have been challenged and amended, based largely on the work from Keneba (FAO/WHO, 1973, Whitehead et al., 1980, Whitehead et al., 1976). A figure of 86 kcal/kg/day was arrived upon after more accurate estimates of breast milk production in healthy infants using the deuterium oxide method (Coward, 1991). Stable isotope techniques have enabled the assessment of energy expenditure in the infant and provided data that also challenges earlier estimates of energy expenditure in the tropics. At all ages, free living Total Energy Expenditure (TEE) when expressed per kg body weight is identical to values obtained in western populations (Vasquez-Velasquez, 1988).
Energy requirement for tissue deposition in growth is estimated to be only 2% of TEE (Prentice et al., 1992, Prentice, 1992b, Prentice, 1992c) and at this level, dietary energy is unlikely to limit growth. Using the data obtained for energy expenditure and breast milk intakes it appears that a figure of 86 kcal/kg is more realistic and the massive shortfall in dietary energy predicted according to the 1973 FAO figure does not happen.

Further evidence that macronutrient deficiencies are not the main cause of growth faltering come from nutritional intervention studies both in Keneba and other developing countries. A 14 year supplementation programme was conducted in Keneba (Prentice, 1992a) with a locally produced porridge, which contained energy, protein, fat and significant amounts of calcium, riboflavin and vitamin C. This was administered daily in sufficient quantities to raise intakes of energy and several micronutrients to within the dietary recommended values. Children from 3 to 12 months of age were given this supplement and observed consuming the meal which was then weighed, so that the energy content of the consumed food could then be calculated. There was however no effect upon the growth of the children in either length or weight over the 14 year period. However, at all ages weight gain will occur, even in obese subjects if energy intake is greater than energy expenditure. The explanation for the failure of weight gain is partly due to children taking correspondingly less breast milk when given the supplement. It was also suggested by the principal investigator of this study that the energy requirements for growth are not as great as previously estimated (Prentice et al., 1992). These results have been mirrored in several studies in different parts of the world ie Bolivia, Congo, Senegal, and New Caledonia (Simondon et al., 1996), who found that substantial increases in energy consumption did not increase weight or linear growth.
It appears that dietary energy and protein requirements appear not to be the main reason for growth faltering in rural West Africa, or there are factors impairing appetite preventing simple energy and protein supplementation from improving growth.

1.2.3 Micronutrient Deficiencies

Much interest has been aroused over the possibility that essential nutrients required in small quantities (micronutrients) are responsible for growth faltering. The knowledge of micronutrient status in The Gambia (involving biochemical measurements, dietary intake and intervention studies with nutrient supplementation) is still incomplete and have produced conflicting results. The studies have largely focused on pregnant and lactating women within the Gambia.

1.2.3.1 Zinc

Among the many biochemical functions of zinc has a role as a co-factor for enzymes involved in chondrogenesis and collagen synthesis (Prentice and Bates, 1994), and nucleic acid synthetase (Golden and Golden, 1981). Studies in the late 70's and early 80's seemed to show that zinc could have a significant effect particularly in linear growth but also on weight gain. The earliest study (Walravens and Hambridge, 1976) upon 68 well grown formula fed infants supplemented with zinc showed a 2cm greater length growth and a 500g weight gain over 6 months but in the males only. The drop out rate from this study was high (40%) leaving the final analysis to a small number (42) which was then not analysed on an intention to treat basis. When the final number of male subjects were subdivided according to sex, the final number of male subjects was only 22 (8 control and 14 supplemented children). There was also
no change in the biochemical zinc status in the final 3 months of supplementation in
the female infants but there was in the males.

Dramatic improvements in rates of linear growth were seen in a zinc
supplementation study of 16 severely malnourished children undergoing nutritional
rehabilitation (Golden and Golden, 1981). In this study the children were allocated to
either a cow’s milk or a soya based rehabilitation diet, but several weeks later both
groups were supplemented with zinc. Both groups significantly increased rates of
weight gain on a similar energy intake implying that the energy requirement for
tissue deposition was either lower or absorption of dietary energy increased with zinc
. Though impressive, these children almost certainly had very low levels of zinc due
to their malnutrition and diarrhoea, which was reflected in plasma zinc levels all
being significantly below the lower limit of normal (9 μmol/l) (Nicholson and Pesce,
1992). Reservations have to be kept with this report on a small number of subjects,
the study was not unblinded and there still remains no good method for assessing
zinc status. Half the group received a soya based rehabilitation diet which was rich in
zinc cheelating phytates and it was this group that grew better than the milk based
formula. Each subject acted as their own control and with such impressive results
crossing over to unsupplementation again would be difficult for ethical reasons.

A second study involving the same group from Denver Colorado (Walravens et al.,
1983) repeated the study in 2-6 year old Spanish American children from a low
socio-economic class. Although 40 children in a pair matched (for zinc status,
anthropometry, sex and zinc intake) double blind placebo control study were selected
there was still a 30% drop out. In this study zinc status improved in response to
supplementation and the boys again showed greater benefit than girls, but the overall
benefit was a modest 1 cm taller at 1 year. There was no benefit with respect to
weight gain.
An intervention study looking at growth as an outcome to supplementation used boys aged 59-95 months in Ontario Canada (Gibson et al., 1989). The children who were chosen were growing below the 25th centile for the expected mid parental height and received regular supplementation for 3 months. There was a moderate increase in linear growth in the group that were zinc deficient (on the basis of a low hair zinc level).

The possibility that zinc deficiency causes infant growth faltering in Keneba was tested (Bates et al., 1993) in a age/sex matched double blind case control study on 110 children (6 months to 2.3 years). Previous studies suggested that there was some evidence of zinc deficiency in Keneba adolescents (Watkinson et al., 1985), and in the face of inadequate measures to assess marginal zinc status the only way to diagnose zinc deficiency is to use an intervention trial. Regular supplementation with zinc only produced a 2% increase in the mid-upper arm circumference, all other anthropometric parameters were unchanged.

The main source of zinc in Gambian infants is breast milk, which has been shown to be richer in zinc at all stages of lactation than milk from women in the UK (Bates and Tsuchiya, 1990). It was therefore suggested that zinc intake in breast fed infants was adequate to meet dietary recommended values (Prentice and Bates, 1994).

The mechanism by which zinc promotes growth is elusive but there is evidence that it may have a significant action by optimising intestinal function in. It has been shown that zinc supplementation, particularly in zinc deficient children improves mucosal integrity without increasing mucosal absorptive capacity (Roy et al., 1992). The most clear demonstration that zinc has an enterotrophic effect in diarrhoeal disease comes from a large double blind case control study involving 937 children with acute diarrhoea from New Delhi (Sazawall et al., 1995). This study showed that
zinc supplementation during diarrhoeal illness could reduce stool output by 39% and reduce the risk of progressing to chronic diarrhoea by 39%.

In summary the literature shows that zinc supplementation can promote linear growth when there is pre-existing zinc deficiency. Zinc deficiency is also linked to defective small bowel function. However, in rural Gambia supplementation with zinc alone cannot promote growth probably because the supplies of zinc from breast milk are appear to be adequate (Prentice, 1992).

1.2.3.2 Calcium

Ninety nine percent of all body stores of calcium exist within the skeleton as hydroxyapatite crystals. Low dietary calcium has been linked to poor skeletal growth. Dietary surveys on Keneba infants suggest that in the first year of life dietary calcium intake is close and possibly below the bone accretion rate (Prentice and Bates, 1994). Breast milk calcium is also 25% lower than UK levels (Prentice et al., 1994), suggesting that even not allowing for the unknown levels of insensible calcium loss, dietary calcium levels are extremely low. However, the period of maximum growth faltering does not co-incide with the times of greatest calcium deficiency. Growth faltering begins at 3-4 months of age when the infant is only consuming 16% of the dietary calcium above bone accretion rates (due mainly to the very high skeletal demands in early infancy). After 9 months of age, when the rate of decline in growth velocity is the greatest, calcium intakes are 51% greater than minimum requirements (Prentice and Paul, 1990). It appears that there are physiological systems operating to optimise dietary calcium intakes for biological utilisation by minimising urinary excretion (Prentice et al., 1995).

The large scale infant supplementation study that took place in Keneba gave a weaning food supplement designed to supplement energy and protein also contained
significant amounts of calcium. The mean consumption of the supplement increased
the daily calcium consumption by 200 mg per day, equal to the minimum daily
calcium requirement (outside of puberty). Despite being given with substantial
amounts of fat, protein and carbohydrate no increase in growth above controls could
be detected.
If calcium deficiency is contributing to growth faltering in infancy then it would
seem to be as a secondary event, limiting catch up rather than the initiating event.

1.2.3.3 Iron
Rural Gambian communities are endemically iron deficient (Topley, 1968) with a
high prevalence of hookworm and malaria together with low dietary intakes of
riboflavin and vitamin C (which are involved with the correct utilisation and
absorption of iron (Powers et al., 1983). The Keneba clinic data show that by 15
months of age 42 % of children have a haemoglobin less than 10 g/dl and 10% have
a haemoglobin less than 9.0 g/dl (Dale, 1993). The published reports on iron status
and growth have focused on 4-12 years old children and adults, particularly pregnant
women. Studies on iron status rarely consider the under two year olds. A double
blind placebo controlled trial supplementing iron and iron plus riboflavin was carried
out in adult men and children aged 4-12 years old in Keneba and surrounding study
villages(Powers et al., 1983). Unfortunately, though anaemic children were recruited,
an estimated prevalence of anaemia in the population from which the sample was
drawn was not reported. Out of the 80 anaemic children recruited 83% had
hookworm infection twenty-six percent had an MCV below 77µl, 13% had evidence
of glucose-6-phosphate dehydrogenase deficiency, and 8% sickle cell trait. Iron
supplementation did not improve weight gain in these children, but the addition of
riboflavin substantially improved iron status.
The effect of iron supplementation on promoting childhood growth has also been looked at in preschool children and 8-13 year olds. The study indicated that the capacity of iron to induce acceleration in growth velocity depended on the age of the child. Two other studies on school age children have indicated some benefit. In the first study sixty children from Kwale, Mombassa Kenya with mild anaemia (Latham et al., 1989) were sex matched and paired for haemoglobin concentration before being divided in to groups receiving iron supplement or a placebo. Iron status was not measured in this study, but because of the very high prevalence of hookworm infection and red cell indices, it can be assumed that iron status was low. The results showed that over 15 weeks of daily iron supplement with iron there was a 3.1 % improvement in weight for age and 3.7 % in height-for-age improvement in the treatment group above controls. There were similarly modest, but statistically significant increases in triceps and subscapular skinfold thickness (0.7 and 0.8 mm respectively) and in mid-upper arm circumference (0.4 cm).

A second study in school aged children in Java (Chwang et al., 1988) divided 119 children in to four groups (anaemic and normal haemoglobin each with a placebo control and iron supplementation group). The supplement continued over 12 weeks. Anthropometry at the start and end of the study indicated that only the anaemic group received some benefit from iron supplementation. Height improved by 0.1 z-score and weight by 0.15 z-score. In the normal group iron supplementation produced no improvement in growth. This study suggests that iron can improve growth if there is iron deficiency.

A third study in Indonesia supplemented iron and vitamin C to pre-school children (Angeles et al., 1993) who were stunted and underweight ( z-scores between -2 and -3 for both weight and height for age) and anaemic. In this double blind case control study lasting 2 months the effect of iron plus vitamin C was compared against
vitamin C alone in a two month supplementation. The iron supplemented group increased length by 0.37 z-scores above the control group. This is approximately twice the improvement seen in the most notable of the previous studies, and can be partly explained by the very low iron status (mean ferritin 6.2µg/L in the treatment group) and partly by more illness episodes in the control group (2.5 times more respiratory infections and 3 times more diarrhoea). Some improvement in the nutritional status of the control group occurred, perhaps due to the deworming procedure, which was part of the protocol. Further studies have looked at the effect of iron on promoting catchup growth in preschool children and found no benefit (Gerschoff et al., 1988).

The mechanisms by which iron promotes growth are not clearly defined. It is possible that the effect on growth is secondary to the treatment of anaemia (Chwang et al., 1988) improving oxygen delivery and raising the anaerobic threshold of work and thus improving work efficiency. However increased iron can increase oxidative capabilities by haem containing enzymes and even improve appetite.

Iron can have a quite separate effect on the intestinal mucosa (Berant et al., 1992), which as demonstrated in a cohort study of 10-39 month old infants with iron deficiency anaemia (mean haemoglobin 7.9 g/L, mean corpuscular volume 60.1 fl and serum ferritin 7.3µg/L) from Haifa, Israel. Each of the children acted as their own controls and had a permeability test with lactulose and rhammose both before and again after the correction of their iron deficiency anaemia. The effect of iron was to completely normalise the lactulose / rhammose ratio which was high in iron deficiency. The effect was on the rhammose (monosaccharide) which was low in iron deficiency, rather than on the lactulose. The low initial rhammose absorption is consistent with reduced mucosal mass (villous atrophy).
Treating iron deficiency states can reduce morbidity even when there is no anaemia (Kuvibidila et al., 1989) and many animal and human experiments have shown that iron deficiency states impair cellular immune function but leave humoral immunity intact.

Iron therefore appears to have growth promoting qualities that are most marked when iron deficiency is severe (Angeles et al., 1993). However iron supplementation can only recover a small part of the growth deficit seen in most developing communities.

1.2.3.4 Folate

Information about the role of folate in infant growth can be extrapolated from a study by Bates et al which involved supplementing 89 pregnant or lactating women in Keneba (Bates et al., 1986). Without folate supplement there was wide seasonal fluctuations in red cell folate with high / normal levels in the rainy season when fruits and bush leaves are eaten, and low levels for rest of the year. Breast milk folate fluctuated very little. It seems that the infants needs take preference over the mother’s own requirements who maintains breast milk folate levels by deterioration of her own folate status. There was no evidence of megaloblastosis which would indicate severe folate deficiency but in half of those supplemented with 50 µg/day red cell folate levels were still below the lower limit of UK normal values(Bates et al., 1994b).

Folate status has been measured in 8-14 year old children from Keneba (Bates et al., 1994a) as part of a multinutrient supplementation studies investigating effects on neuromuscular function. Blood samples, taken at the baseline and six weeks later, indicated that folate levels were adequate. The intake of most micronutrients show a large seasonal fluctuation (Bates and Powers, 1989). In this study the measurements were made at the very end of the rainy season when fruit and fresh vegetable foods
have not been eaten for several months. Therefore the results suggest that there is no folate deficiency in school age children.

1.2.3.5 Riboflavin

Riboflavin deficiency is endemic in West Kiang province of The Gambia with infants being born with biochemical evidence of riboflavin deficiency (judged by a low erythrocyte glutathione reductase activity coefficient or EGRAC)(Bates et al., 1982). As part of the supplementation programme aimed at increasing energy and protein intakes, infants also received extra riboflavin (total of about 170µg per day). This was still insufficient to correct biochemical deficiencies of riboflavin. Through the period of peak growth faltering supplemented children had significantly higher levels of riboflavin yet still grew as poorly as the controls.

Fifty-nine pregnant and lactating women in Keneba were compared to an equal number from the UK (Bates and Villard Mackintosh, 1992) for dietary intakes of riboflavin and biochemical profiles of riboflavin status. The Gambian women had riboflavin intakes well below the dietary recommended values, with correspondingly low EGRAC levels. Despite this finding there was no difference in the breast milk riboflavin status between Gambian mothers and UK controls. Bates et al have shown that even in significant maternal riboflavin deficiency riboflavin is preferentially transported in to the milk in sufficient quantities that were able to correct the EGRAC until weaning foods are introduced at 4 months. Severe riboflavin deficiency prior to weaning induces a unique form of enteropathy in a rat model (Williams et al., 1995), but it would appear that up to the age of weaning, Gambian children are unlikely to suffer from significant riboflavin deficiency. The Keneba macronutrient supplementation trial also included riboflavin and as already reported did not improve growth. However the red cell EGRAC levels were not measured.
1.2.3.6 Vitamin A

Vitamin A has profound effects upon the immune system with beneficial effects on morbidity and mortality. Bates and Prentice (Bates et al., 1994b) have demonstrated a significant difference in vitamin A intake between rainy and dry seasons in The Gambia, using a study population of pregnant and lactating women from Keneba. The plasma carotenoids fluctuate seasonally with a very high peak levels from April through to August (corresponding to the mango season), but the plasma retinol is static and within normal limits and seems to show very little seasonal variation. This seems to be an acceptable situation as there were no cases of clinical vitamin A deficiency (night blindness or xerophthalmia) detected. Furthermore, breast milk vitamin A levels were within normal limits.

As there is no dietary, biochemical or clinical evidence of vitamin A deficiency, it is unlikely that vitamin A deficiency is a major factor in causing growth faltering in breast fed Gambian children. However it has been shown that supplementing vitamin A to levels in excess of the daily recommended value significantly reduces morbidity from infectious diseases, which in turn has a beneficial effect on growth. Several large scale studies from India, Nepal and Cape Town South Africa (Hussey and Klein, 1990, Rahmathullah et al., 1990, West et al., 1991) have shown dramatic reductions in morbidity and mortality (49%, 30% and 45% respectively) especially from diarrhoeal diseases (Rahmathullah et al., 1990) and measles. It is well established that morbidity adversely effects growth but no anthropometry or growth data were presented in the previous studies (Martorell et al., 1980, Rousham et al., 1998).

1.3.2.7 Other micronutrients
Bates surveyed the status of a variety of other micronutrients in school children aged 8-14 years in Keneba (Bates et al., 1994a) at the end of the hungry season when nutritional stores are likely to be depleted. A mild deficiency of red cell ascorbate was found, but thiamine (red cell transketolase activity) and pyridoxine status were normal.

1.3.2.8 Conclusions about Micronutrient status in the rural Gambian village community

Knowledge about micronutrient status in infancy is far from complete, although it may be possible to cautiously extrapolate some of the information obtained in adults and older children. It would appear that iron and calcium deficiency is probably the severest and most prevalent micronutrient deficiencies but they do not appear to be the primary cause of growth faltering. Iron supplementation studies have showed the greatest effects upon growth have some serious confounding factors (i.e. significantly greater morbidity episodes in control groups and high drop out rates) making interpretation difficult. The general consensus seems that iron supplementation in iron deficiency states (which produces the greatest benefit) can only explain a small proportion of the observed growth faltering.

Little is known about micronutrient requirement in disease. Children in the tropics are subject to more frequent bouts of illness which may alter nutrient requirements. But on the basis of the best available evidence (nutritional supplementation studies) it would appear that micronutrient deficiencies do not act as major factors in causing growth faltering.

It is also possible that systemic inflammation associated with infectious diseases is the primary cause of growth faltering and that a variety of nutrients are affected secondarily by effects on intake and interactions with the disease process.
1.3 Intestinal Disease

The impact of intestinal disease upon world wide infant mortality is difficult to ignore since three million children each year die from diarrhoeal disease. Half die from the complications of acute watery diarrhoea, the rest from chronic diarrhoea (Campbell and Gove, 1996). Diarrhoeal disease was implicated in causing growth faltering and malnutrition in the seventies and eighties (Cole and Parkin, 1977, Rowland and Barrell, 1980), but other infectious diseases particularly malaria and pneumonia were also linked to growth faltering.

Diarrhoea has been claimed to be the main causal disease in the development of growth faltering in West Africa and Uganda by Cole and Parkin (Cole and Parkin, 1977). It was speculated that in the absence of diarrhoea growth in the first 18 months of life would approximate to NCHS standards. This was supported by reports from Guatemala (Martorell et al., 1975), Uganda (Whitehead et al., 1976) and from an Urban Township in The Gambia (Rowland et al., 1988).

1.3.1 Diarrhoea may be an insensitive marker of Intestinal disease

Between 1977 (Cole and Parkin, 1977) and 1991 (Lunn et al., 1991a) the prevalence of childhood diarrhoea (greater than three lose stools per day) had decreased from 17% to 9%. Despite the 40% reduction in the time spent with diarrhoea there was no improvement in growth. This has been reported in detail by Poskitt et al (Poskitt et al., 1999).

Diarrhoea though may be an insensitive marker of intestinal disease partly because of difficulties in the definition of diarrhoea, and because of changes in fluid balance alter weight without having a long-term effect on growth. Although diarrhoeal disease does not seem to cause long term growth faltering this does not exclude
enteropathy as a cause of growth faltering. The previously cited paper by Lunn et al 1991 studied 119 children aged 2-15 months to compare lactulose-mannitol permeability against weight and length gain. Intestinal permeability had a strong inverse relationship with both weight and height gain predicting 43% of the growth faltering. The technique of dual sugar permeability of which the lactulose / mannitol (L:M) test is an example, relies upon the different pathways for absorption of the two sugars, although there is uncertainty to the exact pathway through the intestinal epithelial barrier in health (Travis and Menzies, 1992). Mannitol is a monosaccharide (molecular mass 182 Dalton) that is passively absorbed, probably in a transcellular fashion. Lactulose is a disaccharide (molecular mass 342 Dalton) and appears to be absorbed in a paracellular fashion. Normal healthy gut epithelium provides a barrier that is almost impermeable to disaccharides such as lactulose, so that only small quantities of an oral dose are absorbed in health. However, diseased mucosa provides breaks in the integrity of intercellular tight junctions, and damaged epithelial cells offer more sites for lactulose transfer. Absorbed lactulose and mannitol are not metabolised, have a small and identical volume of distribution and are excreted unchanged in to the urine (Elia et al., 1987). A reduction in mucosal mass will also reduces mannitol absorption. Hence the L:M test can be thought of a test of both mucosal integrity and absorptive surface area (DuPont, 1995). By keeping the recovery ratios of both lactulose and mannitol a more robust test is achieved by correcting for dosing errors, incomplete collection of urine, gastric emptying and altered small bowel transit time.

The previously mentioned Keneba study by Lunn et al showed that infants had raised permeability ratios sufficient to cause growth faltering for 75% of the time (Lunn, P. G. personal communication) but diarrhoea was only present for 9% of the time. Once
acquired the lesion was carried until the child was fifteen months of age without resolving, although the severity of enteropathy measured by the L:M test fluctuated. It is not known what happens to L:M permeability beyond this time. This persistence of enteropathy despite resolution of diarrhoea is also demonstrated by studies in children with severe protein-energy malnutrition and diarrhoea in The Gambia (Sullivan et al., 1992). In this study 20 infants with marasmus or kwashiorkor and at least three weeks of diarrhoea, were compared to age matched controls with marasmus but no diarrhoea. The study aimed to characterise the intestinal lesion in persistent diarrhoea and malnutrition and the choice of controls aimed to assess the impact of the symptom of diarrhoea upon mucosal morphology. Small intestinal histology of the intestinal lesion in the group without diarrhoea was similar to those children without diarrhoea, and there was no correlation between mucosal morphometry on the one hand and severity of malnutrition, presence of intestinal parasites (Giardia and strongyloides), plasma albumin (a marker of protein-energy malnutrition) on the other. Moreover, the intestinal lesion which was assessed by histology and intestinal permeability persisted despite resolution of the diarrhoea and the onset of weight gain. Similar observations have been reported by others (Brewster et al., 1997, Lebenthal, 1984, Rossi et al., 1980). It is clear that chronic enteropathy can exist independently of diarrhoea, but during bouts of severe diarrhoeal illness, there are correlations between disease severity and permeability (Kukuruzovic et al., 1999, Hamilton et al., 1987).

In summary, diarrhoea is an insensitive marker of chronic intestinal disease. There is strong evidence that some form of intestinal injury, measured by the L:M test is intimately involved in growth faltering and malnutrition. The next section will
discuss the possible aetiopathological events in small bowel disease acquired in the tropics.

1.3.2 Intestinal disease in the tropics

Having already discussed the impact of acute watery diarrhoea on infant morbidity it is necessary to discuss the possible effect of environmentally acquired small bowel disease (small bowel enteropathies that are geographically restricted to the tropics) upon growth.

The term tropical enteropathy was first used by Lindenbaum following his studies of American Peace Corp workers in Pakistan (Lindenbaum et al., 1966b). He demonstrated a lesion of the small bowel associated with a lymphocytic infiltration of the small bowel ("jejunitis"), acquired when American volunteers took up residence in Pakistan. The abnormality was associated with malabsorption. Further studies demonstrated that the problem was more widespread in the indigenous population of eastern Pakistan (Lindenbaum et al., 1966a, Lindenbaum, 1968) and occurred in the absence of diarrhoea.

Baker and Mathan in 1972 investigated differences in intestinal structure and function between tropical enteropathy and tropical sprue by assessing intestinal structure (Baker and Mathan, 1972). This study was based in India and used three study groups: a group of 71 healthy adults from a low socioeconomic group living in a periurban area; a group of 398 rural village dwellers that included infants children and adults; and 90 patients with tropical sprue. Tests of absorptive function were carried out in all three groups (xylose, fat, and a B12 absorption tests). A jejunal biopsy performed on the ward controls and those with tropical sprue. The xylose absorption / excretion was most impaired in the tropical sprue group, intermediate in the village controls, and best of all in the healthy adults from the urban dwelling. All

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three groups showed a high percentage of abnormal xylose excretion (<25%) with the mean xylose excretion being only 12% in the sprue patients with only 2 out of 90 excreting more than 25% of the oral dose. The change in intestinal function with age was examined in the village dwellers. 92% of children under 5 years of age had an abnormal xylose excretion test but only 50% of those above 5 years showed the same degree of impaired absorptive function. In the first group (healthy ward controls) the mean xylose excretion was abnormal in only 13%. Vitamin B$_{12}$ absorption was assessed by faecal balance and a Schilling test. In all three groups the B$_{12}$ absorption was relatively well preserved, (although 75% of the sprue patients demonstrated a mild abnormality). This indicates that in both tropical sprue and tropical enteropathy the distal small bowel function is well preserved. Biopsies were graded on villous height, crypt volume and cellular infiltrate from I (mild) to IV (severe). In group one (ward controls) 92% were abnormal, (mainly grade I), but in the tropical sprue group all biopsies were abnormal (mainly grade II and III). The details of histology are not given in great detail but it would seem that both tropical enteropathy and sprue are very similar in histological appearance both functionally and histologically with the sprue being more severe than the former. In summary this paper reports a high incidence of small bowel disease (enteropathy) that principally affects the proximal small bowel and is worse in subjects of low socioeconomic status.

A study on 112 children under four years of age in Brazil related malabsorption (xylose excretion) and histological evidence of tropical enteropathy to social deprivation (Fagundes-Neto et al., 1984). There was a positive association between poor socioeconomic conditions and poor xylose excretion and histological abnormalities. The study involved patients with chronic diarrhoea and did not include children who were asymptomatic. The histology again was similar to the Mathan and Baker report but details are few.
Evidence of small bowel dysfunction in children with growth failure has come from field studies at The Dunn Nutrition Unit Keneba (Erinoso et al., 1992). Using the lactose breath hydrogen test it has been shown that by two years of age the prevalence of lactose maldigestion (defined as a rise of >20 ppm of hydrogen above baseline after an oral load of lactose) was 76%. The severity of the lactose malabsorption was not related to anthropometric indices (weight for age) nor the presence of diarrhoea. Though there is a genetically determined loss of lactase activity, this appears to begin in early adulthood rather in the first two years of life, suggesting that what is observed in the Keneba population is a secondary product of small bowel damage and villous atrophy (Vis and Brasseur, 1992).

Intestinal “dysfunction” has been described by Khin-Muang and Pereira (Khim-Maung et al., 1990) amongst rural Burmese village children, on the basis of an abnormal hydrogen breath test after a standard rice based meal. Those children greater than three years of age that had a late (4 hour) rise in breath hydrogen above baseline, were noted to have slower linear growth over the previous six month period than those who did not produce a higher breath hydrogen concentrations. There is some evidence that high levels of breast milk in the first year of life are protective against intestinal damage (Watkinson, 1981, Erinoso and Weaver, 1992, Costa Fuchs et al., 1996).

Modern histological techniques of have provided insights in to the small bowel enteropathy associated with malnutrition. The early case reports by Schneider et al are limited by the small size and crude nature of the histological assessment used (Schneider and Viteri, 1972). In this study 11 children with kwashiorkor were biopsied before treatment, as well as during and after recovery. At all stages of treatment the appearance was one of crypt hypertrophy and villous atrophy. The
lamina propria contained a cellular infiltrate which was greater than that observed in “Western” control subjects but exactly the same as the four local controls. More detailed small bowel morphometry and immunohistochemistry was described by Sullivan in a series of papers from The Gambia. The biggest study involved 40 children with severe protein energy malnutrition (<75 % weight for height) and with a history of greater than two weeks diarrhoea (Sullivan et al., 1991d). The enteropathy in these malnourished children appeared identical in those with or without diarrhoea and the severity of histological abnormalities bore no relationship to the extent of malnutrition or the speed of recovery. The enteropathy persisted despite the resolution of diarrhoea and the onset of catch up growth (Sullivan et al., 1991b). The small bowel histology described in the Sullivan series was similar to that described by Schneider. The central features of all cases was crypt hypertrophy, villous atrophy and an infiltrate in intraepithelial lymphocytes. Therefore there is evidence from many developing countries that impaired intestinal function together with mild to moderate small bowel histological abnormalities are common. The three conditions of tropical enteropathy, tropical sprue and the enteropathy of malnutrition have been discussed together because of functional and histopathological similarities. The next section considers whether similarities could represent a common cause or immunological response.

1.3.3 Pathoaetiology of enteropathies seen in the tropics

A detailed discussion of gastrointestinal immunology is beyond the scope of this thesis but recent advances in the understanding of enteropathy in general has provided valuable insights in to enteropathies associated with tropical life. Studies that have looked at intestinal biopsies from asymptomatic residence living in the tropics and reported varying degrees of villous shortening, crypt hypertrophy,
cuboidal transformation of enterocytes, increased lamina propria infiltrate with inflammatory cells and increased intraepithelial lymphocyte counts (Baker and Mathan, 1972, Fagundes-Neto et al., 1984). These changes do not in themselves indicate a single pathology as almost identical histology is seen in other small bowel disorders with well defined aetiologies such as food allergies (coeliac disease and cow’s milk protein enteropathy), Giardiasis, graft versus host disease, transplant rejection and pouchitis. These histological features seem to represent is a common immunological event triggered by a variety of stimuli (MacDonald, 1992). In vitro studies using foetal explants have capitalised on the fact that before 12 weeks there are no intestinal T-lymphocytes, these migrate in from 12-14 weeks. Incubation of foetal small bowel cultures with stimuli known to activate CD4 T-lymphocytes (pokeweed mitogen or anti-CD-3) lead to villous atrophy and crypt hypertrophy together with evidence of T-cell stimulation (CD-25 expression, and the production of proinflammatory cytokines such as IL-2 and interferon gamma). The development of enteropathy has a dose dependant relationship to the local concentration of proinflammatory cytokines, which is blocked in a similar dose dependant way by immunosuppression (Lionetti et al., 1993, MacDonald, 1992). None of these immunological and structural changes can only take place after 14 weeks gestational age when intestinal lymphocytes are well established.

The histopathology of coeliac disease shows a great deal of homology to tropical enteropathy. Coeliac disease shows strong HLA phenotype restriction (DQ-2) and it is the presence of HLA DQ-2 that allows the class II molecule on the surface of antigen presenting cells to bind the gliadin epitope. This activates a series of inflammatory events in the T-helper cell and to produce to the histological lesion described above. Failure to correctly present gliadin to T-helper cells prevents their
activation and small bowel morphology remains normal (Trejdosiewicz and Howdle, 1995).

*Giardiasis* induced villous atrophy has also been shown to be mediated by CD4 + T-lymphocytes. Congenitally athymic nu / nu (T-lymphocyte depleted) mice when infected with *Giardia muris* have very little intestinal damage until they are transfused with splenic cells from immunocompetent mice. After transfusion the gut developed villous atrophy and crypt hypertrophy (Farthing, 1993), similar to that seen in tropical enteropathy, again suggesting that the presence of intestinal T-lymphocytes is critical to the development of these changes.

The role of the T-lymphocyte appears to be central to the lesion of many small bowel enteropathies, but the cellular activation can come from a variety of stimuli ie allergic, infective, graft versus host disease etc.

Further evidence that a common immunological response is responsible for causing the small bowel enteropathies described above has come from detailed timed morphometric assessment of gut biopsies in patients suffering from tropical sprue. Marsh et al have shown from studies carried out in Vellore, India, that light microscopic changes of villous atrophy and crypt hypertrophy and clinical steatorrhoea came 3 weeks before an infiltration with intraepithelial lymphocytes. This again suggests that the lesion of tropical sprue may be secondary to the presence of T-lymphocytes. (Marsh et al., 1983). This may be equally true for tropical enteropathy, in that a variety of primary insults acting on the small intestinal mucosa can trigger a common immunological response leading to mucosal damage.

HIV causes a chronic enteropathy (Keating et al., 1995), but HIV and AIDS are very rare in The Gambia. It is estimated that the prevalence of HIV is less than 1% in The Gambia and it is mainly HIV 2, which is less virulent than HIV 1, which is more commonly seen in industrialised countries.
Whether the abnormal lactulose : mannitol permeability described by Lunn et al in Keneba infants is due to tropical enteropathy is uncertain, but there are various epidemiological factors that suggest that this is possible.

1.3.4 Social deprivation and enteropathy

Lindenbaum (Lindenbaum et al., 1966b) provided the first evidence of a link between unhygienic conditions of social deprivation and gut histological and functional abnormalities.

It has already discussed that socioeconomic status strongly influences child growth (Karlberg et al., 1994, Martorell et al., 1975) but it also influences intestinal structure and function. Baker and Mathan (Baker and Mathan, 1972) and Lindenbaum (Lindenbaum, 1968), demonstrated that the poorest rural village dwellers had the greatest impairment of small bowel function (assessed by lower urinary xylose and higher faecal fat excretion) than the urban dwellers who were considered to be less deprived. In both of these studies there was evidence of widespread intestinal disease even in the controls but the more deprived village dwellers had the worst indices of small bowel function. Impaired absorptive function was more readily demonstrated in those with more severe histological abnormalities of villous atrophy and crypt hypertrophy.

Although it has already been stated that diarrhoea is an insensitive marker of enteropathy, it may however, be a relatively specific indicator of intestinal disease. Subsistence farming societies such as Keneba still demonstrate an increasing diarrhoea frequency at a time of year when children are left in the care of young nurse maids and the mothers go to the fields to farm at the start of the rainy season. The child is almost certainly exposed to increased risk of infection (Rowland and Barrell, 1980) probably through microbial contamination of the weaning food.
Barrell and Rowland have shown that the practice of preparing weaning foods once daily, is associated with dangerously high bacterial counts (Barrell and Rowland, 1979). The environmental conditions in the rainy season particularly favour the proliferation of coliform bacteria even in short periods of time, making this period of time away from the mother particularly harmful to the child’s growth. Children that are exclusively breast fed throughout this time are relatively spared from the bouts of diarrhoea that occur during the farming and rainy season (Watkinson, 1981).

Adoption and migration studies give further support to the view that environmental factors, apart from simple dietary problems, are involved in the development of growth faltering and malnutrition. Martorell et al concluded from several studies that true successful nutritional rehabilitation is possible only when issues of poverty are adequately addressed and resolved (Martorell, 1994). Such situations exist when children from the tropics are adopted into a western environment. From these studies it is clear that more of the accrued height deficit can be recuperated if the environment is changed, rather than attempts to rehabilitate the child within the environment where the stunting developed (Winick et al., 1975, Proos et al., 1992).

Identifying specific environmental factors that actually cause growth faltering is difficult. Many studies point to factors that are only proxy measures for the true causative variables ie maternal education. This is often cited as a predictor of malnutrition in the young child (Vella et al., 1992), who benefits by a higher standard of hygiene, a balanced diet and a higher likelihood of receiving childhood vaccinations.

Growth faltering becomes most pronounced at an age when the child is introduced to weaning foods and develops developmental milestones of mouthing grasped objects (4-5 months of age). If the child lives in a “dirty environment” the child will quickly be exposed to harmful enteric pathogens.
If the child comes from a family with a mother of very high parity (>10) growth can be depressed before the weaning period (Prentice et al., 1987). Birth weights of babies born to such mothers are significantly lower than those born to mothers with less than 9 children, and the probable mechanism for this is decreased milk intake per child (Prentice, 1986).

Growth after 6-12 months of age is increasingly under the control of growth hormone (Karlberg et al., 1994) which is influenced by the "emotional environment of the child". Children who are exposed to a stressful environment have significantly impaired growth hormone secretion (Skuse et al., 1996) which in turn could lead to impaired growth.

1.4 Growth and Systemic inflammation

The concept of frequent activation of the acute phase response causing a decline in nutritional status has been called "immunostimulation" or "immunological stress" (Solomons and Mazariegos, 1993). The theory makes use of observations from poultry husbandry which have long realised that chicks raised in unsanitary conditions grew less well than their counterparts in the clean environments. Growth rates could be brought up to the same rates if the "dirty chicks" were fed antibiotics. The "clean chicks" had no benefit from the addition of antibiotics to their feed. There are few studies that have looked at the effect of feeding young children antibiotics at the peak of growth faltering i.e. 6-12 months. Few studies have shown that the addition of antibiotics in a low dose daily is of benefit in promoting growth i.e. Rosenberg et al (Rosenberg et al., 1974). Robinson showed that supplementation with aureomycin to the weaker of a twin pair caused significantly faster rates of growth (Robinson, 1952). These reports suggest that bacteria may be involved in childhood growth retardation, but these results have been challenged.
Evidence for the frequent activation of the acute phase response has been reported by Mejia and Chew (Mejia and Chew, 1988) who found in a large cross sectional survey of Guatemalan children that two thirds of children had either clinical or laboratory evidence of systemic illness (raised white cell counts or erythrocyte sedimentation rate). Solomons et al have found that 25% of poorly growing preschool children from peri-urban areas have evidence of raised soluble cytokines (Valdez C, Solomons, N. W., Klasing, K.C. unpublished findings). Lunn et al have shown that plasma cortisol levels for Keneba village children rise with age and inversely correlate with growth velocity, whereas insulin levels are positively correlated to growth velocity and these fall with age (Lunn et al., 1979). Cortisol : insulin ratios were good predictors of the acute phase response. These results were taken as representing activation of the acute phase response with a consequent effect upon growth velocity.

The evidence that bacteria are responsible for “immunostimulation”, however is not strong and there are studies supplementing antibiotics that have not produced beneficial effects upon growth. Respiratory and gastrointestinal infections have already been shown to impair growth, but parasites like malaria are also likely candidates for causing a strong acute phase response in young children (Hurt et al., 1994). A mucosal source of immunostimulation is likely because of the frequent reports of the raised IgA levels in malnutrition (Rafi et al., 1976). These are secreted in to body fluids and suggest a microbial mucosal challenge, leading to a co-ordinated IgA mucosal response (Prentice et al., 1991). The most important effects of an acute phase response appears to be the generation of fever (Stettler et al., 1992), a catabolic state and cachexia (Roubenoff, 1994). These are brought about by proinflammatory cytokines IL-1, IL-2, TNF-α and IFNγ released from a variety of leucocytes (Cederholm et al., 1997). Interestingly one of the explanations as to why the macronutrient supplementation did not promote
growth in the Keneba infant supplementation study was a poor appetite causing the children to substitute calories from breast milk with the supplement given (Prentice et al., 1993).

In summary, chronic activation of the acute phase response in children from the tropics could be an additional factor that impairs growth. The assumed avenue of bacterial invasion is via a mucosal surface, probably the gastrointestinal tract, which is affected by a chronic enteropathy. Other infections, like malaria are also important and will need to be quantified in the following chapters.

1.5 Hypotheses

From the above section it has been shown that there is already good evidence of a highly prevalent enteropathy in the tropics, although little is known about the aetiology. The likelihood is that this enteropathy is inflammatory, but this has never been demonstrated clearly, using new techniques of mucosal immunology. The evidence that gut barrier function is impaired comes almost entirely from studies using dual sugar permeability tests. The claim that this represents a generalised leakiness of luminal contents is disputed. The hypothesis of this thesis is as follows: Growth faltering in Gambian children under 15 months of age is due to a chronic enteropathy leading to a breakdown of gut barrier function, causing systemic inflammation.

The three main aims of this study are as follows:

1. To demonstrate that impaired gut barrier function is related to poor growth (including beyond 15 months of age).

2. That impaired gut barrier function is persistent and associated with systemic inflammation.
3. To characterise the enteropathy and provide insights into aetiology and possible mechanisms.

More specific aims are indicated in the individual chapters.
Chapter 2.0

Methodology

Three separate groups of study subjects were enrolled in order to examine different aspects of the thesis aims. The Keneba longitudinal cohort (2.1) formed the main study to examine the effect of breakdown of intestinal barrier function upon growth and systemic inflammation. Secondly the pathoaetiological mechanisms of the enteropathy were examined in a ward based case series (2.2), and lastly the effect of enteropathy upon nutritional status beyond 2 years of age was examined in a cross sectional study of village inhabitants (2.3).

2.1 The Keneba Longitudinal study

2.1.1 Subject Recruitment

Since 1947 a longitudinal survey of health demography and nutrition has been carried out in Keneba and in the two neighbouring villages of Manduar and Kanton Kunda. All births are notified by traditional birth attendants and the children are visited in the first 24-48 hours after birth where they are examined, weighed, measured and gestational age is scored using the Dubowitz system. The study was explained to the mother at this visit and consent obtained through a trained translator who explained to her that she was entitled to withdraw at any time and it would not effect her right to free medical care.

2.1.2 Study clinics
When the Dunn Nutrition Unit took over the Keneba survey in 1974, infant and child welfare clinics were started (in addition to the acute medical service) for children under two years of age. The children were screened for disease and poor growth, and vaccinations were administered according to the World Health Organisation “Expanded Programme of Immunisation” (EPI). Children are thus seen routinely at: 4, 8, 12, 16, 24, 36, 44, 52, 64 weeks and also at 18 months and 2 years. On each of these occasions a haemoglobin measurement is made via a capillary puncture.

Because one of the study aims was to relate persistent enteropathy to a sustained acute phase response (through a breakdown of gut barrier function) it was felt necessary to study the children frequently for two reasons:

i) To ensure that intercurrent illnesses would not unduly influence summary statistics. For example, the effect of a single bout of mild malaria which can cause a very marked elevation of acute phase proteins, but only transiently effect growth would disproportionately influence the analysis of acute phase response. By making multiple biochemical measurements these effects are minimised, and summary statistics of these measurements would reflect the putative “underlying chronic” inflammatory disease.

ii) The effect of the different variables studied can be quantified in terms of long- and short-term impact upon growth.

For ethical reasons it was decided that the study clinic visits should occur at the same time as the child was called for vaccinations and health clinic visits and not more often to prevent unnecessary intrusion.

Children were recruited at 8 weeks of age at a time when most children are growing well (around the UK 50th centile for weight).

Children attended the study clinic at 4, 8, 12, 16, 24, 36, 44, 52, 64 weeks. At these visits a clinical examination was performed and height and weight were recorded.
Blood was drawn by capillary puncture (except at 8, 44 and 64 weeks when it was planned to measure endotoxin levels). A haematocrit was measured and a blood film was made on every occasion.

A lactulose-mannitol test was carried out at each clinic visit using the technique described by Lunn et al (Lunn et al., 1991b). In brief a standard solution was made up of 0.5 g mannitol and 2g lactulose per 10 mls of water, 2 mls per kilogram were given to the child using a syringe with a soft plastic tube attached. A 24 hour urine bag (Holister UK) was fixed with adhesive to the infant who was constantly observed and the urine was collected through the drainage tube as soon as it was passed. Urine contaminated with faeces was discarded after the volume was recorded. Feeds were withheld for the first hour after the lactulose-mannitol was given and the child was allowed to feed freely after this.

2.1.3 Subject details

In all 73 subjects were recruited details are given in table 2.1 below:

Table 2.1 Subject details for the Keneba longitudinal cohort study

<table>
<thead>
<tr>
<th>Age at recruitment (months)</th>
<th>males / females</th>
<th>birth weight</th>
<th>birth height</th>
<th>age at leaving at recruitment</th>
<th>weight z-score at recruitment</th>
<th>height z-score at recruitment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.42 (0.98) 36 / 37</td>
<td>-1.23</td>
<td>-0.52</td>
<td>10.7 (3.8)</td>
<td>-0.53 (1.0)</td>
<td>-0.70 (0.88)</td>
<td></td>
</tr>
</tbody>
</table>

Values given are mean with standard deviation in parenthesis
Subjects were followed from 2 months of age and discharged at 15 months of age. Because of the longitudinal nature of the study, the cohort effect meant that some children towards the end of the project were followed for a shorter length of time from those at the beginning. The mean age at discharge was 10.7 (SD 3.8) months.

2.2 Fajara subjects who underwent endoscopy

2.2.1 Recruitment and subject details

Children were recruited from the outpatients department and children's ward at MRC Tropical Medical Research Unit, Fajara. Children with protein energy malnutrition or severe growth faltering (defined as falling away from the 70% weight for age line) with no signs of catch up after treatment of any associated medical condition, and or response to nutritional advice.

2.2.2 Subject details

Details of nutritional and clinical status are presented in table 6.1. In all 55 subjects were recruited including 5 well nourished children (who underwent intestinal biopsy because of chronic non-specific abdominal pain). Informed consent through a translator was obtained for a endoscopy with small bowel and gastric biopsies under deep intravenous sedation. Separate permission for this part of the project was granted from the joint M.R.C./Gambian Government ethical committee.

2.3 Subjects from cross sectional permeability survey

2.3.1 Recruitment and subject details (See table 2.2)

Approximately 30 individuals from each age group were randomly selected from the Keneba data base for potential recruitment to the cross sectional permeability study.
(see chapter 3.4). 41% of the recruits were from Keneba, 30% from Kanton Kunda and 29% from Manduar. After explanation of the study aims and requirements, permission was granted by the village elders and consent was obtained individually from each subject, or subjects parent as appropriate. For the secondary aim of examining the effect of season upon intestinal function beyond 5 years of age only 57 of the previous group consented to the study.

2.4 Questionnaire Methodology

2.4.1 Dietary survey

As the infants entered the study at eight weeks of age the mothers were visited in their compounds or the fields twice weekly and the dietary questionnaire (see Appendix 3) was used to establish which food types had been given to the child in the last two to three days. Particular attention was paid to whether or not the child was taking anything other than breast milk and the mother was questioned as to what the weaning foods (monos) were made with.

Every effort was made to question the mother, so multiple visits were made if necessary, and only as a last resort were the nurse maids questioned if the mother was could not be contacted. The field workers were trained in administering the questionnaire, which was checked for inconsistencies and completeness on the day it was finished. Incomplete or inconsistent forms were returned to the administering field worker for corrections. Recall bias was minimised by limiting the time between questionnaires to 3 days. The questionnaire was administered on bank holidays as normal.

Subjects who joined the study late were excluded from the analysis of gut permeability and weaning because of uncertainties when weaning would have started and with what
foods before study data was collected. Subjects with incomplete data collection were also excluded. Water intake was also included in defining when introduction of weaning foods commenced.
Table 2.2 Subject nutritional characteristics at the time of the first clinic visit - cross sectional survey of intestinal permeability.

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>Weight z-score</th>
<th>95% confidence interval</th>
<th>Height z-score</th>
<th>95% confidence interval</th>
<th>BMI z-score</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-5</td>
<td>-1.67</td>
<td>-2.0 to -1.3</td>
<td>-1.76</td>
<td>-2.1 to -1.4</td>
<td>-0.6</td>
<td>-1.0 to -0.23</td>
</tr>
<tr>
<td>5-10</td>
<td>-1.18</td>
<td>-1.6 to -0.8</td>
<td>-0.9</td>
<td>-1.3 to -0.5</td>
<td>-0.9</td>
<td>-1.3 to -0.5</td>
</tr>
<tr>
<td>10-15</td>
<td>-1.75</td>
<td>-2.1 to -1.4</td>
<td>-1.4</td>
<td>-1.7 to -1.0</td>
<td>-1.5</td>
<td>-1.8 to -1.2</td>
</tr>
<tr>
<td>15-20</td>
<td>-1.65</td>
<td>-2.0 to -1.3</td>
<td>-1.2</td>
<td>-1.5 to -0.8</td>
<td>-1.2</td>
<td>-1.5 to -0.8</td>
</tr>
<tr>
<td>20-30</td>
<td>-0.70</td>
<td>-1.1 to -0.4</td>
<td>-0.7</td>
<td>-1.0 to -0.4</td>
<td>-0.6</td>
<td>-0.9 to -0.2</td>
</tr>
<tr>
<td>30+</td>
<td>-1.1</td>
<td>-1.49 to -0.75</td>
<td>-1.1</td>
<td>-1.4 to -0.7</td>
<td>-0.8</td>
<td>-1.1 to -0.4</td>
</tr>
</tbody>
</table>
2.4.2 Morbidity survey

At the same time as mothers were visited for the dietary questionnaires, the morbidity questionnaire (Appendix 3) was administered. The questionnaire was administered twice weekly by a trained field worker and the forms were checked for completeness and consistency on the day of completion. Incorrect forms were returned the next or the same day for correction.

A definition of diarrhoea was taken from Rowland et al (Rowland et al., 1978) who recorded from the mother the presence of “Konobayo”, the Mandinka term for diarrhoea (a change in bowel habit towards increasing looseness and frequency. This has previously been validated against short-term growth faltering and rota virus infection (Rowland et al., 1985).

The presence of fever, cough, vomiting and skin infections was also recorded.

2.5 Clinical Methodology

2.5.1 Anthropometric standards

As this was a longitudinal study it was decided appropriate to use the 1990 UK growth standards expressed in standard deviation (z-scores) units (Cole et al., 1995, Cole, 1995). Table 2.1.4-I compares the World Health Organisation (NCHS) standards with the Tanner-Whitehouse and the 1990 UK growth standards.
Table 2.3 Comparison of commonly used growth standards (weight)

<table>
<thead>
<tr>
<th>World Health Organisation (NCHS)</th>
<th>Tanner Whitehouse standards</th>
<th>1990 UK standards</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of median and z-score equivalent.</td>
<td>SD (z) score</td>
<td>SD (z) score</td>
</tr>
<tr>
<td>100% (0.0 z-score)</td>
<td>50th centile</td>
<td>0.0</td>
</tr>
<tr>
<td>80% (-2.0 z-score)</td>
<td>3rd centile</td>
<td>-2.0</td>
</tr>
<tr>
<td>70% (-3.0 z-score)</td>
<td>&lt; 3rd centile</td>
<td>-3.0</td>
</tr>
</tbody>
</table>

The definition of malnutrition (marasmus) is taken from Waterlow (Waterlow, 1991) as below 4 standard deviations for weight from the NCHS standards. The presence of oedema, pluckable hair and dermatitis allow the diagnosis of kwashiorkor or marasmic kwashiorkor in the wasted child (< -4 weight z-score).

2.5.2 Anthropometric techniques

Infants were weighed on the same Secca baby scale (Secca UK) to the nearest 10g naked and an average of 3 weights was recorded.

Length was measured on a Harpenden stadiometer according to the technique of Jellife and Jellife. An average of three readings were taken by two trained field workers.

2.5.3 Blood collection
Capillary blood (600μl) was collected in to Becton Dixon heparinized blood bottles at all clinic visits except 8, 44 and 64 weeks and promptly centrifuged for 10 minutes to separate the cells from the plasma. At the same time a blood film was made and studied for malaria parasites and a haematocrit was measured. At 8, 44 and 64 weeks blood was collected in to endotoxin free tubes for endotoxin and anti-endotoxin core antibody estimates. Before venepuncture the skin was thoroughly cleaned with alcohol and a sterile butterfly canula was used to draw out 4 mls of blood. Samples were centrifuged for 15 minutes to separate the cells from the plasma.

All samples were stored at -40 °C until shipped back to the UK for analysis.

2.5.4 Stool samples

Samples of stool were collected in to universal containers and then aliquots were taken off for *Giardia* immunofluorescence, viral electron microscopy biochemistry and frozen at -40 °C for shipment back to the UK. A fourth aliquot was used for immediate examination for ova cysts and parasites.

2.5.5 Lactulose-mannitol permeability test

The test solution contained 200 mg of lactulose (Duphalac, Duphar labs, Southampton and 50 mg of mannitol (Sigma) per ml water and administered 2 ml of solution per kg body weight (accurately measured). Urine was collected over the next 5 hours in a urine bag fitted with a drainage tube (Hollister U-bag, Abbot Labs, Queensborough, Kent). Urine bags were inspected regularly and any urine produced was drained into collecting bottles containing 2-3 drops of chlorhexidine gluconate (0.2% w/v) as a bacteriostat. The total 5 hour volume was recorded and an aliquot taken and stored at -20 °C prior to shipment back to the UK. Infants were not allowed to breastfeed or eat for one hour after the test solution was given.
2.5.6 Endoscopic small bowel biopsy

An Olympus XP21 paediatric endoscope was used. Macroscopic appearances of the oesophagus, stomach and duodenum to level D2-3 were noted. Gastric biopsies taken were and tested for urease activity. Those who were positive were put on a two week course of anti-Helicobacter pylori treatment. Paired biopsies were collected from the second part of the duodenum where possible, and orientated and mounted on filter paper or on the side of a cryo tube by a trained field worker. Samples were fixed in 10% formal-saline (for haematoxylin and eosin staining), 2.5% glutaraldehyde (for electron microscopy), and snap frozen in liquid nitrogen (for immunohistochemistry) where they were stored prior to shipment back to the U.K. on dry ice.

2.5.7 Sedation protocol

Children were assessed (clinically and haematologically) before endoscopy, which was not carried out in cases of either severe anaemia or respiratory distress. Intravenous sedation with diazepam (0.1 mg/kg), hyoscine (10μg/kg) and ketamine (10 mg/kg) was used to achieve a level of non-communicative sedation. Oxygen was given via nasal speculæ and oxygen saturation was monitored throughout the procedure and recovery. Full resuscitation facilities were available at each procedure.

2.6 Laboratory Methods

2.6.1 Urine biochemistry

Urine stored at -20 °C was defrosted and then centrifuged for 10 minutes at 2000 rpm to separate out sediment. Sample dilutions were then made on a Hamilton autodilutor.

2.6.1.1 Mannitol measurement
An automated enzymatic assay described by Lunn et al (Lunn et al., 1989) was used. The main reagent buffer was 0.3 M Tris buffer adjusted to pH 8.6. NAD (Boehringer) at 10 mg/ml was added immediately before use. Approximately 100μl of sample was placed in a Cobas-Rio (Roche) sample cup and loaded on to the analyser. The assay was calibrated using 250, 500 and 1000 mg/l mannitol standards (Sigma). During the assay 10μl of sample was added to 100μl of NAD / Tris buffer reagent. After mixing the absorbance at 340 nm was recorded and 10 μl of mannitol dehydrogenase (0.5 unit) was then added. The rise in absorbence at 340 nm was followed for 10 minutes by which time the reaction was completed. The change in optical density was a measure of the amount of NAD converted to NADH and thus directly proportional to the mannitol content of the sample.

2.6.1.2 Lactulose measurement
An automated enzymatic technique described by Northrop et al (Northrop et al., 1990) was used to estimate urinary lactulose concentration. This assay is based on the rate of rise of NADPH (measured at 340 nm) being proportional to the urinary lactulose concentration and is able to distinguish between free lactulose, lactose, glucose and fructose in the urine.

Two Cobas-Bio sample cups were set up for each urine. Into the first 50μl of sample was pipetted, with 50μl of triethanolamine (TE) / MgSO₄ buffer and 25 μl of β-galactosidase in TE / MgSO₄ buffer. The mixture was incubated for 2 hours at 37 °C to hydrolyse lactose and lactulose to their constituent monosaccharides. A further 50μl of sample followed by 75μl of TE / MgSO₄ buffer, but no β-galactosidase was added to the second cup which was used to measure free urinary glucose and fructose. A cocktail of hexokinase (Sigma) and glucose-6-phosphate-dehydrogenase (Sigma) in TE / MgSO₄ buffer was placed in the main reagent reservoir and a suspension of
phosphoglucoisomerase (PGI) in the start reagent reservoir. The analysis was performed in two stages. The first step was performed before PGI was added so the increase in NADPH observed was proportional to the glucose concentration. In samples not treated with β-galactosidase this was proportional to the free urinary glucose concentration, but in the β-galactosidase treated samples, it was both a measure of both free glucose and glucose produced by the hydrolysis of lactose. Thus a subtraction of the free from the combined value gave the lactose concentration. The second stage of the reaction was then performed after addition of PGI. In samples not exposed to β-galactosidase, the further increase in NADPH is a measure of free fructose where as in the β-galactosidase treated samples the increase in NADPH is a measure of combined free fructose and fructose liberated by hydrolysis of lactulose. Subtraction of the free from the combined gives the lactulose concentrations in the original sample.

2.6.2 Haematology and blood chemistry

2.6.2.1 Plasma proteins

Plasma proteins were measured using immunoturbidometric techniques using a Cobas Bio (Roche) centrifugal analyser. All assays used Dako antibodies and standards except α₁-antichymotrypsin which used a Serotec (Kidlington, Oxon) calibrator.

2.6.2.2 Plasma endotoxin and endotoxin core (IgG) antibody

A commercially available limulus amebocyte lysate ELISA assay was used (COASET for endotoxin antibody and COATEST for plasma endotoxin, both manufactured by Endosafe Inc, Charleston, USA, distributed by Chromogenix, Molndal, Sweden). Concentration of endotoxin was measured on completion at 405 nm. Endotoxin free
pipettes and ELISA plates were used, and blood was collected in endotoxin free tubes (Chromogenix).

2.6.2.3 *Giardia*-specific IgM ELISA

*Giardia* antigen was extracted from Portland strain 1 trophozoites (about $2 \times 10^4$ in 50µl phosphate buffered saline, pH 8.5) grown in axenic culture (Goka et al., 1986). Microplate wells were coated with antigen by incubating at 4 °C for 16 hours. Plates were washed three times with phosphate buffered saline (pH 7.2) containing 0.05% Tween using an automated ELISA plate washer (Dynatech Laboratories). Plasma was assayed at 1/200 dilution in phosphate buffered saline (pH 7.2) containing 1% bovine serum albumin (final volume 50µl) were incubated with antigen for 1 hour at 37 °C. After another wash 100µl of anti-IgM horseradish peroxidase conjugate diluted 1/1000 in phosphate buffered saline was added to each well. After 1 hour incubation and a final wash 150µl of o-phenylenediamine was added to each well. After a 30 minute incubation at room temperature, the reaction was stopped by the addition of 25µl 2.35 M sulphuric acid. Optical density was read at 492 nm on a Labsystem Multiscan MCC/340 plate reader (Basingstoke, Hants).

From this present study it was ascertained that *Giardia* infection was unlikely at 8 weeks of age, and therefore the mean optical density reading (plus 1 standard deviation) represented the positive threshold indicative of recent infection.

2.6.2.4 Full blood count

A Coulter counter was used to provide automated white cell differential and platelet counts. Manufacturers standards were used to calibrate results.

2.6.2.5 Blood film for examination of malaria parasites
A capillary blood sample was collected on to a microscope slide, smeared and allowed to dry on a heated surface. The field stain technique was used with added saponin to lyse red cells and positively stain malaria parasites. Blood films were classified as either positive or negative. Blood films were examined at every clinic visit.

2.6.3 Stool

2.6.3.1 Light microscopy for ova cysts and parasites

Faecal smears were made and left to dry before being fixed with acetone and studied by two trained laboratory technicians who were blinded to each others' results. In cases of discrepant results a third technician was asked to give a final verdict.

2.6.3.2 *Giardia* immunofluorescence microscopy

Fresh samples of stool were frozen to -40 °C and flown back to the UK in dry ice. A suspension was made in phosphate buffered saline and applied to a multi well microscope slide which was allowed to dry before being fixed in 100% pure acetone. 10μl of anti-*Giardia* FITC conjugated polyclonal antibody (Cell labs UK) was then added to the fixed faecal preparation and the slide was incubated in a humidity chamber at 37 °C for 30 minutes. The slide was gently washed in a bath of phosphate buffered saline for 5 minutes to remove the unbound antibody and excess faecal debris. A coverslip was mounted using the immunofluorescence mounting medium (Cell Labs UK).

A positive control slide (Cell Labs UK) was used to identify *Giardia* cysts. Each sample of stool was studied blindly and examined in triplicate. A single *Giardia* cyst on any of the three slides was counted as a case of infection.

2.6.3.3 *Cryptosporidium parvum* detection by microscopy
Faecal smears were fixed in methanol for 2 minutes and air dried and stained with full strength Ziehl-Nielsen carbol fuschin for 10 minutes without heating. Smears were then rinsed in running tap water, decolourized with 1% acid alcohol for 1 minute, washed in water and counterstained with malachite green for one minute before being rinsed again in water, air dried and examined under oil immersion.

2.6.3.4 Faecal neopterin

Experiments at aiming to develop a stool inflammatory marker using various monoclonal antibodies against TNF-α and IFN-γ, were conducted at The Dunn Nutrition Unit. It was decided to use an alternative non-cytokine based marker. Recovery of either TNF-α or IFN-γ spike (the addition of a known quantity of cytokine to the stool sample) lead to very low and variable antigen recovery, making interpretation of results problematic. Neopterin was found in relatively high concentrations (x100 greater than serum or urine) with > 90% recovery of sample spiking, and therefore chosen in preference to a cytokine.

Samples of whole stool collected and frozen in Keneba were defrosted at room temperature and 0.1-0.2g was added to a weighed Sarstedt tube. The tube was re-weighed to accurately calculate the weight of whole stool added. The stool was then freeze dried over 48 hours and the dry weight of the stool was calculated. 0.5 ml of saline was added to the dry stool and the sample was aggressively agitated for 30 minutes. The tube was then centrifuged at 3500 rpm for 20 minutes and the supernatant was removed and frozen until further analysis could be carried out.

Faecal neopterin concentration was determined using a commercial radioimmunoassay kit (Henning, Germany). Fifty-μl of faecal extract fluid was incubated with 100μl of neopterin for 1 hour at room temperature, then 100μl of 125I labelled neopterin was
added and incubated for a further 1 hour. The antigen-antibody complexes were precipitated by a combined double antibody polyethylene glycol (PEG 60 gL⁻¹) precipitation. After centrifugation at 200g for 10 min the precipitate was assayed in a gamma counter. The detection limit of the method was 1 nmol L⁻¹ and the normal range of serum neopterin in healthy volunteers is 4.2-10.5 nmol⁻¹.

2.6.3.5 Faecal electron microscopy for viruses
Handling of all stool samples was done in a lamina flow cabinet, with appropriate safety wear (laboratory coat, perspex goggles and latex gloves). Using a disposable transfer pipette 0.1 ml of stool was added to 0.9 ml of phosphate buffered saline and vigorously homogenised. One drop of the emulsion was placed on to a formvar / carbon grid and allowed to air dry. Excess fluid was removed using a filter paper blot. A drop of 0.1M phosphotungastic acid stain was added to the dry grid and blotted off. Examination of at least 5 grid squares at × 30,000 magnification was performed by Mr Graham McPhail, Dept Virology, St Bartholomew’s Hospital.

2.6.4 Small bowel morphometry and immunohistochemistry
2.6.4.1 Sample sectioning
Formal fixed specimens were sent to the department of histopathology at Great Ormond St where multiple 5μm sections were cut and stained with haematoxylin and eosin.
Sections for immunohistochemistry were cut on a cryostat at 6μm at -20 centigrade and then left for one hour to dry before fixing for 10 minutes in 10 % formal saline.
Sections for cytokine staining were cut at 10μm thickness and immediately fixed in 10% formal saline also for 10 minutes.

2.6.4.2 Immunoperoxidase

After the sections were fixed the following procedure was followed.

1. Washed with tris buffered saline.
2. Incubated with 10% horse serum to block non specific tissue binding of secondary and tertiary antibodies.
3. Washed with tris buffered saline.
4. Primary antibody added and incubated for one hour.
5. Step 3 repeated
6. 3% hydrogen peroxide diluted in methanol was added for 5 minutes to reduce the background peroxide activity.
7. Step 3 repeated
8. Secondary, biotinylated antibody was added and incubated for 30 minutes.
9. Step 3 repeated.
10. Avidin peroxidase complex was added and incubated for 30 minutes.
11. Step 3 repeated
12. A di-amino-benzamine (DAB Sigma UK) urea solution was added to the slides as indicator and reagent for the peroxidase bound to the antigen on the tissue surface.
    The slide was studied frequently for a brown colour change and after 10-15 minutes the slides are washed in tap water to terminate the reaction.
13. Counter staining was with Meyers (Sigma UK) medium.
14. Slides were dipped in a 1% hydrochloric acid in ethanol bath to reduce the staining intensity and improve epithelial definition.
15. Tissue sections were dehydrated by placing the slides in increasing concentrations of ethanol from 70-100%.

16. Intensity of the counter staining was further reduced by washing the slides in “Histoclear” for one minute.

17. The sections were covered with a DPX mounted coverslip.

Cytokine immunohistochemistry, particularly IFN-γ staining, showed significant matrix staining that was not present in the no primary antibody control slides. For IFN-γ, and to a lesser extent TGF-β, there was significant extracellular binding to glycosaminoglycans within the matrix and basement membrane, as is recognised from in vitro studies (Lortat-Jacob and Kleinman, 1991). This was seen in all patients studied, including UK controls, and appears to reflect the complex cytokine-rich environment within the intestinal mucosa (Walker-Smith and Murch, 1999; Fiocchi 1997). While pre-incubation of specimens with heparanase II or chondroitinase ABC at 37°C for 2 hours removed most of the matrix-associated cytokine (Murch and MacDonald 1993), it also significantly disrupted tissue morphology. As individual cells with strongly-stained cytoplasm could in any event be detected readily against the matrix-associated cytokine, we elected not to pretreat in this way.

2.6.4.3 Small bowel Morphometry

Measurement of villous height, crypt depth, crypt/villous height and depth ratio and mucosal thickness was obtained using an Olympus BH microscope and “Imigan 2” image analyser via a video link.
1. Crypt depth was measured by setting the mouse controlled cursor at the level of the basement membrane of the crypt epithelium and then recording the distance when the cursor is reset at the crypt villous junction. This reading is repeated on separate tissue sections and the average of 10 is recorded, although on some tissue sections only 5-6 readings could be obtained for technical reasons. A times 40 objective was used giving a magnification of x 400.

2. Villous height, was obtained by measuring from the crypt villous junction to the top of the apical villous epithelium. This was repeated as before 5-10 times on separate sections, the average was recorded as the reading for that subject. A x 40 objective was used.

3. Crypt depth / villous height ratio was derived from the ratio of the two above readings.

4. Mucosal thickness was derived from the sum of 1 and 2.

5. A x100 objective and oil emersion lens was used giving a final magnification of x1000. Intraepithelial lymphocytes could be easily identified because of a darker staining nucleus lying in a different focal plane to the surrounding epithelial cells. Cells attached to the basement membrane were also counted if they were of lymphocyte morphology. The final count was expressed per 100 epithelial cells over the area of 500 epithelial cells.

Lamina propria cell numbers were counted with an eyepiece graticule, calibrated with a stage micrometer. All lymphocytes, granulocytes, macrophages and eosinophils within an area of 0.1 mm² were counted. This was repeated for deep (pericryptal) and superficial (villous) lamina propria. An average of 5 readings for each crypt compartment was taken (both superficial and deep).
2.6.4.4 Quantification of Immunohistochemistry

Each subject had a control slide prepared with out a primary antibody applied. This slide underwent peroxidase staining to insure minimum background staining was achieved. These sections were then counted for "positive" staining and then this was subtracted from the final cell count on those sections that had been exposed to a primary antibody.

IELs lying within the epithelium were counted and expressed per 100 epithelial cells. Cells lying within the lamina propria were counted over a 4 x 4 grid using an eye piece graticule as outlined above. A positive cell was one that stained obviously darker than the background. Cells were expressed per mm² and also as a percentage of lamina propria cells.

2.7 Statistical Analysis: software used

Data Desk 6.0 for the apple Macintosh; Excel 5 spreadsheet and Cricket Graph for graphical representation. Details of appropriate statistical methods are included in the relevant chapters.
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Chapter 3.0
Enteropathy and growth

The main theme of this thesis is the cause of growth faltering and how it relates to small bowel barrier function. There are currently two issues of international debate that are relevant to this thesis.

Firstly there appears to be a global decline in the morbidity and potentially the prevalence of diarrhoeal disease. Secondly there is increasing evidence that long-term growth faltering is not due to episodes of acute diarrhoeal illness. Previous Keneba studies have shown that small bowel function is closely related to long-term growth, but it is not known whether or not this still applies in the face of a changing global and local Keneba pattern of diarrhoeal disease.

As has been shown already in chapter 1.0 enteropathy (measured both functionally and by histopathology) has been described in both children and adults. Currently there are no data on how small bowel function affects growth beyond 5 years of age.

This chapter aims to establish:

1. Whether or not the lack of relationship between diarrhoeal disease and growth still exists in the cohort of Keneba children.

3. Whether or not small bowel function as assessed by the L:M permeability test still relates to growth in the context of a potential significant reduction in diarrhoea morbidity.

4. Whether an alternative measure of enteropathy (faecal neopterin) will significantly predict growth in Keneba children.

5. What effect enteropathy (measured by the L:M test) has on nutritional status beyond 5 years of age.
Each of these is considered in four consecutive sections (3.1, 3.2, 3.3 and 3.4)
Chapter 3.1

Diarrhoea morbidity and growth of Keneba infants

3.1 Background and aims

As has been mentioned in the literature review it now appears that recurrent acute diarrhoeal diseases are probably not the cause of long-term growth faltering in the tropics (Briend et al., 1989, Moy et al., 1994a). However, there is still evidence that diarrhoeal disease may be the an important cause of growth faltering (Lutter et al., 1989). Diarrhoeal episodes however, are still regarded as an important indicator of intestinal disease. Poskitt et al (Poskitt et al., 1999) has described a fall in the prevalence of acute diarrhoeal disease based on clinic data but we do not know what the prevalence is from a community based survey. In view of the fact that there is evidence that diarrhoeal disease patterns are changing, there is a need to ascertain whether or not the previously described lack of association between diarrhoeal disease and intestinal permeability still holds true.

The aim of the first section of this chapter was to use a community based diarrhoea survey (rather than a clinic based one by Poskitt et al) to estimate the subject prevalence of diarrhoea for the cohort of Keneba children and to related this to intestinal permeability and biochemical markers of systemic inflammation (acute phase reactants and immunoglobulins). This would confirm or refute the lack of association between growth and acute diarrhoeal disease in Keneba children.

3.1.2 Methods
Patients see section 2.1.3

Diarrhoeal survey see section 2.4.2.

Lactulose-mannitol permeability see 2.5.5

Anthropometry see section 2.5.2

Biochemistry see section 2.6

3.1.3 Statistical analysis

The main unit of measurement to quantify diarrhoea used was the mean monthly subject diarrhoea prevalence, which is the mean days with diarrhoea for each child standardised to a 30.45 day month. Diarrhoea was defined as (see section 2.4.2) the mothers’ (or main child carer’s impression of change in bowel habit towards increase in stool frequency and looseness. This definition has been previously used in The Gambia by Rowland et al (Rowland et al., 1985, Rowland et al., 1986)

Associations between continuous variables were tested using linear regression. Growth was defined as a change in height or weight standard deviation score over the whole of the study period (response variable) expressed as a monthly rate and a summary of intestinal permeability, acute phase proteins and immunoglobulins were the predictor variables. Summary of predictor variables was done by taking the mean of all measurements across the study period.

Normality was tested using Bartlett’s test. Log transformation was undertaken in non-parametrically distributed variables.

For all analyses one subject was excluded who had a subject prevalence for diarrhoea >4 standard deviations from the cohort mean. No specific cause for the very heavy burden of diarrhoea in this child was identified, and he subsequently developed kwashiorkor and died at 18 months of age. He was negative for HIV antibodies at that time.
3.1.4 Results

Seventy-one children were studied, but 5 from the study cohort were excluded as they were followed for less than 3 months. The mean duration of follow up was 7.5 months with a standard deviation of 2.8 (range 13-3.5 months). There was a clear seasonal pattern to the diarrhoea but this did not strictly follow the rainy season (late June to September). Fig 3.1 shows the change in diarrhoea prevalence over a 12 month period. October to February, during the cool dry season, has the lowest rates of diarrhoea with the average child having diarrhoea about 5% of the time. A sustained rise in diarrhoea morbidity above the October to February rates is seen between May and June, before the rains begin but this escapes significance ($P = 0.09$).

The highest incidence of diarrhoea comes during the heaviest rains in August and September rising to a peak mean monthly prevalence of 18% in August more than 3 fold higher than the period between October to February. However the diarrhoea prevalence seems to rise at least 4 months before the rains start and not fall until the August peak has finished.

The mean diarrhoea prevalence across the whole year is 0.83 days per week (SD 0.67) with a range of 0.1 - 3.6 days / week. This is equal to 12% of the time across a calendar year slightly higher than the value of 9.8% previously quoted by Lunn et al (Downes et al., 1991).

Significant relationships exist between number of days with diarrhoea (after log transformation) and number of days with vomiting ($r = 0.49, P < 0.05$), but not with fever, which correlated more strongly with vomiting ($r = 0.76, P < 0.001$).

Change in weight and height z-score was not significantly associated with the average amount of time a child had diarrhoea per month.
There was also no association between the mean monthly subject prevalence for diarrhoea and the mean L:M permeability over the survey period. This was repeated for % recovery of both lactulose and mannitol and again there was no significant association.

Comparisons between permeability values for each child were made by summarising the serial estimates in to a mean for each child. There was no significant relationship between permeability and average days with diarrhoea per week by linear regression. Measurements of immunoglobulins, albumin, Giardia-specific IgM, haematocrit, alpha-1-antichymotrypsin and CRP estimates were summarised by taking the mean values for individual subjects across the survey period and compared to the mean number of days with diarrhoea per month for each child. Immunoglobulin subclasses IgG and IgM were significantly related to the diarrhoea morbidity ($r = 0.70\ P = 0.02$; $r = 0.65\ P = 0.03$ respectively) but IgA was not ($P = 0.07$). Days with diarrhoea were not significantly related to mean C-reactive protein haematocrit, ACT but approached significance for Giardia-specific IgM ($P = 0.06$).

3.1.5 Discussion

This survey confirms the commonly held belief that diarrhoeal disease is still very prevalent in West Africa. Published data from similar surveys carried out in the USA suggest that children between 6-18 months have 1-3 attacks of diarrhoea per year lasting up to 3 days giving a total of 9 days or 2% over one year (Roubenhoff and Rosenberg, 1991). This is substantially less than the 12 % estimated by this survey. This study has not shown any evidence of a decrease in the subject prevalence since 1994 (Lunn et al., 1995) however much of the reduction reported by Poskitt et al came before 1994 when this time. Differences in the methodology and definition of
diarrhoea may explain the divergence in the results reported by Poskitt et al and this present study. It is likely that a twice weekly visit to the compound would gain a higher estimate than at a 2 monthly clinic visit. This study also used a less rigid definition of diarrhoea than the study by Poskitt et al, which would tend to lead to a higher estimate of diarrhoea prevalence.

Seasonal climatic and social changes are important in the pattern of diarrhoeal morbidity with peak rates coming during August (the month of maximal rain fall). However, the seasonal rise in diarrhoea begins in May and June before the rains commence, but during the period when mothers are away from the children preparing the fields for farming. During the pre-rainy season rise in diarrhoea, the young children are left in the care of a child nurse maid who may only be 7 years old (Weaver and Beckerleg, 1993, Beckerleg, 1993). Infants could be left in the care of nurse maids for long periods of time with the food being prepared in the morning and continuing from the same bowl for the entire day. By evening the bacterial loads are extremely high and the consumption of the gruel puts the infant at risk of gastrointestinal infection (Rowland and Barrell, 1978).

This study has confirmed the previous reports that there is no association between diarrhoeal disease and growth and diarrhoeal disease and intestinal mucosal damage assessed by L:M permeability. There are at least two possible explanations. Firstly L:M rises in a non-specific way in any small bowel inflammatory condition, but is probably not affected by colonic pathology (Elia et al., 1991), which may cause diarrhoeal disease. Secondly, the lack of diarrhoeal disease in coeliac disease is well established, so that even histologically grossly abnormal mucosa can exist in a subject without diarrhoeal symptoms for years. Potentially the same scenario exists for tropical enteropathy. Therefore diarrhoeal disease may be both an insensitive and non-specific
marker of small bowel mucosal damage, or that there were other confounding causes of acute phase response.

Mean immunoglobulin levels are significantly related to diarrhoeal prevalence with higher values seen in children with the greater diarrhoea morbidity, indicating that diarrhoeal disease is associated with detectable systemic inflammatory activity. However the acute phase proteins CRP and ACT were not related to diarrhoea prevalence suggesting that full scale activation of the reticulo-endothelial system does not occur with most bouts of common diarrhoea in Gambian children.

*Giardia*-specific IgM mean levels had a positive relationship to subject diarrhoea prevalence that approaches significance and supports the idea that *Giardiasis* is an important cause of diarrhoeal disease. The role of *Giardia* in causing enteropathy and growth faltering in rural Gambia will be discussed in chapter 4 in more detail.

### 3.1.6 Conclusion

Diarrhoeal disease is not associated with long-term growth performance in Gambian infants, neither is it related to the degree of small bowel mucosal damage as assessed by the L:M test. However mean levels of circulating immunoglobulins (IgM and IgG in particular) are positively related to subject diarrhoea prevalence, indicating that a level of systemic inflammation follows even a common bout of diarrhoea.
Mean days with diarrhoea as a percent of a standardized month
August '96-July '97

Fig 3.1
Chapter 3.2

Lactulose-mannitol permeability and growth

3.2.1 Background and aim

The previous section of this chapter demonstrated a lack of association between diarrhoeal disease and both long-term growth and intestinal permeability. Since it has been first reported that the pattern of diarrhoea morbidity has changed in Keneba there have been no studies to verify the association between small bowel permeability and growth as it was first described by Lunn et al (Lunn et al., 1995).

In the original study by Lunn only the association between long-term growth and L:M permeability was tested. Currently there are no studies that have related the effect of changes in L:M to short-term growth.

In this chapter the aim is to verify or refute the association between intestinal permeability and growth both in the long and short-term.

3.2.2 Methods

Patients see section 2.1

Lactulose-mannitol permeability test 2.5.5

Anthropometry see section 2.5.2

Biochemistry see section 2.6

3.2.3 Statistical Analysis
Age correction of growth over the long-term is done by the method described by Lunn et al (Lunn et al., 1995). Growth is a change in weight (kg) (or height, cm) over the study period expressed as kg (or cm) / month. Rates of growth are highly age dependant with younger children growing faster than older, and the association is non-linear. Therefore a stepwise regression model was used to correct for the effect of age upon growth by including age (at leaving study) and age at leaving study squared, cubed and to the power of 4 as factors in the regression model. By including age these age terms it was possible to remove the variability in growth velocity due to age, taking in to account the non-linearity of the association. A predicted growth velocity for each child can then be calculated following this method of age correction, and this is used for the final regression analysis.

Short-term growth was examined in two ways by breaking up changes in weight in to the following age categories:

8-12 weeks, 12-24 weeks, 24-36 weeks, 36-44 weeks, 44-52 weeks.

1) Seasonal adjustment

For each of these age categories “normal” mean average values for weight were extracted from the Keneba clinic database (kindly supplied by Dr T.J. Cole) in a seasonally stratified form, to be used as normative data against which growth for each child was compared. Subtracting the expected weight (from the Keneba normative data) from the observed weight (that measured in this study) the sum gave a final age and seasonally adjusted value against which small bowel function (L:M permeability) could be tested.

Only seasonally stratified weight gains are currently available and therefore, to examine height growth, a second manner of analysis was needed.

2) Seasonally unadjusted
Growth over the time period for each age band was defined as the change in kg or cm expressed as a monthly rate. This value is calculated for each age band and treated independently and is then compared against L:M permeability by linear regression. For definition of permeability summary values for individual children see section 3.1.3.

3.2.4 Results

3.2.4.1 Permeability trends

Across the whole study period 71 children were followed and a total of 278 successful L:M test and 300 weight and height measurements. Children were followed for a mean of 7 months (range 3-15 months).

Fig 3.2.1 shows that there is a degree of variability in the mean L:M values with age in the first year of life but the overall trend shows an increase ($P < 0.0002$). Twenty-six of the 71 children were followed from 8 weeks to at least 1 year, the mean group permeability values for these children is similar to that shown in fig 3.2.1. The overall mean L:M value is 0.31 (SD 0.25) which is three times higher than the UK normal value of (< 0.1), but lower than previously estimated values by Lunn et al in 1991 (mean L:M 0.38, SD 0.3). Even the baseline values at 2 months of age (0.2) is twice as high as that seen in the UK and rises to 0.46 by one year of age.

Fig 3.2.2 shows the contribution of each of the component sugars to the overall L:M ratio. There is a significant fall in the mannitol recovery from 8 to 44 weeks and then a degree of recovery. The normal percentage recovery of mannitol in this age group is 11-15 %, therefore even at the highest value at 8 weeks (3.7 %) indicates a substantial impairment of small bowel absorptive capacity. For the sake of clarity so that both sugar recoveries could be displayed on the same plot lactulose recovery has been
multiplied by a factor of 10. The normal recovery of lactulose in healthy UK children is <0.3% of the oral dose, therefore Keneba children will have lactulose recovery within the normal range until they reach 44 weeks of age. Fig 3.2.2 shows that the reason for the high L:M ratios at 24-26 weeks is due to a simultaneous nadir in the mannitol recoveries and a peak in the lactulose recoveries. The mean recoveries of lactulose and mannitol was 0.21 (SD 0.21) and 3.1 (SD 2.1) respectively, compared with values of 0.34 (SD 0.29) and 4.4 (SD 3.1) reported by Lunn et al 1991.
Figure 3.2.1

Changes in mean L:M ratios with age

Means and standard error bars show numbers in age category

n = numbers in age category
Fig 3.2.2  Age changes in recovery of oral doses of lactulose and mannitol in the first year of life

- % recovery of lactulose
- % recovery of mannitol

Error bars are standard deviations.
3.2.4.2 Long-term growth and L:M permeability

Figure 3.2.3 demonstrates the scatterplot of age corrected height growth over the whole study period against the mean value of all the L:M ratios. It can be seen that there is a significant negative relationship between growth over the study period and L:M ($P < 0.003$, $r = -0.46$), with approximately 21% of the growth accounted for by permeability. A similar plot is seen for height growth over the same time period although only approximately 16% is accounted for by L:M. The values reported by Lunn et al were 43% for height and 39% for weight. There was a significant, negative association between longterm linear growth and percentage recovery of lactulose ($r^2 = 12.9\%$, $P = 0.002$).

3.2.4.3 Short-term growth

Table 3.1 gives the number of paired permeability and growth numbers for each age category. Each data point is independent and only represents a single child across that age band.

Table 3.1

Number of paired growth readings in each age category

<table>
<thead>
<tr>
<th>Age Band (weeks)</th>
<th>8-12</th>
<th>12-24</th>
<th>24-36</th>
<th>36-44</th>
<th>44-52</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>43</td>
<td>49</td>
<td>49</td>
<td>26</td>
<td>23</td>
</tr>
</tbody>
</table>

There were no significant relationships between L:M and growth until the 36-44 week age group when seasonally corrected weight gain had a significant negative association ($P < 0.02$, $r = -0.55$). At this age, height gain was also negatively associated with L:M
There were no other associations between short-term height growth and L:M, % recovery of lactulose or mannitol at any other time point. Mannitol recovery tended to be more significantly associated with short-term growth than % recovery of lactulose. Seasonally corrected weight gain at 12-24 and 24-36 weeks was positively associated ($P = 0.01, r = 0.56$ and $P = <0.02, r = 0.49$ respectively). Fig 3.2.4 a and b show changes in monthly growth rate and how L:M permeability changes with time in two Keneba children, note the almost mirror images of L:M and weight gain. Not every child produced such close relationships between growth and permeability (as only approximately 20% of the variability in growth is explained by L:M), but similar patterns were seen in 15 of the Keneba children.

3.2.5 Discussion

This study has shown that the same association exists between L:M and long-term growth that was initially reported by Lunn et al (1991). However the association with height growth is not as strong ($r = -0.47$ in this present study compared to -0.66 by Lunn et al 1991) and neither were the permeability values as high ( L:M 0.31 compared to 0.38). Lactulose recovery was also lower (0.21 compared to 0.34) but mannitol recovery was slightly higher in the previous Keneba study. Percentage recovery of individual sugars in this manner is subject to many methodological confounding factors such as incomplete urine collection, or incomplete oral dosing. Before direct comparison is made with the earlier Lunn and Downes study is made, standardisation of sugar concentrations against urinary creatinine concentration needs to be considered. Measurement of urinary creatinine concentration was not attempted in this study. However the data suggests that as well as falling diarrhoea prevalence in Keneba (ie socioeconomic improvements, improvements in sanitation and diet) L:M permeability is also improving. Compared to UK normal levels
however the L:M ratio is grossly elevated, due to a low intestinal absorptive capacity, and after 11 months of age an increase in lactulose absorption.

The lack of association between diarrhoea and short-term growth has been clearly established (Briend et al., 1989, Moy et al., 1994b) and confirmed in the previous section of this chapter. This part of the study shows that summary values of small bowel function, measured by sugar permeability tests is significantly related to growth between 2-15 months of age. This study is limited due to small sample size, making short-term growth comparisons difficult. However, it appears that short-term weight faltering is associated with poor small bowel absorptive capacity (measured by mannitol uptake) and impaired mucosal barrier function (measured by lactulose recovery) tending to affect growth later (after 9 months of age).

3.2.6 Conclusion

Long-term growth (from 2 to 15 months of age) is associated with impaired small bowel permeability in Keneba children but not to the extent that was noted previously by Lunn et al. (1991). Short-term growth is more often associated with decreased gut absorptive capacity than increased permeability.
Fig 3.2.3

Scatterplot of age corrected height growth vs intestinal permeability

$P = 0.003$, $R^2 = 21\%$
Fig 3.2.4 a  Weight gain in month before permeability reading and intestinal permeability in a single individual

![Graph showing weight gain and intestinal permeability over age.]

Fig 3.2.4 b  Weight gain and gut permeability changes with age (single individual)

![Graph showing weight gain and gut permeability over age.]

Weight gain per month

Age (years)

Weight gain (kg)

Age (years)
Chapter 3.3

Enteropathy and growth

Intestinal inflammation, Lactulose : mannitol permeability and growth

3.3.1 Background and aims

The previous section of this chapter has shown that dual sugar permeability is significantly associated with long-term growth of Keneba children. In this section the relationship between a novel marker of gut mucosal inflammation and growth will be examined.

Faecal cytokines have previously been used to quantify the degree of gut inflammation in infective diarrhoea and inflammatory bowel disease (Nicholls et al., 1993, Raqib et al., 1995). Neopterin is classified as a pteridine molecule and is formed as a breakdown product of cyclic guanosine triphosphate which is involved in the synthesis of tetrahydrobiopterin, an essential cofactor for nitric oxide synthetase. Neopterin is specifically released from activated phagocytic cells (macrophages, dendritic cells and neutrophils) and its’ production is predominantly influenced by local levels of IFN-γ (Andert and Muller, 1995). It has been shown that in various inflammatory conditions serum and urinary levels of neopterin rise (Haupt et al., 1995, Fuchs and al, 1992, Diamondstone et al., 1994, BoronKaczmarska et al., 1994, Zangerle et al., 1994), and it is assumed that local mucosal production and release of neopterin in to the stool should therefore represent local cell mediated immune activity within the gastrointestinal tract.
The aim of this section of the chapter was to quantify the relationship between growth across the study period and mean faecal neopterin concentration, as a marker of mucosal inflammation. The secondary aim was to compare the range of L:M permeability to faecal neopterin levels within each individual subject.

3.3.2 Methods

3.3.2.1 Laboratory and clinical methods

Summary measurements of faecal neopterins, growth and L:M permeability were made upon the Keneba longitudinal cohort, see section 2.1.

Patients 2.1.3

L:M permeability test 2.5.2

Stool collection 2.5.4, Faecal neopterin measurement 2.6.3.4.

3.3.2.2 Statistical methods

Mean growth is defined in 3.2.3 and serial estimates of L:M were summarised as a mean of all readings across the whole study period (see 3.2.3). Significance of trends were compared by linear regression after log transformation of non parametrically distributed variables.

3.3.4 Results

Predicted long-term height and weight gain were negatively associated with mean faecal neopterin concentration ($r = -0.29, P < 0.009$ and $r = -0.36, P < 0.007$ respectively).

For the secondary analysis, assessing the association between intestinal permeability and faecal neopterin concentrations, three outliers were excluded, as the final
regression equation was heavily influenced by these three subjects. All had a standardised residual greater than 3.0, these results were therefore excluded from the study. All three had clinical explanations for their classification as outliers. All had a period of persistent diarrhoea (>2 weeks) during the study period, one subsequently dying of persistent diarrhoea and malnutrition (kwashiorkor) at 18 months of age. Another had in addition to persistent diarrhoea, severe recurrent impetigo. There was a positive correlation between L:M permeability and mean faecal neopterin concentration but this did not reach statistical significance (*P* = 0.11). Figure 3.3 demonstrates the changes in L:M permeability and faecal neopterin concentration in two individual subjects, and it can be seen that they are not followed as closely as growth and L:M. Similar graphs were seen in approximately 5 children. Both percentage recovery of mannitol and lactulose were significantly, positively correlated with mean faecal neopterin concentration after the same three outliers were excluded (*r* = 0.26, *P* <0.04 and *r* = 0.25, *P* <0.04 respectively).

3.3.5 Discussion

Faecal neopterin is still an experimental tool for assessing mucosal inflammation. It is not known if dietary pteridines influence the final faecal concentration, whether pteridines are excreted in to the gut during systemic inflammation, or if maternal illness whilst breast feeding will likewise alter faecal neopterin concentration. There are no published studies that have measured faecal neopterin concentration and we are not aware of this having been done before. Although faecal neopterin concentrations have not been validated against intestinal inflammation assessed by histology and immunohistochemistry, an inverse relationship to growth would be expected if intestinal inflammation was linked to growth faltering. The data from this study is consistent with this view. The mean levels of neopterin are measured in μg/l in faecal...
extracts and this is 100 fold higher than is seen in plasma or urine and suggestive of a very active cell mediated inflammatory process in the gut mucosa. Faecal chemistry however will not differentiate between small or large bowel inflammation and may reflect colonic pathology more strongly.

Faecal neopterin concentration is not related to L:M permeability, but is related positively to both lactulose and mannitol recovery in a positive direction with an equal strength of association. It is somewhat surprising that mannitol uptake is related to faecal neopterin concentration in a positive manner as experimental evidence suggests that cell mediated inflammation causes villous atrophy and hence a lower intestinal absorptive capacity. However, if a large percentage of faecal neopterin originated from the colon, it could explain this apparent anomaly.
3.3.6 Conclusion

Faecal neopterin, which is an experimental tool to measure mucosal inflammation, shows a significant negative association with growth. More studies are needed to establish the source and significance of faecal neopterin.
Fig 3.3 a) Changes in L:M permeability and stool neopterin concentration with age in two Keneba children.
Chapter 3.4

Long-term effect of enteropathy on nutritional status - from childhood to adulthood.

3.4.1 Background and aim

Chapter 1 has highlighted the fact that enteropathy has been reported from many third world countries, in both adults and children. There is very little data available on how small bowel enteropathy relates to growth in the tropics beyond 2 years of age, nor how age itself affects small bowel permeability. Age related changes in gut function also give some clues to the aetiology of the enteropathy. It has already been noted that L:M permeability in young children varies with season, but it is not known what effect season has on small bowel function in later childhood and in the adult years.

The aims of this section are threefold:

1. To describe the changes in small bowel function with age.
2. To quantify the effect of enteropathy upon nutritional status and growth beyond 2 years of age.
3. To assess the impact of seasonality upon gut function beyond 2 years of age.

3.4.2 Methods

Subject details and recruitment see 2.3.

In brief 162 inhabitants from the 3 study villages: Keneba, Manduar and Kanton Kunda aged 2-60 years were recruited. Forty-one % of the subjects were from Keneba, 30% from Kanton Kunda and 29% from Manduar. Research clinics were held in May
1997 (dry season) and August 1997 (wet season). Subjects underwent the L:M test if they were in good health and had been free from diarrhoea for the previous week. Subjects were briefly examined prior to receiving the L:M solution and then all urine passed in 5 hours was collected. Subject details and dosing schedule of lactulose and mannitol are shown in table 3.4.1.

The dose of permeability sugars varied with age but was constant at 2g of lactulose and 0.5g of mannitol per 10 ml of water (see table 3.4.1).

For details of anthropometry, L:M test and laboratory details of urinary lactulose and mannitol assays see the appropriate sections in chapter 2.0.

Separate ethical permission was obtained for this part of the study.

Table 3.4.1
Dosing schedule of lactulose and mannitol in each age group

<table>
<thead>
<tr>
<th>Age group(years)</th>
<th>2-5</th>
<th>5-10</th>
<th>10-15</th>
<th>15-20</th>
<th>20-30</th>
<th>30+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age</td>
<td>3.2</td>
<td>8.0</td>
<td>13.4</td>
<td>18.1</td>
<td>25.8</td>
<td>48.5</td>
</tr>
<tr>
<td>(years)</td>
<td>(0.6)</td>
<td>(1.0)</td>
<td>(1.6)</td>
<td>(0.7)</td>
<td>(5.1)</td>
<td>(6.4)</td>
</tr>
<tr>
<td>Dose of L/M (mls)</td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
</tbody>
</table>

3.4.3 Statistical analysis

For the effect of age upon L:M permeability and nutritional status cross sectional data was collected. For the effect of enteropathy upon growth and the effect of season upon small bowel function longitudinal data were collected.

Analysis of the effect of age upon L:M, percentage recovery of lactulose and mannitol was by ANOVA and regression analysis.
To quantify the effect of enteropathy upon nutritional status and growth the independent effects of age and sex had first to be removed. Weight, height or BMI was expressed as a z-score (from the Cambridge 1990 standards) but this was not sufficient to remove the effect of age. This was done by including sex and age, $a^2$, $a^3$ and $a^4$ as factors in the linear model for ANOVA or stepwise regression as in section 3.1.3. The reason for including different forms of age was the non-linear association that age had with nutritional status.

The significance of change in weight across the rainy season was tested using the paired t-test.

3.4.4 Results

Figure 3.4.1 shows the changes in weight, height and BMI z-scores with age across the study period. There are 2 periods when Gambians showed considerable catch up growth but never beyond a weight-for-age $z$-score of -0.9 and -0.75 for height. The first of these time periods was between 4 and 8 years of age, the second between 17-19 years was probably due to the prolongation of the pubertal growth spurt. Both of these periods of catch up was associated with a fall in the BMI $z$-score, indicating that height gain is at the expense of a degree of wasting. This suggests that there is still evidence of nutrient limitation.

3.4.4.1 Changes in Intestinal function with age

Figure 3.4.2 shows the changing pattern of L:M permeability with age (ANOVA $P < 0.0001$). L:M permeability is particularly high in young children under 5 years and gradually improves but never reaches the normal UK value of $< 0.1$.

Figures 3.4.3 shows the change in percentage recovery of both lactulose and mannitol with age. The percentage recovery of mannitol is approximately one third of that seen
in the UK and never seems to recover with age. However lactulose recovery improves significantly with age (ANOVA $P < 0.0001$) to reach the UK normal range of $< 0.3$ at 10-15 years of age.

3.4.4.2 The effect of enteropathy upon nutritional status and growth.

At both the dry season and rainy season clinic visits height $z$-score is significantly related to L:M after correcting for gender and age ($P < 0.02$, $r = -0.3$ and $P < 0.007$, $r = -0.4$ respectively). The percentage recovery of mannitol was never significantly related to any anthropometric variable. The percentage recovery of lactulose is significantly related to height only in the dry season ($P < 0.0001$), whereas weight and BMI $z$-scores were not significantly correlated with any of the parameters of gut function ($P > 0.3$) at either the wet or dry season clinics.

Growth was quantified as a change in weight, height and BMI $z$-score between the 2 visits (approximately 3 months apart). $\delta z$-scores for all 3 variables were highly significant ($P < 0.0001$). The mean L:M, % recovery of both lactulose and mannitol was used as predictors to form regression analyses with $\delta z$ (height, weight or BMI $z$-score). There were no significant relationships between weight ($P > 0.1$), height ($P > 0.5$) and BMI ($P > 0.2$) and any of these measures of intestinal permeability when corrected for age and sex.

3.4.4.3 The effect of season upon small bowel function

Table 3.4.2 shows the changes in L:M between wet and dry season.

It can be seen that there is no overall deterioration in L:M permeability but mannitol recovery rises slightly between the two measurements ($P < 0.01$).
Table 3.4.2 Dry and wet season L:M permeability values (mean and 95% confidence intervals)

<table>
<thead>
<tr>
<th>Season</th>
<th>L:M</th>
<th>95% CI</th>
<th>% recovery</th>
<th>95% CI</th>
<th>% recovery</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry</td>
<td>0.22</td>
<td>0.1 - 0.4</td>
<td>0.26</td>
<td>0.11 - 0.41</td>
<td>5.3^a</td>
<td>5.2 - 5.5</td>
</tr>
<tr>
<td>Wet</td>
<td>0.20</td>
<td>0.0 - 0.4</td>
<td>0.29</td>
<td>0.10 - 0.47</td>
<td>6.2</td>
<td>6.0 - 6.4</td>
</tr>
</tbody>
</table>

^P < 0.01 all other comparisons P > 0.5

3.4.5 Discussion

This section of the study has shown that mucosal integrity (measured by the recovery of lactulose) improves with age, but absorptive capacity is always significantly impaired and shows no improvement with age. The relationship between permeability and height even during adult years, indicates that enteropathy has probably been a growth limiting factor throughout the life. However the magnitude of effect appears to be less strong in the older subjects (regression analysis for height r = 0.3 for 2-60 year olds in this part of the study and -0.45 for children under 15 months from the Keneba cohort). Lactulose recovery improves steadily with age, and although there is still a significant correlation between wet and dry season levels, the overall trend is for improvement.

The absence of deterioration in L:M between wet and dry seasons is surprising, but potentially the timing of the dry season measurement co-incides with the beginning of the pre-rainy season trough in gut function. To examine whether or not there is a seasonal change in gut function the study would need to be repeated with a dry season estimation of permeability in January or February.
3.4.6 Conclusion

There is evidence that mucosal integrity improves with age but never reaches normal UK levels. Gut absorptive capacity appears to be function at approximately one third of that seen in the UK, and fails to improve with age. Enteropathy (measured by the L:M test) limits growth throughout life, but is a stronger influence in the younger age group (under 5 years of age). An effect of season upon L:M could not be demonstrated in this study.
Figure 3.4.1  Changes in anthropometric indices with age Keneba, Kanton Kunda and Manduar

- WT Z-SCORE
- HT Z-SCORE
- BMI Z-SCORE

n=162
Age Related Changes in Lactulose Permeability Ratio in Rural Gambians

Figure 3.4.2

Age Group in years

Lactulose mannitol ratio

Error bars SE of mean

Upper limit of UK normal
Figure 3.4.3

Age related changes in recovery of mannitol and lactulose

**Mannitol**

UK normal range 11-15%

**Lactulose**

UK upper limit of normal

Error bars = SEM
Chapter 4.0

Causes of chronic enteropathy

Section 4.1
Dietary associations with chronic enteropathy

Chapter 3 showed that a chronic enteropathy, but not acute diarrhoeal disease, is related to growth in Keneba children, although to a lesser extent than previously estimated. This chapter goes on to examine some of the possible causes of this enteropathy.

4.1.1 Background

Many investigators have clearly demonstrated a chronic small bowel enteropathy in children and adults living in situations of poor sanitation (Baker and Mathan, 1972, Lindenbaum et al., 1966b, Cook and Lee, 1966, Schneider and Viteri, 1972). The commonest causes of chronic small bowel enteropathy in the UK are post enteritis syndrome and food allergy (cow's milk or soya protein sensitive enteropathy), both of which have been linked to changes in dual sugar permeability. There are no studies in the published literature that have investigated the role of different food types at weaning on the development of subsequent small bowel enteropathy in the tropics.
Food intolerance tends to develop in the first few months of infancy (Sicherer et al., 1998, Goldman and Proujansky, 1986), often in association with an acute gastroenteritis, producing the post enteritis syndrome. The commonest dietary protein to develop a hypersensitivity reaction to (in the UK) is cow’s milk protein. Treatment is with dietary exclusion until tolerance is re-established. It has been reported in earlier Keneba studies that cow’s milk is an infrequent part of the diet in rural Gambia (Hudson, 1992). However, since these earlier studies the International Trypanotolerance Co-ordination (ITC) has kept a large herd of cattle around Keneba and now milk is sold cheaply in the village. It is not known how frequently unmodified cow’s milk protein is included in the weaning diet of Keneba children since the arrival of the ITC cattle herd. If significant numbers of children are taking unmodified cow’s milk as part of the weaning diet this may then represent a risk factor for post-enteritis cow’s milk protein intolerance. This enteropathy may potentially be detected by an increase in the L:M permeability ratio in those children who take cow’s milk at a young age (when the risk of developing an enteropathy is highest).

The second commonest cause of acquired chronic enteropathy in the UK is likely to be infection. Previous Keneba studies have indicated that *Giardia lamblia* is commonly isolated from faeces of children from tropical environments (Gupta, 1980, Farthing, 1993). It is estimated that 45% of children with persistent diarrhoea and malnutrition were also infected with *Giardia lamblia* (Sullivan et al., 1991a). However, the prevalence of *Giardia* infection in Keneba is unknown. The impact of *Giardia* upon growth has recently been examined by Lunn et al (Lunn et al., 1999 in press) using serology as a diagnostic tool, but the effect of *Giardia* on growth and gut function has not been confirmed by faecal microscopy which is generally regarded as the gold standard.
Since the early bacteriological studies in Keneba (Rowland and McCollum, 1977) Cryptosporidium parvum has been discovered to be an enteric pathogen and identified as the causative agent of 9% of the cases of diarrhoea in the peri-urban area of Bakau and Fajara in The Gambia (Adegbola et al., 1994). The climatic and socio-economic environment of these peri-urban areas are very different to Keneba, and there are no studies that have examined the prevalence of Cryptosporidium in a rural part of The Gambia.

It has been 22 years since a study has been published reporting the incidence of viral gastro-intestinal infection. As the frequency of acute diarrhoeal disease has fallen since then, there is a need to assess whether viral gut infections in Keneba are related to small bowel function.

The aims of the first part of this study were two fold. Firstly to ascertain the frequency of each of the common food types in the weaning diet, with particular reference to cow’s milk in the Keneba cohort. Secondly, at 6 months of age to compare intestinal permeability, plasma biochemical profile and nutritional status in children exposed to cow’s milk protein with those unexposed to cow’s milk protein. Section 4.1 will examine the dietary associations with growth and enteropathy, section 4.2 will investigate the association between gut pathogens and enteropathy. These two issues are presented in two separate sections.

4.1.2 Methodology

4.1.2.1 Clinical and laboratory methodology

Subject details, recruitment and outline of study procedure, see section 2.1 under Keneba cohort.

Dietary survey see 2.4.1.
4.1.2.2 Statistical Methods

Children who were still exclusively breast fed at 3 months of age were defined as late weaners and those who started before 3 months of age were defined as early weaners. Weaning was defined as the consumption of any other food type (excluding water) in addition to breast milk. Comparison of median values for intestinal permeability, nutritional status and biochemical profile between those children who weaned early and those that weaned late was made at the 24 week clinic visit (Mann Whitney-U test).

4.1.3 Results

4.1.3.1 Age of onset of weaning (see table 4.1)

20 children joined the study just under 8 weeks of age, of these 12 (60 %) were exclusively breast fed, the remaining 8 (40 %) were taking additional water, 5 (25%) were taking weaning foods.

Forty-four children with a complete dietary record were followed between 2-3 months of age. Of these 36 (80 %) were taking something other than breast milk, 14 (32 %) had taken water on at least one occasion and milk (both fresh and sour) was taken regularly (at least once a week) by 16 (36 %) children, whilst semi-solid weaning foods were taken less frequently (12 children, 28 %) and comprised of egg, maize and findo.
It therefore appears that approximately 40% of children were weaned by 8 weeks and 80% by 3 months of age. Water was given first before weaning foods and egg was given as a preferential source of dietary protein in these very young children.

4.1.3.2 Dietary Food types

See table 4.1

At 3-4 months all of the 64 children with a complete dietary record were breast fed. Fifty-seven (80%) were taking weaning foods and 26 were regularly taking water. Fruits were never eaten even in the early rainy season (June-July) when there were abundant supplies of mango and later oranges. Fish was rarely added to the weaning foods which appeared to be made up of a variety of cereal bases: Millet; maize; findo; wheat flour and rice.

From 6 months onwards the diet becomes somewhat more varied although all of the 64 children were still taking breast milk. Rice and maize was consumed more frequently but with similar proportions of other staple cereals. Fruit still was mentioned in less than 1% of the responses. Fish and adult style food (leaf sauces, bread and doughnuts) were given to the child more often than at 3-4 months.

It therefore seemed that Keneba children still had a monotonous cereal based diet at 6 months of age and vegetables and fruit were rarely eaten at this age. The main cereals eaten by children seem to be maize and millet, rather than rice which is preferentially eaten by adults (McCrae and Paul, 1996a).

By 6 months 20% of children were taking eggs and 60% milk regularly (on a weekly basis). Other dietary components had not substantially changed from previous dietary surveys conducted by Downes et al (Downes et al., 1992), for example children rarely ate fish, groundnuts or meat.
4.1.3.3 Intestinal permeability, acute phase reactants and nutritional status in early and late weaners at 6 months of age.

Table 4.2 summarises the results of L:M permeability ratios, nutritional status and relevant biochemistry at 24 weeks of age for the late and early weaners. 12 children had complete dietary records up to 3 months of age and were exclusively breast fed, 52 were taking weaning foods by this stage. There was a tendency for L:M permeability to be greater in the early weaning group which did not reach statistical significance ($P = 0.2$). Nutritional status was significantly better in the late weaning group of children who were heavier, taller and less wasted than their peers who started weaning sooner. Plasma IgG levels were significantly raised in the late weaning group, but all other plasma proteins, including acute phase reactants were unchanged.

4.1.3.4 Cow’s milk protein permeability, nutritional status and growth at 6 months

Cow’s milk (either fresh or sour) was consumed as part of the weaning diet by 10 children who entered the study at 8 weeks and had a complete dietary record to beyond 3 months of age. Only 5 children with a complete record over the same time period never had any cow’s milk but were taking other weaning foods. These children were not however matched for age of weaning more closely than both those who were weaned on to cow’s milk protein or weaned on to a cow’s milk free diet before 3 months of age.

Children who received no cow’s milk were on average 480g heavier, 1 cm taller and had a greater BMI than those who were feeding on cow’s milk (See table 4.3). Weight was the only parameter that was statistically different ($P < 0.04$). L:M was greater in the group who had an early introduction of cow’s milk protein, both the percentage recoveries of lactulose and mannitol were raised in this group but none reached statistical significance. Of the plasma biochemistry performed (see table 4.4)
immunoglobulin subtypes (IgA and IgM) and acute phase reactants (CRP and ACT) were greater in the early weaning group compared to the late weaning group at 6 months of age. None of these differences were however statistically significant apart from IgM ($P<0.02$), which was higher in the early weaning group.

4.1.4 Discussion

This survey highlights again that Keneba children have a monotonous cereal based diet. When non-cereal products are introduced they are done so infrequently and to few children. The staples of maize and millet are a superior source of protein and micronutrients than rice, with a good protein / energy balance. Some evidence of dietary change has been detected in Keneba reflecting the increased prosperity brought to the area, with cow’s milk forming a more frequent component of the diet which was previously reported as unusual (Prentice, 1992a). Furthermore eggs and wheat flour (in the form of bread or dough nuts) are not uncommon child foods. If this dietary trend is genuine, it may be due to two separate factors. Firstly socio-economic improvements in Keneba and the availability of cheap milk from the large dairy herd based at the International Trypanotolerance Co-ordination (ITC) in Keneba have encouraged a modification of weaning foods. Secondly an earlier Keneba health education programme (Hoare, 1994) encouraged the addition of milk and egg to the mono to increase its’ energy density, which previously was a watery pap low in calories (Hudson et al., 1980).

The chosen methodology, given the sample size, was not suitable for providing a statistically significant answer to the question of whether the early introduction of cow’s milk protein is associated with small bowel enteropathy. The chance of detecting a 0.5 SD difference in L:M ratio between those children weaning early and late, with $\alpha \leq 0.05$ and 80% power ($\beta=80\%$), would require a
sample size of 90 (2 groups of 45). Given that a cohort of 72 yielded unequal group sizes of 10 and 5 in the early and late cow’s milk weaning groups respectively, the overall cohort size would have to be approximately 600 children. The age of weaning is a significant confounder in this study, and children would need to be age-matched more closely than was done in this study. It is therefore not possible to say if early introduction of cow’s milk protein is associated with subsequent enteropathy on the bases of this data. A larger study matching children by age of weaning would be needed to meet the study aims set out in 4.1.1.

The early introduction of weaning foods may be an epiphenomenum, as the child that already has established growth faltering, would potentially be given early weaning foods in an attempt to improve nutrition.

4.1.5 Conclusion

Early weaning (under 3 months of age) in the unhygienic setting of Keneba village was associated with a poorer nutritional status at 6 months of age. The early introduction of unmodified cow’s milk may be associated with enteropathy and evidence of systemic inflammation and poorer nutritional status, but a larger study, where children are matched for age of weaning would be needed to confirm these findings.

Some changes in the diet in infancy including cow’s milk have occurred since earlier surveys and now appear to have become a more important part of the infant diet in Keneba than previously reported.
Table 4.1

Mean number of occasions that each food type is taken (as monos or otherwise) by age group on at least one occasion per week (%)

<table>
<thead>
<tr>
<th>Age (mo)</th>
<th>n</th>
<th>Milk (fresh or sour)</th>
<th>Millet</th>
<th>Findo</th>
<th>Maize</th>
<th>wheat flou</th>
<th>Groundnuts</th>
<th>Rice</th>
<th>Sorghum</th>
<th>Egg</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3</td>
<td>44</td>
<td>16 (36)</td>
<td>3 (6.8)</td>
<td>1 (2.2)</td>
<td>1 (2.2)</td>
<td>1 (2.2)</td>
<td>2 (4.4)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>7 (16.9)</td>
<td>nil</td>
</tr>
<tr>
<td>3-4</td>
<td>64</td>
<td>28 (51)</td>
<td>6 (11)</td>
<td>1 (2.2)</td>
<td>3 (6)</td>
<td>3 (6)</td>
<td>4 (7.2)</td>
<td>1 (2.2)</td>
<td>2 (3)</td>
<td>13 (23.6)</td>
<td>Veg oil 3 (6.8)</td>
</tr>
<tr>
<td>4-6</td>
<td>66</td>
<td>40 (58.9)</td>
<td>17 (26)</td>
<td>3 (4)</td>
<td>13 (20)</td>
<td>8 (12)</td>
<td>4 (6)</td>
<td>7 (11)</td>
<td>5 (8)</td>
<td>13 (19.5)</td>
<td>fruit 13, fish 40</td>
</tr>
<tr>
<td>6-10</td>
<td>66</td>
<td>64 (97)</td>
<td>17 (25)</td>
<td>10 (15)</td>
<td>46 (69.7)</td>
<td>22 (33)</td>
<td>9 (14)</td>
<td>15 (22)</td>
<td>42 (63)</td>
<td>7 (11)</td>
<td></td>
</tr>
<tr>
<td>10-15</td>
<td>39</td>
<td>39 (100)</td>
<td>5 (12.8)</td>
<td>14 (36)</td>
<td>37 (93)</td>
<td>36 (90)</td>
<td>15 (38)</td>
<td>23 (58)</td>
<td>36 (90)</td>
<td>39 (100)</td>
<td>Fruit, fish, oils and a</td>
</tr>
</tbody>
</table>

Veg and palm oil, vegetable oils and a few other foods.
Table 4.2
Permeability and plasma IgG levels at 6 months of age in those children who started weaning before and after 3 months of age.
Means and standard deviation shown

<table>
<thead>
<tr>
<th>Time of weaning</th>
<th>L/M ratio (n)</th>
<th>IgG (g/L)</th>
<th>zwt</th>
<th>zht</th>
<th>zBMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early</td>
<td>0.25 (52)</td>
<td>5.9 (1.3)*</td>
<td>-1.4</td>
<td>-1.24</td>
<td>-0.98</td>
</tr>
<tr>
<td></td>
<td>(0.13)</td>
<td>(1.03)**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Late</td>
<td>0.21 (12)</td>
<td>7.05 (1.7)</td>
<td>-0.3</td>
<td>-0.50</td>
<td>-0.04</td>
</tr>
<tr>
<td></td>
<td>(0.22)</td>
<td>(1.05)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

t-test for difference between early and late weaning groups.
* p=0.05
** p=0.005
Ψ p=0.02
Table 4.3

Nutritional status at 6 months of age in children weaned on to cow’s milk before and after 3 months of age

Medians with range shown

<table>
<thead>
<tr>
<th>Age at weaning on to cow’s milk</th>
<th>Weight (kg)</th>
<th>Height (m)</th>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 3 months</td>
<td>7.17¹</td>
<td>0.64</td>
<td>17.6</td>
</tr>
<tr>
<td>n=5</td>
<td>(6.9-7.6)</td>
<td>(0.6-0.7)</td>
<td>(15.9-16.2)</td>
</tr>
<tr>
<td>&lt; 3 months</td>
<td>6.69</td>
<td>0.63</td>
<td>16.27</td>
</tr>
<tr>
<td>n=10</td>
<td>(5.7-7.5)</td>
<td>(0.6-6.7)</td>
<td>(15.3-18.7)</td>
</tr>
</tbody>
</table>

¹ Mann Whitney U test P = 0.04
Table 4.4

Intestinal permeability, acute phase reactants and immunoglobulins in children weaned on to cow’s milk protein before and after 3 months of age. Medians with range shown.

<table>
<thead>
<tr>
<th>Group</th>
<th>L:M</th>
<th>% recovery of lactulose</th>
<th>% recovery of mannitol</th>
<th>IgG (g/L)</th>
<th>IgA (g/L)</th>
<th>IgM (g/L)</th>
<th>CRP (mg/L)</th>
<th>ACT (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 3 months</td>
<td>0.17</td>
<td>0.08</td>
<td>2.21</td>
<td>6.36</td>
<td>0.30</td>
<td>0.54</td>
<td>1.43</td>
<td>0.29</td>
</tr>
<tr>
<td>n=5</td>
<td>(0.08-0.36)</td>
<td>(0.01-0.2)</td>
<td>(0.8-3.1)</td>
<td>(2.7-8.2)</td>
<td>(0.2-0.7)</td>
<td>(0.2-0.9)</td>
<td>(0.3-49.5)</td>
<td>(0.2-0.6)</td>
</tr>
<tr>
<td>&lt; 3 months</td>
<td>0.2</td>
<td>0.1</td>
<td>2.48</td>
<td>5.8</td>
<td>0.41</td>
<td>0.88</td>
<td>4.25</td>
<td>0.41</td>
</tr>
<tr>
<td>n=10</td>
<td>(0.02-0.3)</td>
<td>(0.007-0.4)</td>
<td>(1.3-4.8)</td>
<td>(4.6-11.7)</td>
<td>(0.2-1.1)</td>
<td>(0.7-1.9)</td>
<td>(0.2-15.4)</td>
<td>(0.2-0.6)</td>
</tr>
</tbody>
</table>

*Mann Whitney U P < 0.02, all other comparisons P>0.05*
Causes of Chronic Enteropathy

Section 4.2

Parasites and viruses associated with chronic enteropathy

4.2.1 Background and aims

In the previous section of this chapter we demonstrated that early weaning was associated with a tendency for intestinal permeability to be higher and nutritional status to be worse compared to children who weaned after 3 months of age. Unmodified cow's milk taken before 3 months may be implicated in causing enteropathy either through an allergic mechanism, or unidentified infective organisms present in milk. In this chapter other, infections associated with chronic enteropathy will be examined.

Histopathological case reports of small bowel mucosal biopsies collected in the tropics have shown common histopathological abnormalities (Baker and Mathan, 1972, Schneider and Viteri, 1972, Sullivan et al., 1991d, Gracey, 1996, Klipstein et al., 1968, Garcia, 1968), but the clinical significance (in terms of nutritional status) and aetiology of these changes is uncertain and have not been fully investigated. In any unhygeinic environment the infant is frequently challenged with infection (Ahmed et al., 1993, Vis and Brasseur, 1992), and other environmental contaminants, both of which could be linked to the development of enteropathy.

Previous Keneba studies have conducted bacterial screening of village children, and these have are infrequently isolated infective agents from stool cultures, despite the presence of large numbers of microbial contaminants in the environment and in the food (Barrell and Kolley, 1982, Rowland et al., 1978). It is now known that
diarrhoeogenic *E. coli* types are involved in acute gastrointestinal infections and are linked to enteropathy (Sullivan et al., 1994, Kakai et al., 1995, Khalil et al., 1993). As the diarrhoeogenic *E. coli* types have only recently been isolated, there is no data available as to how prevalent chronic carriage with these organisms is. The identification of this important subgroup of gut pathogens requires specific gene probes and there were not the resources to undertake these investigations during this study.

Viral infections were found to be very common in a cross-sectional study performed by Rowland and Barrell (Rowland and Barrell, 1978), occurring in about 98% of Keneba residents screened. However, there was no difference between those with and without diarrhoea. It is not known whether chronic carriage of viruses in Keneba is associated with enteropathy. It is also not known whether there has been a change in the prevalence of viral infection to accompany the other changes that have been noted in the previous chapters (ie decreasing diarrhoea prevalence, improvements in small bowel permeability and changes in dietary practices).

*Giardia lamblia* infection is found worldwide, but more commonly so in developing countries (Farthing et al., 1986a). *Giardiasis* has been associated with malabsorption, small bowel mucosal damage and decrease in brush border disaccharidase activity (Chavez et al., 1995, Farthing, 1993, Katelaris et al., 1995), and therefore is a potential candidate for producing enteropathy. However, in a large cohort study in Guatemala (Farthing et al., 1986b) there was no effect of *Giardia* infection upon growth until the second year of life. *Giardia* infection also appears to have variable effects upon the mucosal structure. In one paediatric endoscopic case series there were a range of abnormalities reported, including normal mucosal morphology in up to 45% of children (Hjelt et al., 1993). *Giardiasis* is the commonest intestinal parasite infection in Keneba (Keneba clinic data base), but its’ prevalence is unknown in children under
15 months of age. Lunn et al (Lunn et al., 1999) has demonstrated from Giardia serology that a rise in IgM titre is associated with short-term growth faltering, but not with long-term growth faltering. It is widely accepted however that the gold standard for diagnosis of Giardia infection is stool microscopy with the aid of specific immunofluorescent antibodies (Mayer and Palmer, 1996, Winiecka Krusnell and Linder, 1995). There have not been any studies in rural Gambia that have examined the role of Giardiasis, as assessed by stool microscopy, on intestinal function and growth. 

Cryptosporidium parvum was reported as a causative agent of acute gastroenteritis in 1976 (Brunser et al., 1997), and subsequently has been associated with chronic diarrhea. A study in the periurban area of The Gambia demonstrated that approximately 9% of cases of acute watery diarrhea and 3% of control children had Cryptosporidium parvum particles in the stool (Adegbola et al., 1994). There are no studies defining the prevalence of Cryptosporidium in rural parts of The Gambia, nor how infection relates to intestinal permeability and growth.

The aim of this study was to examine the subject diarrhea prevalence, growth and intestinal permeability in children with the following gastro-intestinal infections:

1 Viruses

2. Giardia lamblia

3. Cryptosporidium parvum

4.2.2 Methods and subject details

Subject recruitment and details, see section 2.1.3

Diarrhoea morbidity see section 2.4.2.

Anthropometry 2.5.2

L:M permeability test 2.5.2 and laboratory methods.

Stool collection 2.5.4, microscopy 2.6.3.1.
Faecal electron microscopy for virus particles 2.2.3.5.
Lactulose and mannitol assays, see section 2.6.1.
Plasma protein estimation, 2.6.2.
Faecal microscopy for C. parvum 2.6.3.3
Giardia immunofluorescence 2.6.3.2

Giardia-specific IgM immunofluorescence 2.6.2.3

A cross-sectional survey of 84 randomly selected samples across the age range of the study cohort, including wet and dry season were taken as an initial screen to assess the mean prevalence of gastrointestinal viral infection.

A case of Giardiasis was defined on the basis of finding a Giardia part in the stool under conventional or immunofluorescent microscopy. The stool samples underwent multiple microscopic examination, and any discrepancies in results were settled in the following ways. Immunofluorescent microscopy with specific anti-Giardia antibodies was used as the final reference standard. In the case where immunofluorescence was not performed and there was a discrepancy in results between two lab technicians, then a third faecal smear for light microscopic examination was performed by a third technician. This was taken as the final verdict.

For the estimation of the prevalence of Giardia infection by immunofluorescent microscopy, an even spread of samples were selected across the calendar year. Once arranged in to month of sample collection a random selection of samples were collected, from 59 subjects.

4.2.3 Statistical Analysis

Long-term growth was compared between cases and controls using a similar method to 3.2.3. The weight and height for age z-scores were significantly greater at enrolment in those children who went on to become infected with Giardia. To remove any bias that
this might create a correction using the method of Wright et al was used (Wright, 1995). This involves calculating a “thrive index” for each child. Firstly a predicted final height or weight z-score is calculated by multiplying the early (8 week) z-score by the regression coefficient (B) of the regression line of weight (or height) z-score against age, this was repeated for each child. The difference between the final observed and predicted z-scores is denoted the thrive index (TI).

A similar technique for analysis of short-term growth to chapter 3.2.2 was used, but normal growth free of *Giardia* infection was derived from those children who had a full compliment of stool examinations and were consistently free from *Giardia* infestation. The decline in weight and height standard deviation score across the same age bands for the children infected with *Giardia* was subtracted from the normative (*Giardia* free) data. Each individual time band was treated as independent of surrounding time bands. The null hypothesis was that there was no difference in growth between cases and controls, i.e. the difference between expected and observed growth should be approximately 0. The alternative hypothesis, (H₁) would be accepted if the difference between observed and expected growth ≠0 (H₀=0, H₁≠0). The mean was regarded as being not significantly greater than 0 if the 95 % confidence interval crossed 0.

Comparison of gut permeability between those children infected and those children uninfected with *Giardia*, was assessed using the mean L:M, % recoveries of both lactulose and mannitol at 24, 36, 44 and 52 weeks (mean point estimate).

4.2.4 Results

4.2.4.1 Intestinal viral infection
The initial screen of 84 samples (from 40 subjects) yielded an estimated frequency of viral isolates of 18%, lower than that reported by Rowland et al (94%). Table 4.5 lists the isolates. There was no *Rota virus* and only 5 out of the 15 isolates (6%) are considered to be gut pathogens. *Enterovirus* was the most common isolate and at the age groups studied it was not possible using electron microscopy to differentiate between polio vaccine and wild type *Enterovirus*. In the UK a viral infection as a cause of acute gastroenteritis in infancy is approximately 40% (Walker-Smith and Murch, 1999b), with enteroviruses isolation being extremely rare, even in children who have recently been vaccinated with live attenuated polio. In a French hospital based survey, “pathogenic” viruses were found in 20% of well control children (Puel et al., 1983).
Table 4.5

Viruses isolated on faecal electron microscopy

<table>
<thead>
<tr>
<th>Virus type</th>
<th>Frequency isolated (%</th>
<th>Number of particles per × 30,000 grid (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enterovirus</strong></td>
<td>9</td>
<td>From 1-5 up to 10-15</td>
</tr>
<tr>
<td></td>
<td>(11.0)</td>
<td></td>
</tr>
<tr>
<td><strong>small round structured virus</strong></td>
<td>4</td>
<td>5-10</td>
</tr>
<tr>
<td></td>
<td>(4.8)</td>
<td></td>
</tr>
<tr>
<td><strong>Microsporidium</strong></td>
<td>1</td>
<td>1-5</td>
</tr>
<tr>
<td></td>
<td>(1.2)</td>
<td></td>
</tr>
<tr>
<td><strong>Bacteriophage</strong></td>
<td>1</td>
<td>1-5</td>
</tr>
<tr>
<td></td>
<td>(1.2)</td>
<td></td>
</tr>
</tbody>
</table>

* Considered to be pathogenic

It therefore appears that the viral carriage rate in the Keneba cohort was extremely low, especially for pathogenic viral species, and therefore unlikely to be a uncommon cause of chronic enteropathy. Therefore, time consuming faecal electron microscopy was not carried out on the rest of the cohort.

4.2.4.2 *Giardia lamblia* intestinal infection

See Appendix 5 for photomicrograph of immunofluorescence of faecal smear with *Giardia* cysts.
A total of 496 stool specimens were collected 313 were studied under immunofluorescent microscopy and 396 under conventional microscopy. One hundred and sixty-nine samples were studied by both techniques and from this sub group it was estimated that the sensitivity of the conventional microscopy was 77% with a specificity of 90.4%.

On average 5-6 stool samples were collected over the study period giving a mean time period between stool samples of 7 weeks, of the 73 children recruited 2 withdrew as the mother left Keneba. Eleven children did not provide stool specimens within 48 hours for one of the study clinics and were therefore excluded from the analysis. Thirty-eight children consistently had negative stool samples at every clinic visit. Nineteen children were infected with *Giardia* at a median age of 9 (range 2-14) months. Of those infected *Giardia* was isolated on just one occasion in the majority, clearing spontaneously without treatment (12/19 or 60%), the remainder (8/19 40%) were persistent and cysts were excreted for a mean duration of 12 weeks (range 6-20 weeks). One child was re-infected after spontaneously clearing the infestation. Only two children met the criteria for treatment (because of diarrhoea) and received a week of metronidazole. One succeeded in clearing the infection and one failed and continued to carry *Giardia* cysts asymptotically.

Infection with *Giardia* occurred all year long with a notable increase in the pre-rainy season, and reached a peak in August-September, at the time of maximal rain fall (see figure 4.2.1).
Figure 4.2.1

Period prevalence of *Giardia* infections by month in Keneba, N=59

![Bar chart showing period prevalence of *Giardia* infections by month in Keneba.]

Table 4.6 shows the difference in the mean subject prevalence of diarrhoea and vomiting in those children who were never infected with *Giardia* compared to those that were. There were no differences in prevalence of either diarrhoea or vomiting in the months before and after *Giardia* infection was confirmed. There is a slight trend...
for the control group to have a slightly higher diarrhoea and vomiting morbidity, but this was not statistically significant.

Table 4.6

Subject prevalence for diarrhoea and vomiting in children with *Giardia* infection (days of diarrhoea, or vomiting, per week in the month before and after isolation of *Giardia*.)

<table>
<thead>
<tr>
<th>Group</th>
<th>mean days with diarrhoea per week</th>
<th>95% Confidence interval</th>
<th>Mean days of vomiting per week</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (never had <em>Giardia</em>)</td>
<td>0.80</td>
<td>0.71 - 0.85</td>
<td>1.62</td>
<td>1.51 - 1.73</td>
</tr>
<tr>
<td>Month before <em>Giardia</em> isolated</td>
<td>0.70</td>
<td>0.31 - 1.07</td>
<td>1.19</td>
<td>0.61 - 1.75</td>
</tr>
<tr>
<td>Month after <em>Giardia</em> isolated</td>
<td>0.70</td>
<td>0.20 - 1.10</td>
<td>1.03</td>
<td>0.32 - 1.73</td>
</tr>
</tbody>
</table>

Mann Whitney U P>0.2 for all comparisons between cases and controls

ii) *Giardia* infection and long-term growth

At enrolment there was a significant difference in both height and weight between those children who subsequently developed *Giardia* infection and those that did not
Mean heights and weights for cases and controls were 5.40 kg (56.4 cm) and 4.98 kg (57.5 cm) respectively (Comparison of weights and heights respectively $P < 0.04$ weight and $P = 0.05$). Comparison of the predicted 52 week weight once the difference in weight at enrolment was corrected (using a predicted height or weight described above in the calculation of the thrive index), showed no difference between groups. Table 4.7 shows the differences in corrected heights and weights between cases and controls at 6, 9, 11 and 12 months of age. There is no significant difference between either group at any age, although children infected with *Giardia* tended to be heavier than controls.
Table 4.7
Difference in corrected weight (kg) and height (cm) between children infected with *Giardia* (by stool isolation) and controls (means and 95% confidence intervals)

<table>
<thead>
<tr>
<th>Age</th>
<th>Cases (infected)</th>
<th>Controls (infection free)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weight</td>
<td>Height</td>
</tr>
<tr>
<td></td>
<td>(kg)</td>
<td>(cm)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 weeks</td>
<td>6.96 (6.5-7.4)</td>
<td>64.0 (20-107)</td>
</tr>
<tr>
<td></td>
<td>[n=18]</td>
<td></td>
</tr>
<tr>
<td>36 weeks</td>
<td>7.30 (6.7-7.7)</td>
<td>67.0 (21-114)</td>
</tr>
<tr>
<td></td>
<td>[16]</td>
<td></td>
</tr>
<tr>
<td>44 weeks</td>
<td>7.70 (7.1-8.1)</td>
<td>70.0 (19-119)</td>
</tr>
<tr>
<td></td>
<td>(19-122)</td>
<td>(7.22-8.08)</td>
</tr>
<tr>
<td>52 weeks</td>
<td>8.14 (7.15-8.5)</td>
<td>71.0</td>
</tr>
</tbody>
</table>

*P > 0.1 (t-test) for all comparisons*

Using the children who never sustained a Giardia infection to form normative data for weight and height gain as discussed in section 4.2.2.3 the mean height and weight standard deviation score (plus 95 % confidence intervals) were:

-0.27 - 0.16 (height, cm)

-0.23 - 0.27 (weight, kg)

As both confidence intervals cross 0 there is no significant difference in mean weight and height gain between those children infected and uninfected with Giardia at any age.

iv] Giardia infection and gut permeability

Mean permeability values with 95 % confidence intervals for cases and controls are given below. There are no differences at any age between children infected or uninfected with Giardia but the numbers are small (n < 10).
Table 4.8

Mean L:M permeability values by age between children infected (cases) and uninfected (controls) with *Giardia lamblia* (by stool microscopy). Means and 95% confidence intervals shown.

<table>
<thead>
<tr>
<th>Permeability</th>
<th>24 weeks controls</th>
<th>36 weeks controls</th>
<th>36 weeks cases</th>
<th>44 weeks controls</th>
<th>44 weeks cases</th>
<th>52 weeks controls</th>
<th>52 weeks cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>L:M</td>
<td>0.24 (-0.07 - 0.53)</td>
<td>0.21 (-0.15 - 0.75)</td>
<td>0.32 (-1.0 - 3.34)</td>
<td>0.44 (-0.1 - 0.89)</td>
<td>0.35 (-0.56 - 1.28)</td>
<td>0.54 (-0.1 - 0.89)</td>
<td>0.40 (-0.36 - 1.16)</td>
</tr>
<tr>
<td>% recovery of lactulose</td>
<td>0.19 (-0.11 - 0.48)</td>
<td>0.17 (-0.04 - 0.58)</td>
<td>0.27 (-1.10 - 1.50)</td>
<td>0.15 (-0.26 - 0.64)</td>
<td>0.18 (-0.66 - 1.21)</td>
<td>0.32 (-0.23 - 0.89)</td>
<td>0.32 (-0.44 - 1.08)</td>
</tr>
<tr>
<td>recovery of mannitol</td>
<td>2.77 (2.47 - 3.07)</td>
<td>2.09 (2.49 - 4.64)</td>
<td>2.50 (2.19 - 2.81)</td>
<td>2.03 (-1.0 - 1.64)</td>
<td>1.94 (1.48 - 2.4)</td>
<td>2.95 (2.02 - 3.89)</td>
<td>2.71 (2.14 - 3.26)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P > 0.2* (t-test) for all comparisons between cases and controls
v] *Giardia* infection and *Giardia* specific IgM

The previous cut-off used to define an infection was given by Lunn et al (Lunn et al., 1999), who defined recent *Giardia* infection on the basis of the 8 week mean *Giardia* IgM ELISA optical density reading (equivalent to an optical density reading of 150 units). The rationale for using this cut off is that at 8 weeks of age few children are taking weaning foods sufficiently often to make them likely to contract a *Giardia* infection.

Figure 4.2.2 is a cumulative frequency curve of the proportion of children who have demonstrated on at least one occasion a positive IgM titre against *Giardia*. The frequency rises sharply over the first 6 months so that 96% of children have serological evidence of past infection by 9 months of age.

**Figure 4.2.2**

*Cumulative frequency curve of age of child to first show positive Giardia IgM*
There were no significant differences in the mean *Giardia*-specific IgM titres over the study period in those children who had a positive stool microscopy (250 ± 116 OD units) and those who were consistently negative at 52 weeks (222 ± 102 OD units). At 24, 36, 44 and 52 weeks of age there was also no difference in the mean *Giardia*-specific IgM titres between cases and controls, as defined by faecal microscopy. There is a rise in the *Giardia*-specific IgM titres from baseline in both cases and controls (by stool microscopy). Figure 4.2.3 shows that there are clear points of seroconversion in controls which are never detected as *Giardia* parts by stool microscopy. *Giardia*-specific IgM showed a mean rise of 132 (SD 63.8) OD units before or after a positive stool microscopy in all but 2 of the 19 children with faecal cysts.
Figure 4.2.3 Five Keneba children who consistently were negative on stool microscopy for *Giardia* cysts.
A rise in *Giardia* IgM titre of greater than 50u above 150 u (equivalent to 1 SD above the 8 week, or unexposed, baseline) was taken as evidence of re-infection. Having identified the time point of infection on the basis of this criteria, weight gains the month before and after this time point were compared in each subject. Between 2 and 9 months of age the median number of such events was 2 per child. Table 4.9 shows that there were no significant differences in rates of height and weight gain before and after a point of infection, as diagnosed by serology.
Table 4.9

Weight and height gains per month before and after serological evidence of infection with *Giardia* by specific IgM.

Means and 95% CI shown in parenthesis (n=60)

<table>
<thead>
<tr>
<th></th>
<th>Weight gain (kg/month)</th>
<th>Height gain (cm/month)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Month before</td>
<td>0.29</td>
<td>1.66</td>
</tr>
<tr>
<td></td>
<td>(0.21-0.31)</td>
<td>(1.44-1.94)</td>
</tr>
<tr>
<td>Month after</td>
<td>0.31</td>
<td>1.54</td>
</tr>
<tr>
<td></td>
<td>(0.29-0.38)</td>
<td>(1.28-1.80)</td>
</tr>
</tbody>
</table>

*P > 0.5* (t-test) for comparisons of both weight and height

4.2.4.3 *Cryptosporidium parvum* intestinal infection

Only 3 cases out of the cohort were infected with *C parvum* and all were transitory and did not cause chronic diarrhoea (less than 2 weeks duration). 500 stool specimens were screened for *C parvum* infection.

4.2.5 Discussion

This study has confirmed that children are frequently challenged with faecal-oral infections. Other agents not studied in this section could potentially cause enteropathy and be transmitted by this route e.g. *Helicobacter pylori* (Thomas et al., 1999, Thomas and Pretolani, 1994) and diarrhoeogenic *E coli*.

The previous survey on enteric viral infections by Rowland and Barrell (Rowland et al., 1978) estimated a 94% prevalence of enteric viral infection in both children with and with out diarrhoea. This present study estimated the prevalence of viral infections
across both wet and dry seasons and found it to be much lower (18% compared to 94%). The earlier study by Rowland and Barrell (Rowland et al., 1985, Rowland et al., 1978) showed a complete absence of *Rota virus*, and the most frequent viral isolate being *Enterovirus*. Both these findings were replicated in this present study. Wild *Enterovirus* species are indistinguishable from polio vaccine strains, which are administered 4 times in the first year of life to Gambian children. If 6% is considered as the final prevalence rate of pathogenic viral strains, then viral infection is unlikely to be a major aetiological agent in the generation of chronic enteropathy. However, if the chronicity of the enteropathy is due to a post infectious food intolerance, then it is possible to speculate that viral infections initiated the enteropathy during the first year of life.

*Giardia* infection is frequent and recurrent in Keneba children, but seems to produce a very different spectrum of disease to that seen in the tropical traveller. It seems that detection of *Giardia* cysts in the stool was not associated with bouts of diarrhoea and vomiting. In the majority of cases, carriage of *Giardia* cysts cleared spontaneously. There was a non-significant trend to gain less weight in the month before infection, but there was evidence of catch up in the month after. The final result is that there was no long-term weight or height deficit. Microscopy seemed to be less sensitive a tool at diagnosing infection. With most children getting infected (diagnosis by serology) 1-2 times in the first 9 months of life, it is arguably, somewhat meaningless to classify a child as infection free if his/her stool was free of *Giardia* cysts at any particular point in time. In a community where growth faltering is so pervasive it cannot be said that *Giardia* infection is of no consequence to long-term growth until an intervention trial has been performed. An intervention study in Guatemala showed that eradication of *Giardia* in children aged between 2-5 years, resulted in only a small (1 cm) benefit in height gain (Gupta, 1980). The eradication of *Giardia* resulting in a modest
improvement in linear growth has been also demonstrated in Australia (Kay et al., 1977). No such intervention studies have been performed in The Gambia.

*Cryptosporidium parvum* infection is much more prevalent in other parts of West Africa, and even parts of the developed world (Pettoello Mantovani et al., 1995, Rodriguez-Hernandez et al., 1996), than it appears to be in The Gambia. The low prevalence of *C. parvum* infection suggests it is unlikely to be an important cause of enteropathy, unless, like viruses, it can initiate a post-enteritis-like enteropathy through some kind of food intolerance.

This study has not investigated the role of bacterial infections in causing chronic enteropathy in Gambian children. Potentially, they along with the more infrequent infections (ie *C. parvum*), all contribute to the defective gut function. Perhaps repeated transitory infection, as is seen with *Giardia lamblia*, may act as an initiating event for a post-enteritis food intolerance.

4.2.6 Conclusion

The incidence of the infectious agents studied is much lower than the incidence of enteropathy, with the exception of *Giardia lamblia*. Enteric infections start early and could initiate a chronic post-enteritis enteropathy, but no one single infective agent can explain the range of growth seen in this study.
Chapter 5.0

Gut barrier function and it’s relationship to growth, systemic inflammation and small bowel enteropathy

In chapter 3 it was estimated that approximately 22% of height or weight growth could be predicted by the L:M permeability test. In this chapter further specific evidence will be sought that impaired gut barrier function (ability to exclude immunogenic antigen) is implicated in growth faltering and activating systemic inflammatory mechanisms.

5.1 Background and aims
Small bowel enteropathy as measured by the dual sugar permeability test is responsible for a significant proportion of poor growth that is observed in the rural Gambia. It has been proposed that the two component sugars used in the L:M test represent different aspects of small bowel function (see 1.3 theory of L:M test). Lactulose being too large a molecule to pass across intact mucosa is only absorbed in small quantities (< 0.1% of the oral dose) unless the mucosa is diseased. Mannitol absorption and urinary excretion in The Gambia is fairly constant at 2.5-3% of the oral dose, and does not vary with age (after 3 months). Therefore, the high L:M ratio is explained by the increasing percentage of the oral dose of lactulose recovered in the urine, suggesting an impairment in mucosal barrier function. However, the claim that lactulose uptake represents impaired gut barrier function is disputed (Weaver and Coombs, 1988), and it is suggested that luminal macromolecules are taken up in very different ways to lactulose.
The lumen of the gastrointestinal tract acts as a major body store for bacterial lipopolysaccharide such as endotoxin. Therefore, in the otherwise healthy child, plasma endotoxin is most likely to have its’ origin in the gastrointestinal tract. Endotoxin can directly stimulate macrophages and T-lymphocytes in to proinflammatory activity, leading to a systemic acute phase response. Severe endotoxaemia can lead to multi-organ failure through intense and widespread activation of immune cells, with resulting massive proinflammatory cytokine production. It has been shown that children in the tropics suffer from frequent illnesses, and often immunoglobulins and acute phase reactants are elevated. It is not known if impaired gut barrier function leads to chronic low level endotoxaemia, which could explain the observed acute phase response, and in turn could explain growth faltering.

Malaria transmission is seasonal in The Gambia, with peak transmission occurring in October, just after the rains have fallen. Malaria parasitaemia is known to activate the acute phase response, but it is not known to what extent malaria parasitaemia contributes to systemic inflammation in Gambian children.

The aims of this chapter were to test three interrelated hypotheses linking impaired gut barrier function to growth faltering:

1. That impaired gut barrier function (permeability) leads to the systemic absorption of endotoxin (which can be measured directly, or by a specific antibody response).
2. That plasma endotoxin and endotoxin antibodies are significantly associated with an acute phase and systemic immunoglobulin response.
3. That these markers of systemic inflammation (immunoglobulin sub-classes, c-reactive protein and ACT) are associated with growth impairment.
A secondary aim is to measure the contribution of malaria parasites to the observed acute phase response.

5.2 Methods

5.2.1 Clinical and laboratory methodology

In brief, children in the Keneba longitudinal cohort study (see 2.1) had growth, intestinal permeability and plasma acute phase reactants, immunoglobulins and endotoxin and endotoxin core antibodies checked every 7 weeks. Growth over the study period was related to summary measures of each of these biochemical variables. For more details see the appropriate sections in chapter 2.0.

Subjects 2.1

Lactulose-mannitol permeability 2.5.5

Anthropometry 2.5.2

Biochemistry (immunoglobulins, CRP, ACT, endotoxin and endotoxin antibody) 2.6

Full Blood counts and malaria thick films 2.6.2.4

5.2.2 Statistical Methodology

Long-term growth is defined in 4.2.3 as is the methodology for age correction of height and weight.

5.3 Results

5.3.1 Profile of systemic markers of inflammation

5.3.1.1 Haematological markers

A mechanical fault in the coulter counter meant that only 80 (out of a potential 210) blood counts were analysed. The results are given in table 5.1. Mean haemoglobin is below the UK normal range (>11.5 g/dl) with a low mean corpuscular volume of 71 fl
(sd 8.9) (UK normal range 77-95), indicating a potential iron deficiency state. The total white cell count was within the UK normal range but with a mean lymphocyte count 2 fold higher. Two thirds of children have a total white cell count > 11.5 × 10^9/l indicating systemic inflammation and acute phase response.

Platelet counts were also elevated with the mean lying outside the UK normal range, and showed levels that are often observed in inflammatory conditions such as Crohn’s disease or ulcerative colitis.
Table 5.1 Haematological profile of Keneba children under 15 months of age n=80
Means and standard deviations given in brackets

<table>
<thead>
<tr>
<th>Haemoglobin</th>
<th>Total white cell count</th>
<th>% Granulocytes</th>
<th>% Lymphocytes</th>
<th>Platelet count</th>
</tr>
</thead>
<tbody>
<tr>
<td>(g/dl)</td>
<td>(x10^9/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.4</td>
<td>10.9</td>
<td>32</td>
<td>60</td>
<td>507</td>
</tr>
<tr>
<td>(1.1)</td>
<td>(3.5)</td>
<td>(14.5)</td>
<td>(16.2)</td>
<td>(155)</td>
</tr>
</tbody>
</table>

Normal ranges: Haemoglobin 11.5-15.5 g/dl; total wcc 6.0-11.5 x10^9/l; granulocyte % 54-62; lymphocytes % 25-33 %; platelet count 150-400 x10^9/l.
5.3.1.2 Plasma biochemistry

Mean CRP, ACT and immunoglobulin subclasses are summarised in table 5.2 below.

<p>| Table 5.2 Biochemical markers of systemic inflammation |
|---------------------------------------------|-----------------------------------------------|-------------------------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>95% confidence interval</th>
<th>UK normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin g/l</td>
<td>35.2</td>
<td>34.9 - 35.4</td>
<td>39-42 g/l</td>
</tr>
<tr>
<td>CRP mg/l</td>
<td>5.4</td>
<td>5.2 - 5.7</td>
<td>&lt; 5 mg/l</td>
</tr>
<tr>
<td>ACT mg/l</td>
<td>0.33</td>
<td>0.11 - 0.56</td>
<td>&lt;0.1 mg/l</td>
</tr>
<tr>
<td>IgG g/l</td>
<td>7.1</td>
<td>6.8 - 7.3</td>
<td>Age dependant see below</td>
</tr>
<tr>
<td>IgA g/l</td>
<td>0.48</td>
<td>0.26 - 0.70</td>
<td>Age dependant see below</td>
</tr>
<tr>
<td>IgM g/l</td>
<td>0.82</td>
<td>0.60 - 1.04</td>
<td>Age dependant see below</td>
</tr>
</tbody>
</table>
CRP was > 5 mg/l for 25 % of the time (95% CI 4 - 48%) and >10 mg/l for 17 % (95 % CI -4 - 39%) of the time. ACT is an acute phase reactant with a long half life of approximately 4 days. The mean levels obtained in this study were approximately 50% lower than those obtained in previous Keneba studies.

Albumin levels were significantly below UK normal range, with complete separation of 95% confidence limits from the UK normal range.

Immunoglobulin levels were highly age dependant in the UK, with IgA and IgM mean levels reaching adult levels by about 6 and 2 years respectively. IgG levels decline in the first 6 months of life until the rate of synthesis is greater than the rate of decline, usually about 12 months of age, and adult levels are reached at approximately 12 years of age. Gambian children, however, did not demonstrate any decline in IgG titres, figure 5.1 shows the trend in mean IgG levels in Keneba children compared to UK children. Comparing the Gambian and U.K. mean levels by constructing a 95% confidence interval for the Keneba values shows the difference between the two is highly significant \(P < 0.008\) at each time point shown in figure 5.1. Although the mean Gambian IgG levels were significantly greater than U.K. mean levels, the U.K. range is very wide, so that Gambian mean levels were always situated in the upper quartile of the UK range.

5.3.2 Plasma endotoxin and endotoxin core antibody levels

The variance in endotoxin antigen levels is high at all ages and no clear age or seasonal trend is noted \(P > 0.3\). Endotoxin antigen level is positively correlated with endotoxin antibody titres \(r = 0.32, P < 0.01\) by regression analysis.

Endotoxin IgG levels fall in the first 3 months of life (fig 5.2), then progressively rise (ANOVA \(P < 0.0001\)) above the levels reported in acutely unwell malnourished UK
adults with malignancy, (range 59-158 MU/ml) (Welsh et al., 1998). Endotoxin antibody levels begin rising and peak in October then slowly fall to a nadir in April.

5.3.2.1 Relationship to growth

Regression analysis of both predicted height and weight gain with mean endotoxin antigen levels over the whole of the study period was negatively correlated (r = -0.3, P < 0.02). Endotoxin antibody levels were more closely associated with height (r = -0.65, P < 0.0001) and weight growth (r = -0.63, P < 0.0001).
Fig 5.1

Changes in mean IgG levels with age in Gambian and UK children

Plasma IgG (g/l)

Age (weeks)

UK IgG mean levels (g/l)
Gambian mean IgG
Fig 5.2

Changes in endotoxin antibody concentration with time

Error bars SEM
5.3.2.2 Relationship to gut permeability

Endotoxin antigen and endotoxin core antibody were significantly related to lactulose recovery ($P < 0.02$ and $P < 0.003 r = 0.36$), but not to mannitol uptake. The L:M permeability was also positively related to endotoxin antibody ($P < 0.008, r = 0.34$, but not endotoxin antigen ($P < 0.7$).

5.3.2.3 Relationship to markers of systemic inflammation

Endotoxin antibody levels are positively correlated with both immunoglobulin subclass levels (IgG, IgA and IgM) (see table 5.4) and endotoxin antigen ($r = 0.33, 0.28$ and $0.2$ respectively) (see table 5.3). CRP levels were also positively correlated with both endotoxin antigen and antibody ($r = 0.20$ for both $P = 0.1$). ACT had no association with endotoxin levels.

5.3.3 Systemic inflammation: associations with growth, intestinal permeability and malaria

5.3.3.1 Systemic inflammation and association with growth

Regression analysis of age corrected height and weight gain against markers of systemic inflammation showed significant associations between all subclasses of immunoglobulins ($r = 0.63, 0.64$ and $0.33$ for IgG, IgA and IgM respectively, $P \leq 0.006$). The association between weight gain and CRP approached significance ($P < 0.06$), but height gain showed no such relationship to CRP ($P = 0.17$). ACT mean levels were not associated with either height or weight gain.

5.3.3.2 Systemic inflammation and gut permeability
Table 5.5 shows the results of regression analysis between immunoglobulins, acute phase reactants and intestinal permeability. The main associations with permeability are immunoglobulins, due mainly to the variability of the % uptake of lactulose. There is a tendency only for CRP to be positively associated with permeability.

5.3.4 Malaria infection and acute phase response

Only 6 cases of proven malaria were observed in the children during their time on the study. Only half of those were associated with fever and the corresponding CRP level was raised at a mean of 24 mg/l, which was significantly greater than the mean study CRP level of 5.4 mg/l \((P < 0.0001)\). In the other 3 cases asymptomatic parasitaemia was detected and all acute phase reactants and immunoglobulins were within the normal range. During the study period there was a particularly low prevalence of malaria due to the relatively low levels of rain fall.

5.4 Discussion

This chapter has shown that Gambian children have a significant elevation of systemic inflammatory markers (immunoglobulins and acute phase reactants). Platelet numbers were significantly elevated with the mean lying outside the UK normal range and CRP levels were frequently elevated above the UK normal range. In the two rainy seasons that this study ran through, there was a very low level of malaria parasitaemia, suggesting that malaria was probably not the main reason for the elevated inflammatory markers observed. Mean Gambian immunoglobulin levels are substantially higher than those seen in the UK, and IgG levels fail to follow the normal UK pattern, possibly due to endogenous production by the infant overtaking the rate of decline of maternal antibodies sooner than in the UK. The evidence for raised systemic inflammatory markers therefore seems to show frequent low level CRP elevation,
constant high immunoglobulin levels (all subclasses), raised white cell and platelet counts.

The cumulative effect of low level activation of systemic inflammation upon growth is likely to be greater (Tracey et al., 1988) than short lived peaks of very acute phase response. Potentially a low level of inflammation in a chronically inflamed small bowel could lead to significant nutritional deficit, either directly or indirectly via translocated bacteria or toxins, and have a greater effect upon growth than transitory high peak levels seen in pneumonia or malaria. Raised platelet numbers, immunoglobulins and CRP are non-specific markers of inflammation. However, associations with intestinal permeability suggest an intestinal source of endotoxin stimulating systemic inflammation. It is possible now for the first time, in children from a tropical environment, to describe an association between intestinal disease, the translocation of known gut derived immunogenic antigens, acute phase response and growth faltering. The associations between immunoglobulin levels and endotoxin antibody levels and growth is greater than between permeability alone, suggesting that systemic inflammation may be closer to the causative pathological mechanism than permeability itself. This will be discussed further in chapter 7.0, where the inter-relationships between dual sugar permeability, endotoxin levels and systemic inflammatory responses will be quantified and related to final height and weight.

5.5 Conclusion

Gambian children show evidence of systemic inflammation, which has to a large extent, its’ origin in the gastrointestinal tract, via impairment of gut barrier function. This impairment of barrier function has a strong, negative association with growth performance.
Table 5.3
Regression analysis between endotoxin antigen and antibody and L:M permeability. Regression coefficient ($r$) and $P$ values shown.

<table>
<thead>
<tr>
<th></th>
<th>L:M % recovery</th>
<th>lactulose % recovery</th>
<th>mannitol % recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endotoxin antigen</td>
<td>0.05</td>
<td>0.32</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>0.95</td>
<td>&lt;0.02</td>
<td>&gt;0.6</td>
</tr>
<tr>
<td>Endotoxin antibody</td>
<td>0.32</td>
<td>0.35</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>&lt;0.007</td>
<td>&lt;0.004</td>
<td>&gt;0.6</td>
</tr>
</tbody>
</table>
Table 5.4

Regression analysis between endotoxin antigen and antibody with markers of systemic inflammation

*P* values and regression coefficients (*r*) shown

<table>
<thead>
<tr>
<th></th>
<th>Endotoxin antigen</th>
<th>Endotoxin antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>r</em></td>
<td><em>P</em></td>
</tr>
<tr>
<td>IgG</td>
<td>0.31</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>IgA</td>
<td>0.25</td>
<td>0.06</td>
</tr>
<tr>
<td>IgM</td>
<td>0.14</td>
<td>0.30</td>
</tr>
<tr>
<td>CRP</td>
<td>0.20</td>
<td>0.11</td>
</tr>
<tr>
<td>ACT</td>
<td>0.05</td>
<td>&gt;0.60</td>
</tr>
</tbody>
</table>

166
Table 5.5
Regression analysis between markers of systemic inflammation and intestinal permeability
$P$ and $r$ values given

<table>
<thead>
<tr>
<th></th>
<th>L:M</th>
<th>% recovery of lactulose</th>
<th>% recovery of mannitol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r$</td>
<td>$P$</td>
<td>$r$</td>
</tr>
<tr>
<td>IgG</td>
<td>0.69</td>
<td>&lt;0.0001</td>
<td>0.25</td>
</tr>
<tr>
<td>IgA</td>
<td>0.65</td>
<td>&lt;0.0001</td>
<td>0.19</td>
</tr>
<tr>
<td>IgM</td>
<td>0.49</td>
<td>&lt;0.0001</td>
<td>0.01</td>
</tr>
<tr>
<td>CRP</td>
<td>0.27</td>
<td>0.07</td>
<td>0.0</td>
</tr>
<tr>
<td>ACT</td>
<td>0.06</td>
<td>&gt;0.3</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Chapter 6.0

Descriptive study of enteropathy and potential pathoaetiological mechanisms

Chapter 3.0 has demonstrated that chronic enteropathy is negatively related to growth performance in Gambian infants. Chapter 4.0 failed to identify infective causes of chronic enteropathy but identified a potential role of early weaning and cow's milk protein with subsequent impairment of small bowel function (however further studies would be needed to verify these findings). This chapter will examine in detail the histopathological and immunological characteristics of the enteropathy seen in Gambian children, which is identified by dual sugar permeability. Evidence will also be sought for small bowel mucosal disease contributing to systemic inflammation. From these data potential mechanisms for the enteropathy will be hypothesised.

6.1 Background and aims

This present study has confirmed that raised dual sugar permeability is significantly associated with poor growth, in Gambian children in Gambian children. It is not known what these abnormalities of gut function represent at a histopathological or cellular level. Sullivan et al (Sullivan et al., 1991c) examined the relationship between L:M and a 3 dimensional model of morphometry in children with severe protein energy malnutrition and found there were no correlations. However there are no studies that have examined the relationship between L:M permeability and nutritional status in children from tropical environment with less severe degrees of malnutrition.
against traditional linear morphometry. Dual sugar permeability has been shown to
detect certain abnormalities such as villous shortening in coeliac disease (Menzies et
al., 1979) and crypt hypertrophy in cow’s milk protein sensitivity. But it is not known
if component sugars (lactulose or mannitol) can specifically predict any one aspect of
morphometric pathology, such as crypt hypertrophy or villous atrophy in Gambian
children.

It has been shown from a mouse model (Hermiston and Gordon, 1995) that abnormal
permeability may itself cause enteropathy. A strain of mice was raised that expressed a
dominant gene defective for cadherins (a protein forming the major part of epithelial
tight junctions). The homozygote form was lethal, but a chimeric form was compatible
with life. In the chimeric form the gastrointestinal tract produced patchy areas of
defective cadherin expression with associated underlying mucosal inflammation. Areas
of mucosa with normal cadherin and hence normal tight junction structure had no
evidence of mucosal inflammation. From this model it was suggested normal inter-
epithelial adhesion was essential for effective barrier function, which in turn was
critical to prevent mucosal inflammation.

Chapter 4 failed to find a correlation between infection with intestinal parasites and
viruses with growth and permeability. But there was some evidence that early weaning
was associated with impaired small bowel function and adverse nutritional status at 6
months of age. The presence of enteropathy (broadly described as tropical enteropathy)
has been confirmed in Gambian infants (Sullivan et al., 1991d), but many of these
morphometric features are shared by other types of enteropathy, such as coeliac
disease, food sensitive enteropathies, graft versus host disease. All these diseases have
a common mechanism involving lamina propria T-cells producing pro-inflammatory
cytokines, but there is little data to support this hypothesis in Gambian children.
T cell production of pro-inflammatory cytokines has been shown to be critical to producing small intestinal damage, previously regarded as the result of infecting organisms (Pender et al., 1998, Pender et al., 1996, Lionetti et al., 1993). It is now becoming clear that enteropathy is not only related to abnormal cell mediated immune activity but can also be due to a defective anti-inflammatory response (Groux et al., 1997, Powrie et al., 1996). Many of the actions of proinflammatory cytokines such as IFN-γ or TNF-α can be opposed by anti-inflammatory cytokines such as TGF-β or IL-10. The balance of cytokine production by T-cells in vivo is critical in determining whether or not cell mediated inflammation occurs in response to bacteria in the gut lumen. Particularly important are Th3 cells producing TGF-β and promoting oral tolerance. There is no data available on how the density of either pro or anti inflammatory cells relate to gut permeability, nor how changes in cell density relate to deteriorating nutritional status in Gambian children.

Chapter 5.0 has demonstrated that Keneba children have chronic low level activation of the acute phase response and early maturation of immunoglobulin levels suggesting systemic inflammation. Systemic inflammation is known to impair appetite, calorie intake and cause a negative nutrient balance (Keusch, 1991). The gastrointestinal tract is a major site of protein turnover, accounting for up to 60% of the body total in one study alone (Brunser et al., 1991). The potential for the gastrointestinal tract to cause nutritional disease therefore is very great. It is not known however in Gambian infants to what extent the gastrointestinal tract contributes to systemic acute phase and immunoglobulin responses.

For ethical reasons it is not possible to obtain gut biopsies from well children. Therefore a separate group of patients was recruited from the WEC Mission Hospital in Sibanor and The Tropical Medical Research Unit at Fajara The Gambia. As will be detailed below all of these patients had potential gastrointestinal disease.
The aims of this study were:

1. Define the mucosal inflammatory cell populations and morphometric appearance across a range of nutritional states and compare these with healthy UK children.
2. Quantify the changes in pro and anti-inflammatory cell numbers with deteriorating nutritional status.
3. Quantify the contribution of mucosal inflammatory cell populations to systemic inflammation.

6.2 Methods

6.2.1 Subjects

Patients were recruited from the outpatient clinics at The WEC Mission Hospital in Sibanor and The Tropical Medical Research Unit at Fajara The Gambia Between August and September 1997. Patients had to meet the following criteria (Summary given in table 6.1):

1. Weight z-score > -2 (ie above UK 3rd centile), but with non specific abdominal pains and free of diarrhoea.

2. Weight z-score < -2 but > -4 with declining weight on subsequent clinic visits despite treatment of any obvious underlying medical conditions (ie malaria or pneumonia) and the giving of nutritional advice.

3. Weight z-score <= -4.

4. UK control children were also recruited from The University Department of Paediatric Gastroenterology at The Royal Free Hospital, London. All children were
under 3 years of age and weights were above the UK 3rd centile. Children were investigated for possible enteropathy (ie poor growth, vomiting or suspected abdominal pain) but were found to have normal small bowel biopsy appearance with no evidence of inflammation on immunohistochemistry (HLA-DR negative on crypt epithelium). No gastrointestinal diagnosis was finally made.

Ethical permission for this part of the study was separately obtained from the Joint MRC and Gambian Government medical research ethics committee. Parental consent was obtained from the mother by a trained field worker in the appropriate local language. Forty children underwent upper GI endoscopy under deep sedation with a full resuscitation team available.

6.2.2 Endoscopic assessment

Biopsies were taken from the second part of the duodenum under direct vision. Samples were fixed in 10% formal saline for morphometric assessment and snap frozen in liquid nitrogen for subsequent immunohistochemistry.

Morphometry see section 2.2.2.4
Immunohistochemistry see section 2.2.2.2
Quantification of immunohistochemistry see section 2.2.4
Lactulose-mannitol test see section 2.2.1
Biochemistry for immunoglobulins and acute phase reactants see section 2.2.2

6.2.3 Statistical analysis

Continuous variables were tested for normality and were log-transformed if distribution was non-parametric. Comparison between continuous variables was made
using linear regression after checking that a scatterplot demonstrated a true relationship between response and predictor variables. Statistical significance was assessed by the Mann Whitney U test for non-parametric variables and t-tests for parametric variables. The significance in trends of categorical data (trend analysis) was tested using ANOVA.

6.3 Results

6.3.1 Morphometry

See Appendix 5 for photomicrographs of morphometric appearance.

All the Gambian children, regardless of nutritional status, showed significant small intestinal enteropathy by UK standards. The Gambian biopsies all showed mild / moderate villous shortening and crypt hypertrophy. There were a range of severities of villous atrophy and crypt hypertrophy and inflammatory cell infiltration of the epithelium and lamina propria. The histological appearance of the enteropathy had many similarities to protein sensitive enteropathies such as cow’s milk protein or coeliac disease.

The degree of enteropathy, as assessed by morphometry alone, did not correlate with current nutritional status (see Table 6.2). No significant differences between the clinical groups was seen for any of the morphometric parameters studied (villous height, crypt depth and IEL numbers), and there was no correlation with the presence or absence of diarrhoea. Comparison of mean values for villous height and crypt depth with published values for European and high socio-economic class Brazilian children (Walker-Smith, 1988b, Fagundes-Neto et al., 1997) confirmed that all three groups of Gambian children had enteropathy with crypt depth and villous height lying outside the normal ranges. The median IEL count of 40% was within the range characteristic of coeliac disease (Savidge et al., 1996).
6.3.2 Immunohistochemistry

See Appendix 6 for photomicrographs of immunohistochemistry.

6.3.2.1 Mucosal lymphocyte population and nutritional status

Overall the density of CD3 T cells within the small intestinal lamina propria was 4-5 times higher in Gambian children than UK controls (see Table 4). These were split roughly equally between CD4^ helper and CD8^ cytotoxic/suppressor T cells. CD3, CD4 and CD8 cell density tended to increase with worsening malnutrition, although this trend did not reach significance (e.g. regression of CD3 cells/mm^2 against weight z-score, \( r = 0.3 \) \( P = 0.07 \)). Expression of the early T cell activation marker CD25 (the \( \alpha \) chain of the IL-2 receptor, also expressed by activated macrophages) was detected about 15 to 30 times more frequently than in UK controls (Table 4). However, in contrast to overall T cell numbers, the density of CD25^ cells fell significantly with worsening malnutrition (CD25 cells/mm^2 against height z-score, \( r = -0.55 \), \( P <0.04 \)).

Numbers of IEL’s were markedly higher than in UK controls. The Gambian mean value lying more than 2 SD above the UK normal and in the range seen in coeliac disease (Walker-Smith, 1988a). Immunohistochemical analysis of IEL populations showed significant increase in the % \( \gamma\delta \) T cells (see table 6.3), well above the UK normal of approximately 10% (Walker-Smith, 1988b), with the higher numbers in the children with a weight less than -2 weight for age z-score. By contrast the expression of the cytotoxic granule perforin, a marker of activated cytotoxic T cells, was increased in all Gambian children, and actually tended to be lower in the more malnourished children.

Further evidence of epithelial activation was demonstrated by the uniform expression of HLA-DR antigen on crypt epithelium in all groups of Gambian children, in striking contrast to normal children from the UK children (Walker-Smith and Murch, 1999c, Walker-Smith and Murch, 1999a). The density of CD19^ B cells within the lamina propria was 2-3 times higher in Gambian children than UK controls. Similarly the density of mature plasma cells (syndecan-1^) was at least 25-30 times higher in the
Gambian children. However, in contrast to T cell numbers, the density of both B cells and plasma cells tended to decrease with worsening nutritional status, although these trends did not reach statistical significance.

6.3.2.2 Cytokine immunohistochemistry

Table 6.4 summarises the density of cytokine producing cells in the lamina propria of the 34 Gambian children, who had sufficient frozen sections available for analysis. The distribution of cytokine immunoreactivity within the mucosa was broadly similar to European children, although staining for IL-10 and TGF-β within the epithelium tended to be stronger. Although there were no differences in the overall cell numbers within the lamina propria for any individual cytokine between the three Gambian clinical groups, all three had numbers of cytokine-immunoreactive cells 5-20 fold above UK controls for both pro-inflammatory (IFN-γ and TNF-α) and regulatory (IL-10 and TGF-β) cytokines. A trend was seen towards decreasing density of cytokine-immunoreactive cells with worsening nutritional status, and numbers of TNF-α⁺, IFN-γ⁺ and TGF-β⁺ (but not IL-10⁺) cells fell with increasing malnutrition (Table 6.4).

Although cells producing pro-inflammatory cytokines showed reduction in those children with worse nutritional status (e.g. \( r = 0.62, P < 0.04 \) comparing IFN-γ⁺ cells and height z-score), the decline in TGF-β⁺ cells was much more marked. Over the range of nutritional status from height z-score +0.5 to -4.5, IFN-γ⁺ and TNF-α⁺ cell numbers fell by 20%, compared to a 45% drop in TGF-β⁺ cells, leaving a relative excess in the numbers of pro-inflammatory cells. When the balance between regulatory and proinflammatory cytokine-producing cell numbers was examined, there was a significant relationship between both the TGF-β : TNF-α and TGF-β : IFN-γ ratios and nutritional status. This was stronger for height than weight z-scores (TGF-β : TNF-α ratio against height z-score, \( r = 0.48, P = 0.03 \)). Overall, in the relatively well nourished Gambian children with height z-score > -2 the ratio of regulatory (TGF-β + IL-10) to proinflammatory (IFN-γ + TNF-α) cytokine cell numbers was approximately 3:1, but as the children became more malnourished and height z-score fell to < - 4, the
ratio reversed so there were more pro-inflammatory than regulatory lymphocytes (Figure 6.1).

UK children (note only 4 of the UK control children had cytokine immunohistochemistry performed) had significantly less cytokine producing cells (both proinflammatory, T-regulatory and Th3) in the small bowel mucosa, with a regulatory : proinflammatory cytokine ratio of approximately 1:1. This suggests that upregulation of the anti-inflammatory (Th3) response in a tropical environment may be clinically important, as the overall numbers of TNF-α and IFN-γ positive cells were within the range found in active Crohn’s (Murch et al., 1993) or coeliac disease (Breese et al., 1994a).

6.3.3 Intestinal permeability

Permeability of the small intestine was substantially higher in the Gambian infants than UK controls, (Table 6.5). However, in this study there was no overall relationship between nutritional status (both height and weight z-score) and the L:M ratio, nor with the percentage recoveries of lactulose and mannitol in the 5 hour urine collection (see table 6.5). There was however, a clear relationship between the density of lymphocytes within the mucosa and overall permeability. Regression analysis (Table 6.6) showed increase in the L:M ratio to correlate positively with lamina propria CD3+ T cells, total IEL’s and perforin+ IEL’s and negatively with CD19+ B cells and the IL-10 : IFN-γ ratio. Thus increased gut permeability was seen in children with more T cells than B cells, in particular activated epithelial cytotoxic T cells and with a relative excess in the number of IFN-γ compared to IL-10+ cells within the lamina propria.

On assessment of lactulose recovery alone, as an index of paracellular permeability, the only significant correlation was again a negative one with the ratio of IL-10+ cells to both IFN-γ+ and TNF-α+ (r = 0.51 P < 0.02 and r = 0.35 and P = 0.05 respectively). There were no associations between morphometric analysis and lactulose recovery. Neither were there any significant relationships between mannitol permeability and any immunohistochemical or morphometric parameter.
6.3.4 Morphometry, immunohistochemistry and systemic inflammation

Associations between markers of systemic inflammation and CD25, TNF-α and IFN-γ and CD3 cell numbers demonstrated no correlations between plasma immunoglobulin levels and any single inflammatory cell population. However, there were significant relationships between mean lamina propria CD3 cell densities \( r = 0.4, P < 0.04 \) and also with the early activation marker CD25 \( r = 0.44, P < 0.05 \) and plasma CRP concentration. After adjustment for multiple comparison by inclusion of all investigated variables in to a single regression equation only CD3 cell numbers remained significant. A similar process was performed for markers of B-cell lineage and activation (CD19 and syndecan-1) that had negative associations with acute phase reactants. Syndecan-1 mean cell density had a strong negative relationship to mean CRP levels \( r = -0.62, P = 0.008 \).

6.4 Discussion

For ethical reasons it was not possible to collect small bowel biopsies from well children in the Keneba cohort. Extrapolating the findings from this group of patients in to the typical child living in the environment of Keneba has to be done with caution. However, nearly half of the study children came from rural communities (including Keneba) and the range of nutritional status represented by this study and that found in Keneba overlaps.

It has been noted that by UK standards a significant enteropathy exists in Gambian children before the onset of severe protein energy malnutrition (marasmus and kwashiorkor). This challenges the commonly held belief that the enteropathy of tropical malnutrition is a distinct entity, but rather it appears to be on a continuum with tropical enteropathy. The morphometric appearance of intestinal biopsies obtained from these children do not change with nutritional status, neither does the presence or absence of diarrhoea alter gut morphology. However, at a cellular level certain
populations of inflammatory cells (ie CD 19 and CD3) are closely related to nutritional status and small bowel function. This study confirms a T cell mediated inflammatory enteropathy exists in Gambian children across a wide range of nutritional states, with expression of mucosal inflammatory markers in the range of untreated coeliac disease and inflammatory bowel disease (Breese et al., 1994b, Murch et al., 1993).

Morphometric appearances, however, suggest that the enteropathy is more like cow’s milk protein sensitivity or coeliac disease, rather than inflammatory bowel disease. It is intriguing to see such high numbers of T_{H1} cells without the hallmark features of Crohn’s disease, such as granulomas and ulceration. A possible reason for this is the presence of equally high number of anti-inflammatory producing cells (TGF-β and IL-10), elevated x6-10 above UK controls. The parallel increase in the anti-as well as the pro-inflammatory cytokines and the absence of neutrophils is suggestive of a chronic inflammatory enteropathy. The activity of the anti-inflammatory cells may prevent the inflammatory activities of IFN-γ and TNF-α causing ulceration and granuloma formation, may be insufficient to prevent villous atrophy.

There are very consistent changes in inflammatory cell populations in the enteropathy seen in these Gambian children as nutritional status declines. Malnutrition causes a fall in the numbers of B-lymphocytes (CD19 and syndecan-I+) and a rise in the numbers of T lymphocytes (CD4, CD8 and γδ IEL’s). This dichotomy in responses between T and B lymphocytes in severe malnutrition has never been described before in the intestinal mucosa and may be due to an excess of pro-inflammatory cytokines inhibiting T_{H2} pathway, allowing a T-cell response to predominate.

Cytokine production was characterised at the protein and not the mRNA level. Using immunohistochemistry it is not possible to define securely cytokine producing lymphocytes from other types of cells. However, any positively staining cells that were obviously of non lymphocyte morphology were excluded (macrophages, fibroblasts etc.). With these reservations we found a consistent trend in mucosal cytokine expression with worsening nutritional status. For the first time the imbalance between T_{H1} and T_{H3} cytokines has been observed in the enteropathy present in severe malnutrition. Whether the bias towards T_{H1} cytokine production is a cause or result of
malnutrition or even an epiphenomenon, cannot be assessed from this data. The body of evidence in human and animal work suggests that unopposed Th1 (pro-inflammatory) cytokine activity leads to enteropathy in a dose dependant manner (epithelial damage, lamina propria matrix disruption, villous atrophy and crypt hypertrophy) (Lionetti et al., 1993, Pender et al., 1996, Pender et al., 1998, Thompson et al., 1996). Potentially a vicious cycle of intractable enteropathy and malnutrition could develop (Fig 6.3). A child becomes malnourished because of an acute illness or food shortage, with resulting increase of the Th1 cytokine activity in the mucosal microenvironment and a tendency towards gut damage. This could in turn lead to malabsorption, inflammation, anorexia and further nutritional deterioration. The cycle establishes a spiral of progressive nutritional deterioration and enteropathy.

Presumably other, as yet undefined control mechanisms are brought to bear upon the dominating Th1 response, in order to control the enteropathy. If this were not the case and malnutrition did stimulate a self-perpetuating cell mediated inflammatory reaction in the lamina propria, then potentially relatively mild illnesses could lead to intestinal failure. Potentially the child with severe intractable diarrhoea and malnutrition may be in a situation similar to this. If the imbalance between Th1 / Th3 cytokines is an aetiological association with malnutrition, then a selective nutritional immunosuppression of Th3 cells is taking place (see figure 6.2). Further work is needed to confirm or refute these preliminary findings.

A close association between intractable diarrhoea and malnutrition has long been recognised (Gracey, 1996, Walker-Smith and Murch, 1999b), and when complicating marasmus and kwashiorkor is associated with high mortality rates (de Onis et al., 1993). This study does not offer new therapeutic options but poses the question of whether there is a role for immunomodulation in cases of persistent diarrhoea and malnutrition who fail to respond to a hypoallergenic diet (usually comminuted chicken). Further nutritional options would be the preferred route such as extensive protein hydrolysation through fermentation. Local immunosuppression with topical corticosteroids such as fluticasone would be attractive because of low systemic absorption, but would be prohibitively expensive. Oral corticosteroids are readily
available, but a high risk option because of occult infection. Currently much of the high mortality rates in the management of tropical malnutrition is thought to be due to case mismanagement (i.e. parenteral iron, sodium overload, undiagnosed sepsis) (Gove et al., 1999, Campbell and Gove, 1996) and poor maternal education preventing subsequent relapses (Ketema and Lulseged, 1997). These issues need to be addressed as first priority in malnutrition management, before any trials of immunomodulation are considered.

It has been noted that the overall histopathological appearance and the morphometric scores are similar to those of dietary protein sensitive enteropathy. Recent advances in the understanding of mucosal T-cell control has clarified the events that lead to the loss of oral tolerance which is a critical event in food sensitive enteropathy. In particular the role of cells producing TGF-β (Th3 cells) and IL-10 (Tr1 cells) (Powrie et al., 1996, Groux et al., 1997, Walker-Smith and Murch, 1999c) is critical. Mice deficient in either cytokine spontaneously develop severe mucosal inflammation (Kuhn et al., 1993, Boivin et al., 1995), and transfer of either regulatory lymphocyte type prevents experimental colitis, with the relative proportion of transferred Th1 and regulatory lymphocytes determining the extent of mucosal inflammation (Powrie et al., 1996, Groux et al., 1997, Groux and Powrie, 1999), (Strober et al., 1997).

Both TGF-β and IL-10 producing cells are thought to be critical in the maintenance of oral tolerance to dietary antigens. Tolerance to low dose dietary antigen is an active process, in which TGF-β-producing Th3 cells are generated in Peyer’s patches in response to local TGF-β expression, and then mediate “bystander tolerance” within the mucosa when they encounter their antigen. By contrast, tolerance to high-dose antigen requires the induction of T cell anergy, following antigen presentation to mucosal lymphocytes by intact epithelium, which does not express co-stimulatory ligands (accessory molecules that increase the affinity of receptor-ligand binding affinity and alter event involved in cellular activation) in health (Friedman and Weiner, 1994). Importantly, anergised T cells are now recognised to produce IL-10 (Groux and Powrie, 1999). Such tolerance inducing mechanisms can be broken in two major ways, both of which are likely to be relevant in Gambian children: firstly, by skewing of the
Peyer’s patch microenvironment towards T\textsubscript{H}1 rather than T\textsubscript{H}3 cell generation following pathogen-induced expression of IL-12 (Strober et al., 1997), which is likely to happen in Gambian children exposed to frequent challenges ie with \textit{Giardiasis}, and secondly by disruption of the epithelial barrier to allow paracellular passage of intact antigen (Friedman and Weiner, 1994). The antigen is then presented to T cells by activated subepithelial macrophages and dendritic cells which, in contrast to enterocytes do express costimulatory ligands, and thus cause T cell activation rather than anergy.

This study has validated the use of the L:M test in field trials as a reasonable test of mucosal disease. A raised L:M permeability was significantly correlated with numbers of T cells and inversely correlated with the ratio of IL-10 / IFN-\gamma cell numbers and numbers of B cells. These findings are supportive of the picture of an enteropathy caused by cell mediated inflammation, where oral tolerance is being “stressed” or partially lost due to the falling numbers of anti-inflammatory cells producing cytokines such as IL-10. These results may explain the link between gut leakiness and mucosal inflammation in children from the tropics, and provides an analogue with the cadhedrin deficient mice. In this case the cause of barrier disruption is probably loss of oral tolerance mechanisms.

CRP is routinely used in clinical practice to follow the activity of inflammatory bowel disease. This study has shown that approximately 20 % of the variability in plasma CRP levels can be explained statistically by proximal small bowel inflammation in these Gambian children. CRP is a non specific marker of inflammation and will rise in a wide range of infective conditions common in the tropics. Common illnesses like malaria and pneumonia would tend to raise the acute phase proteins to high levels for a short period of time. But this study has demonstrated that small bowel inflammatory activity potentially contributes to systemic acute phase response.

6.5 Conclusions
A T cell mediated enteropathy exists in Gambian children that may arise from the partial loss of oral tolerance mechanisms. Malnutrition is associated with significant changes in the inflammatory cell populations (increase in CD4, CD8, γδ IEL and fall in numbers of B cells) that are not reflected in changes in small bowel morphology. Malnutrition is associated with a relatively selective suppression of \( T_{h3} \) cytokines leading to an imbalance between pro and anti-inflammatory cytokines, which may perpetuate the enteropathy. Small bowel mucosal inflammation is significantly associated with plasma CRP levels, suggesting a possible causal association.

Table 6.1 Subject nutritional status by group

Figures given are means and (SD)

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Weight z-score</th>
<th>Height z-score</th>
<th>BMI z-score</th>
<th>Number with diarrhoea (%)</th>
</tr>
</thead>
</table>
| weight z-score  
> -2 (4)  
-0.3 (0.37)  
0.33 (2.9)  
-2.2 (1.2)  
0 |
| weight z-score  
-2 to -4 (11)  
-2.9 (0.95)  
-1.5 (0.87)  
-2.8 (1.1)  
7 (65) |
| weight z-score  
< -4 (25)  
-5.5 (1.1)  
-2.8 (1.5)  
-5.4 (1.5)  
18 (70) |
Table 6.2

Morphometry by categories of weight z-score in Gambian children
values given means ± (SD)

<table>
<thead>
<tr>
<th>Clinical group (n)</th>
<th>Crypt depth(μm)</th>
<th>Villous height (μm)</th>
<th>Villous / crypt ratio</th>
<th>IEL (per 100 epithelial cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight z-score</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; -2</td>
<td>269 (50)</td>
<td>261 (70)</td>
<td>1.0 (0.5)</td>
<td>43.2 (7.3)</td>
</tr>
<tr>
<td>(4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight z-score</td>
<td>280 (79)</td>
<td>241 (77)</td>
<td>1.0 (0.7)</td>
<td>37.8 (8.8)</td>
</tr>
<tr>
<td>-2 to -4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight z-score</td>
<td>286 (60)</td>
<td>256 (61)</td>
<td>0.92 (0.3)</td>
<td>42.7 (10.4)</td>
</tr>
<tr>
<td>&lt; -4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(27)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

ANOVA P > 0.1 for all categories
Table 6.3
Small bowel mucosal inflammatory cell densities (cells per mm² unless stated) in Gambian and UK children (mean and ±SD given)

<table>
<thead>
<tr>
<th>Group</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>CD19</th>
<th>syndecan-1</th>
<th>CD25</th>
<th>IEL-perforin+(%)</th>
<th>IEL-γδ+%</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK controls</td>
<td>346</td>
<td>308</td>
<td>190</td>
<td>176</td>
<td>ND</td>
<td>29</td>
<td>3.6</td>
<td>&lt;10%</td>
</tr>
<tr>
<td>(19)</td>
<td>(170)</td>
<td>(112)</td>
<td>(102)</td>
<td>(109)</td>
<td></td>
<td>(22)</td>
<td>(3.4)</td>
<td>see text</td>
</tr>
<tr>
<td>Weight z-score &gt; -2</td>
<td>1088</td>
<td>535</td>
<td>377</td>
<td>346</td>
<td>498</td>
<td>578</td>
<td>39.3</td>
<td>28.4</td>
</tr>
<tr>
<td>(3)</td>
<td>(240)</td>
<td>(110)</td>
<td>(37.5)</td>
<td>(50.4)</td>
<td>(n=1)</td>
<td>(110)</td>
<td>(11.6)</td>
<td>(n=1)</td>
</tr>
<tr>
<td>Weight z-score -2 to -4</td>
<td>1089</td>
<td>776</td>
<td>451</td>
<td>672</td>
<td>362</td>
<td>461</td>
<td>56</td>
<td>46</td>
</tr>
<tr>
<td>(8)</td>
<td>(278)</td>
<td>(380)</td>
<td>(192)</td>
<td>(291)</td>
<td>(191)</td>
<td>(226)</td>
<td>(28)</td>
<td>(15)</td>
</tr>
<tr>
<td>Weight z-score &lt; -4</td>
<td>1371</td>
<td>706</td>
<td>426</td>
<td>434</td>
<td>235</td>
<td>370</td>
<td>43</td>
<td>45.2</td>
</tr>
<tr>
<td>(23)</td>
<td>(327)</td>
<td>(363)</td>
<td>(267)</td>
<td>(214)</td>
<td>(124)</td>
<td>(189)</td>
<td>(33)</td>
<td>(29)</td>
</tr>
</tbody>
</table>

Comparing Gambian Vs UK controls Mann Whitney U[^a] < 0.01, [^b] < 0.02; [^c] > 0.1; [^d] < 0.0001, ND = not done
Table 6.4 Cytokine producing cell densities (per mm$^2$) in the lamina propria mean and SD given

<table>
<thead>
<tr>
<th>group</th>
<th>IFN-γ</th>
<th>TNF-α</th>
<th>TGF-β</th>
<th>IL-10</th>
<th>TGF-β / TNF-α</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SD</td>
<td>SD</td>
<td>SD</td>
<td>SD</td>
<td>SD</td>
<td></td>
</tr>
<tr>
<td>Normal (2)</td>
<td>388</td>
<td>40</td>
<td>403</td>
<td>379</td>
<td>206</td>
<td>203</td>
</tr>
<tr>
<td>n=1</td>
<td>1.76</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight z-score &gt; -2 (10)</td>
<td>451</td>
<td>192</td>
<td>409</td>
<td>177</td>
<td>288</td>
<td>192</td>
</tr>
<tr>
<td>n=1</td>
<td>1.1</td>
<td>0.68</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight z-score -2 to -4 (22)</td>
<td>453</td>
<td>237</td>
<td>361</td>
<td>202</td>
<td>321</td>
<td>208</td>
</tr>
<tr>
<td>n=1</td>
<td>1.0</td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UK controls (4)</td>
<td>21.6 *</td>
<td>9.9</td>
<td>45.5 *</td>
<td>12.6</td>
<td>60.4 *</td>
<td>15.6</td>
</tr>
<tr>
<td>n=1</td>
<td>43. *</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.0001 compared to all Gambian groups. * P < 0.05 BY ANOVA. All other between group differences P > 0.05
The relationship between height SDS and the ratio of TGF-β: TNF-α cell numbers in the lamina propria (n=29)

R squared = 21.5% ; P = 0.01
Table 6.5
Intestinal permeability according to weight z-score in Gambian children

<table>
<thead>
<tr>
<th>Group</th>
<th>L:M</th>
<th>% recovery of lactulose</th>
<th>% recovery of mannitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight z-score &gt; -2</td>
<td>0.53</td>
<td>0.13</td>
<td>6.3</td>
</tr>
<tr>
<td>(4)</td>
<td>(0.4-1.3)</td>
<td>(0.006-0.9)</td>
<td>(0.3-12.1)</td>
</tr>
<tr>
<td>Weight z-score -2 to -4</td>
<td>0.47</td>
<td>0.05</td>
<td>3.3</td>
</tr>
<tr>
<td>(11)</td>
<td>(0.02-2.20)</td>
<td>(0.008-0.90)</td>
<td>(0.83-11.7)</td>
</tr>
<tr>
<td>Weight z-score &lt; -4</td>
<td>0.73</td>
<td>0.03</td>
<td>1.50</td>
</tr>
<tr>
<td>(26)</td>
<td>(0.14-2.2)</td>
<td>(0.001-0.44)</td>
<td>(0.11-7.6)</td>
</tr>
</tbody>
</table>
Table 6.6
Regression analysis between inflammatory cells densities and L:M permeability

<table>
<thead>
<tr>
<th>Surface marker</th>
<th>(n)</th>
<th>r</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3 (T-cell)</td>
<td>38</td>
<td>0.47</td>
<td>&lt;0.007</td>
</tr>
<tr>
<td>CD19 (B-cell)</td>
<td>32</td>
<td>-0.75</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IL-10/IFN-γ</td>
<td>33</td>
<td>-0.52</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>IEL (H+E)</td>
<td>41</td>
<td>0.73</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>IEL perforin+</td>
<td>30</td>
<td>0.39</td>
<td>&lt;0.03</td>
</tr>
</tbody>
</table>
Cycle of intractable enteropathy in protein energy malnutrition

- Worsening malnutrition
- Malabsorption, anorexia, acute phase response
- Pro-inflammatory bias in small bowel
- Increasing small bowel mucosal damage
Chapter 7.0
Summary of results and final Conclusions

7.1 Background and aims
Chapters 1.0 through to 6.0 have established that a chronic enteropathy significantly impairs growth in Gambian children. The screening test for the enteropathy (the L:M test) has been validated against histology and immunohistochemistry, which suggest a cell mediated inflammatory process. Significant associations between growth and stool neopterin concentrations also point towards mucosal inflammation. Systemic inflammatory markers, particularly plasma immunoglobulins have a very close relationship to final height and weight gain. This study suggests that the small bowel may be a significant cause of this systemic inflammatory response, either directly by the production of inflammatory mediators, or indirectly by allowing the passage of toxins in to the circulation. Plasma endotoxin and endotoxin antibodies, have strong relationships to growth, intestinal permeability assessed by the L:M ratio, and some markers of systemic inflammation. This is consistent with the main hypothesis of this thesis, that in these Gambian children a breakdown of gut barrier function allows the translocation of endotoxins in to the systemic circulation. This in turn causes inflammation with subsequent growth impairment. The overall association of plasma endotoxin antibodies and growth is stronger than any previously measured dietary or physiological even. Furthermore, a mechanism involving translocation of endotoxin from the gut in to the systemic circulation has been demonstrated. However, of all the parameters that have a significant growth limiting effect, it is uncertain to what extent they represent the same pathological events in the individual child, or are measuring different, unrelated mechanisms of growth impairment. For
example systemic endotoxin probably exerts a significant proportion of its’ growth limiting effect through activation of systemic inflammatory processes, rather than acting independently of them.

The aim of this chapter is to estimate the degree of interrelationship between the various factors identified as having a significant growth limiting effect upon Keneba children. Particular attention will be paid to the inter-relationship between L:M, faecal neopterin concentration, plasma endotoxin and markers of systemic inflammation and their effect upon long term growth. How these findings relate to the original hypothesis and main thesis aims will be discussed in the following order, relating the observed findings to the original aims, namely:

1. That an enteropathy leading to a breakdown of gut barrier function is associated with growth faltering.

2. Impaired gut barrier function is associated with an acute phase response.

3. To characterise the enteropathy and provide insights into aetiology and immunopathological mechanisms.

The above aims will be dealt with separately in sections 7.2 and 7.3.

Finally, the implications of the results obtained from chapters 3.0-6.0 will be discussed.

7.2 Statistical methods

To assess the degree of overlap in the mechanisms that various parameters measured (i.e. L:M permeability, plasma endotoxin IgG, plasma endotoxin antigen etc) have on growth faltering the following method based on partial regression was used (see figure 7.1). The response variable is the age corrected predicted height gain. Two variables b and d that have significant associations with growth are considered. Potentially these associations may be interrelated, representing a common pathological event. Separate
regression analysis for each variable give $r^2$ values of $r^2b$ and $r^2d$ respectively. The greatest $r^2$ value is then included as the first value in the partial regression equation, i.e. $r^2b$ in a simple linear correlation equation as before. When variable $r^2d$ is included in the regression equation there is a new $r^2$ value of $r^2bd$. The change in $r^2$ ($\delta r^2$) gives an indication of the additive effect of the new variable upon growth.

$$\delta r^2 = r^2bd - r^2b$$

An estimate of the inter-dependence of the two mechanisms upon growth is given by the overlap:

$$\text{overlap} \% = 1 - (\delta r^2)$$

The magnitude of any single mechanism upon growth is given by the size of the individual $r^2$ value, whilst the degree of overlap between the two variables is given by the $\delta r^2$ value, expressed as a % overlap. Separate analysis using predicted weight and height gains gave very similar results and therefore were not repeated.

7.3 Results

7.3.1 Enteropathy with mucosal inflammation is associated with impaired gut barrier function and growth faltering.

The mean individual L:M ratios and faecal neopterin concentrations after log transformation had an estimated overlap of 87% upon age corrected height gain (fig 7.2). Mean individual endotoxin antibody concentration had a considerable overlap with L:M and % recovery of lactulose (93% and 96% respectively) (fig 7.2a). The effects of endotoxin upon growth showed a similar degree of interdependence, with an estimated overlap of 94% with lactulose recovery (fig 7.2b).

The degree of overlap between endotoxin antigen and antibody reached 98%, when related to predicted height and weight gain.
7.3.2 Impaired gut barrier function is associated with acute phase response and growth faltering.

The degree of overlap between L:M and plasma immunoglobulins and their effect upon growth is similar for each of the subclasses IgG, IgA but less for IgM, although the overall proportion of growth variability explained varies. The overlap was estimated to be 93%, 95% and 75% (for IgG, IgA and IgM respectively).

A 98% overlap on final age corrected height occurred between mean subject endotoxin antigen and IgG (94% and 91% for IgA and IgM respectively). As the acute phase reactants CRP and ACT were only weakly associated with growth, the separate effects of acute phase reactants and endotoxin was not examined.

7.4 Discussion

7.4.1 Thesis aim 1 (section 1.5): Impaired gut barrier function is associated with growth faltering beyond 15 months of age.

The original study by Lunn et al was repeated (chapter 3.0) because of the reported changes in diarrhoea prevalence potentially representing changes in the microbiological environment of Keneba. It was found that mean L:M permeability was approximately half of the earlier value, reported by Lunn et al (1991). Comparing the association with growth in the Lunn et al (1991) study and this present study, shows a similar relationship between L:M and growth up to 15 months of age, but this association is approximately half as strong.

Chapter 3.4 has demonstrated that small bowel function measured by the L:M test never reached adult levels, but improved with age, due to a decrease in the % absorption / excretion of oral lactulose. Even at 30-40 years of age there was a significant association between nutritional status and final attained height, suggesting
that whatever aspect of intestinal disease that L:M permeability measures, it was exerting its' effect throughout the growing period.

Endotoxin is derived from bacterial cell walls of gram negative bacteria, and the major site of body endotoxin store is in the lumen of the gastrointestinal tract (predominantly large bowel). Endotoxin will most likely enter the circulation via translocation through the mucosa of the gastrointestinal tract. Although both endotoxin and endotoxin core antibody were both measured in section 5.3.2, stronger relationships between growth and systemic acute phase response were seen with endotoxin antibody rather than the antigen. This may be due to the short t\textsubscript{1/2} of endotoxin, which will cause a greater sample variance when compared to endotoxin antibody. There were highly significant inverse associations between growth and mean individual endotoxin titres, and these associations almost completely overlap with mean individual L:M values. This suggests that L:M is an indicator of small bowel integrity (Elia et al., 1987), and the growth limiting mechanism measured by the L:M test may exert its’ effect upon growth via systemic endotoxin translocation.

Therefore, this thesis has provided evidence of impaired gut barrier function by confirming an abnormally high dual sugar permeability ratio which is related to poor growth. This study has also showed for the first time, that high circulating levels of endotoxin antibodies exist in rural Gambian children and have a profound effect upon growth. Furthermore chapter 6.0 has demonstrated that cell mediated inflammation in the small bowel mucosa is associated with an abnormal L:M permeability ratio and is probably the mechanism by which endotoxin enters the systemic circulation.

7.4.2 Thesis aim 2 : Impaired gut barrier function is associated with systemic inflammation.
Having provided evidence that growth faltering is associated with impaired gut barrier function, section 5.3 demonstrated that systemic inflammatory markers are raised. White cell and platelet counts are significantly higher than those seen in the UK, and CRP shows chronic low level activation. Mean individual immunoglobulin titres are also high and have a strong negative association with growth over the whole study period. Section 7.3 then examined the degree of overlap between plasma endotoxin antibody and total plasma IgG in their combined effects upon growth. The two mechanisms were found to have a 96% homology. This provides evidence towards supporting the hypothesis, that impaired intestinal barrier is present and exerts a negative effect upon growth by allowing the translocation of antigenic toxins, that stimulate systemic inflammation.

Systemic inflammation seems to be demonstrated predominantly via immunoglobulins, rather than acute phase reactants (CRP and ACT). Potentially the very high levels of immunoglobulins are an adaptive response to chronic endotoxaemia. This in turn leads to rapid neutralisation of immunogenic molecules such as endotoxin, preventing a very intense acute phase response.
7.4.3 Thesis aim 3: To characterise the enteropathy and provide insights into aetiology and mechanisms

Chapter 3.3 employed a novel marker of intestinal mucosal inflammation, neopterin, which suggested that in the Keneba children mean individual levels of faecal neopterin are inversely associated with growth. There is a relatively large overlap between the effect of both small bowel permeability and faecal neopterin, suggesting the possibility that a large proportion of faecal neopterin is derived from small bowel mucosa undergoing cell-mediated inflammation. Further studies are needed in order to ascertain the site of gastrointestinal neopterin synthesis/excretion.

Chapter 6.0 provided a detailed descriptive account of morphometric and immunohistochemical characteristics of the enteropathy that is present in Gambian children across a range of nutritional states. For ethical reasons very few children with good growth and absence of intestinal symptoms could be recruited. However, those children who were recruited included children in a good nutritional state. The pattern of cytokine expression and lineage of inflammatory cells is suggestive of abnormal cell-mediated inflammation, with skewing towards TH1-dominated activity as nutritional status deteriorates. The down regulation of TH3 cytokines that occurs with increasing expression of TH1 cytokines is suggestive of partial loss of the oral tolerance mechanisms, such as may occur in dietary protein allergy (type 4 hypersensitivity).

Chapter 6.0 also provided evidence that the lactulose-mannitol test is able to predict abnormal cell-mediated immune activity in the small bowel mucosa. The main strength of association between dual sugar permeability and inflammation comes from the percentage recovery of lactulose. Chapter 7.0 has indicated a significant association between L:M (and particularly lactulose recovery) and endotoxin antibody
concentrations, suggesting the dual sugar permeability test is a measure of small bowel mucosal integrity. This is supportive of the view that lactulose uptake is largely disease dependant and occurs via aberrant paracellular pathways or effete enterocytes (Travis and Menzies, 1992). It is therefore with some justification that L:M permeability can be used as a measure for small bowel permeability.

The estimated prevalence of various infectious agents (excluding non pathogenic viruses and *Helicobacter pylori*) is at most estimated to reach approximately 40% in various tropical populations (Matthews et al., 1996, Rowland and McCollum, 1977, Gorbach, 1972, Rowland et al., 1978, Sullivan et al., 1994, Vis and Brasseur, 1992, Mahmud et al., 1993, Gupta, 1980, Kakai et al., 1995). The prevalence of enteropathy is assumed to be 100% by 1 year of age (taking a normal L:M of <0.1), leaving a large proportion of children in whom their enteropathy cannot be explained by identifiable infection. Early weaning has been identified as a potential risk factor for enteropathy and growth faltering in chapter 4.1. A food sensitive enteropathy may explain why there is such a large gap between the prevalence of enteropathy on the one hand and the prevalence of identifiable gastrointestinal infection on the other.
7.5 Conclusions

Growth faltering in Gambian infants is associated with a chronic small bowel enteropathy that is caused by a cell mediated immune reaction against as yet unidentified agents. These agents are likely to be infectious or dietary luminal protein antigens, immunological and morphometric similarities suggest these antigens may act in a manner similar to a post enteritis syndrome. The mechanism by which enteropathy limits growth is likely to involve systemic inflammation, derived either from mucosal inflammation or impaired barrier function allowing immunogenic luminal antigens to reach the systemic immune system. This study needs to be repeated in a larger number of infants in different geographical settings to establish whether these findings are generally applicable to all children in developing countries, or are only specific to The Gambia. It is not possible to implicate enteropathy measured (by the employed methods) in causing growth faltering without an intervention study to treat enteropathy and then observe a positive effect upon growth.
Figure 7.1
Venn Diagram model of partial correlations. $x_1$ and $x_2$ squared correlation coefficients between growth and 2 predictor variables.

A = residual unexplained variation
B + C = variation in growth explained by variable $x_1$
C + D = variation in growth explained by variable $x_2$
B + C + D = overall variation in growth explained by variable $x_1$ and $x_2$
D = variation in growth due to $x_2$, independent of $x_1$
C = overlap in effect of variable $x_1$ and $x_2$ upon growth
B = variation in growth due to $x_1$ independent of $x_2$

Total area = 1
A + B + C + D = 1
Figure 7.2
Variation in growth explained by faecal neopterin and L:M ratio
\[ x_1 = \text{Faecal neopterin concentration}, \quad x_2 = \text{L:M ratio} \]
Figure 7.3
Venn diagrams showing variation in growth explained by endotoxin IgG, L:M and % recovery of lactulose

Figure 7.3a
Mean subject endotoxin IgG and L:M

Figure 7.3b
Mean subject endotoxin IgG and % recovery of lactulose
Appendix 1

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Nutrient requirements for growth, pregnancy and lactation: the Keneba experience.


Energy requirements of children: is growth faltering a consequence of inadequate energy?


Appendix 2

Ethical Approval

Separate ethical approval was obtained for conducting the Keneba longitudinal study, the cross-sectional intestinal permeability survey and the hospital based study at Fajara and Sibanor where intestinal biopsies were collected. There is a two stage process for obtaining ethical permission for medical research in The Gambia. Firstly a detailed proposal of each study undergoes peer review at a scientific co-ordinating committee. The purpose of this is to ensure a workable study with achievable scientific aims is being considered. Secondly the proposals are submitted to a joint ethics committee formed by The Gambian government (including a representative from the Ministry of Health), lay members of Gambian society and members of the Medical Research Council of the United Kingdom. Here the importance of the question raised is considered against the cost to the individual study subject (in terms of time and investigative procedures). Unnecessary blood sampling may lead to the rejection of the project. The Keneba longitudinal study had to be submitted through ethical review on 2 occasions as the frequency of blood sampling was regarded as excessive. More information was provided, Keneba already samples children at 4-12 weekly intervals to screen and treat iron deficiency anaemia, the study did not involve any further blood samples other than these.

At Keneba agreement was reached between the community, via the village elders and the MRC Dunn Nutrition Unit as early as 1974. The village agrees to participated with the medical research in return for free medical care (3 resident paediatricians, free drugs, maternity care etc). Each individual study is presented to a meeting of village
elders who could object to the study if they so choose, but in fact never have. Then
each individual subject (via the mother) has to provide informed consent before being
enrolled in to the study. Refusal of consent did not effect the right to free medical care
as long as the individual remained a Keneba resident.
Subjects from Sibanor and Fajara were symptomatically unwell and benefited from
diagnosis only available via endoscopy ie eradication of \textit{H pylori} or treatment of
biopsy proven \textit{Giardia}. Free treatment was given to the children who were enrolled in
to this study.
During the period of the studies presented in this thesis (February 1996-November
1997) discussion of the Helsinki Declaration (an international agreement on ethical
standards for medical research) and how this applies to research in the tropics took
place (Crawley and Hoet, 1998). More specifically controversy over intervention trials
for vertical transmission of HIV in the tropics arose (Angell, 1997), however the
studies reported in this thesis were observational and therefore did not come under the
same criticism.
# Appendix 3

**Weekly Diet Record**

<table>
<thead>
<tr>
<th>Child’s name:</th>
<th>Number:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mother’s name:</td>
<td>Number:</td>
</tr>
</tbody>
</table>

**Compound:**

**Week Beginning:**

1) Is the child with mother (M), Nursemaid (N), Relative (R), other (O) ?

2) Is the child breast-feeding this week? (Y/N)

3) Is the child taking foods other than breast-milk ?

4) In the last week has the child taken: (Y/N)

- **Water**
- Cow’s-milk, fresh (ninsi kekeo), alone or in mono ?
- Cow’s milk, sour (ninsi nono kumongo), alone or in mono ?
- Millet (sanyo mono) ?
- Sorghum ?
- Rice (Manimono) ?
- Findo (Findomono) ?
- Corn / Maize (Tubanymono) ?
- Wheat flour (Farinoo) ?
- Groundnut (Tiakere churo)
- Other (specify ie fish, meat, egg, fruit) ?

Field workers signature: ________________________________

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Appendix 4

Daily Morbidity Record

Child’s name: ____________________________ Number: ____________________________

Mother’s name: ____________________________ Number: ____________________________

Compound: ____________________________

Week Beginning: ____________________________

Has the child any of these symptoms (Y/N):

<table>
<thead>
<tr>
<th>Sun</th>
<th>Mon</th>
<th>Tue</th>
<th>Wed</th>
<th>Thur</th>
<th>Fri</th>
<th>Sat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diarrhoea</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vomiting</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cold/cough</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin/ear infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

If Yes for diarrhoea :

<table>
<thead>
<tr>
<th>Number of stools / day</th>
<th>Sun</th>
<th>Mon</th>
<th>Tue</th>
<th>Wed</th>
<th>Thur</th>
<th>Fri</th>
<th>Sat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood or mucus in stool</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Y/N) ?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Using oral rehydration solution (Y/N) ?</th>
<th>Sun</th>
<th>Mon</th>
<th>Tue</th>
<th>Wed</th>
<th>Thur</th>
<th>Fri</th>
<th>Sat</th>
</tr>
</thead>
</table>

Temperature

Clinic visit

Is the child with mother (M), nursemaid (S), relative (Y/N) ?

Field worker’s signature: ____________________________

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Appendix 5

Top figure: photomicrograph of *Giardia lamblia* cysts labelled with immunofluorescent antibodies.

Middle figure: photomicrograph showing high numbers of intraepithelial lymphocytes

Bottom figure: photomicrograph of small bowel mucosa from a subject with weight faltering. Note partial villous atrophy and crypt hypertrophy.
Appendix 6
Photomicrographs of immunohistochemistry

UK controls (A-C) and Gambian children (D-J). Peroxidase immunohistochemistry delineates positively staining cells brown.

A. CD3+ cells. Relatively low density in lamina propria and epithelium compared to D,E.
B. IFN-γ immunoreactive cells in UK control. Note staining in subepithelial basement membrane and some matrix immunoreactivity in lamina propria, but significantly less than in Gambian biopsies- see 1.
C. IL-10 immunoreactive cells at low density within the lamina propria of Gambian child with severe growth faltering.
D. CD4+ cells at high density within the lamina propria of Gambian child with severe growth faltering.
E. Large numbers of CE8+ intraepithelial lymphocytes in the same child.
F. High density of γδ intraepithelial lymphocytes despite normal villous architecture in marasmic child.
G. Strong HLA-DR expression in surface epithelium of well grown Gambian child. This was seen in all Gambian children, unlike in UK children where epithelium does not stain positive for HLA-DR.
H. TGF-β cells in lamina propria of non-marasmic Gambian child.
I. Strong IFN-γ immunoreactivity in epithelium, basement membrane and within laminal propria of marasmic Gambian child. Note preserved villous architecture.
J. IL-10 immunoreactivity in serial section to I, showing substantial increase in IL-10+ cells above UK control- see C- but relative paucity compared to IFN-γ.
Appendix 7

Lactulose mannitol permeability results for children undergoing intestinal biopsy

<table>
<thead>
<tr>
<th>Number</th>
<th>% recovery of mannitol</th>
<th>% recovery of lactulose</th>
<th>L:M ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>101</td>
<td>4.60</td>
<td>0.028</td>
<td>0.24</td>
</tr>
<tr>
<td>104</td>
<td>10.9</td>
<td>0.001</td>
<td>0.04</td>
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<td>105</td>
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<td>0.01</td>
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<td>126</td>
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<td>0.66</td>
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<td>**</td>
<td>**</td>
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<td>133</td>
<td>7.06</td>
<td>0.090</td>
<td>0.46</td>
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<td>1.74</td>
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<td>0.75</td>
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** Volume of urine not accurately recorded and therefore percentage recovery of individual sugars not possible. Calculation of final L:M ratio is however possible.