

Pak Leong Lim^a
Yu Lung Lau^b

^a Clinical Immunology Unit,
Chinese University of Hong Kong;
^b Department of Paediatrics,
University of Hong Kong

Occurrence of IgG Subclass Antibodies to Ovalbumin, Avidin, and Pneumococcal Polysaccharide in Children

Key Words

Avidin
IgG subclass antibodies
Ovalbumin
Pneumococcal polysaccharide

Abstract

The serum levels of the four subclasses of IgG to three different antigens – ovalbumin (OVA), pneumococcal polysaccharide (PNC), and avidin – were determined by enzyme-linked immunosorbent assay in three age groups of healthy children: 1.2–2.5 years (group A, n = 20), 6–7 years (group B, n = 21) and 15–17 years (group C, n = 20). The anti-OVA response in all groups was dominated by IgG1, IgG2, and IgG4 antibodies which generally increased in levels from group A to group B, but then declined significantly in group C. In all age groups, the IgG2 and IgG4 as well as the IgG1 and IgG3 responses were correlated; an association was also observed between IgG1 and IgG2 in groups B and C, but not in group A. The anti-PNC response was restricted to the IgG2 and IgG4 subclasses; correlation between these subclasses was seen only in group B children. The more predominant IgG2 antibodies increased dramatically in concentration from group A to group B, and then less remarkably in group C. Occurrence of the IgG1 anti-PNC antibodies was different which resembled the anti-OVA response. Responses to avidin, a protein derived from the same source as OVA, were different from the anti-OVA responses. These were low and confined to the IgG1 and IgG2 subclasses only and mainly to the younger children. A small group (n = 8) of 6- to 7-year-old atopic children who had a history of asthma or eczema appeared to have responses similar to their healthy counterparts.

Introduction

There is recent interest in the measurement of total serum levels of the four subclasses of IgG in human beings because perturbations of these levels can have serious clinical implications [1]. The importance of such analyses is seen in patients who had normal total IgG levels, but lacked antibodies of one or more of the IgG subclasses [2, 3]. These patients suffered from various kinds of recurrent infections,

implicating the important protective role of these subclasses, especially IgG2 [3]. The role of IgG4 antibodies is least understood, but these proteins seem essential to health [4] and appear to be associated with allergy [5].

Useful information has been obtained from measuring the IgG subclass levels to various antigens. The antigens most commonly used are of two types: those derived from infectious agents – e.g., pneumococcal polysaccharide (PNC) and hepatitis B antigen – and the dietary proteins e.g.

ovalbumin (OVA) and lactoglobulin. Interestingly, different antigens appeared to stimulate different subclasses of antibodies. Thus, the antibodies formed against PNC in normal adults were almost exclusively of the IgG2 subclass [6], whereas anti-OVA antibodies belonged mostly to the IgG4, IgG1, and IgG2 subclasses [7]. In contrast, the antibodies induced by rubella virus in infected patients were mostly IgG3 [8], while the response of infants vaccinated with hepatitis B antigen was predominantly IgG1 and IgG4 [9].

We have carried out similar IgG subclass determinations in healthy children using three types of antigen. Three widely separated age groups were used to gain an insight into the development of these antibodies. Unlike previous studies which examined the responses to OVA and PNC in separate individuals, we studied these responses in the same subjects to see the relationship between the responses. Avidin was included, since this heat-labile protein, which is derived from the same source as OVA (egg albumin), had not been studied previously and could, moreover, provide an interesting comparison with OVA. Also included in the present study was a small group of children who had a history of asthma or eczema to see whether their antibody profiles were different from those of age-matched normal children.

Materials and Methods

Subjects

The children participating in this study were from the same cohort described previously [10, 11], recruited from child health clinics, kindergartens, and schools. A standard questionnaire was used to screen these children for histories of allergies and infections. The healthy children were randomly selected from three age groups: 1.5–2.5 years (group A, $n = 20$), 6–7 years (group B, $n = 21$), and 15–17 years (group C, $n = 20$). Eight children, 5–7 years of age, who had a history of eczema (i.e., having erythematous and scaly skin lesions with or without lichenification) or asthma (with reversible airway obstruction) were also included in the study (group D). These children had elevated IgE levels: 977 ± 169 (SE) IU/ml (range 47–3,426); all except 1 > 100 IU/ml.

Antigens

OVA and avidin were purchased from Sigma Chemical (St. Louis, Mo., USA), and PNC was prepared from cell lysates of *Streptococcus pneumoniae* type 3 (ATCC strain) as described previously [12].

Immunochemicals

Monoclonal antibodies specific for the four subclasses of human IgG were obtained from Sigma: anti-IgG1 (clone HP-6001), anti-IgG2 (clone HP-6014), anti-IgG3 (clone HP-6050), and anti-IgG4 (clone HP-6025). Peroxidase-labeled goat antimouse IgG (whole molecule) antibody was also obtained from Sigma.

Enzyme-Linked Immunosorbent Assay

The method used was essentially as described [12]. Briefly, Immulon 2 microplates (Dynatech, Alexandria, Va., USA) were coated with antigen (1–5 $\mu\text{g/ml}$). Serial twofold dilutions (starting at 1:250) of the test serum were incubated with the antigen for 1 h at 37°C. Following extensive washing, the subclass-specific monoclonal antibody (1:1,000 dilution) was added and allowed to react for 1 h at 37°C. The plate was washed, and peroxidase-labeled goat antimouse IgG (whole molecule) antibody was added. After incubation for 1 h at 37°C and subsequent washing, the assay was developed using *o*-phenylene diamine (30 min, 37°C) and the results read using an MR 710 reader (Dynatech). The results were expressed as the absorbance (OD 490 nm) of the serum diluted at 1:1,000 (for IgG1, IgG2, and IgG4 activities to OVA) or at 1:250 (for all other activities). The reason for using the greater dilution in some cases is because saturation binding was observed at higher concentrations. The readings were corrected against a standard made up of pooled normal sera, and a buffer control (in triplicates) was also used on every plate. Nonresponders were considered those with absorbance values $\leq$ mean of buffer control + 2 SD (0.05 in most cases).

Statistical Analysis

Comparison of data between paired groups for difference or association was performed by the Wilcoxon rank test or regression analysis using the Stat-Works program (version 1.1; Cricket Software, 1985). In these analyses, all nonresponders were regarded as having O.O absorbance units.

Results

Sera of individuals of the four groups of subjects (Groups A–D, see Materials and Methods) were assayed for IgG subclass antibodies to OVA, avidin and PNC. The results are shown in figures 1 and 2. The antibody responses are expressed as absorbance units determined from 1:250 or 1:1,000 serum dilutions. The higher dilution was used for the IgG1, IgG2, and IgG4 responses to OVA, since higher activities were seen in these as compared with the other responses (see Materials and Methods). Total IgG antibodies to the three antigens were not determined.

Antibodies to OVA

In general, high levels of anti-OVA antibodies were found in the IgG1, IgG2, and IgG4 subclasses in all age groups. Significantly lower levels of IgG3 antibodies were present. For all subclasses, the best response was seen in the group B children.

IgG1 antibodies were present in 90% of the group A children at high levels (median 0.74 units). These were found in all children in group B at levels (0.79 units) not significantly different from those in group A. However, these levels dropped significantly in the oldest age group (group C, 0.09 units), and fewer of these subjects (70%) responded.

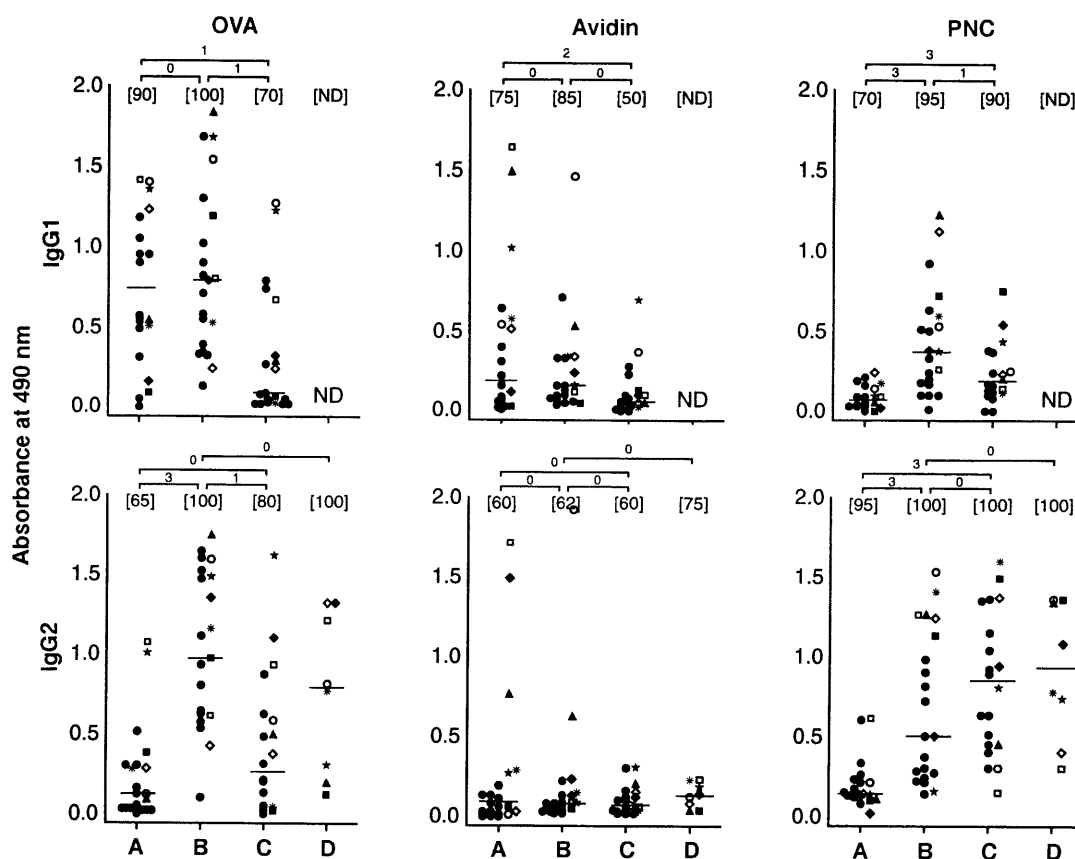


Fig. 1. Ig1 and IgG2 antibody levels (absorbance units) to OVA, avidin, and PNC in individual subjects. A, B, and C = 1.5- to 2.5-year-old, 6- to 7-year-old, and 15- to 17-year-old healthy children, respectively; D = 6- to 7-year-old children with asthma or eczema. ND = not done. Some individuals are indexed, shown by a common symbol used throughout the panels for each group (including those in figure 2). Bars denote the median in each group and the numerical values in brackets the responders (%). Other numerical values denote the test of significance analysis between groups: 0 = $p > 0.05$; 1 = $0.01 < p \leq 0.05$; 2 = $0.005 < p \leq 0.01$; 3 = $p \leq 0.005$.

Low levels (0.12 units) of IgG2 antibodies were found in group A children only 65% of whom responded. Group B children, on the other hand, developed high concentrations of these antibodies (0.97 units), significantly different from those in the younger children, and all subjects responded. A significant decrease in antibody levels (0.26 units) was seen in the older children (group C, 80% responders). The atopic children (group D) had slightly lower levels (0.79 units) of antibodies as compared with their healthy counterparts (group B), but the difference was not significant.

Although the IgG3 levels were relatively low in all age groups, the distribution of these antibodies was similar to that of IgG1. Thus, the response increased marginally from group A (0.08 units, 75% responders) to group B (0.15 units,

100% responders), but decreased significantly in group C (0.05 units, 60% responders).

The occurrence of IgG4 antibodies was similar to that of IgG2. Thus, low levels (0.05 units) were found in 55% of group A children, but these increased dramatically in group B (1.68 units; 100% responders), followed, however, by a significant decrease in the oldest children (group C; 0.38 units, 85% responders). As with IgG2, the IgG4 levels (1.47 units) found in the atopic children (group D) seemed depressed, but the difference was not significant as compared with those in normal age-matched children (group B).

The similar patterns of responses seen among the IgG subclasses suggest that some of these responses could be correlated with one another. Indeed, a positive correlation

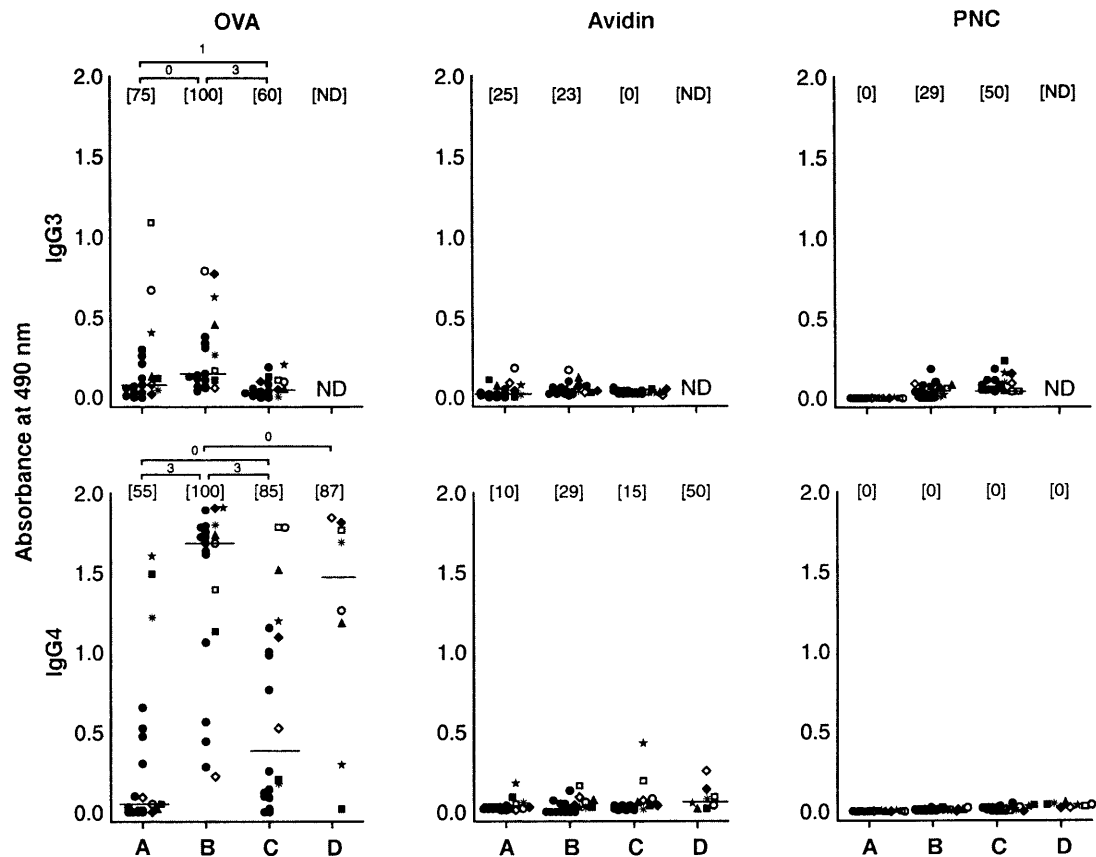


Fig. 2. IgG3 and IgG4 antibody levels in the individuals described in figure 1.

was seen between IgG2 and IgG4 in all age groups (group A: $r = 0.47$, $p = 0.04$; group B: $r = 0.70$, $p = 0.0004$; group C: $r = 0.81$, $p = 0.0001$; group D: $r = 0.85$, $p = 0.007$) and between IgG1 and IgG3 (group A: $r = 0.63$, $p = 0.003$; group B: $r = 0.51$, $p = 0.002$; group C: $r = 0.68$, $p = 0.001$). In addition, an association was found between IgG1 and IgG2 ($r = 0.75$, $p = 0.0002$) as well as between IgG1 and IgG4 ($r = 0.80$, $p = 0.0001$) in group C. Group A is different in that these associations were not seen. In group B, while IgG1 and IgG2 ($r = 0.48$, $p = 0.03$) or IgG3 ($r = 0.51$, $p = 0.02$) were correlated, the IgG1 and IgG4 responses ($r = 0.17$, $p = 0.47$) were not.

Antibodies to Avidin

The responses to avidin differed from those seen with OVA: (1) the responses were significantly lower, and (2), these were confined mainly to the IgG1 and IgG2 subclasses, with IgG4 notably absent. However, the occurrence of

the IgG1 antibodies – the major subclass for this antigen – was similar to that of the IgG1 anti-OVA antibodies. Thus, similar levels of antibodies were found in group A (0.19 units, 75% responders) and group B (0.16 units, 85% responders), while markedly lower responses were seen in the oldest children (group C, 0.03 units, 50% responders). Low levels (0.07–0.13 units) of IgG2 antibodies were found in 60–75% of the children throughout the ages.

The IgG1 and IgG2 responses were correlated for both group A ($r = 0.66$, $p = 0.002$) and group B ($r = 0.89$, $p = 0.0001$).

Antibodies to PNC

The responses to PNC resembled the antiavidin responses in that these were restricted to the IgG1 and IgG2 subclasses. These responses, however, differed in other ways: (1), anti-PNC responses were found predominantly in IgG2 (rather than IgG1), and (2), the distribution of these

(IgG2) antibodies among the different age groups was different. Thus, the youngest children (group A) developed very little of these antibodies (0.14 units, 95% responders), but the concentration of these increased dramatically in the 6- to 7-year-old children (group B; 0.50 units, 100% responders). Still higher levels (0.85 units) were found in the oldest age group (group C, all responders), although the difference from those of group B was not significant. The small group of atopic children examined (group D) showed higher levels (0.93 units) than their healthy counterparts (group B), but the difference was not significant.

The IgG1 response was slightly different from that of IgG2, resembling, in fact, the IgG2 anti-OVA response. Thus, the youngest children (group A) had very low activities (0.07 units, 70% responders), but these increased significantly in the older children (group B; 0.37 units, 95% responders). However, these then decreased significantly (0.19 units, 90% responders) in the next age group (group C).

The difference in occurrence between the IgG1 and IgG2 antibodies is reflected by the lack of correlation between these activities in group A ($r = 0.41$, $p = 0.07$) and group C ($r = 0.27$, $p = 0.25$) children. Correlation was, however, seen in group B ($r = 0.60$, $p = 0.005$).

Antibodies to Both OVA and Avidin

The high IgG1 responders to avidin in group A also produced good IgG1 responses to OVA ($r = 0.53$, $p = 0.02$). However, there was no such association in group B children ($r = 0.28$, $p = 0.21$).

Antibodies to Both OVA and PNC

A cursory investigation of the antibody activities to OVA and PNC from the same individual in atopic children (group D) revealed an interesting finding. The better IgG2 responders to OVA, who also showed high IgG4 responses to this antigen, produced little IgG2 antibodies to PNC. Conversely, the better responders to PNC made only modest amounts of anti-OVA antibodies. Regression analysis, however, revealed no inverse correlation between the anti-PNC responses and the IgG2 ($r = 0.59$, $p = 0.13$) or IgG4 ($r = 0.46$, $p = 0.25$) anti-OVA responses.

In age-matched normal children (group B), many of the good IgG2 responders to PNC also showed excellent IgG2 and IgG4 anti-OVA responses. As a group, however, there was no correlation between the anti-PNC responses and the IgG2 ($r = 0.20$, $p = 0.38$) or IgG4 ($r = 0.03$, $p = 0.90$) anti-OVA responses.

Discussion

Consistent with a previous study in adults [7], the anti-OVA response in all our study populations was found largely in the IgG1, IgG2, and IgG4 subclasses. The IgG3 levels were relatively low; although this could be due to the poor activity of the reagent (isotype specific) antibody used, Husby et al. [7] also found an absence of IgG3 antibodies in their subjects. It should be noted, nevertheless, that antibody subclass quantitation, in general, depends very much on affinity and specificity of the reagent antibody used; the anti-IgG2 monoclonal antibody (HP6014), for example, is known to be biased somehow in its reactivity by the type of light chain present in the target protein.

In general, the anti-OVA antibodies increased in concentration from infants (1.5–2.5 years, group A) to the older children (6–7 years, group B), but these decreased significantly in the oldest individuals (15–17 years, group C). This differs from the Japanese children studied by Okahata et al. [13] in whom anti-OVA antibodies (IgG1, IgG4, total IgG, and IgA) were found to peak in levels at an earlier age (1 year). The reason for the discrepancy is not clear, but may be analogous to our earlier finding that the total IgG1 and IgG2 levels in our children matured at later ages than in Caucasian children [11].

Unlike previous studies, we followed the different IgG subclass activities for each antigen in each individual. By so doing, we found that group C individuals were unique in that all four subclass antibodies to OVA appeared to be coordinately expressed, i.e., the good responders for IgG1 also produced high levels of IgG2, IgG3, and IgG4. In contrast, group A children were different in that the IgG1 and IgG2 or IgG4 responses were not correlated. However, in these children, as well as in children of other age groups, there was a good association between IgG1 and IgG3 and between IgG2 and IgG4 responses. The latter was also observed in atopic children.

The IgG1-IgG3 and IgG2-IgG4 linkages break down, however, in the anti-PNC and antiavidin responses. Here, IgG1 and IgG2 antibodies were found almost exclusively in the absence of the other subclasses. A similar isotypic restriction was observed previously in the anti-PNC response [6].

We found a progressive acquisition of IgG2 anti-PNC antibodies in our children as they grew, similar to the development of total IgG antibodies to this antigen observed previously [14]. IgG1 anti-PNC antibodies, on the other hand, followed a different pattern of distribution among the age groups, similar to that of the anti-OVA response. Thus, the level of antibodies dropped significantly in the oldest age

group from the group before. This unusual bias towards IgG2 production with increasing age is interesting both teleologically and from the point of view of gene regulation. The lack of correlation between the IgG1 and IgG2 responses in group C differentiates these children from those in group B and also the anti-PNC response from the anti-OVA response in group C children.

The results obtained with avidin were unexpected. Since it is a protein like OVA and comes from the same source [15], it would be expected to behave like OVA. Instead, it resembles PNC more with regard to isotypic restriction. This suggests that the carbohydrate moiety attached to the polypeptide chains of the molecule [16] is immunodominant or more resistant to degradation than the protein components. Indeed, the generally poor response seen with avidin in our subjects most probably results from the extensive degradation or heat denaturation of the molecule. Alternatively, antigenic fragments of avidin which are orally absorbed could be neutralized (bound) by biotin present in the body. Another possibility is that biotin present in the test serum blocks the reagent avidin used in the immunoassay. This, however, is unlikely, since no biotin was detected in our samples (data not shown).

Avidin, while resembling PNC in isotypic restriction, behave more like OVA in the distribution of responses generated. Indeed, the good antiavidin responders seen in group A were also good responders to OVA. Avidin is thus a very unique immunogen.

The decline in antibody response to both OVA and avidin towards adulthood is presumably the result of oral tolerance being developed against these antigens – their constant ab-

sorption from the oral tract [17]. In contrast, acquisition of anti-PNC antibodies in our children is probably the result of repeated exposure to *S. pneumoniae* through natural infection (rather than from vaccination). It should be noted, however, that a dominant epitope in PNC is phosphorylcholine [12], and antibodies to this can be induced by other phosphorylcholine-bearing organisms.

A small group of atopic children were included in the study to see whether they had abnormal antibody responses to OVA and PNC which are thymus dependent and thymus-independent antigens, respectively [12]. Previous studies revealed an impairment of cell-mediated function [18] and an elevation of IgG4 antibodies to OVA and other thymus-dependent antigens [13, 19] in atopic individuals. We did not find an elevated IgG4 anti-OVA response in our atopic children but, on the contrary, the levels of these antibodies as well as those of the IgG2 subclass were marginally depressed. This discrepancy from previous findings may stem from the fact that the children participating in our study were older. These children, on the other hand, appeared to have elevated levels of IgG2 anti-PNC antibodies and an inverse correlation between the anti-PNC and anti-OVA responses. However, these unusual observations are not substantiated at the moment, and larger groups of subjects would be required to verify these.

Acknowledgements

We thank D. Mahayani for technical assistance and Jenny Mok for typing the manuscript.

References

- Schur PH: IgG subclass: A review. *Ann Allergy* 1987;58:89–100.
- Umetsu DT, Ambrosino DM, Quinti I, Siber GR, Geha RS: Recurrent sinopulmonary infection and impaired antibody response to bacterial capsule polysaccharide antigen in children with selective IgG-subclass deficiency. *N Engl J Med* 1985;313:1247–1251.
- Shield JPH, Strobel S, Levinsky RJ, Morgan G: Immunodeficiency presenting as hypergamma-globulinaemia with IgG2 subclass deficiency. *Lancet* 1992;340:448–450.
- Beard LJ, Ferrante A: IgG4 deficiency in IgA-deficient patients. *Pediatr Infect Dis J* 1989;8:705–709.
- Layton GT, Stanworth DR: The quantitation of IgG4 antibodies to three common food allergens by ELISA with monoclonal anti-IgG4. *J Immunol Methods* 1984;73:347–355.
- Freijd A, Hammarstrom L, Persson MAA, Smith CIE: Plasma anti-pneumococcal antibody activity of the IgG class and subclass in otitis-prone children. *Clin Exp Immunol* 1984;56:233–238.
- Husby S, Oxelius V-A, Teisner B, Jensenius JC, Svehag S-E: Humoral immunity to dietary antigens in healthy adults. *Int Arch Allergy Appl Immunol* 1985;77:416–422.
- Skvaril F, Schild U: Characterisation of the subclasses and light chain types of IgG antibodies to rubella. *Clin Exp Immunol* 1984;55:671–676.
- Rossi ME, Azzari C, Resti M, Appendino C, Pezzati P, Vierucci A: Selectivity in IgG subclass response to hepatitis B vaccine in infants born to HBsAg-positive mothers. *Clin Exp Immunol* 1988;72:196–200.
- Lau YL, Jones BM, Yeung CY: Biphasic rise of serum immunoglobulins G and A and sex influence on serum immunoglobulin M in normal Chinese children. *J Paediatr Child Health* 1992;28:240–243.
- Lau YL, Jones BM, NG KW, Yeung C-Y: Percentile ranges for serum IgG subclass concentrations in healthy Chinese children. *Clin Exp Immunol* 1993;91:337–341.

- 12 Lim PL, Choy WF: A thymus-independent (type 1) phosphorylcholine antigen isolated from *Trichinella spiralis* protects mice against pneumococcal infection. *Immunology* 1990; 60:443–448.
- 13 Okahata H, Nishi Y, Mizoguchi N, Yumiba C, Fuji H, Ueda K: Development of serum *Derma-tophagoides farinae*, ovalbumin- and lactalbumin-specific IgG, IgG1, IgG4, IgA and IgM in children with bronchial asthma/allergic rhinitis or atopic dermatitis. *Clin Exp Allergy* 1990;20: 39–44.
- 14 Windebank KP, Faux JA, Chapel HM: ELISA determination of IgG antibodies to pneumococcal capsular polysaccharides in a group of children. *J Immunol Methods* 1987;104:143–148.
- 15 White HB III, Whitehead CC: Role of avidin and other biotin-binding proteins in the deposition and distribution of biotin in chicken eggs. *Biochem J* 1987;241:677–684.
- 16 Budavari S, (ed): *The Merck Index*, ed 11. Rahway, Merck, 1989.
- 17 Newby TJ, Stokes CR: *Local Immune Responses in the Gut*. Boca Raton, CRC Press, 1984.
- 18 Rees J, Friedman PS, Mathews JNS: Contact sensitivity to dinitrochlorobenzene is impaired in atopic subjects. *Arch Dermatol* 1990;126: 1173–1175.
- 19 Falconer AE, Friedman PS, Bird P, Calvert JE: Abnormal immunoglobulin G subclass production in response to keyhole limpet haemocyanin in atopic patients. *Clin Exp Immunol* 1992;89: 495–499.