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Distinctive development of IgG4 subclass antibodies in the primary and secondary responses to keyhole limpet haemocyanin in man

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SUMMARY

The human primary and secondary IgG subclass antibody responses to keyhole limpet haemocyanin (KLH) have been measured by ELISA using IgG subclass-specific monoclonal antibodies. KLH-specific IgG1 and IgG2 antibodies were detected 3 weeks after primary immunization, and IgG1, IgG2 and IgG4 antibodies after secondary immunization. IgG3 antibodies were observed less frequently in both primary and secondary responses. Unlike the other subclasses, IgG4 antibodies developed very slowly during the primary response, with no antibody detected at 3 weeks and often with only low titres 1 year after immunization. In one individual, this IgG4 primary response peaked around 10 months, but there was considerable variation between individuals. Comparing primary and secondary responses, the greatest increase in KLH antibody was for the IgG4 subclass (45-fold rise), followed by IgG1 (7·3-fold rise), whilst IgG2 and IgG3 KLH-specific antibodies did not show a significantly increased secondary response. There was no detectable IgG4 antibody response when secondary immunization was performed 1 month after the primary, even though IgG1, IgG2 and IgG3 antibodies were present. Reasons for the different time–course of IgG4 anti-KLH development and the isotype-related differences in 'memory' responses are discussed.

INTRODUCTION

There are four subclasses of human IgG, the heavy-chain constant regions of which are encoded by distinct genes on chromosome 14. The differing amino acid sequences of their heavy chains imparts each subclass with distinct properties, most importantly those relating to the activation of biological effector mechanisms (Burton, Gregory & Jefferis, 1986). This is of particular interest when it is realized that antibody activity is not evenly distributed over the four IgG subclasses, and different types of antigen elicit characteristically different IgG subclass profiles. For example, anti-carbohydrate antibodies are usually IgG2- or, less often, IgG1-restricted (Yount *et al.*, 1968; Bird *et al.*, 1984; Amlot *et al.*, 1986), red cell and viral antibodies are predominantly IgG1 and IgG3 (Skvaril, 1986), whilst injected protein antigens, e.g. tetanus toxoid and phospholipase A2 (PLA2) in bee venom, induce IgG1 and IgG4 antibodies preferentially (Seppälä *et al.*, 1984, Aalberse, Gaag & Leeuwen, 1983).

There is evidence that the IgG subclass response is modified by repeated antigenic stimulation (Devey, Wilson & Wheeler, 1976). In a group of novice beekeepers, the proportion of IgG4 in the total IgG anti-PLA2 antibody was found to increase with time and the number of bee stings (Aalberse *et al.*, 1983). However, a detailed analysis of the human primary and secondary antibody response is lacking. For many natural antigens it is difficult to follow a defined primary and secondary exposure, as previous antigenic encounters by ingestion, infection or immunization cannot be excluded. Keyhole limpet haemocyanin, KLH, a high molecular weight glycoprotein extracted from the lymph of an inedible Pacific ocean shellfish, has been used to assess immune function as a thymus-dependent protein neo-antigen (Amlot *et al.*, 1986; Curtis *et al.*, 1970).

The development and distribution of antibodies of each human IgG subclass in primary and secondary responses to KLH after intradermal injection, allowing an interval of 1 year between primary and secondary immunization, has been studied and is reported here. A highly distinctive pattern of IgG4 KLH antibody development was observed.

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MATERIALS AND METHODS

Subjects

Fourteen laboratory staff (five female, nine male; average age 31 years, range 22–43 years) were immunized. These subjects were included in a previous report (Amlot *et al.*, 1986). A further nine subjects (mean age 50 years) were immunized subcutaneously to follow the time–course of IgG4 antibody development to KLH in the year following primary immunization.

Antigens

Keyhole limpet haemocyanin (KLH) was purified from an ammonium sulphate slurry (Pacific Biomarine Lab. Inc., CA), according to Herscowitz, Harold & Stavitsky (1972), and was filter sterilized and stored in vials as a solution at 30 mg/ml with 0·01% merthiolate at 4°. It was shown to be sterile, pyrogen and endotoxin free.

Immunisation schedule

Serum was analysed from subjects bled before (Day 0) and 21 days after both primary and secondary immunization, the latter being approximately 1 year later. On both occasions subjects were immunized intradermally with KLH (0·2 mg in 0·1 ml of saline) in the upper outer arm. Incidentally to this report, subjects were also immunized in the other arm with 0·2 mg of DNP–Ficoll. These time-points for IgG subclass analysis were chosen after consideration of previous data analysing the development of the total IgG response to KLH (Amlot *et al.*, 1986).

Anti-KLH antibody ELISA

Purified KLH was coated overnight to flexible Falcon PVC plates at 1 mg/ml in borate-buffered saline (BBS), pH 8·2. After washing in phosphate-buffered saline (PBS)/0·05% Tween 20, the wells were filled with 100 μl of PBS/Tween containing 3 mg/ml of bovine serum albumin (BSA) (Sigma Chemical Co. Ltd, St Louis, MO). Doubling dilutions of serum samples were made, starting with a 1/10 dilution.

For the assay of IgG antibody to KLH, the plates were incubated for 2 hr at 18–20°, washed in PBS/Tween and then incubated for a further 2 hr with 1/500 dilution of rabbit anti-human IgG conjugated to horseradish peroxidase (HRP) (Dako Ltd, High Wycombe, Bucks) in PBS/Tween/BSA, before washing and developing the plates with orthophenylene diamine (OPD; Sigma) as substrate.

For the assay of individual IgG subclass KLH antibodies, the sera were incubated for 4 hr on the KLH-coated ELISA plates at 20°, washed, and incubated overnight at 4° with 1/200–1/500 dilution of IgG subclass-specific mouse monoclonal antibodies as ascitic fluids; NL16 for IgG1, GOM2 for IgG2, ZG4 for IgG3 and RJ4 for IgG4 (Lowe *et al.*, 1982). These monoclonal antibodies have been shown previously to be isotype-specific in ELISA (Lowe *et al.*, 1982) (donated by Dr N. R. Ling, University of Birmingham). In addition, human/mouse chimaeric anti-NP (4-hydroxy-5-nitrophenylacetic acid) antibodies of each human IgG subclass (European Collection of Animal Cell Culture, Porton Down, Sussex) (Bruggemann *et al.*, 1987) have been used in NP-specific ELISA to check the specificity of each ascitic fluid. After washing, the plates were incubated with 1/1000 dilution of rabbit anti-mouse IgG conjugated to HRP (Dako), preabsorbed against human IgG in PBS/Tween/BSA for 1 hr at 37° and developed with OPD as substrate.

A standard serum was included on every ELISA plate. One-thousand units per millilitre were arbitrarily assigned as the value of IgG, IgG1, IgG2 and IgG4 anti-KLH in the standard serum, and 100 U/ml as the value of IgG3 anti-KLH. These arbitrary assignments allowed the sensitivity (defined as twice the background optical density) of all subclass assays to be of a similar order of magnitude (log scale) and were approximately

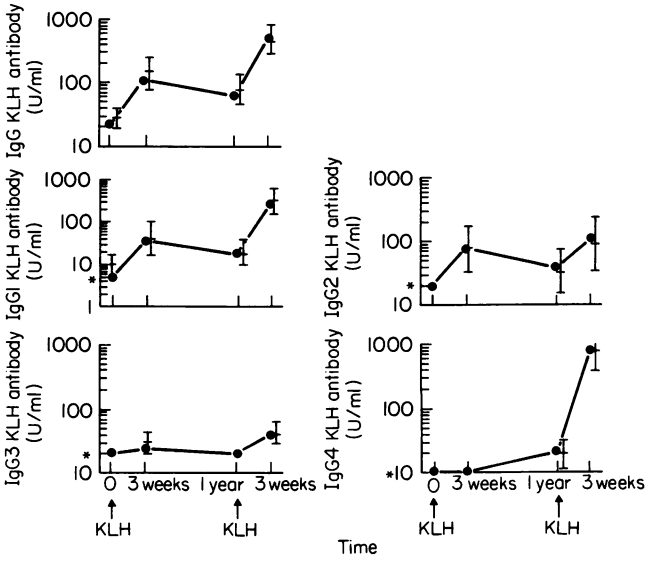

Figure 1. Quantitative changes in serum anti-KLH with time for IgG, IgG1, IgG2, IgG3 and IgG4. The median response is shown together with geometric means and their 95% confidence limits ($\pm SE \times t_{n-1}$) at each time-point. * Indicates the limit of sensitivity within each ELISA. NB: the units for different IgG subclasses are not comparable.

5 U/ml for IgG1, 10 U/ml for IgG4 and 20 U/ml for IgG3 and IgG2. Standard curves relating the mean optical density at 492 nm for duplicate dilutions of the standard serum on each plate to the U/ml of standard serum were constructed and the U/ml of the relevant anti-KLH isotype in the unknown sera were determined from these.

Within the IgG, IgG1, IgG3 and IgG4 isotype-specific ELISA, the dilution curves for different sera were all approximately parallel to each other and to the standard, but within the IgG2 KLH antibody ELISA the slope of the dilution curves varied between sera. This variation may have resulted from differences in the avidity of IgG2 KLH antibodies (Peterfy, Kuusela & Mäkelä, 1983). To minimize these effects, IgG2 antibodies were quantified near the detection limit of the ELISA (Lew, 1984).

Statistical analysis

Individual anti-KLH responses within each isotype were compared after log transformation using a paired *t*-test. Similarly, the ratios of antibody titres at different times during the response were compared by paired *t*-tests after log transformation.

RESULTS

Changes within each IgG subclass of anti-KLH antibodies during a primary and secondary response

The quantitative distribution and development of IgG, IgG1, IgG2, IgG3 and IgG4 KLH antibodies from 14 subjects is shown in Fig. 1. The median response is shown and, at time-points where all or most subjects have a detectable response, the geometric mean of the KLH antibody titre is also shown with its 95% confidence limits.

Prior to immunization. Sera from 13/14 subjects contained significant titres (i.e. greater than twice the background) of IgG

Table 1. Geometric mean and median KLH antibody titre 3 weeks after immunization

	Primary		Secondary			
	Geometric mean	Median	Geometric mean	Median	Ratio	*P**
IgG	128	108	445	496	3·5	<0·001
IgG1	40	35	288	250	7·2	<0·001
IgG2	76	77	80	105	1·1	NS
IgG3	29	24	41	40	1·4	NS
IgG4	17†	21†	783	785	46	<0·001

*Comparison of secondary and primary titres by a paired *t*-test.
†Primary IgG4 response at 1 year. No detectable primary IgG4 response at 3 weeks.

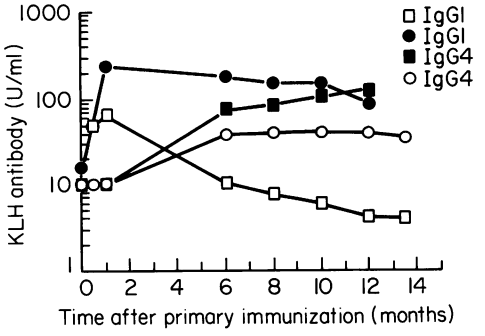

Figure 2. Detailed time-courses for the late primary IgG1 and IgG4 response in two individuals. The limits of sensitivity of each IgG subclass were approximately 5 U/ml for IgG1 and 20 U/ml for IgG4.

KLH antibodies, and approximately half of these had detectable IgG1 and/or IgG2 KLH antibodies. No reactivity was detected in these sera when the ELISA plates were not coated with KLH. Such 'natural' antibodies have been encountered previously and appeared to be cross-reacting antibodies with extremely low binding avidities and/or other ligand-binding properties because they are not inhibited by a vast excess of free KLH (Amlot *et al.*, 1986).

Primary response. Three weeks after primary immunization, KLH-specific antibody was detected within the IgG1, IgG2 and IgG3 isotypes in 92%, 85% and 61% of positive IgG antibody responders, respectively. However, IgG4 antibodies were completely undetectable at this time in any subject.

One year following primary immunization, there were significant falls in IgG, IgG1, IgG2 and IgG3 KLH-specific antibodies ($P < 0{\cdot}05$ by paired *t*-tests), although total IgG, IgG1 and IgG2 antibodies were still raised compared to pre-immunization levels ($P < 0{\cdot}05$ by paired *t*-tests). In contrast, IgG4 antibodies, undetectable at 3 weeks, clearly increased over the same period, even though titres were generally low (median 21 U/ml).

The absence of a detectable IgG4 anti-KLH antibody response 3 weeks after primary immunization might theoretically result from competitive inhibition in the ELISA due to KLH-specific antibodies of other isotypes including IgM. However, when sera taken at 3 weeks and 1 year after primary immunization were mixed and assayed for IgG4 KLH-specific antibodies, there was no alteration in the total IgG4 anti-KLH titre, suggesting that competition in the ELISA was not the explanation for the failure to detect an early IgG4 anti-KLH response.

Secondary response. After a second KLH immunization, anti-KLH antibodies were found in all four IgG subclasses, with frequencies of 100% for IgG1 and IgG4 and 75% for IgG2 and IgG3. When the mean or median titres attained in the primary and secondary responses were compared, clear differences were seen between the IgG subclasses, and these are summarized in Table 1. A comparison of the ratio, for each subject, of the antibody titre in the secondary response at 3 weeks to that of the primary response (at 3 weeks for IgG, IgG1, IgG2 and IgG3 and at 1 year for IgG4) shows a remarkable rise in the secondary IgG4 anti-KLH response, with IgG1 showing a significant but less spectacularly increased secondary response. In contrast, there was no significant increase in the 3-week secondary compared to the 3-week primary antibody mean titre for the IgG2 or IgG3 isotypes.

Detailed time-course of the late primary response

Only two of the subjects with a late primary IgG4 KLH antibody response (Fig. 1) were bled at 4 months post-primary immunization, and there was no detectable IgG4 antibody to KLH in either of these samples.

Nine subjects immunized *subcutaneously* with KLH were analysed in more detail for the development of the late primary IgG4 anti-KLH response. In only 2/9 subjects' sera at 1 year were IgG4 KLH-specific antibodies detected, and in these the IgG4 antibody responses arose late and continued rising throughout the first year, whereas other IgG subclasses tended to peak about 3 weeks post-immunization and to fall thereafter (Fig. 2). The reason for this lower number of positive primary IgG4 responses in this group is unclear: mean serum IgG4 levels were lower in this group (213 μg/ml compared to 482 μg/ml), but mean total IgG anti-KLH responses at 3 weeks were not significantly lower (114 U/ml compared to 128 U/ml). Differences in the route of immunization (subcutaneous compared to intradermal) may have contributed.

Repeat KLH immunization 1 month after the primary

Although a primary IgG4 KLH antibody response cannot be detected before 6 months, it is possible that early re-immunization with KLH could lead to the production of detectable serum IgG4 KLH antibody. To test this, four subjects were re-immunized with KLH 1 month following primary immunization, and two of these four subjects were further immunized with KLH at 3 months. Figure 3 contrasts the IgG1 and IgG4 KLH antibody response in these two individuals. Following secondary KLH immunization at 1 month, no IgG4 antibody response was detectable in the serum of any of the four subjects 2 or 6 weeks later, despite rising IgG1 (4/4 subjects), IgG2 (4/4 subjects) and IgG3 (3/4 subjects) anti-KLH responses. However, IgG4 anti-KLH (2/2 subjects) could be detected 2 weeks following tertiary KLH immunization (14 weeks following primary immunization) at titres not dissimilar from the range of IgG4 antibody titres detected following secondary KLH immu-

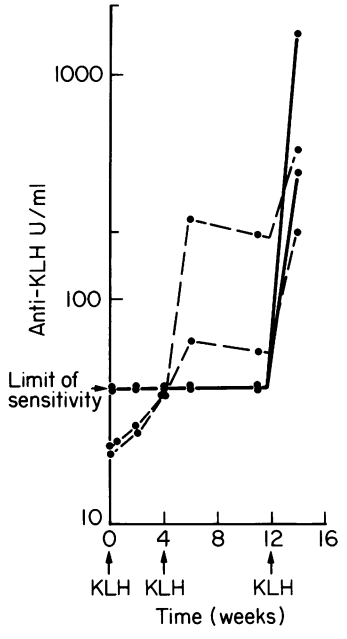

Figure 3. A comparison of the IgG1 (- - -) and IgG4 (——) antibody responses to KLH in two individuals following secondary and tertiary administration of KLH at 1 and 3 months.

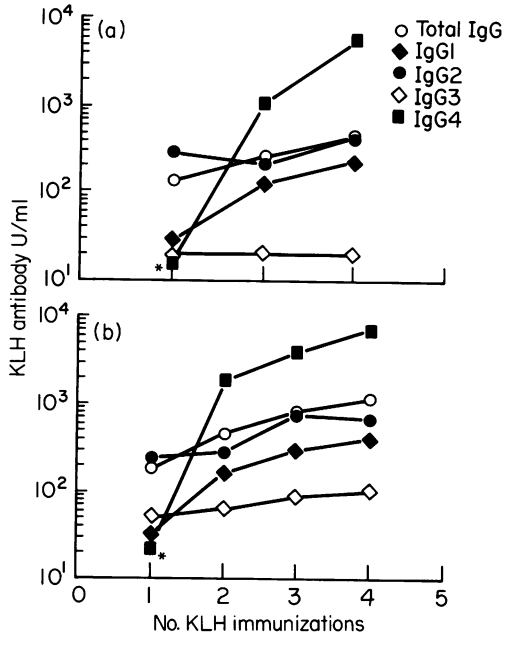

Figure 4. The effect of repeated KLH immunization on the development of KLH antibodies of each IgG subclass in two individuals (a and b). The KLH antibody titre for each isotype 3 weeks following KLH immunization is shown, except for the primary IgG4 response where the 1-year titre is shown (marked *). NB: the different IgG subclass units are not comparable.

nization at 1 year (Fig. 1). The two subjects not re-immunized with KLH at 12 weeks failed to produce IgG4 KLH antibodies at 14 weeks.

Relative change in isotype of anti-KLH response following repeated immunization over a long time period

Following repeated bee stings, individuals are reported to show an increasing proportion of IgG4 in the total IgG antibody (Aalberse *et al.*, 1983). It was of interest to see if the IgG4 antibody response to KLH continued to increase at a relatively much greater rate than that of the other IgG subclasses following repeated KLH immunization over a prolonged time period. Two individuals were immunized repeatedly with KLH, with each administration at least 1 year apart. The resulting IgG subclass antibody titres, 3 weeks after each immunization, are shown in Fig. 4. Direct comparison of the units for each isotype is not possible, but it appears that the increase in titre of total IgG and IgG1 is very similar (as indicated by similar slopes), whilst the increase in IgG4 antibody titre with repeat KLH immunization is greater most particularly between the primary and secondary response, as shown earlier. The titre of IgG2 and IgG3 antibodies increased relatively more slowly than that of IgG. Therefore, with repeated KLH immunization over a long time period, the proportion of IgG4 and IgG1 will tend to increase, whilst the proportion of IgG2- and IgG3-specific antibody will fall.

DISCUSSION

This is a detailed analysis of human IgG subclasses produced during a defined primary and secondary response to intradermal injection of a T-dependent antigen, in this case the glycoprotein KLH, and it has led to two new observations. First, the IgG4 response differs markedly in its development from that of IgG1, IgG2 or IgG3. Second, immunological memory, as measured by an increased secondary/primary response, varies strikingly for different IgG subclasses.

The results presented here have been obtained with antigen-specific ELISA using mouse monoclonal antibodies to detect KLH antibodies of each IgG subclass. It has not been possible to compare the quantity of antibody between the different IgG subclasses because of inherent differences in assay sensitivity using mAb with unique affinities. Despite this, it would appear from the frequency of their detection that IgG1 and IgG2 KLH antibodies predominate early in the primary response and that IgG1, IgG2, and IgG4 are usually present in the secondary response a year later. IgG3 antibodies were observed less often in both responses.

The IgG4 anti-KLH response had a completely different time-course from that of the other subclasses. It was undetectable between 1 and 4 months following primary immunization, but was frequently detected 1 year later. More detailed time-courses showed that IgG4 KLH antibodies rose in titre at 6 months at a time when IgG1 antibody titres were falling. The IgG4 KLH antibody titre reached a peak at 10 months in one subject and was still rising at 1 year in another. In addition, it was not possible to induce a secondary IgG4 antibody response at one month, suggesting that some event required for the maturation of IgG4-committed B cells was delayed.

IgG4 KLH antibodies rose in titre (at least 45-fold) more than any other subclass when primary and secondary responses were compared. The IgG4 KLH antibody titre also showed a relatively greater increase following further KLH immunization than that of any other IgG subclass. This is in agreement with the previously observed predominance of IgG4 following

continued antigenic challenge by other protein antigens, e.g. bee stings and food allergens (Aalberse et al., 1983, Layton & Stanworth, 1984).

The comparison of secondary and primary antibody responses may indicate which IgG isotypes are preferentially produced by 'memory' B cells. The data here suggest that 'memory' is not evenly distributed between the IgG isotypes (at least in the antibody response to KLH). 'Memory', by this definition appears to reside mainly in IgG1 and IgG4 isotypes, while there appears to be little, if any, IgG2 'memory'. IgG2 responses are associated with T-independent antigens and previous studies on human antibody responses to the 'de novo' T-independent antigen, DNP–Ficoll, have suggested that B-cell memory may not be generated to such antigens, at least in the sense suggested here for T-dependent antigens (Amlot *et al.*, 1986; Amlot & Hayes, 1985). Likewise, the index for IgG3 'memory' was very small. Responses to cytomegalovirus and rubella virus showed higher proportions of IgG3 antibody in primary than in secondary infections (Linde *et al.*, 1983; Linde, 1985), which could support the concept that 'memory' may not exist for IgG3 subclass responses. The relative change of 3·5-fold in the total IgG antibodies between primary and secondary responses encompasses these variations within the individual IgG subclasses.

Selective pressure for one or other IgG subclass in the antibody response to antigen may be determined by (i) the type of antigen presentation; (ii) the quality or quantity of T-cell help; and/or (iii) inherent B-cell control mechanisms.

Antigen presentation. About 7 days after immunization with KLH, delayed hypersensitivity reactions occur at the site of intradermal immunization (Amlot *et al.*, 1986), which probably represent the appearance of recirculating KLH-specific T cells released from lymph nodes, leading to the final clearing and degradation of free KLH in the body. Between Days 7 and 14 after immunization there is a sharp rise in IgG antibody, and by the 21st day there is detectable KLH antibody of IgG1, IgG2 and IgG3 subclasses. Sites at which antigen (in the form of immune complexes) can persist for long periods of time are on follicular dendritic cells within germinal centres (Mandel *et al.*, 1980; Klaus *et al.*, 1980). Somatic mutation of IgV genes, IgH class switching and B-memory cell development may all occur within the germinal centres and the localization of antigen at that site is dependent on complement fixation either directly or by immune complexes (MacLennan & Gray, 1986; Rajewsky, Forster & Cumano, 1987). The very prolonged time–course of IgG4 antibody development suggests that this may depend on long-term antigen retention on follicular dendritic cells within the germinal centre. In support of this is the recent observation that IgG4 is the only subclass to be severely depressed in genetically determined complement deficiency involving the classical pathway of C3 activation (Bird & Lachmann, 1988).

T-cell help. There is evidence in the mouse that different lymphokines influence the production of different IgG subclasses (Snapper & Paul, 1987; Bergstedt-Lindquist *et al.*, 1984) and that distinct types of T-helper cells preferentially produce different lymphokines. Thus, in mouse, TH1 cells have been defined which secrete interleukin-2 and interferon-gamma, and contribute to the production of IgG2a responses, whereas TH2 cells, secreting interleukins-4 and -5, preferentially promote IgG1 production (Coffman *et al.*, 1988). Although a similar division of T-helper cells may not hold true for human T cells, T cells with differing lymphokine production have been reported in man (Umetsu *et al.*, 1988; Dohlstein *et al.*, 1988). In man it is not clearly defined to what extent IgG subclass regulation is lymphokine dependent. The ability to provide help for IgE production may correlate with the secretion of particular cytokines by T-cell clones, in particular IL-4 (Del Prete *et al.*, 1988; Pene *et al.*, 1988), and similar mechanisms may regulate IgG4 production. One possibility, consistent with the data presented here, is that the quality of T-cell help available changes during the course of a primary response to KLH and following a secondary response, thus altering the IgG subclasses produced. Qualitative differences between 'naive' and antigen-stimulated helper T cells have been reported in mice responding to KLH: the frequency of IL-4-secreting T cells was low in lymphocytes freshly isolated from mice, although many cells produced IL-2; however, after restimulation with antigen *in vitro*, the relative frequency of IL-4-secreting cells increased (Powers, Abbas & Miller, 1988). Furthermore, T cells have been identified within germinal centres that are phenotypically distinct from recirculating T cells (Poppema *et al.*, 1983) whose lymphokine secretion could play a role in affecting IgG isotype production.

B-cell control. The late primary IgG4 response might simply reflect delayed class switching to this isotype, which might be related to the distal Cγ4 gene position (Flanagan & Rabbitts, 1982). If switching to this gene is a rare event a period of expansion of committed cells might be needed before the antibody could be detected. It is also possible that, some time following the first exposure to antigen, IgG4-committed B cells are generated (perhaps within germinal centres) but do not at this time undergo further maturation. It is only then, during the secondary response, that they differentiate into antibody-secreting cells. This would be consistent with the greatly increased IgG4 KLH antibody titre seen in the secondary response. In this case, the late primary IgG4 KLH antibody response might reflect a low level of stimulation of these committed B cells by a small amount of persisting KLH.

Factors influencing the isotype production by B cells during an immune response are gradually being identified and much of our knowledge depends upon murine experimentation. Man and mouse have a similar number of IgG isotypes, which is probably fortuitous as there is great species variation and the subclass regulation in these two species may be very different. Detailed analysis of primary and secondary responses to defined antigens in man may help define common as well as divergent features of isotype production between mouse and man. The delayed IgG4 KLH antibody response in man is an example of a finding not anticipated from murine studies.

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