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Quantitative and spectrotypic analysis of paternal IgG2a expression in normal and allotype-suppressed mice

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Summary. The synthesis and clonal diversity of IgG2a molecules bearing the paternally inherited immunoglobulin allotype have been examined in the offspring of matings between BALB/c mothers (Igh-1^a) and SJL or C57BL/10 males (both Igh-1^b) using a sensitive quantitative single radial immunodiffusion in gel assay and isoelectric focussing with autoradiography. In normal litters, the first detectable paternally-marked IgG2a is extensively polyclonal in both F_1 crosses (i.e. diversity precedes expression); however, there is a delay of 2–3 weeks in the first appearance of the clonally diverse set of molecules when these are coded by the SJL genome, compared with the C57BL/10. Delayed maturation of allelically-excluded Igh-1^b-expressing B cells in the (BALB/c × SJL)F_1 may explain the unique susceptibility of these offspring to chronic allotype suppression when exposed to maternal anti-Igh-1^b antibodies in early life. We find that, although such suppressed mice may begin life with a (delayed) synthesis of polyclonal IgG2a of paternal allele (Igh-1^b), the condition of chronic suppression later imposed in the majority of mice is associated with spectrotype (clonal) simplicity.

INTRODUCTION

Modulation of immunoglobulin expression in offspring by maternal alloantibody was first described by Dray (1962) who found that exposure of young rabbits to maternal antibody directed against a paternal immunoglobulin alloantigen (allotype—in this case a1) resulted in long-term suppression of expression of the marker in the serum of the offspring. Subsequently, several attempts, all unsuccessful, were made to induce a similar chronic condition in the offspring of allotype-disparate parental mice after immunization of the breeding female (Lieberman & Dray, 1964). Herzenberg *et al.* (1967) described a short-term suppression of Igh-1^b (IgG2a) in alloantibody-exposed (BALB/c × C57BL/10)F_1 mice, but this was probably a phenomenon of target molecule neutralization. It was not until the SJL strain was used as the male Igh-1^b partner that chronic antibody-induced allotype suppression was successfully demonstrated in mice by Jacobson & Herzenberg (1972). Further work on this model showed that suppression was restricted to the Igh-1 locus coding for IgG2a heavy chains and was mediated by T suppressor cells which have been postulated to delete or inactivate allotype-specific (restricted) T helper cells (Herzenberg *et al.*, 1976). The BALB/c × SJL combination remains unique in its susceptibility to allotype suppression, but the underlying reasons for this remain as unclear as the exact mechanisms of suppression itself. Herzenberg & Herzenberg (1974) claim to have found no differences between the suppressible (BALB/c × SJL)F_1 and the non-suppressible (BALB/c × C57BL/10)F_1 in respect of Igh-1^b synthetic capacity, although it has to be said

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that the quantitative and temporal data in this study are not of sufficient detail to be certain of this conclusion.

Important clues to the mechanisms of allotype suppression can be adduced from determining, *vis a vis* antibody synthesis and its diversity, the state of differentiation of the target B cells, and so quantitative and spectrotypic analysis of Igh-1^b synthesis in suppressible and non-suppressible F_1 combinations is a relevant study to undertake. We find that the suppressible F_1 mouse shows an inherent delayed maturation of Igh-1^b (paternal) IgG2a synthesis compared with the non-suppressible (in chronic form) F_1 combination. Under conditions of suppression, the (BALB/c × SJL)F_1 shows a pattern of first, delayed polyclonal synthesis and then polyclonality, alternating, over about the first 6 months, with episodes of clonal restriction. A large proportion of animals then enter a condition of permanent clonal restriction of the suppressed paternal allotype which, in some animals, is extreme to the extent of total suppression but, in others, however, leaves one or a few dominant clones which are expressed throughout the remainder of life.

MATERIALS AND METHODS

Animals

The mouse inbred strains BALB/c, C57BL/10 and SJL have been previously used by us in the context of the suppression model (Appleby & Catty, 1983). The mice were bred and maintained in the department's closed colony under clean, but not germ-free, conditions and were all derived from genetically-monitored, isolator-reared stock.

Suppression

This is generated in (BALB/c × SJL)F_1 (Igh-1^{ab}) allotype heterozygotes by transmission of maternal anti-allotype antibody (anti-Igh-1^b) following the method of Herzenberg & Herzenberg (1978). (BALB/c × C57BL/10)F_1 mice were produced under similar antibody exposure. Control F_1 mice of both hybrids were born to unimmunized BALB/c mothers.

Quantitation of Igh-1^b

The Igh-1^b-marked IgG2a in mouse sera was measured by the single radial immunodiffusion (SRID) assay using thin layer agarose gel as described by Appleby & Catty (1983). A high titre precipitating anti-Igh-1^b alloantibody prepared in BALB/c mice was incorporated in this gel. A serum pool from a large number of 36-week-old (BALB/c × SJL)F_1 mice served as a standard allotype antigen for the assay; results are expressed as the percentage of the Igh-1^b serum level of the standard. Interplate coefficient of variation, determined on 60 SRID plates, was 9·6%.

Haemagglutination inhibition (HAI) was used to detect trace quantities of Igh-1^b, using a technique slightly modified from Mage *et al.* (1973). Sheep red blood cells (SRBC) were coated with highly purified Igh-1^b IgG2a by the chromic chloride method of Goding (1976). The coating antigen was the product of the PC101 plasmacytoma grown in the CB20 (Igh-1^b) congenic strain. This was purified from ascitic fluid by euglobulin precipitation in distilled water and fractionation on CM Sephadex G50 (Pharmacia, Uppsala, Sweden) in a starting buffer of 0·1 M phosphate, pH 5·0, with 0·2 M saline and with a 16 hr linear pH gradient formed by addition of 0·1 M phosphate, pH 9·0, with 0·2 M saline. Igh-1^b-sensitised SRBC were tested for proper coating with a reference anti-Igh-1^b serum. This was then diluted in PBS to two dilutions less than the end point (1:4000). Samples of neonatal and juvenile mouse serum and standard serum were diluted out in Cooke microtitre round-bottom trays in PBS plus 1% fetal calf serum (FCS) diluent. An equal volume (25 μl) of the agglutinating dilution of the anti-Igh-1^b serum was then added to all wells, the mixture shaken and incubated for 30 min at room temperature; 25 μl of 0·5% suspension of coated SRBC were then added. The final test serum dilution showing inhibition of agglutination was taken as the end point, which was compared with that achieved with the standard serum and the titre expressed as a dilution percentage of the standard.

Spectrotypic analysis of allotype

Isoelectric focussing (IEF) and autoradiography (ARG), to provide a visual assay of Igh-1^b clones in sera, were performed as described by Appleby & Catty (1984). Briefly, sera obtained from mice from a few days of age to 18 months were focussed and the plates then exposed to affinity-purified ^{125}I-labelled antibody to Igh-1^b allotype. After extensive washing, the dried gels were developed for autoradiography and then stained for protein (immunoglobulin) bands.

RESULTS

Concentration of serum Igh-1^b

The first appearance and subsequent rise in levels of

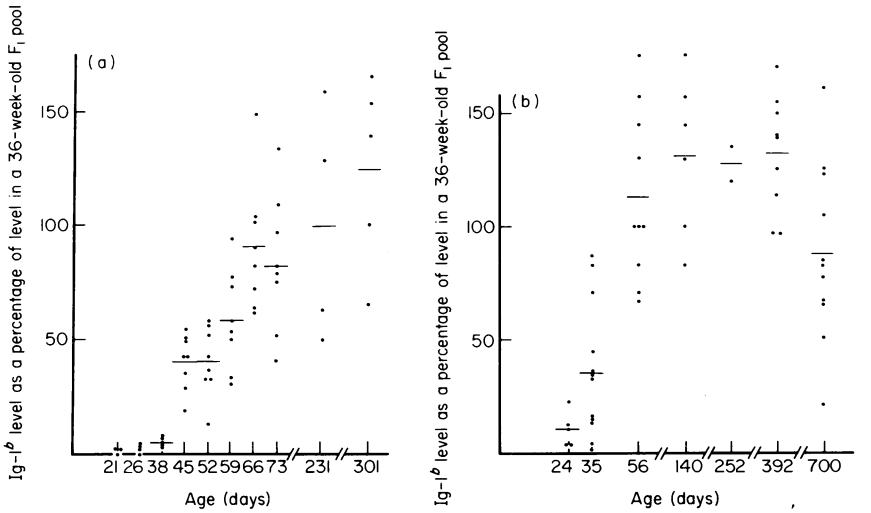

Figure 1. Development of Ig-1^b in (a) normal BALB/c × SJL F$_1$ hybrids and (b) normal BALB/c × C57BL/10 F$_1$ hybrids. Dots represent the concentration of Igh-1^b in the serum of individual mice at the ages after birth indicated. The short bars represent the mean of these values at each age.

Igh-1^b in sera of normal BALB/c F$_1$ hybrids with male SJL and C57BL/10, selected at random from several litters, is shown in Fig. 1a, b. Because of the difficulties and risks of bleeding very small animals, the numbers selected are small; however, it is evident that the SJL hybrid shows a pronounced delay (2–3 weeks) in IgG2a maturation of cells allelically restricted to the paternal allotype, compared with the C57BL/10 hybrid. The SJL genome contributes only trace amounts of Igh-1^b up to at least 5 weeks of age, and the mice are over 2 months old before they reach adult allotype levels. In neonatal and juvenile mice, HAI assays reveal a trace of paternal allotype in the C57BL/10 hybrids by 5 days post-partum and substantial levels by weaning, whereas in the SJL hybrid, nothing could be detected until 16–20 days of age, and this remained less than 1% of adult allotype levels at 24 days of age (Table 1).

It is evident that the IgG2a C_H gene of the SJL genome in the F$_1$ mice is delayed in activation until several weeks after birth, and then only slowly begins expression compared with the equivalent gene system of the C57BL/10 hybrid.

In the next experiment, expression of the paternal allotype was examined in young mice of both hybrids after exposure to the maternal alloantibody suppression routine that generates allotypic suppression in the Igh-1^{ab} hybrids of SJL origin (Fig. 2a, b). In all but a small proportion of the SJL hybrids, as expected, there

Table 1. HAI data on appearance of Igh-1^b in serum of neonatal BALB/c × SJL and BALB/c × C57 F$_1$ mice

Age (days)	BALB/c × SJL*	BALB/c × C57BL/10*
1	0	>0·1
5	0	0·2
8	0	0·4
12	0	0·4
16	>0·1	ND†
20	1·0	12·5
24	0·6	21·2

* Concentration expressed as a percentage of that found in an adult (BALB/c × SJL) F$_1$ serum pool.
† ND, not done.

is a profound and long-lasting allotype suppression—an initial period of variable suppression is followed (by 30 weeks) by a state of permanent chronic suppression. By contrast, the C57BL/10 hybrids show no sign of a long-term effect of exposure to antibody, although a delay in first appearance of allotype to the 5th week, and a slower maturation thereafter, is noted; however, as substantial maternal anti-allotype antibody persists in serum in hybrid Igh-1^{ab} mice to at least 4 weeks of age (Appleby & Catty, 1983), the delayed appearance of free allotype in the C57BL/10 hybrid is probably a simple neutralization effect and not a

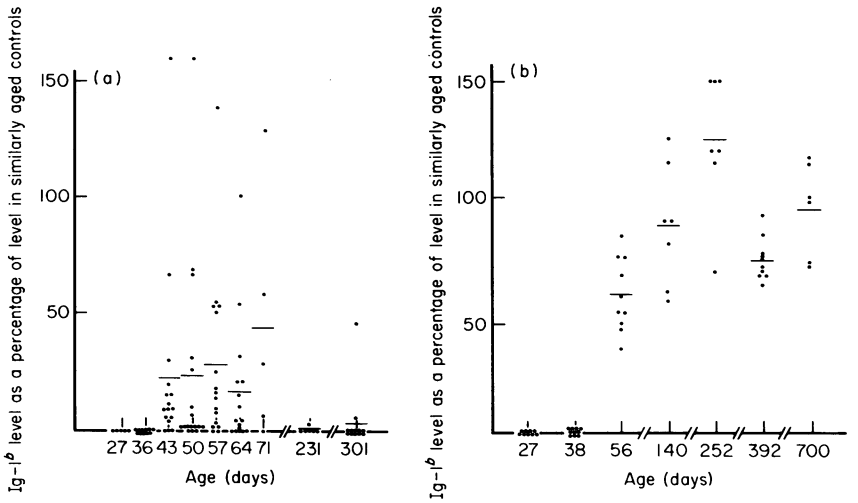

Figure 2. Development of Igh-1^b in (a) BALB/c × SJL F_1 hybrids exposed to maternal anti-Ig-1^b antibody and (b) BALB/c × C57BL/10 F_1 hybrids exposed to maternal anti-Ig-1^b antibody. Dots represent the concentration of Igh-1^b in the serum of individual mice at the ages after birth indicated. The short bars represent the mean of these values at each age.

transient suppression of synthesis, as postulated by Herzenberg *et al.* (1967).

We then undertook a study of suppression in the SJL hybrid in two larger groups of litters exposed to alloantibody and in a control group, with analysis extending to at least 78 weeks or longer. This was designed to assess antibody dose effects, the proportion of mice entering chronic profound suppression and the clonal restrictions on allotype synthesis imposed on the B cells as a result of suppression. In Fig. 3a, the serum Igh-1^b levels are shown in a group of 22 mice entering the experiment and followed for 83 weeks. The BALB/c mothers were preimmunized with Igh-1^b, and were boosted intraperitoneally with anti Igh-1^b 10 days before and again 1–5 days after parturition.

Suppression follows two patterns in this group: after an initial period of allotype synthesis by nearly all subjects (many to adult levels) for up to 11 weeks, some mice then develop chronic total or sub-total suppression. It must be stressed that it is the same mice, by and large, week by week, which show suppression—the pattern is not the result of sudden switch-off in synthesis by some mice and a spontaneous renewal of synthesis by others. The second pattern represents cyclically imposed episodes of partial suppression with intermittent recovery and with a final longer period of normal synthesis beyond 1 year of age. As all SRID assays were done blind and not in weekly groups, the synchronous behaviour evident in this pattern of response is not due to assay variables.

In a control group of 12 SJL hybrid mice followed over 102 weeks (Fig. 3b), the pooled serum from the samples taken after 9 weeks show an almost identical Igh-1^b level to that of the standard 36-week pool. Individually, beyond 6 weeks of age, although a remarkable range of allotype concentration is evident, synthesis occurs in all mice, mostly to achieve levels approaching or above the standard, and the weekly means lie, with one marginal exception, above this line. One can conclude that the patterns of suppression exhibited in the experimental groups of Fig. 3a & c are the result of antibody-induced effects and not the result of naturally variable patterns of paternal allotype expression in the BALB/c × SJL hybrid.

In Fig. 3c, a second group of 30 suppressed hybrids was followed for 78 weeks. In this group, the six BALB/c mothers were selected for high titre antibody before mating to SJL males. The mothers were boosted with Igh-1^b after mating and also after parturition. In addition, the newborn mice were given intraperitoneal injections of DEAE-purified antibody on a number of occasions up to 2 weeks of age. Under this routine, it is interesting to note that, although about half the mice up to 17 weeks of age produced adult levels of allotype, beyond this time an increasing number entered total or severe suppression under

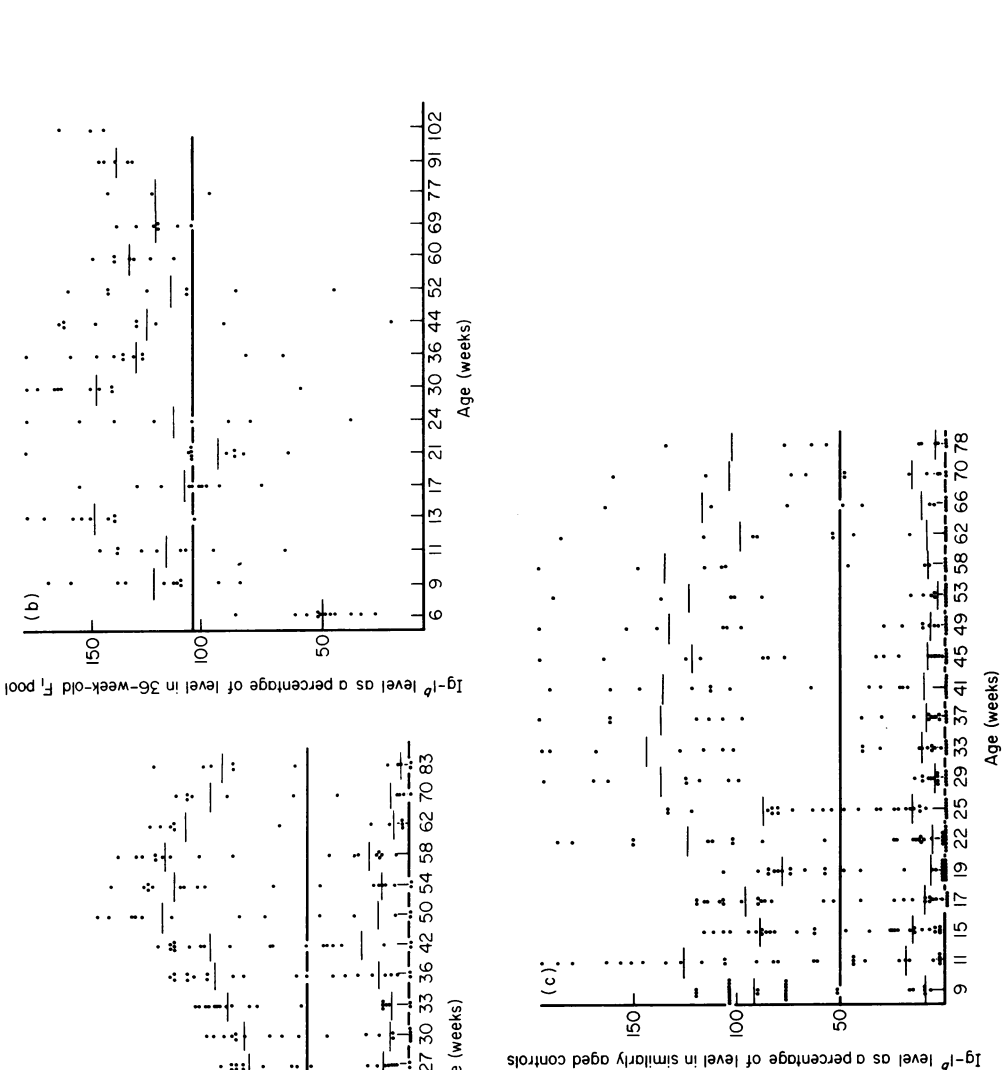

Figure 3. Igh-1^b levels in (a) BALB/c × SJL F$_1$ mice exposed to maternal anti-Igh-1^b antibody, (b) normal BALB/c × SJL F$_1$ hybrids over 2 years, and (c) BALB/c × SJL F$_1$ mice exposed to maternal anti-Igh-1^b antibody. Dots represent the concentration of the Igh-1^b allotype in the serum of individual mice at the ages indicated. (a), (c) The long bar represents the 50% dividing line distinguishing between Igh-1^b suppressed (< 50%) and non-suppressed (> 50%) mice, while the short bars are the mean Igh-1^b concentrations of the suppressed or non-suppressed mice at each age. (b) The long bar represents the overall population mean for serum Igh-1^b concentration in normal BALB/c × SJL F$_1$ mice. The short bars give the mean values at the different ages indicated.

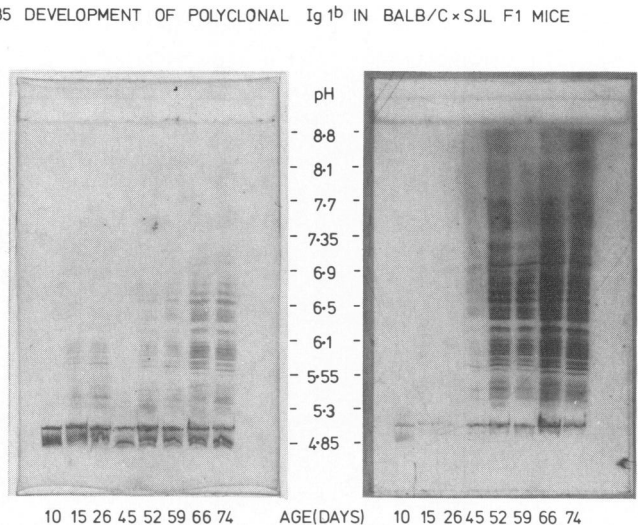

Figure 4. The spectrotype of Igh-1^b revealed by autoradiography using ^{125}I-labelled anti-Igh-1^b, (left) stained plate and (right) autoradiograph. The serum concentration of Igh-1^b, expressed as a percentage of similarly aged controls, is as follows: days 10, 15 & 26, 0%; day 45, 1·9%; day 52, 36%; day 59, 53%; day 66, 83%; day 74, 98%.

these more stringent induction conditions. A proportion of mice remained permanently uninfluenced, however, so there is considerable biological variation in mice of the same genetic constitution in their response to suppressing antibody. In Fig. 3a & c, there is an arbitrary 50% cut-off line to differentiate between suppressed and non-suppressed populations of mice. In order to validate statistically this impression of two populations divided by response, and to determine whether separation of the data at other arbitrary points gave more significant distinctions between the populations, cut-offs at 40%, 50% and 60% were compared by the standard Student's *t*-test. The serum allotype levels were log-transformed to allow the *t*-test to be applied to non-parametric data. Values falling on the cut-off line were taken as belonging to the lower, suppressed population. Analysis showed that cut-offs between 40% and 60% of adult pool Igh-1^b serum concentrations do not critically affect the distinctions between suppressed and non-suppressed populations which remain as highly significant ($P > 0{\cdot}001$) at all three levels.

Clonal analysis of Igh-1^b

In Fig. 4, the synthesis of Igh-1^b is traced against the pattern of total immunoglobulin in the serum of one typical normal SJL F_1 hybrid from 10 days of age. Although immunoglobulins are found on day 15 by protein staining, there is no detectable Igh-1^b until day 45. However, when this allotype is first detectable by autoradiography, it is already extensively polyclonal. This is consistent with the sudden onset of synthesis at about 6 weeks observed in quantitative studies (e.g. Fig. 2a). We conclude that the use of anti-allotype to monitor clonal expression of the paternal IgG2a molecule, uncomplicated by the presence of maternal antibodies, reveals a new and sudden expression of a pre-existing diversity, and not a slow progressive but random output of new variable region sequences (specificities) to be identified as single isolated clones with limited starting complexity, as might be anticipated if diversity was driven by random exposure to antigens after birth. A similar pattern of allotype expression was noted in other control SJL hybrids and also in the C57BL/10 hybrids, although, in the latter case, the synthesis of allotype was detected earlier, but again with initial polyclonality.

The typical spectrotypic pattern of Igh-1^b allotype exhibited in SJL hybrids exposed to the antibody suppression routine (i.e. Fig. 3a b c) is shown for 6–39 weeks in Fig. 5, and from 37–79 weeks in Fig. 6. At 9 and 12 weeks, mice produce polyclonal allotype, but by 18 weeks, in this example, synthesis is suppressed and clonally restricted, only to return at 21 weeks to a polyclonal condition followed by an episode of extreme suppression (29 weeks) and clonal restriction at 39 weeks. The sensitivity of the assay is notable, as

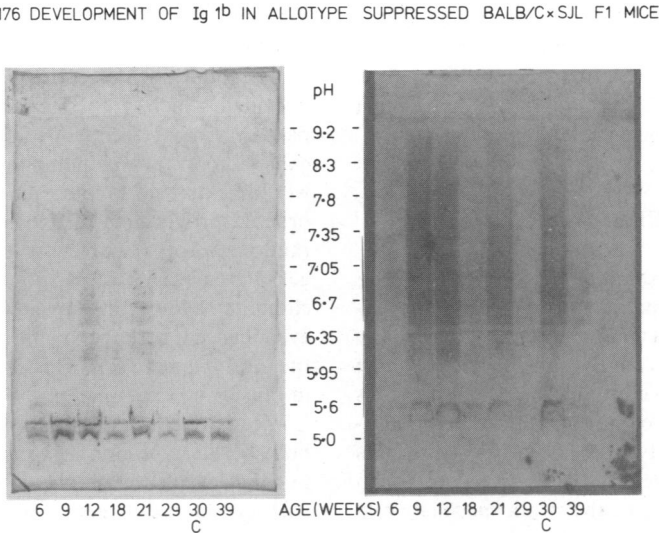

Figure 5. As for Fig. 4, except C = normal control serum. The concentration of Igh-1^b is as follows: week 6, 0%; week 9, 50%; week 12, 50%; week 18, 10%; week 21, 15%; week 29, 0%; week 39, 5%.

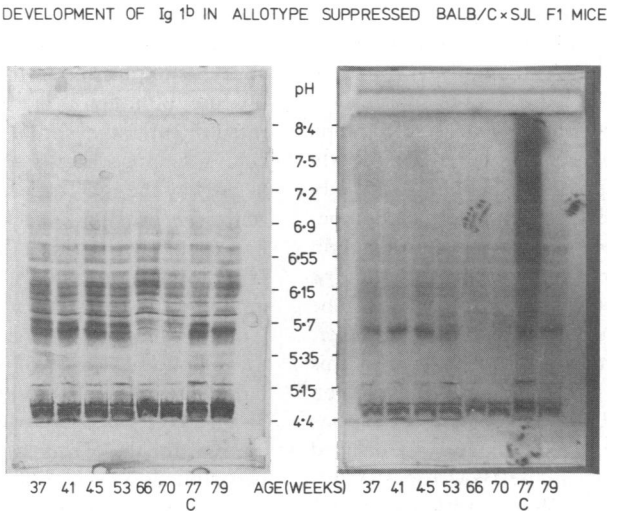

Figure 6. As for Fig. 5, except the concentration of Igh-1^b is as follows: week 37, 5%; week 41, 0%; week 45, 3%; week 53, 4%; week 66, 0%; week 70, 2·5%; week 79, 0%.

the control sample carries intense labelling at concentrations that barely register on the protein-stained plate. The period of extended chronic allotype suppression which is imposed on many mice after 6 months of age is well illustrated in Fig. 6 by a remarkable perseverance of expression of limited clones of allotype, often (as in this case) with a single dominant product which is lost for 6 months and then returns. During this extended period, clonal patterns in other focussed IgG subclasses, or of maternal IgG2a, come and go but the specificity of the allotype labelling gives no hint of these on the autoradiograph. In this plate, the normal polyclonal pattern of allotype of 77-week-old SJL hybrids is shown (c). It is clear that normal synthesis occurs with molecules focussing in the pH 6–8 region. In suppressed mice, where synthesis

is often less than 5% of adult normal levels, the clonal pattern is restricted, both in number and in pH range—usually lying between pH 5·5 and 7·0, and the main bulk of paternal IgG2a expression in the high pH ranges is absent or below detectable levels.

DISCUSSION

In this paper, we have examined the long-term modulating effects of maternal alloantibody on the expression of Igh-1^b target allotype coded for by the paternal IgG2a C_H gene of F_1 Igh-1^{ab} heterozygote mice—this work being a continuation of our previous studies of transmission of passive anti-allotype antibody (Appleby & Catty, 1983, 1984). The results present new quantitative and qualitative data which add to the understanding of the strain specificity and mechanism of murine immunoglobulin allotype suppression.

The first significant finding is the delayed maturation of Igh-1^b B cells in the SJL hybrid, which is a suppressible combination, compared with the C57 hybrid, which shows no long-term suppression effects. This phenomenon aids an understanding of the unique susceptibility of the SJL gene product to suppression in the hybrid. The circumstance of delayed allotype synthesis may assist suppression by maternal antibody in two ways. Firstly, suppressing maternal antibody can survive longer to affect nascent paternal allotype-expressing cells where no serum antigen neutralization occurs. The level of transmission of anti-allotype antibody does not vary significantly between the suppressible and refractory hybrids. Later, however, there is evidence of a more rapid clearance of this antibody in the C57BL/10 hybrid, once transmission from the maternal (BALB/c) milk to the serum has ceased (Appleby & Catty, 1983). Secondly, the delayed onset of Igh-1^b synthesis in the (BALB/c × SJL)F_1 implies a prolonged period of immaturity of allotype-restricted B cells and/or T helper cells—a circumstance which would, by analogy with concepts of tolerance induction in the neonate (Nossal & Pike, 1978), lead to a condition of allotype-positive receptor blockade or stripping which, in this case, would lead to allotype-specific B-cell abortion or anergy instead of antigen-(tolerogen)-specific effects.

The use of isoelectric focussing has enabled an investigation of the clonal development in paternal allotype synthesis and the effects of antibody suppression on clonal expression in susceptible hosts. Although of exquisite sensitivity, the autoradiographic labelling of Igh-1^b molecules in young mouse serum could not detect any juvenile phase, in either hybrid, in which synthesis begins with restricted clonal patterns; there seems, on the contrary, a sudden explosion of polyclonal allotype expression which would be difficult to interpret on the basis of either an ordered programme of expression of diversity, or a random process of diversity expression through antigen-driven events. The spontaneous burst of polyclonality favours, in our view, a sudden maturation event caused, perhaps, by the self-driven expansion of the idiotypic network. This concept will be addressed in our future experiments.

When we come to examine the effects of alloantibody on allotype clonal expression, we can document in considerable detail a chain of events which begins with polyclonal diversity in synthesis, during which both allotype-specific T helper and B lymphocytes are working normally, and there is a balanced activity of T suppressors and T helpers—this phase then shifts to a sequence of suppression with, often extreme, clonal restrictions.

The mechanisms of induction and maintenance of allotype suppression in the SJL hybrid mouse remain obscure, although it is assumed to depend upon the recruitment and eventual dominance of allotype-specific T suppressor cells which are found to influence negatively the Igh-1^b specific helper cell activity (Herzenberg *et al.*, 1976; Black & Herzenberg, 1979). Our finding of clonal restrictions in severely suppressed mice supports a concept of sub-total T helper cell inhibition, leaving a few 'leaks' in the panoply of induced suppression which might possibly arise through local losses of suppression or occasional induction of B cells to synthesis of IgG2a without T-cell help. Once a clone escapes, it is possible to account for the observed restricted clonal dominance persisting as a feature of suppressed animals through successive generations of memory cells. Phillips & Waldman (1977) and Doenhoff *et al.* (1979) have shown that removal of substantial elements of T helper activity by limiting dilution reconstitution, or via partial *in vivo* T-cell depletion, resulted in clonally-restricted responses to specific antigens. The clonal restrictions observed in allotype suppression can be seen as operating also through a reduction in numbers of allotype-specific T helper cells. We note that the persistent restricted clones expressed in chronic murine allotype suppression exist in a pH range usually lower than 7·0, whereas the majority of normal Igh-1^b

polyclonal molecules focus to a higher pH range (Appleby & Catty, 1980, 1984). Antibody of similar restriction and of similar pI value is selectively transferred to the neonatal mouse during suckling, and we suppose that antibody of this character confers some special protective and survival advantages upon the young mouse (Appleby & Catty, 1984). We are intrigued by the possibility that persistent 'escaping' clones of IgG2a of paternal allotype in the suppression model are of particular significance, as responses to critical environmental antigens (e.g. of micro-organisms) and perhaps represent dominant germ line V gene products expressed through early clonal selection in the young mouse and thereby brought forward to a non-suppressible state of development before the alloantibody-dependent induction of suppression becomes pervasive within the B-cell population.

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