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Intraclonal diversification in immunoglobulin isotype secretion: an analysis of switch probabilities

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The production of all immunoglobulin isotypes except IgD was studied in a large number of single lipopolysaccharide (LPS)-reactive B cell clones. The majority, but not all, of the IgM-producing clones were found to secrete one or more other isotypes. IgG_3 and IgG_{2b} were most frequently found while IgA secretion was extremely rare. Many clones produced all four IgG subclasses and the statistical analysis of the data indicates, with a high degree of significance, that single clonal precursors give rise to progenies producing multiple isotypes. By assuming that intraclonal diversification follows the C-gene order in chromosome 12, the absolute switch probabilities of normal, unprimed LPS-reactive B cells can be calculated. The multi-potentiality of C-gene expression was further analyzed at the single cell level: a sizeable fraction of all activated B cells express two different IgG isotypes in the membrane-bound form, indicating consecutive switch events. In contrast, the majority of IgE and IgA secreting cells appear to switch directly from IgM. These results might reflect the functional relevance of S-region homologies in the control of C-gene expression.

Key words: immunoglobulin isotypes/B lympocytes/C-gene expression

Introduction

The control of immunoglobulin C-gene expression by B lymphocytes can be considered on the basis of two major alternatives: either individual B lymphocytes are 'precommitted' to the expression of one C gene (Abney *et al.*, 1978) in addition to μ and δ, or normal B cells are multi-potent as to the isotype they can produce upon activation. If the first alternative is correct, controls of C-gene expression must be studied along the pathways of precursor B cell differentiation where commitment takes place. In contrast, if immunocompetent B cells or their clonal progenies can produce multiple isotypes, then the control of C-gene expression operates along with the mechanisms driving immune responses, in particular growth and maturation of B lymphocytes. Now that molecular biology approaches can be used to analyze physiological situations, it appears of importance to settle this point in order to select the correct experimental systems.

In order to analyze those alternatives, we have studied immunoglobulin isotype production by single clones of B cells reactive to the mitogen lipopolyaccharide (LPS). The cloning by limiting dilution of normal LPS-reactive B lymphocytes has previously allowed for the determination of the absolute frequencies of clonal progenitors giving rise to IgM secreting cells (Andersson *et al.*, 1977) as well as of clones producing the different isotypes (Benner *et al.*, 1981). The same methods were used to demonstrate intraclonal switches from IgM to IgG (Andersson *et al.*, 1978).

This approach presents several advantages in the study of isotype production: (i) the cells involved are normal B lymphocytes, activated directly by the mitogen; (ii) the plating efficiency is very high, since every second or third B cell develops into a clone (Andersson *et al.*, 1977); and (iii) the stimulation is polyclonal, and therefore it proceeds in the absence of V-region selection or any other V-gene related controls.

The results obtained show that single immunocompetent lymphocytes can give rise to progenies producing multiple isotypes with different probabilities, and that single B cells can undergo more than one switch event.

Results

Frequencies of LPS-reactive clones producing the various immunoglobulin isotypes

The frequencies of LPS-reactive clonable precursors giving rise to progenies producing the various isotypes has previously been determined by detecting class-specific plaque forming cells (p.f.c.) (Benner *et al.*, 1981). We have now determined clonal frequencies for all known isotypes (except IgD) using a solid phase enzyme immunoassay, the sensitivity and specificity of which are described in Materials and methods. The isotype-specific assay detects at least 1 ng/ml of any isotype, while the production from single clones under our experimental conditions should be expected to be in the range of 5 – 50 ng/culture: it is therefore suitable for detection of all isotype switches that result in high rate secretion.

Table I shows the summary of such frequency determinations. IgG_3 and IgG_{2b} are the most frequent isotypes, ~ 3 – 4 times more frequent than IgG_1 and IgG_{2a}. A small number of clones secreted IgE, while IgA is detected so rarely that precise frequency determinations cannot be achieved.

The results are in general agreement with previously reported ones: a significant difference is however observed at the level of IgG_1 precursor frequency, higher in our determinations as compared to previous reports (Benner *et al.*, 1981). This can be due to different characteristics and sensitivity of the assays, one measuring numbers of secreting cells at a fixed point in time, the other detecting all molecules accumulated throughout the culture period. In addition, we observed that the amount of IgG_1 was always very low as compared to the amount of the other isotypes, and it probably corresponds to the product of a number of secreting cells too low to be detected or considered in a p.f.c. assay. This is in line with independent observations that secretion of, but not switch to, IgG_1 is selectively impaired in LPS cultures (Forni and Coutinho, 1982).

Production of more than one IgG isotype by single clones: p.f.c. analysis

Independent determinations of clonal frequencies for the various isotypes provide no indications as to the possibility that single clones can produce more than one isotype. We attempted to investigate this possibility by assaying individual

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cultures for the presence of secretors of more than one IgG subclass. For these experiments, a large number of cultures were set containing a small number of cells so that no more than 20–30% of all cultures would contain a precursor giving rise to a clone of immunoglobulin-secreting cells. Under these conditions, the probability of occurrence of more than one clone per culture is negligible, and the finding of p.f.c. of two isotypes in the same culture would demonstrate directly that a single precursor can diversify for isotype production during clonal expansion. All cultures that were found to contain immunoglobulins in the supernatant after 7 days were split and assayed for p.f.c. of two isotypes. Results of a number of such experiments where the simultaneous presence of IgG_3 and IgG_{2b} p.f.c. was tested, are shown in Table II. The number of cultures containing both isotypes is significantly higher than the number of cultures expected to contain more than one precursor. In addition, the two isotypes were significantly associated, which means that clones producing one of these two isotypes have a higher probability of producing the other than clones that do not produce the first isotype.

The distribution of the clone sizes observed in these experiments is shown in Figure 1. Two points can be made: (1) the average clone sizes for a given isotype are similar in clones containing one or both isotypes; and (ii) clones containing both IgG_3 and IgG_{2b} show good correlation between the number of p.f.c. in each class. This could indicate that the switches take place early in clonal development and roughly at the same time for both IgG subclasses, and that the efficiency of terminal maturation to p.f.c. is similar for both isotypes.

Since the simultaneous high rate secretion of two IgG isotypes has been excluded (Pernis *et al.*, 1976), we conclude that a single LPS-reactive B cell precursor gives rise to a progeny that undergoes intraclonal differentiation and diversifies as to the isotype the different members of the clone produce. Furthermore, since it has been previously shown that all IgG p.f.c. arise in single clones which also produce IgM (Andersson *et al.*, 1978; Wabl *et al.*, 1978), these results indicate that in the process of clonal expansion of a single IgM-producing precursor, switches to two IgG isotypes can occur, demonstrating lack of 'precommitment' of immunocompetent cells.

Similar experiments have been performed for other isotype combinations, namely IgG_1 and IgG_3, IgG_1 and IgG_{2b}, IgG_1 and IgG_{2a}. The number of double cultures in these cases was not significantly higher than expected for occurrence of more than one precursor. However, since only 480 cultures were tested for each combination, and due to the low frequency of IgG_1-producing clones, the absolute number of observations was too small to allow any conclusion on the significance of association.

Analysis of all isotypes produced in clonal cultures

The approach used above, while sufficient to demonstrate the production of two isotypes other than IgM by progenies of a given IgM precursor, is obviously limited by the characteristics and sensitivity of the assay, which is dependent on both the kinetics of the response and on the life-span of plasma cells. We have therefore assayed, in a solid phase immunoassay, the isotypes of immunoglobulins accumulated in the supernatant of single clonal cultures at the end of a culture period of 10 days. Table III shows a summary of the results indicating the numbers of cultures containing no im-

Table I. Approximate frequencies of LPS-reactive B cell precursors giving rise to clones producing the various isotypes in C3H/Tif or CBA/J mouse spleen

	IgM	IgG_3	IgG_1	IgG_{2b}	IgG_{2a}	IgE	IgA
Absolute frequencies	1:3–6	1:8–12	1:25–35	1:10–15	1:30–40	1:200	<1:2000
Switch frequencies	–	0.4	0.13	0.32	0.11	0.02	<0.002

Pooled results of four different experiments in which the number of spleen cells per culture was titrated in the convenient range (except for IgA), and of a large number of cultures all containing five spleen cells. The data conformed to the first order term of the Poisson distribution and were treated accordingly to derive the frequencies shown here. Switch frequencies for non-IgM isotypes were calculated on the basis of the observation that IgM production occurs invariably in clones producing any other isotypes.

Table II. Detection of IgG_3 and IgG_{2b} p.f.c. in clones of LPS-reactive B lymphocytes from C3H/Tif and C57BL/6 spleen

Exp.	Cells/ culture	No. cultures assayed	Positive clones			
			Total	IgG_3 only	IgG_{2b} only	IgG_3+ IgG_{2b}
1	2	480	57	11	11	15
2	2	480	87	11	7	6
3[a]	5	480	134	23	28	25
4	5	480	280	44	60	48
	Total	1920	558	89	106	94

[a]C57BL/6.

Statistical evaluations have been performed as suggested by Lefkovits and Waldmann (1979).

(1) For an average number of clones/culture (u) of 0.4 the fraction of responding cultures containing more than one clone is expected to be 0.208. The fraction of doubles out of all clones containing non-IgM isotypes observed in this experiment is 0.325. The difference between the observed and the expected values is highly significant ($\chi^2 = 18.2$).

(2) In the test for independence in the 2 x 2 contingency table, the expected number of doubles in this experiment is 65. The χ^2 of 29.7 indicates a highly significant association.

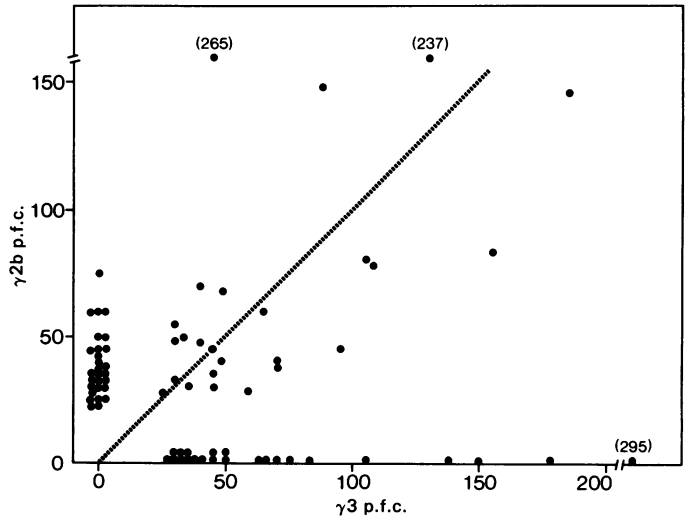

Fig. 1. Clone size distribution of IgG_3 and IgG_{2b} p.f.c., in LPS-activated clonal cultures containing the two isotypes either alone or mixed.

Table III. Analysis of all isotypes produced in clonal cultures

No. cultures analyzed: 2351; No. responding cultures: 1605; No. non-switched cultures (only IgM): 268; No. switched cultures (IgM + non-IgM): 1337.

Cultures containing non-IgM isotypes									
1		2		3		4		5	
γ3	273	γ3+γ1	73	γ3+γ1+γb	e85	all γ	108	all γ+ϵ	1
γ1	59	γ3+γ2b	197	γ3+γ1+γ2a	41	γ3+γ1+γ2b+ϵ	3	all γ+α	1
γ2b	178	γ3+γa	35	γ3+γ1+ϵ	4	γ3+γ2b+γ2a+ϵ	1		
γ2a	64	γ3+ϵ	4						
ϵ	7			γ3+γ2b+γ2a	78				
α	2	γ1+γ2b	35	γ3+γ2b+γ2a	3				
		γ1+γ2a	22						
		γ1+ϵ	3	γ3+γ2a+ϵ	2				
				γ3+γ2a+α	1				
		γ2b+γ2a	38						
		γ2b+ϵ	1	γ1+γ2b+γ2a	17				
		γ2a+ϵ	1						
Total	583		409		321		112		2

Statistical evaluation for expected frequencies of cultures containing more than one clone was calculated from: $F_r = \frac{u^r}{r!} \cdot e^{-u}$

(where u = number of clones per culture; F_r = fraction of responding cultures; F_1, F_2, F_3, F_4, F_5 = fraction of responding cultures containing 1, 2, 3, 4, 5 clones.
The calculations take into account: A. all responding cultures; B. all switched cultures.

	F_1	F_2	F_3	F_4	F_5
A. u = 1.14	0.536	0.301	0.115	0.042	0.007
B. u = 0.842	0.638	0.269	0.075	0.016	0.002
Observed frequencies	0.436	0.306	0.173	0.084	0.0015

χ^2 A = 128.8
B = 654.4

From the test for independence in 2 x 2 contingency tables, only γ3 and γ2b were significantly associated (χ^2 = 13.4).

Table IV. Multiple isotype production by single B cell clones: relative representation of the various isotypes

Isotype	% representation in clones containing				
	1	2	3	4	5
IgG_3	46.8	75.6	92.6	100	100
IgG_1	10.1	32.5	63.6	99.1	100
IgG_{2b}	30.5	66.3	79.2	100	100
IgG_{2a}	11.0	23.5	60.2	97.3	100
IgE	1.2	2.2	3.9	3.6	50
IgA	0.3	0	0.4	0	50

munoglobulins, IgM only, and one, two, three, four, and five non-IgM isotypes in addition to IgM. All the cultures contained five spleen cells, and 32% of them were found to contain no precursors at all.

From the Poisson distribution a number of cultures containing more than one precursor could be expected, but the calculations of these probabilities and the comparison of the expected and observed distribution demonstrate, with a high degree of significance, the possibility of multiple isotype production by single clones and, once more, a significant association of IgG_3 and IgG_{2b}.

Analysis of switch probabilities in LPS-activated B cell clones

The detailed analysis of the results reported in Table III is collected in Tables IV and V. This raises several considerations.

(i) About 20% of all clones produce IgM exclusively. This value is probably an underestimate in view of the probability of the simultaneous occurrence of one IgM-only clone with a switched clone in the same culture: still it shows that a switch is not an obligatory step in the life of a B cell.

(ii) As previously shown (Andersson *et al.*, 1978; Wabl *et al.*, 1978) the production of non-IgM isotypes is invariably accompanied by secretion of IgM, indicating that isotype switches do take place in culture, and that immunocompetent cells that can no longer mature to IgM secretion are not LPS-reactive.

Table V. Analysis of switch probabilities in B cell clones

Isotype	Absolute frequency of switch to this isotype[a]	Frequency of direct switch from IgM[b]	Frequency further switches downstream[c]	Frequency of of unique switches[d]
IgG_3	0.68	1	0.70	0.30
IgG_1	0.34	0.30	0.55	0.13
IgG_{2b}	0.56	0.19	0.30	0.13
IgG_{2a}	0.31	0.16	0.02	0.16
IgE	0.04	0.23	0	0.23
IgA	0.005	0.50	–	0.50

[a]Fraction of cultures containing the indicated isotype out of all switched cultures.
[b]Fraction of cultures containing a given isotype alone or mixed with downtream isotypes out of all cultures containing that isotype.
[c]Fraction of cultures containing a given isotype mixed with downstream isotypes out of all cultures containing that isotype.
[d]Fraction of cultures containing a given isotype and no other non-IgM isotypes out of all cultures containing that isotype.

(iii) Almost half of all clones (583 out of 1337) that produce non-IgM isotypes switch only once, and the frequencies of two, three, or four switch events in the same clone decreases linearly with the number of isotypes detected (Table IV).
(iv) Given the reasonable assumption that all single switches occurred from IgM to the detected isotype, the frequencies of single switches decreases from IgG_3, but it does not follow a 'gradient' along the chromosome: unique isotypes are detected with the order $IgG_3 > IgG_{2b} > IgG_{2a} \geq IgG_1 >$ IgE > IgA.
(v) The fraction of clones maturing to secretion without further switches is very similar for IgG_1, IgG_{2b}, and IgG_{2a}, in spite of the very different frequency of occurrence of these isotypes. This might indicate that we observed real events, rather than accumulations within a given clone.
(vi) The multiplicity of isotype production is primarily achieved by increased frequencies of switches to IgG_1 and IgG_{2a}, as shown by the representation of these isotypes in clones containing two and three non-IgM isotypes.
(vii) Considering now all clones containing any non-IgM isotype, the absolute frequencies of production follow the same order as for single switches (compare column 1, Table IV, with column 1, Table V).
(viii) In contrast, the frequency at which clonal progenies switch to a given isotype directly from IgM (regardless of continuing to switch or not) decays with the distance from $C\mu$ to the respective $C\gamma$ gene (Table V). This rule, however, does not apply to IgE and IgA, suggesting that while many switch events occur from one $C\gamma$ to another, direct switch from $C\mu$ to $C\epsilon$ or from $C\mu$ to $C\alpha$ occurs at a relatively high frequency, and that γ-to-ϵ or γ-to-α switches may be rare at the single cell level. Obviously, multiple isotype production in the same clone may result from independent switches from IgM or from consecutive switches (see below).
(ix) Finally, the probability of clones that have switched to a given isotype expressing other downstream C genes also decreases with the distance from $C\mu$.

From this analysis it appears that the absolute frequency of the clonal production of a given isotype, or the isotype distribution in mitogen-activated p.f.c. in mass cultures, result from several independent probabilities. Maintaining the postulate that backward switches do not occur, and that our assay detects all switches leading to secretion of immunoglobulins, the probabilities of isotype diversification in clones of LPS-reactive B cells are shown in Figure 2.

The figure represents probabilities at the clonal level and not at the single cell level, that is a clone containing IgG_3 and IgG_{2b} is represented as 'passing through' IgG_3 in the probability tree, although these two switch events may well have occurred independently, directly from IgM.

Observations at the single cell level: demonstration of γ-γ switches

In search of the possibility that switch events are not unique in the life history of a B lymphocyte, we have analyzed proliferating B cells by double immunofluorescence for expression of more than one IgG isotype. The double expression was investigated at three levels: (1) double expression of secretory form in the cytoplasm; (2) expression of one isotype on the membrane and another in the cytoplasm; and (3) double expression on the membrane.

The results were as follows: out of a few hundred secreting cells scored for each combination, we found no indications for double secretion of two IgG isotypes, in confirmation of previous results (Pernis *et al.*, 1976), indicating that high rate secretion of two non-IgM isotypes by the same cell is a rare event. On the other hand, when cells were studied for one γ isotype on the membrane, and another in the cytoplasm, a small but consistent proportion (ranging from 2 to 6% in different combinations) of cells secreting a given isotype were found bearing another γ isotype on the membrane. Interestingly, the membrane-bound isotype was invariably coded upstream of the secreted one. In fact we have observed IgG_{2b} and IgG_{2a} secretors bearing membrane-bound IgG_3 or IgG_1, but never IgG_3 secreting cells bearing other isotypes (except IgM) on the membrane; also we never detected IgG_{2b}-containing cells with IgG_{2a} on the membrane.

A much higher proportion of cells expressing two γ isotypes in the membrane-bound form were observed, and the results are summarized in Table VI. In this table, we report results obtained in two polyclonal responses, induced by a T-independent mitogen (LPS) or by T cells specifically activating a large set of B lymphocytes (Augustin and Coutinho, 1980; Coutinho *et al.*, 1981). This point is worth mentioning only as an indication that multiple switch events are not a peculiarity of LPS-induced responses. Preliminary analysis of isotypes in supernatants of T-cell induced B cell clones also show intraclonal diversification, although the absolute representation of the various γ isotypes is sharply different, in analogy with what is observed in mass cultures

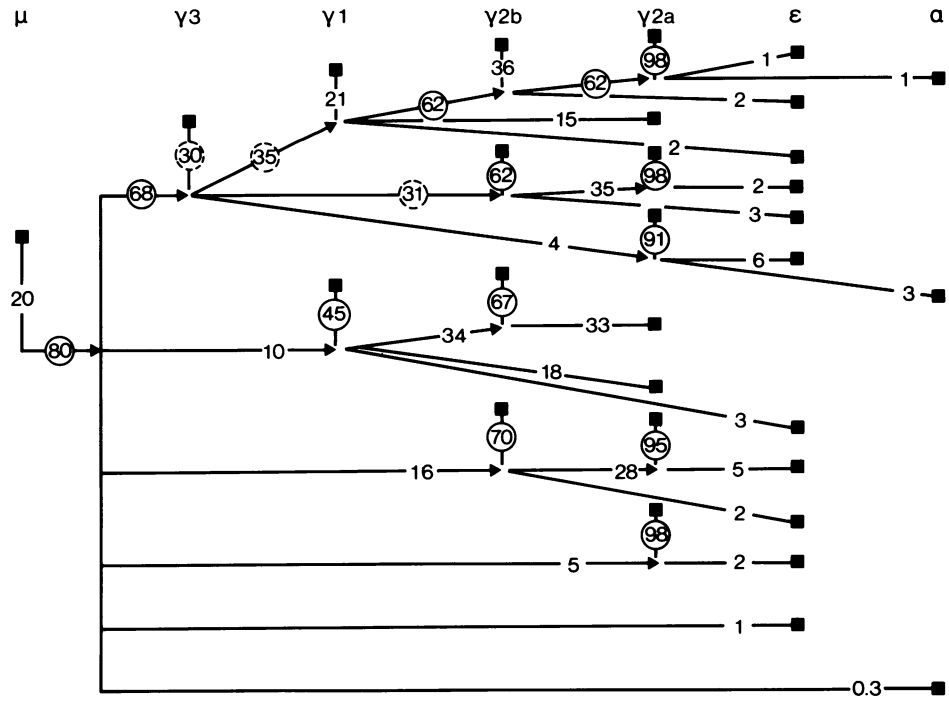

Fig. 2. Schematic representation of switch probabilities in LPS-activated B cell clones. Values enclosed in a circle represent the highest probability in each step. Arrows represent switches, and solid squares the probability of no switch (for IgM) or of no further switches (for non-IgM isotypes).

(Martinez-A. *et al.*, 1980).

Finally, we have also to point out that the majority of IgG-bearing, and all the IgE- or IgA-bearing blasts also express membrane-bound IgM: we cannot exclude, nor prove due to the limitations of immunofluorescent double staining, that cells might exist expressing, at a given point in time, more than two isotypes in the membrane-bound form.

In addition, at day 7 of culture, when the highest numbers of non-IgM secretors are present, only a minority of IgG secretors of any isotype still express membrane IgM (Pernis *et al.*, 1976). On the other hand, all IgE secretors observed in day 7 LPS cultures, and IgA secretors in day 7 helper cultures were IgM-positive on the membrane, and this is also the case for IgE and IgA plasma cells in normal mouse spleen (personal unpublished observations). This observation could support the possibility that a switch to IgE or IgA does occur with high probability directly from IgM.

Table VI. Membrane isotypes on B cells activated *in vitro* by LPS or by specific T-helper cells

	% blasts in culture	
	LPS	T-helpers
μ	89.7	62.0
γ3	5.4	0.1
γ3 + γ1	0.4	<0.05
γ3 + γ2b	0.16	<0.05
γ3 + γ2a	<0.05	<0.05
γ1	8.5	14.9
γ1 + γ2b	0.6	0.22
γ1 + γ2a	0.9	1.1
γ2b	6.9	2.0
γ2b + γ2a	n.d.	n.d.
γ2a	3.2	2.8

Discussion

The present results establish that immunocompetent B cells stimulated by a thymus-independent mitogen into clonal growth give rise to diversified progenies producing multiple immunoglobulin isotypes. Since we are dealing with a very large fraction of all B lymphocytes at high experimental efficiency, these results definitely exclude the possibility that single B cells are 'precommitted' to the expression of one non-IgM isotype only, and that switch events occur in a stimulation-independent manner during B lymphocyte differentiation. This conclusion is further reinforced by the total frequency of clones found to contain non-IgM isotypes, which is much higher than the frequency of cells expressing non-IgM classes in the population that was cloned (Abney *et al.*, 1978; and personal unpublished observations). In addition, the pattern of isotype distribution in these normal populations is quite different from that found in clonal analysis (Benner *et al.*, 1981; this paper). We conclude, therefore, that switch events take place along with cell activation and clonal expansion, and that these experimental conditions are suitable for studies on the control of C-gene expression.

Our experiments have primarily analyzed secretory forms of the various isotypes, and therefore provide indications at two possible levels of regulation: (i) switch in isotype expression; and (ii) terminal maturation to secretion. We have recently found that these are independent events, and that signals controlling terminal maturation to high rate secretion may well operate in a C-gene specific manner (Forni and Coutinho, 1982). All our conclusions are based therefore on the observation of a complex phenotype and may not include switch events in their entirety.

The second major conclusion of these studies is that γ-γ switches observed with low frequency in meyloma lines (Francus *et al.*. 1978; Radbruch *et al.*, 1980; Eckhardt *et al.*, 1982) are frequent events in activated B cell populations. Not only

can clonal progeny diversify in isotype production, but also, already switched cells retain the ability to undergo further switch events and express new non-IgM classes. Whatever the molecular mechanism ensuring switches, these are not unique events in the biography of a single cell. On the other hand, the activation of mechanisms controlling the production of high levels of secretory forms of immunoglobulin may be incompatible with further switches. Incidentally, the finding that secretory isotypes may often be different from those expressed on the membrane, not only reinforces the absence of 'precommitment', but also causes reservations on the physiological significance of putative isotype-specific T cells on the regulation of switch events (Rosenberg and Chiller, 1979).

Consecutive switches along the chromosome might not apply to ϵ and α: high rate secretors of these isotypes both *in vitro* and *in vivo* retain membrane IgM, suggesting that most switches to these C genes occur directly from $C\mu$.

These observations of frequent γ-γ switches, but μ-ϵ and μ-α (rather than γ-ϵ or γ-α), find a reasonable molecular explanation in the homologies of S-regions (Kataoka *et al.*, 1981; Lang *et al.*, 1982). In fact both S_ϵ and S_α are more homologous to S_μ than to any S_γ. This interpretation would indicate that the probability of switch events is largely determined by the probability of recombination between S-regions, which in turn depends directly on DNA sequence, and not on the specificity of putative recombination proteins with two or more DNA-recognition sites. Furthermore, since this is also observed at the membrane level, the same probabilities apply to whatever mechanisms control this type of expression, even if these do not involve DNA recombination and deletion, as recently observed (Alt *et al.*, 1982; Yaoita *et al.*, 1982; Perlmütter and Gilbert, in preparation).

Finally, our results reinforce, at clonal levels, previous conclusions that switches in isotype expression are not the result of random processes occurring with decreasing probability with increasing distance from $C\mu$. It is unquestionable whether the relative position of the respective C genes appears to influence the overall probability of their expression. It is also clear, however, that the frequency of expression of the different IgG subclasses does not follow the gene order in the chromosome (Roeder *et al.*, 1981; Shimizu *et al.*, 1981), but is largely dependent on the quality of the stimulus driving the cell into clonal expansion. We refer here to the expression of γ isotypes in the membrane-bound form, which is strikingly different in a T-independent and in a T-dependent induction. An additional level of regulation is introduced for maturation to secretion (Forni and Coutinho, 1982), holding to the described isotype-selectivity of T-dependent and T-independent specific and polyclonal responses (Slack *et al.*, 1980; Martinez-A. *et al.*, 1980).

In essence, the present results demonstrate the multipotentiality of single B cells for C-gene expression and indicate that such expression is selectively regulated. These regulatory processes seem not to depend on antibody (V gene) specificity, since they operate in our case in polyclonal stimulation, and they might involve (membrane) molecules other than immunoglobulins. The experimental conditions under which the regulatory mechanisms are demonstrated to be operating could provide cell populations suitable for investigations of the molecular basis of C-gene expression and switching in normal B cells.

Materials and methods

Mice

C3H/Tif, CBA/J, and C57BL/6 mice were from Bomholtgaard (Ry, Denmark) or from our breeding colony.

Cell cultures

These have been performed as described by Andersson *et al.* (1977). Spleen cells were cultured in RPMI 1640 supplemented with 2 mM glutamine, 10 mM HEPES, 50 μM 2-mercaptoethanol, antibiotics, and 20% foetal calf serum (GIBCO) and stimulated with 50 μg/ml *Salmonella abortus equi* LPS (DIFCO), in the presence of 3×10^6 rat thymus fillers. Cultures were carried out in Microtiter II tissue culture plates (Falcon) at 37°C in 5% CO_2 for 7 days for experiments where p.f.c. were assayed, and for 10 days in experiments where the presence of secreted immunoglobulins was analyzed in supernatants.

Cell cultures to be analyzed by immunofluorescence were set up from spleen cell suspensions in the same medium as above, at 5×10^5/ml, 2 ml/well in Costar 24 trays. The cultures were stimulated for 3 days with either 50 μg/ml *S. abortus equi* LPS, or with 2×10^5/ml helper cells from C3H/HeJ anti-C3H/Tif cell lines (Augustin and Coutinho, 1980).

Assay for p.f.c.

This was performed as described by Gronowicz *et al.* (1976) using sheep red blood cells coupled with Protein A from *Staphylococcus aureus* (Pharmacia, Uppsala). Anti-isotype specific rabbit antisera were prepared, tested for specificity, and titrated for maximum efficiency as described (Bernabe *et al.*, 1981).

Solid phase enzyme immunoassay

The same antisera used for the plaque assay were extensively absorbed by passage on Sepharose-coupled mouse myeloma proteins of all isotypes except the homologous ones, as well as on Sepharose-coupled $F(ab)'_2$ fragment prepared by pepsin digestion of normal mouse immunoglobulins. These were purified by affinity chromatography on Sepharose-coupled Staphylococcal-Protein A from pooled normal mouse serum. More than one round of absorption was necessary to obtain antisera specific in the assay.

The sensitivity of the assay was greatly improved by coating polyvinyl chloride plates (Microtiter U M24) with sheep antibodies directed against total mouse immunoglobulins, as a specific anchor for immunoglobulins present in the culture supernatants. Bound mouse immunoglobulins were reacted with the specific rabbit anti-mouse isotype antibodies. Detection of bound rabbit antibodies was revealed with a polyspecific goat anti-rabbit immunoglobulin antiserum labelled with peroxidase (Nordic Labs, Tilburg, UK), followed by an enzymatic reaction using o-phenylendiamine (SIGMA) as substrate. The reaction was quantitated photometrically in a Multiskan (Flow Labs, Irvine, UK) at a 450 nm wavelength.

The specificity and sensitivity of the test was controlled as follows: myeloma proteins of the relevant isotype were used at 1 μg, 100 ng, 10 ng, and 1 ng/ml, for assessment of sensitivity. Myeloma proteins of all irrelevant isotypes were used at 10 μg and 1 μg/ml, to assess the specificity. Background non-specific binding was measured in wells where immunoglobulins were omitted. Every antiserum was considered suitable for the test when the binding on irrelevant myeloma proteins gave absorbance values included in the average + 3σ of the background absorbance, while giving a strong signal (between 0.4 and 0.5 OD units) on the relevant isotype at 1 ng/ml.

In the experiments, the supernatant of each culture was incubated on 7 replica plates, each one exposed to a different anti-isotypic antiserum. For the background, one plate (96 wells) per experiment per antiserum was incubated with supernatants of parallel cultures containing only rat thymus fillers. Absorbance values above the average + 3σ of these plates were considered positive.

Immunofluorescence

The same antisera used in the above immunoassay were labelled with either fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate (Nordic Labs, Tilburg, UK). Purification and conjugation of immunoglobulins and staining procedures for membrane-bound and intracytoplasmic immunoglobulins were performed as described (Forni, 1979).

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