ISOTYPE COMMITMENT IN THE IN VIVO IMMUNE RESPONSES

I. Antigen-dependent Specific and Polyclonal Plaque-forming Cell Responses by B Lymphocytes Induced to Extensive Proliferation*

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The control of immunoglobulin (Ig) isotype production has a wide range of implications, ranging from the regulation of gene expression in eucaryotic cells to clinical applications such as allergy and "facilitation." In spite of the rapidly accumulated knowledge (1–3) on the structure and organization of Ig C-genes and flanking "switch regions," there is at present no general agreement on the rules determining the selective expression of one or another isotype in specific antibody responses. A model based on the order of structural C-genes along the direction of transcription (4) and on the repeated finding that all C-genes 5′ to those being transcribed in secretory cells are deleted from the expressed chromosome (5–8) has recently received much attention. This model (9, 10) assumes no specific control of switch recombination, rather, it postulates randomness in these genetic events and, consequently, dictates, that cells in a clonal progeny will successively delete C-genes and express those located most 3′ in the chromosome. Because, in accordance with current molecular models (1) and as shown by direct evidence (11), switch recombination requires DNA replication, it follows that such a model predicts a direct correlation between the number of mitoses undergone by a competent cell and the number of switch recombination events that have taken place in its DNA. This model explains, for example, the high frequency of IgA-secreting cells in some anatomic sites or biological situations by postulating a long process of replication in these cells before being induced to terminal maturation (10).

We have now directly tested these assumptions by making use of a serial transfer system, originally described by Möller (12) and later used to analyze clonal stability of V-gene expression (13). It is particularly important to point out that, in this transfer system, the progenies of immunocompetent B cells are analyzed for their whole life span because the experiment is continued until clonal senescence limits further proliferation. It appeared that the analysis of the isotypes produced by antibody-secreting cells along their whole life-span would provide direct indications as to the validity of that random recombination hypothesis. The results obtained do not support that model.

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Materials and Methods

Experimental Systems. We used serial transfers of normal or hyperimmunized spleen cells (10^7 cells/recipient) with antigen into irradiated (380 rad) recipients. At 7-d intervals, spleen cells recovered from recipients were transferred to new irradiated mice until senescence of the transferred cells was observed. Three to four mice were used in each group at each transfer.

Animals. Male SPF mice of the inbred strain C3H/HeJ were used between 20 and 34 wk of age.

Immunization. Hyperimmunized donors had received three weekly injections of 10^8 SRC and were used 12 d after the last injection.

Cell Transfer. Spleen cell suspensions were prepared in ice-cold (BSS),[1] and clumps were withdrawn by a rapid sedimentation. Plaque-forming cell (PFC) assays were performed in individual cell suspensions that were thereafter pooled before transfer. For adoptive transfer, 5×10^7 of these cells in 0.8 ml BSS were mixed with 0.2 ml packed sheep erythrocytes (SRC), and 0.2 ml of this suspension was immediately injected into the lateral tail vein of mice that had undergone total body x-irradiation (380 rad) 6 h before. 7 d after each transfer, the spleen cells of three or four individuals were investigated for nonspecific and antigen-specific PFC. The same was done with the pool of spleen cells before injection.

PFC Assays. Subclass-specific plaque assays for all secreting cells were done using protein A-coated erythrocytes and rabbit anti-mouse Ig-developing antisera as described in detail (14). After the appropriate absorptions, the antisera were titrated and their specificity assessed in plaque assays using myeloma or hybridoma cells of known subclasses.

Antigen- and class-specific plaque assays were performed with erythrocytes of the same sheep donor used for immunization and the same rabbit anti-mouse Ig-developing antisera as described above, at appropriate dilutions.

All direct PFC, detected without developing antisera, are here referred to as IgM-secreting cells. The numbers of PFC indicated for all other isotypes correspond only to PFC that were developed by addition of class-specific antisera in the plaque assay, i.e., these numbers indicate the excess of PFC detected with antisera after subtracting the total numbers of direct PFC. Because developing antisera inhibit a small fraction of all IgM PFC (5–15%), the numbers of indirect PFC (IgG and IgA) are slightly underestimated. We, however, find this practice better than referring to all PFC detected in the presence of developing antisera as indirect PFC because IgM PFC inhibition varies with the antisera used, time of incubation, and rate of secretion by individual cells.

Results

Antigen-specific Responses of Normal Spleen Cells in Successive Transfers. Normal, non-immune spleen cells were serially transferred, together with antigen (SRC), to irradiated recipients at 7-d intervals until senescence of the transferred cells was observed. At each transfer, spleen cells were assayed for specific anti-SRC PFC and total Ig-secreting cells of all isotypes. To investigate the contribution of the irradiated recipients to the responses detected, the following controls were carried out: mice were either simply irradiated or both irradiated and injected with antigen, and the numbers of splenic specific and nonspecific PFC were analyzed 7 d later. The results are shown in Table I. Under our conditions, a considerable number of nonspecific PFC persisted in the spleen, although the antigen-specific response was negligable. In comparison with the recipients of cells and antigen (see below), the irradiated recipients contributed ~20% of the total nonspecific response, and ~10% of the antigen-specific PFC. The average number of cells per spleen in those receiving no cells was 12×10^6, which is to be compared with an average of 60×10^6 cells per spleen recovered throughout the first six transfers of 10^7 donor cells. We conclude, therefore, that this experimental

[1] *Abbreviations used in this paper:* BSS, balanced salt solution; HRC, horse erythrocytes; PFC, plaque-forming cell; SPF, specific pathogen-free; SRC, sheep erythrocytes.

TABLE I

*Irradiated Host Contribution to Splenic PFC**

Treatment				
Irradiation‡	−	+	+	
Antigen (SRC)§	−	−	+	
Number of cells/spleen	90×10^6	12×10^6	12×10^6	
PFC/Spleen	Nonspecific	Nonspecific	Nonspecific	SRC-specific
IgM	280,633	137,811	66,079	1,519
IgG_3	5,469	2,959	3,401	466
IgG_1	28,097	14,533	38,503	3,775
IgG_{2b}	15,265	45,376	35,782	5,209
IgG_{2a}	28,838	60,914	66,283	5,627
IgA	81,993	109,905	41,952	687
Total	440,293	371,498	252,000	17,283

* Assays were performed 7 d after treatment. These values are the geometric means of four to six mice.

‡ 380 rad.

§ 10^8 SRC, injected intravenously.

system can be used to study the responses of transferred cells, as the large majority of PFC detected are of donor origin. In addition, these "background" control numbers of cells and PFC were deducted from the experimental numbers in each transfer, such that the values dealt with in this report concern exclusively the response of transferred cells.

Fig. 1 shows the number of cells recovered per recipient spleen for the whole period of one such experiment. These remain essentially constant during the first six transfers, with an average number of 48×10^6 cells per spleen (net increases over recipients of no cells). The interval between the 7th and the 8th transfer is 8 d, and not 7 d, which is reflected in a considerably higher number of cells recovered (76×10^6). After the 8th transfer, the number of recovered cells decreased drastically, yielding only 1×10^6 cells after the 9th (and last) transfer.

These results confirm previous observations on clonal senescence (12, 13) that have also reported failure in maintaining proliferating cells for longer than 7–10 transfers. A rough analysis of the results also demonstrates the enormous expansion of the immunocompetent cells originally transferred. Because only a maximum of 20% of all transferred cells successfully home and expand in recipient spleens (15), each transfer represents an expansion of ~20–30-fold in cell numbers (2×10^6 to 50×10^6). This is equivalent to six cell doublings per transfer, which could be serially repeated eight times, indicating a potential of ~40 mitosis for immunocompetent lymphocytes before clonal senescence and extinction. Similar figures have been previously derived, and they indicate that a single lymphocyte can give raise to a progeny of 10^{12} cells. It is important to point out that such very extensive proliferation is antigen dependent. As shown in Table II (and Fig. 1), when cells recovered after the 7th transfer are stimulated with a heterologous antigen (HRC) in irradiated recipients, they proliferate very poorly. This also demonstrates that serially transferred cells have been selected for specificity and that the majority of all proliferating clones are stimulated by the appropriate antigen.

The numbers of antigen-specific PFC at each transfer are also shown in Fig. 1.

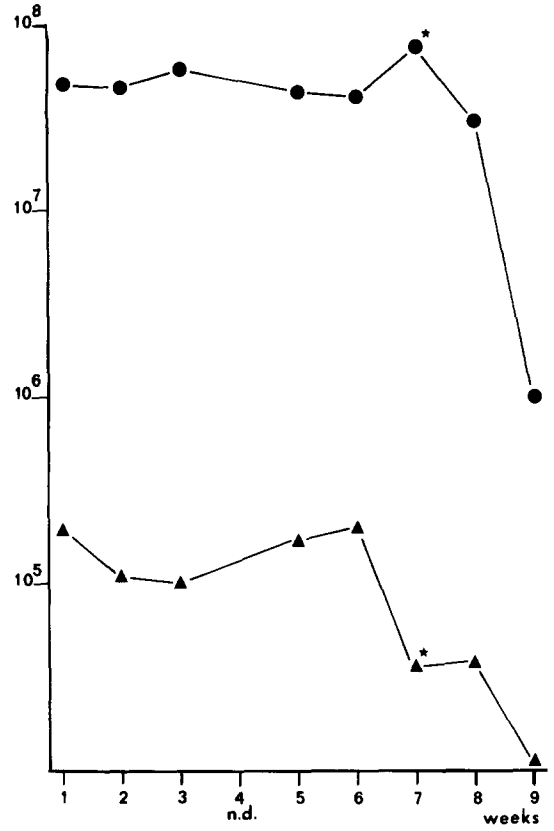

FIG. 1. Serial transfers of spleen cells from normal donors with antigen. Numbers of nucleated cells (●) or SRC-specific PFC (▲) in the spleen of recipient mice 7 d after transfer. These values are geometric means of three or four mice in each experiment. The star indicates that the assays were performed 8 d after transfer.

These roughly follow the same pattern as the total number of cells. After the 7th transfer, the specific response declines rapidly and yields only 6–11% of the original response after the 9th and last transfer. It is noteworthy that the specific response decreases already after the 7th transfer, although the number of spleen cells is still increasing. This is in line with the findings of others (13), and it might indicate that the senescence of antibody-forming function or cells takes place before that of proliferative abilities. Alternatively, the rapid loss of PFC from spleen could be caused by extra splenic location of PFC (16).

Of interest is whether the control of IgC-gene expression in proliferating B cell clones depends on specific regulatory mechanisms or, alternatively, is due to a random process of DNA-recombination and deletion. If the latter were the case, and given the C-gene order originally proposed by Honjo (4) and later repeatedly confirmed (5–8), extensive proliferation of B cell clones should yield a majority of IgA-secreting B cells (9, 10). On the other hand, a specific regulatory mechanism should maintain the antigen-specific subclass pattern throughout the life-span of the proliferating B cell clones. As shown in Fig. 2 and Table III, the subclass pattern of the antigen-specific response remains essentially constant throughout the experiment, with two exceptions:

TABLE II

*Antigen Dependence of Specific PFC Responses by Serially Transferred Cells**

Transfer into recipients	10^7 serially transferred cells + SRC	10^7 serially transferred cells + HRC	10^7 normal spleen cells + HRC
Number of Cells/spleen:	76×10^6	21×10^6	35×10^6
SRC-specific PFC/spleen			
IgM	545	273	525
IgG	33,779	<10	402
IgA	2,115	<10	154
Total	36,439	273	1,081
Total PFC/spleen			
IgM	309,322	217,599	354,373
IgG	242,058	113,360	46,522
IgA	184,074	204,361	158,653
Total	735,454	535,280	559,548

* Serially transferred cells (on the 7th passage) or normal spleen cells were transferred with 10^8 SRC or HRC into 380 rad irradiated recipients. Assays were performed 8 d later, and these values are geometric means of three or four mice.

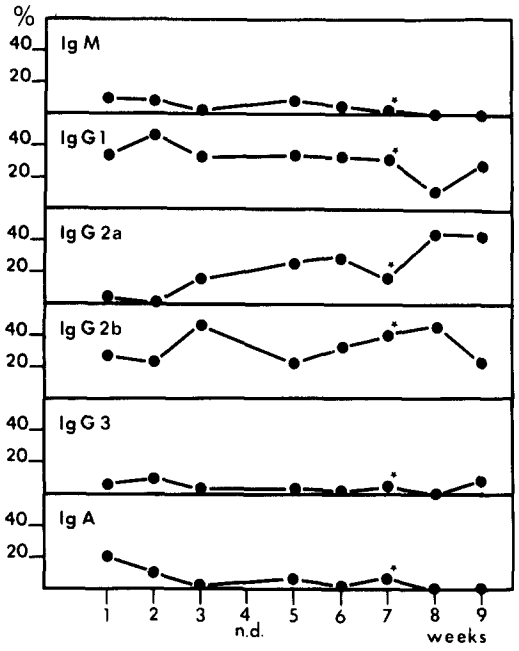

FIG. 2. Isotype distribution of SRC-specific PFC in consecutive transfers.

IgM PFC disappear after the 8th transfer, and IgG_{2a} changes from an isotype of low expression to one of the major antibody classes after the 3rd transfer. As a result, the IgG_1 class is, after the 3rd transfer, no longer the main isotype in the response. Nevertheless, IgG_1 is, together with both IgG_2 subclasses, one of the most represented isotypes throughout the experiment, in contrast to the other three antibody classes, namely, IgM, IgG_3, and IgA, that are poorly or not at all expressed. These results are generally in agreement with previous observations in thymus-dependent immune responses measured in short-term experiments (17–19). In the context of the present

TABLE III

*Isotype Distribution of SRC-specific PFC in Serially Transferred Cells**

Isotype of PFC	Number of transfers							
	1	2	3	4	5	6‡	7	8
IgM	8,9§	7,6	2,0	7,7	3,1	1,5	<0,1	<0,1
IgG_3	5,8	9,5	3,2	2,8	0,7	5,1	0,1	8,2
IgG_1	33,6	47,1	31,5	33,0	32,6	31,4	10,0	26,9
IgG_{2b}	27,2	24,4	47,0	22,7	31,9	40,2	45,6	22,2
IgG_{2a}	4,1	1,0	14,6	26,3	29,2	15,9	44,2	42,7
IgA	20,5	10,4	1,7	7,4	2,6	5,8	<0,1	<0,1
Anti-SRC PFC/spleen	192,724	207,636	100,692	165,851	202,310	36,439	38,705	11,310

* Spleen cells from normal donors were serially transferred with antigen (SRC) to irradiated recipients at weekly intervals. 7 d after each transfer, the number of splenic SRC-specific PFC of all classes were determined in groups of three or four mice.

‡ Assays on day 8 after transfer.

§ Percent distribution of the specific PFC in the indicated Ig classes.

experiments, they demonstrate that isotype patterns are maintained regardless of the extent of proliferation, arguing against a random recombination and deletion model for switch in Ig isotypes.

Antigen-nonspecific Responses of Normal Spleen Cells in Successive Transfers. It has been known for many years (20, 21) that specific immunization results in increased production of Ig that are antigen nonspecific. Recently, this phenomenon has received considerable attention in the context of isotype commitment, in view of observations relating the class of this nonspecific Ig to the dominant isotypes in the specific antibody responses (18, 22). We analyzed this phenomenon under the extreme conditions provided by the serial transfer system.

The total numbers of PFC in each isotype were investigated at each transfer in the same individual mice analyzed for specific responses. The numbers of total PFC in each subclass are shown in Fig. 3 and Table IV. The "nonspecific" subclass pattern remains more or less constant throughout the serial transfers, as for the specific antibody isotype pattern. The bulk of the response is contributed by IgG_1 and IgG_2 as in specific PFC. In contrast to the specific response, however, IgM is well expressed among total PFC, and after the 7th transfer it is actually the most expressed class. Analogously to the specific subclass pattern, IgG_3 and IgA subclasses are little expressed throughout the experiment. The ratio between antigen-specific PFC and total (nonspecific) PFC is within the range of 10–20% from the 1st until the 7th transfer. Thereafter, this ratio decreases to 5–6% (Fig. 4). The increase detected in the last transfer is probably not significant, as clonal senescence results in drastic decreases in both specific and nonspecific PFC, and those ratios are calculated on very low numbers of PFC.

Responses of Hyperimmune Spleen Cells in Serial Transfers. The same system as described above was used with hyperimmune spleen cells. The results support the same conclusions as the experiments with normal, nonimmune spleen cells. Thus, the subclass patterns of both the specific and the nonspecific responses are more or less constant throughout the successive transfers. These responses are also antigen dependent (data not shown), but fewer transfers are practicable as the senescence of the

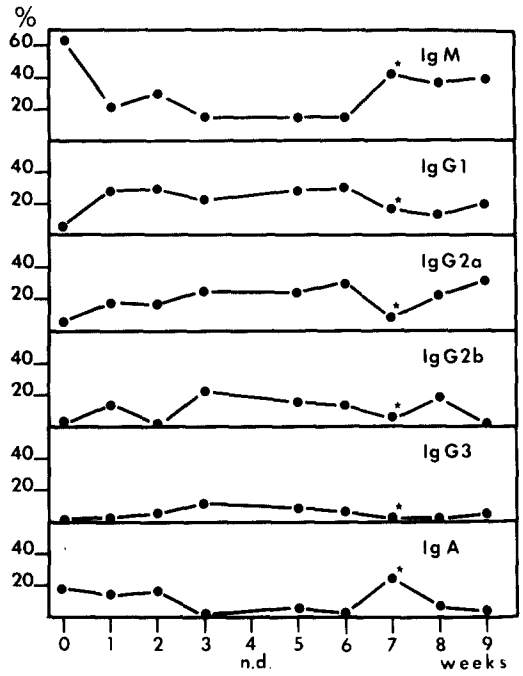

FIG. 3. Isotype distribution of total Ig-secreting PFC in consecutive transfers.

TABLE IV

*Isotype Distribution of Total (Nonspecific) PFC in Serially Transferred Cells**

Isotype of PFC	Number of transfers							
	1	2	3	4	5	6	7	8
IgM	21,4	30,3	15,1	15,2	14,8	42,1	36,5	39,9
IgG_3	2,2	4,9	12,2	9,2	6,9	1,8	2,5	4,6
IgG_1	28,9	30,9	22,8	29,4	31,5	16,8	12,6	20,0
IgG_{2b}	15,3	ND‡	22,2	16,5	13,6	5,0	17,8	ND
IgG_{2a}	18,3	16,8	25,6	23,5	29,9	9,4	23,0	31,5
IgA	13,8	17,0	2,1	6,1	3,3	25,0	7,6	4,0
Total PFC/spleen	1,597,154	528,306	1,033,762	1,469,782	1,020,634	735,454	606,996	75,111

* As in Table III, but total Ig-secreting PFC were assayed.

‡ Not done.

transferred cells occurs earlier. Both the numbers of recovered cells (Fig. 5 a) and antigen-specific PFC (Fig. 5 b) decrease drastically already after the 6th transfer. This is to be expected if we assume that the cells used in the first transfer had already been induced to some degree of proliferation by the priming of the donor. In contrast to the analogous experiment with nonimmunized spleen cells, IgG_{2a} is well expressed already in the hyperimmunized donors and rapidly becomes the dominant subclass in the next transfers (Fig. 6). This confirms that a considerable degree of proliferation has taken place in the hyperimmune donors and suggests that, in part, dominant IgG_{2a} production requires extensive "priming" (19).

The ratios between specific and nonspecific PFC in hyperimmune cells decline during the experiment (Fig. 7), indicating a shorter life-span of the antigen-specific

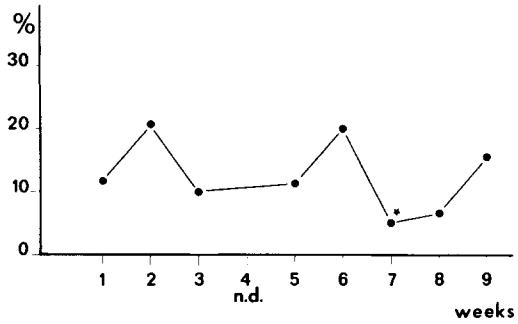

FIG. 4. Fraction of SRC-specific PFC among total Ig-secreting PFC in consecutive transfers.

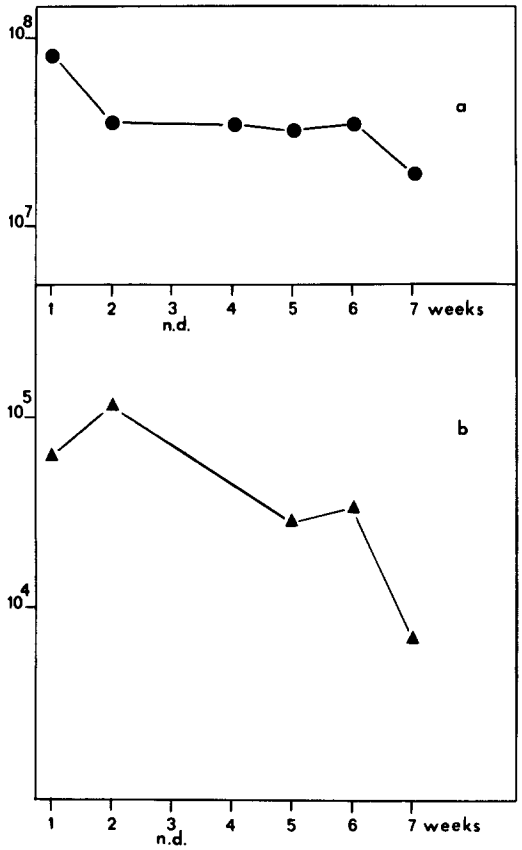

FIG. 5. Serial transfers of spleen cells from hyperimmunized donors with antigen. Number of nucleated cells per spleen (●) and SRC-specific PFC per spleen (▲) of three or four recipient mice 7 d after transfer.

clones as compared with the clones maintained along with the transfers, which do not show measurable binding to the antigen. This might suggest that such nonspecific PFC are drawn from a precursor cell population distinct from the primed, antigen-specific pool.

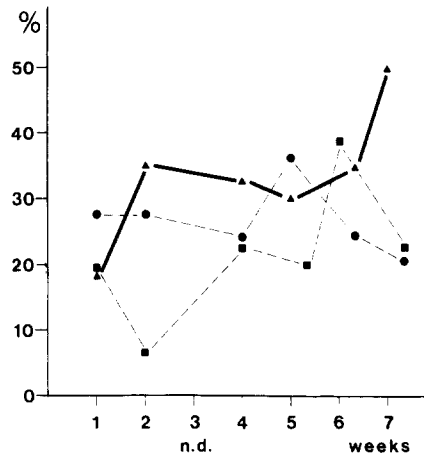

FIG. 6. Isotype distribution of SRC-specific PFC in consecutive transfers of hyperimmune spleen cells. Only IgG_1 and IgG_2 PFC are shown, but their representation at each transfer is indicated as percentage of the total numbers of specific PFC, including all other classes. ▲, IgG_{2a}; ●, IgG_1; ■, IgG_{2b}.

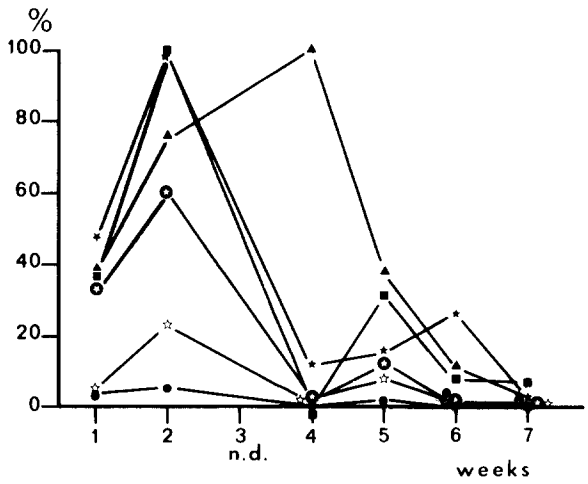

FIG. 7. Fraction of SRC-specific PFC among total Ig-secreting PFC in consecutive transfers of hyperimmune spleen cells. ●, IgM; ★, IgG_1; ■, IgG_{2a}; ▲, IgG_{2b}; ✪, IgG_3; ☆, IgA.

Discussion

Different models have been suggested to control IgC-gene expression in antigen-specific responses. Both specific regulatory mechanisms (17, 18, 23) and random DNA-recombination and deletion (9, 10) have been postulated. The present work was carried out to investigate these possibilities. Normal nonimmune or hyperimmunized spleen cells were serially transferred together with antigen (SRC) into irradiated syngeneic recipients until senescence of the transferred cells was observed. The antigen-specific and nonspecific PFC responses were analyzed at each transfer time in the various isotypes: IgM, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, and IgA.

If specific mechanisms regulate isotype commitment, the subclass-pattern could be expected to remain constant throughout the life-span of the transferred cells. On the

other hand, a regulation based on random processes at the DNA level associated with cell division should yield an accumulation of IgA-secreting cells, provided that the IgC-gene order is $\mu(\delta)$ $\gamma 3$ $\gamma 1$ $\gamma 2b$ $\gamma 2a$ $(\epsilon)\alpha$ (4) and a recombination and deletion switch model (5–8) is postulated.

The data obtained in these experiments strongly suggest the existence of specific regulatory mechanisms because, throughout a period of 9 wk, with an extreme proliferative activity that was limited only by clonal senescence, the subclass-pattern remained roughly constant for both the nonspecific and specific PFC responses. No accumulation of IgA-secreting cells was observed.

The SRC-specific response is mainly restricted to the IgG class, IgG_1, IgG_{2a}, and IgG_{2b}, in particular. In the experiments started with nonimmune spleen cells, IgG_1 was the predominant subclass in the two first transfers, followed by IgG_{2b}, with IgG_{2a} being poorly expressed. After the 3rd transfer, however, IgG_{2a} increased, becoming one of the major subclasses, while IgA decreased from relatively high to low expression. This change in subclass expression probably reflects differences between primary and secondary type responses to an antigen and hyperimmunization of the transferred cells in the adoptive hosts. Thus, in the parallel experiment with hyperimmunized cells, IgG_{2a} is well expressed already in the donors, and it becomes the dominant subclass in the antigen-specific response during the coming transfers. Expression of SRC-specific IgG_{2a} therefore seems to be strongly dependent on the degree of immunization, a phenomenon that was not observed for any other subclass. This could be the result of modifications in the C-gene composition of the responding B cells DNA. Because, however, the changes occur in both directions as to the C-gene order in the chromosome, relative IgG_1 and IgA decreases with concomitant increase in IgG_{2a}, it is more likely that this pattern is determined by the degree of priming of accessory cells or other regulatory mechanisms.

This dependence of subclass expression upon the immunization protocol might be a reason for different antigen-specific subclass patterns previously described in BALB/c mice (19, 24). Several different mechanisms of independent genetic control have been described to regulate both the primary IgG_{2a} anti-SRBC response in BALB/c and C57B1/10 mice as well as hyperimmune anti-SRBC responses in C57B1/6 and A strains (19). Taken together with our studies, these results suggest that the levels of individual Ig classes produced during primary and hyperimmune responses are regulated by complex mechanisms, in clear contrast to the random recombination and deletion models.

Our results, extending these observations to the limit of clonal senescence, reinforce those conclusions. The possible number of serial transfers differs in accordance to the cells used in the first transfer. It was possible to transfer nonimmune spleen cells nine times, while hyperimmunized spleen cells showed a clear clonal senescence already after the 6th transfer. A 7th transfer was not done, although it was performable, as the number of anti-SRC PFC drastically decreased. This number of seven consecutive weekly transfers with hyperimmunized spleen cells is in line with the findings of others (12, 13). The difference in life-span of the transferred cells shows that the vast majority of the proliferating and Ig-secreting cells belong to the transferred clones and that the recipient contribution is probably lower than the controls shown. These results also confirm that there is a limited life-span for antigen-specific PFC clones, but they indicate that the cells participating in the nonspecific response have a comparatively

higher proliferative potential. This can be readily explained by postulating that the antigen-nonspecific clones associated with these responses, although antigen-dependent in their proliferation, are drawn from a precursor cell pool that is not primed in the donors (that has not proliferated), even in conditions of hyperimmunization. This conclusion does not support the mechanism of nonspecific responses proposed by Rosenberg and Chiller (18). On the other hand, our findings are quite compatible with theirs, as to the concordance of isotype expression in antigen-specific and -nonspecific PFC.

Given the conclusion that regulatory mechanisms control isotype expression, their nature is not yet clear. We know, however, that a given antigen yields specific responses with typical subclass patterns (17, 18), often associated with similar nonspecific responses (18), and that isotype specificity is a "carrier-related" property (17) that is also observed in polyclonal responses in the absence of selection for antibody specificities (23). These and other indications suggest that C-gene expression might depend on the nature of the stimulus driving clonal proliferation. The question, however, is not only whether a given B cell will specifically switch to produce a particular subclass, but also whether a given B cell in contact with a mitogen or antigen and specific T cells will secrete antibodies at all. That is, the regulation of terminal maturation for the high rate production of secretory Ig molecules cannot be dissociated from the control of isotype expression whenever the experiments such as ours measure antibody secretion. Now that we have shown that isotype patterns are maintained regardless of the extent of clonal proliferation, these questions can better be asked in short-term experimental systems, offering higher analytic potentialities, such as the study of single B lymphocyte clones.

Summary

The random recombination and deletion hypothesis for the control of isotype commitment in antibody responses was directly tested in a serial transfer system in vivo. Normal or hyperimmune spleen cells were used in weekly serial transfers with antigen into irradiated recipients until clonal senescence was observed. Antigen-specific and -nonspecific plaque-forming cells of all isotypes were determined at each transfer time. No major changes in the isotypes of specific antibodies were observed for the whole life-span of the transferred cells (9–10 wk), and no indication was obtained for the accumulation of cells transcribing the most 3′ members of the C-gene cluster with sustained proliferation. Rather, the dominant isotypes were found throughout the response to be IgG_1, IgG_{2b}, and IgG_{2a}.

The results imply isotype-specific regulatory mechanisms in the control of Ig class production. These appear to operate as well in the antigen-nonspecific component of the immune response.

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