Immunology 1998 **93** 149–153

Low antigen dose favours selection of somatic mutants with hallmarks of antibody affinity maturation

A. GONZÁLEZ-FERNÁNDEZ*† & C. MILSTEIN† *Universidad de Vigo, Area de Inmunología, Facultad de Ciencias, Lagoas Marcosende, Vigo, Pontevedra, Spain, †Medical Research Council, PNAC Division, Laboratory of Molecular Biology, Hills Road, Cambridge, UK

SUMMARY

The immunization schedule is critical for the derivation of high-affinity antibodies, low antigen dose being particularly favourable for the development of a more efficient memory response. To analyse the molecular events underpinning this preference, we analysed the early maturation of the response to the hapten 2-phenyloxazolone (phOx) using low and high doses of immunogen. The phOx response is initially dominated by antibodies expressing the VkOx1-Jk5 light chain and the hallmark of the early stages of maturation is the substitution of His 34 by Asn or Gln increasing affinity 10- or eightfold, respectively, and of Tyr 36 by Phe. High-affinity antibodies express mutations at both sites. We cloned and sequenced VkOx1-Jk5 light chains from antigen-specific B cells taken 14 and 21 days after immunization with high and low antigen doses. We found that overall, the derived sequences were more mutated both at longer times and at higher dose. At day 14, His 34 was more frequently mutated at the higher than at the lower dose, while at day 21 the reverse was true. On the other hand, the His 34/Tyr 36 mutation pair was more frequent at low than high doses at both 14 and 21 days. Furthermore, at both times, the low immunization protocol yielded double mutants in cells with a lower mutation background. It appears therefore that while the higher dose may favour the acquisition of individual critical mutations, low-dose immunization favours the selection of a more focused mutational pattern, whereby advantageous mutations are associated with a low mutational background.

INTRODUCTION

During the course of hyperimmunization, the affinity, as well as the quantity, of the generated antibodies increase. This maturation process is influenced by the immunization protocol since lower antigenic doses give rise to antibodies of higher affinity.[1,2] Since those early studies, we have learned that the affinity maturation of antibodies relies on two overlapping strategies for the somatic derivation of new antibody structures. The first involves the somatic hypermutation of an antigen-selected and -restricted repertoire of antibody genes, and the second on the shift of the early repertoire.[3,4] While little is understood concerning the nature of the latter, much is known about the nature of the intrinsic pattern of hypermutation.[5,6] Furthermore, antigen-selected B cells first proliferate in germinal centres in an unmutated form and then hypermutate and die by apoptosis unless they are selected by antigen to differentiate further into plasma cells and memory cells.[3,7–12] Thus, antigen selection plays two roles: the first is the selective proliferation, the second is the rescue from apoptosis.[11,13–15] In this paper we analyse the effects that different doses of antigen has on the generation and selection of high-affinity antibody mutants. For this study we chose the response to the hapten 2-phenyloxazolone. This response is dominated by VkOx1-Jk5 light chain. Seven days after an intraperitoneal immunization, the light chain is expressed in an unmutated form. However, by day 14 a number of mutant forms are detected, predominantly the substitution of His 34 by either Asn or Gln.[3] In high-affinity antibodies, this substitution is usually accompanied by a Tyr 36 for Phe mutation. While the single change His to Asn or Gln improves antigen binding by a factor of 10 or 8, respectively, the putative functional advantage of the Tyr for Phe replacement is not known.[4,14] We have now analysed the extent of hypermutation and of the His 34 and Tyr 36 substitutions at days 14 and 21 following immunization with 10 and 100 μg of protein-coupled hapten.

Received 29 July 1997; revised 3 November 1997; accepted 4 November 1997.

Abbreviation: phOx, 2-phenyloxazol-5-one.

Correspondence: Dr A. González-Fernández, Universidad de Vigo, Area de Inmunología, Facultad de Ciencias, Lagoas Macrosende s/n, 36200 Vigo, Pontevedra, Spain.

MATERIALS AND METHODS

Mice

Groups of BALB/c mice were preimmunized with the carrier chicken serum albumin and a week later were immunized

intraperitoneally (i.p.) with 10 or 100 μg of 2-phenyloxazol-5-one (phOx) chicken serum albumin/alum precipitate plus 10^9 heat-inactivated *Bordetella pertussis*. Spleen cells were obtained 2 and 3 weeks after immunization from pools of three mice, and mononuclear cells were purified by density gradient. Cells were incubated with magnetic beads coated with oxazolone–bovine serum albumin (BSA), prepared and washed as described.[13] Bound cells to the beads were collected.

DNA purification

Cells were washed in phosphate-buffered saline (PBS) twice, resuspended in 10 μl of DDW, frozen in liquid nitrogen and kept at −70° until further use. Twenty microlitres of K-buffer (10 mM Tris–HCl, pH 8.3; 50 mM KCl, 1·5 mM $MgCl_2$, 0·5% Tween-20; and proteinase K) was added to the pellet and incubated for 1 hr at 56°, followed by incubation at 99° for 30 min. The tube was centrifuged, the supernatant (containing the DNA) was transferred to a different tube, and kept at −20° until used.

DNA amplification

Genomic DNA was amplified by polymerase chain reaction (PCR) using the primers VkOxBACK and Jk5FOR which contain *Eco*RI and *Bgl*II sites, respectively, and amplification was carried out as previously described.[16] The PCR product was purified from the agarose gel with the Gene Clean II kit (Bio 101), digested with *Eco*RI and *Bgl*II and cloned into M13mp18 (already digested with *Eco*RI and *Bam*HI). VkOx1 clones were selected by hybridization to oligonucleotide VkOX149,[13] and were sequenced by standard procedures using Sequenase (United States Biochemical, Cleveland, OH) with the −40 universal primer M13. The PCR error was around $0{\cdot}8 \times 10^{-3}$.

Statistical analysis

Statistical analysis was performed with 'SYSTAT' to compare the significance of the data found in all groups. We have done a transformation of data for normalization of distribution and we accomplished an unbalanced two-way analysis of variance (ANOVA) for the factors: day (14; 21) and dose (10 μg; 100 μg), and the variables: mutations of His 34, mutations of Tyr 36 and total number of mutations. A P value $<0{\cdot}05$ was considered significant.

We also calculated Kendall correlation coefficients of the combination Asn/Gln 34 and Phe 36 in all groups.

RESULTS

Groups of BALB/c mice were preimmunized with carrier protein to avoid T-cell help being a limiting factor.[17,18] Intraperitoneal immunization was with 10 or 100 μg of carrier hapten and the spleen B cells taken 14 or 21 days later. DNA was extracted from anti-phOx-specific B cells, purified using hapten-coated magnetic beads, and the VkOx1 region was amplified using specific primers. The fragments were then cloned into M13 and the sequences were compared (Table 1).

Most sequences were mutated under all conditions. However, the proportion of unmutated sequences was higher at day 21 than at day 14, presumably reflecting the waning of the germinal centre reaction (Fig. 1a). On the other hand, the most mutated clones (five or more mutations) at either high or low antigen dose, were more common at day 21. In addition, either at day 14 or at day 21, such clones were better represented at the higher antigen dose. Indeed a plot of the frequency of mutations accumulated amongst mutated clones, shows a consistent trend (Fig. 1b). Both at days 14 and 21, there was a larger proportion of highly mutated sequences derived from the high-dose than those derived from the low-dose immunization protocol. The average accumulation of mutations confirmed this trend, especially when only clones carrying more than one mutation were computed (Table 2). Thus, at least within the limits of antigen dose and time postimmunization tested, there seemed to be a positive correlation; the higher the immunizing dose, the larger the accumulation of mutations. Statistical analysis shows the significant effect of the dose in the accumulation of mutations, considering either all clones or only those carrying more than one mutation ($P<0{\cdot}05$). In addition, either at high or low immunization dose, highly mutated sequences were more represented at day 21 than at day 14.

We then analysed the distribution of sequences containing the mutation of His 34 to either Asn or Gln. At day 14, mice immunized with the 100 μg yielded a larger proportion of clones containing such substitutions than those immunized with 10 μg (Table 2). This was true even when, to account for the increase of mutations accumulated following the higher dose protocol, the frequency of the mutation was calculated as a fraction of all mutations. In contrast, at day 21 there was a higher proportion of clones with the diagnostic His 34 mutation at the low than at the high dose (the interaction among day and dose is significant with $P=0{\cdot}023$). The fraction of the His 34 mutation among all mutations, was twofold higher in the low-dose than in the high-dose data.

The substitution of Tyr 36 for Phe is another characteristic mutation found in high-affinity anti-phOx antibodies.[3,13] Although the possible advantage of this mutation has not been elucidated, the fact is that all, or almost all, high-affinity antibodies, particularly at later stages of maturation, carry the double His 34/Tyr 36 mutations. Although the number of clones carrying the Tyr for Phe substitution was higher at day 21 (Table 2), when computed relative to the number of all mutations, the frequency was rather similar throughout, although marginally higher in the 10 μg dose. However, the frequency at which the Asn (or rarely Gln) 34 and Phe 36 substitution pair appeared was higher at the low dose, both at day 14 and even more so at day 21 (Table 2). The coefficient analysis shows the clear significance ($P<0{\cdot}005$) of the association of both mutations at low dose either at 14 or 21 days. To be noted, is that with low-dose immunization, at day 21 all the mutations to Phe found in clones containing more than one mutation, also expressed the relevant His 34 mutation. Most suggestive is the fact that sequences expressing the characteristic mutation pair, were otherwise less mutated when the low immunization dose was used (Table 3). Indeed at day 14, the substitution pair was found in sequences containing a total of at most four mutations at low dose, while at high dose, they contained up to nine mutations (Table 1).

DISCUSSION

We analysed the responses at days 14 and 21 because we subscribed to the view that the germinal centre reaction peaks

Table 1. Mutations found in the VkOx1 gene

Day 14 (10 μg)				Day 14 (100 μg)				Day 21 (10 μg)				Day 21 (100 μg)			
Clone	Mutations*			Clone	Mutations*			Clone	Mutations*			Clone	Mutations*		
C5	5			C1	0			C1	6	N	F	C4	0		
C6	3			C2	4	N		C5	0			C5	4		
C8	0			C3	4		F	C6	5	N		C8	8	N	F
C9	1			C4	0			C8	1			C9	2		
C10	2			C5	4			C11	3			C10	5	N	
C11	4	N	F	C7	2	N	F	C16	5			C11	0		
C12	1		F	C8	5	N		C18	1			C12	1		
C14	3	N	F	C10	1			C19	4	N		C16	8	N	F
C15	0			C11	5			C20	4	N	F	C19	6		
C33	2	N	F	C17	4	N		C21	0			C20	0		
C34	2			C18	1			C22	1			C40	6		
C35	1			C19	2			C26	0			C41	0		
C36	4	N	F	C20	4	N		C27	0			C42	0		
C37	3			C22	9	N	F	C29	4	N	F	C43	8	N	F
C38	2			C23	3	N		C31	2	Q		C44	6		F
C39	0			C24	2			C32	7	N	F	C45	6	N	F
C40	2			C25	4	N		C34	1			C46	8	N	F
C41	0			C26	2			C35	0			C47	6		
C42	0			C27	1			C43	4	N	F	C48	5		
C43	4			C33	1			C44	1	N		C49	0		
C44	2	N		C34	4	N		C45	0			C50	0		
C45	5			C35	1		F					C51	1		
C46	2			C37	1							C52	0		
C47	6			C38	6	N	F					C53	6	N	F
C48	2			C39	7	N	F					C55	6		F
C49	3			C40	4							C56	1		
C50	1														
C51	4	Q	F												
C52	4	N													
29	68	7	6	26	81	11	6	21	49	9	5	26	93	7	8

*Mutations: number of mutations found in each individual clone.
N and Q: substitution of His 34 by Asn or Gln; F: substitution of Tyr 36 by Phe.

Table 2. Summary of accumulation of mutations in the VkOx1 gene from antigen-selected B cells

	Mutations	Clones mutated		Mut./Clone†		Asn/Gln 34‡		Phe 36‡		Asn/Gln 34+Phe 36
Day 14	>1	All	>1	All	>1	All	>1	All	>1	>1
10 μg*	64	24	20	2·8	3·2	29 (10)	35 (11)	25 (9)	25 (8)	25
100 μg	75	24	18	3·4	4·2	46 (14)	61 (15)	25 (7)	28 (7)	22
Day 21										
10 μg	44	15	10	3·3	4·4	60 (18)	80 (18)	34 (10)	50 (11)	50
100 μg	87	18	15	5·2	5·8	39 (7)	47 (8)	44 (9)	57 (9)	40

*Amount of 2-phenyloxazolone injected into mice.
†Mut./Clone, average mutation per mutated clone.
‡Fraction (%) of clones that includes one or both of indicated substitutions. In parenthesis is the fraction (%) of total mutations represented by the indicated substitutions. All, clones mutated; >1, only clones carrying more than one mutation.

during the 2nd week postimmunization, and that in the absence of further antigenic stimulation, begins to wane by day 21.[19–21]

The results of this paper support such a view, because the number of unmutated cells is higher at 21 days than at 14 days postimmunization. Indeed, in the case of the oxazolone response, 45 days after immunization, the number of mutated sequences returned to almost background levels.[13] However, our results also suggest that the germinal centres remaining

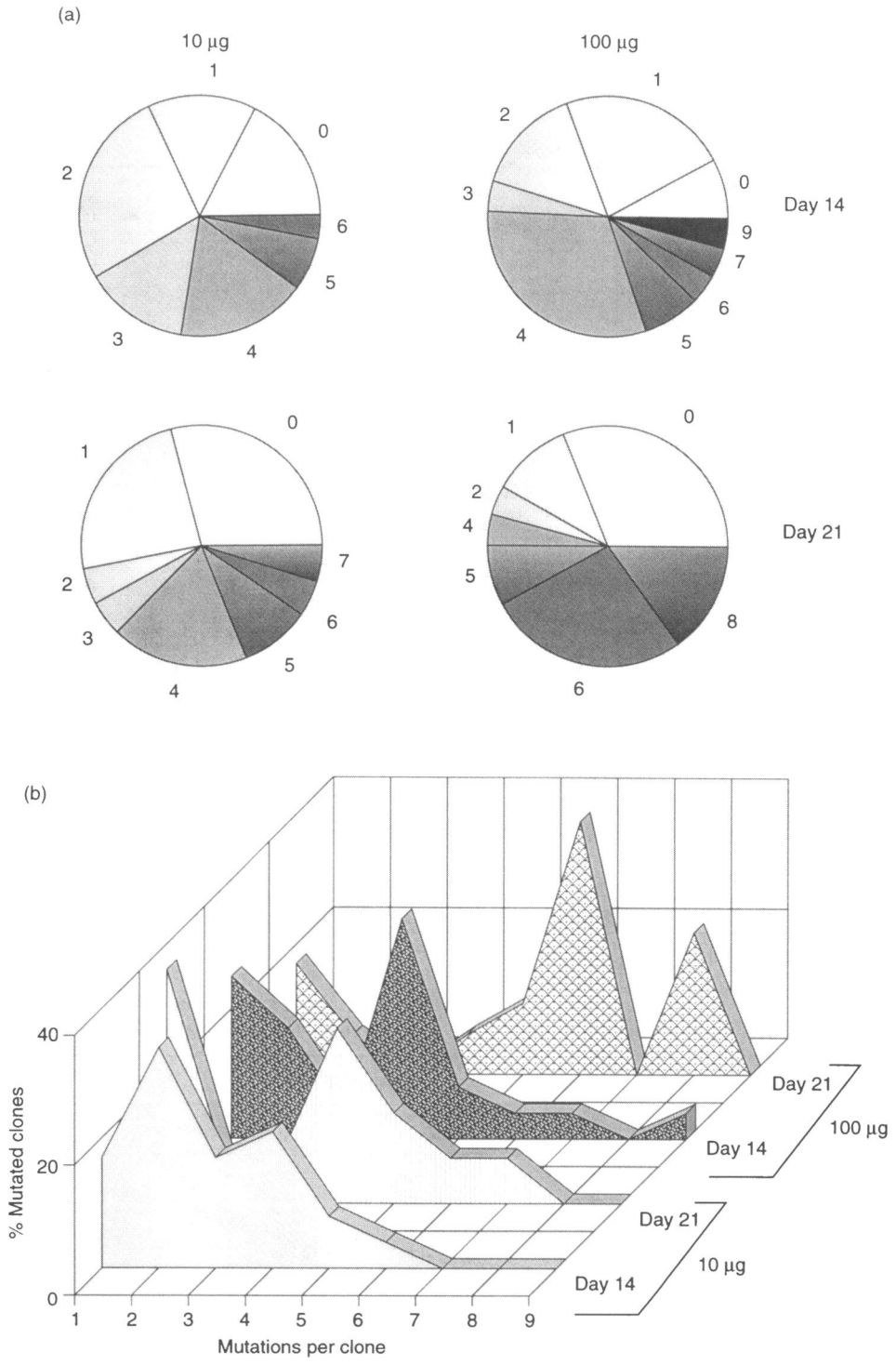

Figure 1. Frequency distribution of clones with respect to the number of mutations they carry. (a) Pie-graph depicting the proportion (%) of sequences with the indicated number of mutations. (b) Graphic comparison of the fraction of mutated sequences that have accumulated the indicated number of mutations following different immunization protocols.

after day 14 are also active during the 3rd week. There are two reasons in support of this statement. The first is the increase in highly mutated sequences with time and the second is the marked increase in the proportion of cells carrying the double mutation at His 34 and Tyr 36, characteristic of high-affinity phOx antibodies.

The most mutated clones were more frequent at high dose than low dose. While it is possible that the intrinsic rate of mutation increases with the amount of antigen present, this is unlikely because transgenes that do not bind antigen at all (passengers), mutate quite efficiently.[6] It is more likely that at higher doses, individual cells have a better probability of

Table 3. Average number of mutations in clones with substitutions for the pair Asn/Gln 34 and Phe 36

	Day 14	Day 21
10 μg	3·4	5
100 μg	6	7·3

undergoing a larger number of cycles of mutation. Indeed, it seems that multiple mutations require independent cycles of hypermutation separated by antigen selection. Thus cells incorporating five or more mutations, should have undergone several such cycles.[22] Higher concentrations of antigen may increase the stochastic probability of multiple mutations.

The number of sequences carrying the critical His 34 mutation was larger at low than at high antigen concentration at day 21, but the reverse was true at day 14 (Table 2). We cannot exclude that this apparent shift has a trivial explanation, but we take the view that it reflects a dynamic process. The more a cell mutates, the larger is the probability for specific mutants to appear. Thus, the proliferation of suitably mutated cells would occur earlier. If so, why is the picture reversed at day 21? We suggest that this is because the probability of introducing deleterious mutations also increases, and that the best adapted sequences are those expressing the maximum number of useful mutations with the lowest number of hindering or potentially deleterious mutations. Thus, more focused mutations may take longer to arise, but when they do, they are preferentially selected. This may not be valid for all responses, but in the phOx response it is supported by several observations. Even at day 14, when the His 34 mutation is more frequent at high dose, the substitution pair at residues 34 and 36 is more frequent at low dose. In addition, the highest fraction of the His 34 mutation relative to background mutations, was found with low dose at day 21. Finally and most important, the Asn (or Gln) and Phe mutation pair, the hallmark of the most common high-affinity anti-phOx antibodies, occurred with a lower mutation background, when the low-dose protocol was used, regardless of time postimmunization (Table 3).

Thus, the results of this paper throw some light on the dynamics of the hypermutation and antigen-driven selection during the affinity maturation of antibodies and may provide a rational molecular explanation of the advantages of low-dose immunizations for the derivation of high-affinity antibodies. Taken together, our results support the notion that while high-dose immunization gives rise to a larger accumulation of mutations, selection for high-affinity antibodies is less stringent. Thus, immunization with low-antigen doses favours selection of a memory cell pool displaying a more focused set of somatic mutants, because the genes with the lowest load of irrelevant or potentially deleterious mutations are more efficiently selected.

ACKNOWLEDGMENTS

We thank the National Foundation of Cancer Research (USA), Xunta de Galicia and Ministerio de Educación y Ciencia (Spain), who contributed support to this work. We thank Teresa Langford for help with mice, Richard Pannell for help with the preparation of phOx-beads, and Joaquín Prado for advising on statistical analysis. We also thank Beatriz Goyenechea and Gottfried Fischer for comments and helpful discussions.

REFERENCES

1. Goidl E.A., Paul W.E., Siskind G.W. & Benacerraf B. (1968) The effect of antigen dose and time after immunisation on the amount and affinity of anti-hapten antibody. *J Immunol* **100,** 371.
2. Siskind G.W. & Benacerraf B. (1969) Cell selection by antigen in the immune response. *Adv Immunol* **10,** 1.
3. Griffiths G.M., Berek C., Kaartinen M. & Milstein C. (1984) Somatic mutation and the maturation of immune response to 2-phenyloxazolone. *Nature* **312,** 271.
4. Berek C. & Milstein C. (1987) Mutation drift and repertoire shift in the maturation of the immune response. *Immunol Rev* **96,** 23.
5. Betz A.G., Neuberger M.S. & Milstein C. (1993) Discriminating intrinsic and antigen-selected mutational hotspots in immunoglobulin V genes *Immunol. Today* **14,** 405.
6. Betz A.G., Rada C., Pannell R., Milstein C. & Neuberger M.S. (1993) Passenger transgenes reveal intrinsic specificity of the antibody hypermutation mechanism: Clustering, polarity, and specific hot spots. *Proc Natl Acad Sci USA* **90,** 2385.
7. Liu Y.J., Joshua D.E., Williams G.T., Smith C.A., Gordon J. & MacLennan I.C.M. (1989) Mechanisms of antigen-driven selection in germinal centres. *Nature* **342,** 929.
8. Apel M. & Berek C. (1990) Somatic mutations in antibodies expressed by germinal centre B cells early after primary immunization. *Int Immunol* **9,** 813.
9. Jacob J., Kelsoe G., Rajewsky R. & Weiss U. (1991) Intraclonal generation of antibody mutants in germinal centres. *Nature* **354,** 389.
10. Kuppers R., Zhao M., Hansmann M.L. & Rajewsky K. (1993) Tracing B cell development in human germinal centres by molecular analysis of single cells picked from histological sections. *EMBO J* **12,** 4955.
11. MacLennan I.C.M. (1994) Germinal centres. *Annu Rev Immunol* **12,** 117.
12. Shokat K.M. & Goodnow C.C. (1995) Antigen-induced B-cell death and elimination during germinal-centre immune responses. *Nature* **375,** 334.
13. Rada C., Gupta S.K., Gherardi E. & Milstein C. (1991) Mutation and selection during the secondary response to 2-phenyloxazolone. *Proc Natl Acad Sci USA* **88,** 5508.
14. Ziegner M, Steinhauser G. & Berek C. (1994) Development of antibody diversity in single germinal centers: Selective expansion of high-affinity variants. *Eur J Immunol* **24,** 2393.
15. Lindhout E., Mevissen M.L., Kwekkeboom, J., Tager J.M. & De Groot C. (1993) Direct evidence that human follicular dendritic cells (FDC) rescue germinal centre cells from death by apoptosis. *Clin Exp Immunol* **91,** 330.
16. González-Fernández A. & Milstein C. (1993) Analysis of somatic hypermutation in mouse Peyer's patches using immunoglobulin κ light chain transgenes. *Proc Natl Acad Sci USA* **90,** 9862.
17. Katz D.H., Paul W.E., Goidl E.A. & Benacerraf B. (1970) Carrier function in anti-hapten immune responses. Enhancement of primary and secondary anti-hapten antibody responses by carrier preimmunization. *J Exp Med* **132,** 261.
18. Kallberg-E., Gray-D & Leanderson-T. (1995) The effect of carrier and carrier priming on the kinetics and pattern of somatic mutation in the Vκ Ox1 gene. *Eur J Immunol* **25,** 2349.
19. Kelsoe G. (1995) *In situ* studies of the germinal center reaction. *Adv Immunol* **60,** 267.
20. Berek C. & Ziegner M. (1993) The maturation of the immune response. *Immunol Today* **14,** 400.
21. Weiss U., Zoebelein R. & Rajewsky K. (1992) Accumulation of somatic mutants in the B cell compartment after primary immunization with a T cell-dependent antigen *Eur J Immunol* **22,** 511.
22. Kepler T.B. & Perelson, A.S. (1993) Cyclic re-entry of GC B cells and the efficieny of affinity maturation. *Immunol Today* **14,** 412.

