

Somatic diversity of the immunoglobulin repertoire is controlled in an isotype-specific manner

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We have studied two aspects of the IgE immune response. First, we have compared the kinetics of the IgE response to the T cell-dependent antigen ph-Ox coupled to ovalbumin with that of the IgG1 response and we have assessed the quality of the IgE response. Second, we have studied the generation of somatic diversity, understood as the combined effect of somatic mutation and the selection of D(iversity) and J(oining) elements, in germinal center B cells at the molecular level, using the germ-line sequence of the prototype anti-ph-Ox heavy chain variable element V_HOx1 as reference. We evaluated sequences derived from μ -, γ 1- and ϵ -variable elements and showed that somatic diversification was different for all isotypes studied. We further compared the IgE responses of wild-type mice with those of mice expressing a truncated cytoplasmic IgE tail (IgE^{KVK Δ tail}). IgE^{KVK Δ tail} mice showed a more diverse sequence pattern. We corroborated previous results suggesting that short CDR3 regions are indicative for high-affinity antibodies by measuring relative affinities of phage-expressed Fab fragments with prototype long and short CDR3 regions. Therefore, the composition of the antigen-receptor is responsible for the selection process and the expansion of antigen-specific cells, leading to an isotype-specific antibody repertoire.

Key words: Affinity maturation / IgE memory / Germinal center B cell / Repertoire shift / Oxazolone

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1 Introduction

The humoral immune response plays a critical role in the containment and elimination of many pathogenic organisms. It does so by first generating a broadly reactive, not very specific, but often effective, IgM-dominated primary response [1]. Later, the response becomes more focused, reflected in a switch [2, 3] from the IgM-based to an IgG-, IgA- or IgE-mediated response and an increased affinity of the antibodies. Class switch and affinity maturation are exquisitely T cell dependent, and take place in a specialized microenvironment, the germinal center [4–7]. During these processes, two other important events take place: the generation of short- and long-lived antibody-forming cells (AFC, also known as plasma cells) [8], ensuring an immediate and a longer-lasting specific antibody level in the serum, and the generation of immunological memory [9], which allows a

rapid, high-affinity response to a secondary encounter with the pathogen. In principle, immune memory has a T and a B cell component. B cell memory is thought to rely on the selection and persistence of a B cell subset that has acquired affinity-enhancing somatic mutations. Previously, we showed for murine IgE [10] that the generation of a class-switched, affinity-maturated repertoire is dependent on the expression of the membrane-bound isotype during the immune response. Similar results were reported for murine IgG1 [11] and later for human IgG2a [12]. However, the nature and effects of the signals generated by the antigen receptor are mostly unknown. We [10] and others [11] showed that the 28-amino acid-long cytoplasmic tail of murine IgE and IgG1, respectively, strongly influenced both the number of cells that were recruited in the IgE- or IgG1-AFC pool and the quality of the response.

We have now studied the influence of the cytoplasmic domain of Ig receptors on the somatic diversity of the antibody repertoire. The immune response was monitored over a 49-day period after immunization with the T cell-dependent hapten 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (ph-Ox) with particular attention for the quality and quantity of serum antibodies, and the gener-

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Abbreviations: **AFC:** Antibody-forming cell **ph-Ox:** 4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one **IgE^{KVK Δ tail}:** Truncated cytoplasmic IgE tail **WT:** Wild type

ation of diversity at the molecular level in germinal center B cells. We studied these aspects of the immune response also in mutant mice with a truncation of the cytoplasmic tail of IgE (IgE^{KVKΔtail}), rendering this tail similar to that of IgM and IgD, and compared them to wild-type (WT) IgE and IgG1 and IgM responses.

2 Results

2.1 IgE immune response in answer to immunization with ph-Ox

We first analyzed the IgG1- and IgE-mediated immune response in WT BALB/c mice. Mice were immunized with the T cell-dependent hapten ph-Ox coupled to ovalbumin [13] over a 49-day period. The immunization protocol is schematically depicted in Fig. 1. IgG1 antibodies were readily found a few days after immunization (Fig. 2a), rose further after a first booster immunization and remained on a high level. Strong support exists in the literature that during this phase-affinity maturation takes place [4–7, 14] and long-lived plasma cells are generated [8]. Specific IgE antibodies were only detectable after the first booster immunization, peaked at day 21, and then declined rapidly, until the next encounter with antigen (Fig. 2b). These results clearly show that IgE responses are short-lived. This may be partly due to the very short half-life time of IgE [15, 16] but it also is reflective of a poor recruitment of long-lived plasma cells in the IgE immune response (Fig. 2b, and unpublished

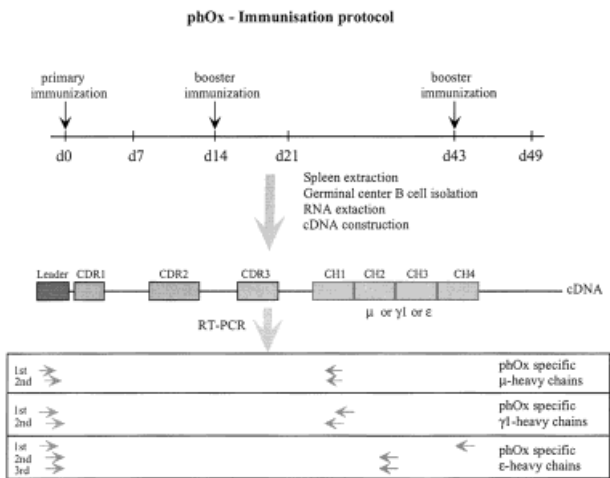


Fig. 1. Scheme of the ph-Ox specific amplification of variable heavy chains of different isotypes. Primer positions correspond to the original location. μ- and γ1-heavy chains were amplified by a standard nested PCR protocol. ε-heavy chains could only be amplified after a triple nested protocol.

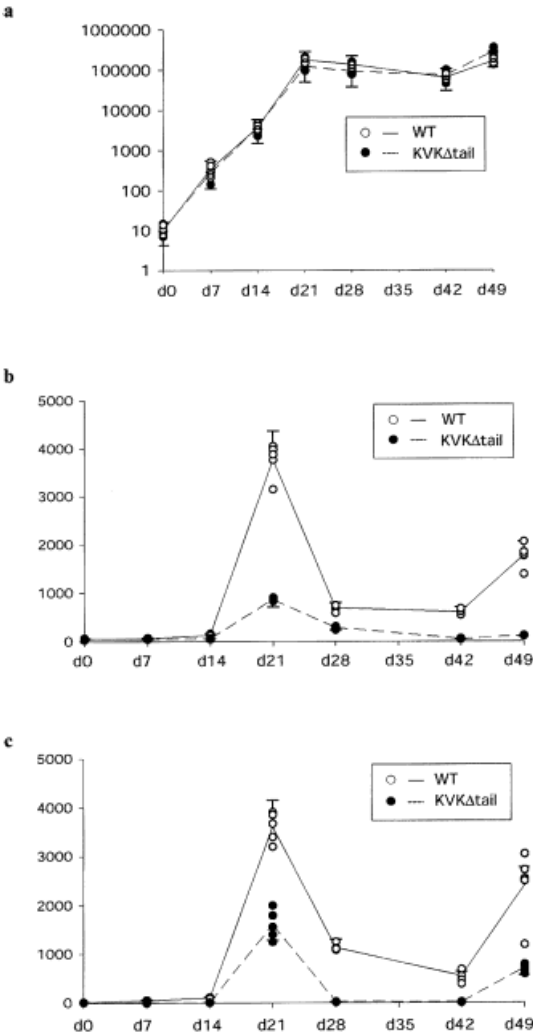


Fig. 2. ph-Ox-specific IgG1 and IgE serum levels. Mice (five animals per group) were immunized with ph-Ox₁₁-OVA and received booster immunizations 2 and 6 weeks later. Mice were bled at the indicated time points and analyzed individually. Results are expressed as the serum dilution where half-maximum absorbance was obtained for IgG1 (a), IgE (b) and IgE after depletion of the serum with protein G-Sepharose (c).

observations). The responses therefore reflect the efficiency of the recruitment of short-lived IgE AFC. These AFC have not necessarily undergone extensive somatic mutations [8]. IgE responses do show “memory” in the sense of a swift response to secondary and tertiary immunization [8]. In mice with a deletion of the 25 C-terminal amino acids of the IgE cytoplasmic tail (IgE^{KVKΔtail}) [10] specific IgG1 titers were similar to those in WT mice, as expected; however, IgE titers were 70% lower than in WT mice after immunization with ph-Ox. Titers only increased slowly, indicative of a poor recruitment of AFC in the immune response.

2.2 Determination of the relative affinity of ph-Ox-specific IgE antibodies

Next, we assessed the relative affinity of anti-ph-Ox specific IgE antibodies in two ways. Because IgE antibody levels are measured in about a 1,000-fold excess of IgG1 antibodies we first depleted the serum antibodies with protein G-Sepharose for competing excess IgG antibodies (a reversed “classical” affinity measurement) [16]. Depletion of the IgG did not markedly increase the titers in WT mice, indicating that IgE antibodies of sufficient affinity that were not influenced by competing IgG1 antibodies, were produced (Fig. 2c). In IgE^{KVKΔtail} mice specific IgE responses were sensitive to IgG depletion by protein G (Fig. 2c). The latter result implies that the affinity of the IgE antibodies was sufficiently low to be influenced by competing IgG1 antibodies. These results were confirmed by a second approach where the relative affinity [17] of anti-ph-Ox-specific IgE and IgG1 antibodies was measured using “low-haptenated” and “high-haptenated” ELISA plates [17]. In WT mice we could not detect differential binding to the differently haptenated ELISA plates. In IgE^{KVKΔtail} mice, a twofold difference in titer between the highest- (ph-Ox₃₅-BSA) and the lowest haptenated ELISA-plate (ph-Ox₁₁-BSA) was seen at each time point at which specific IgE was detected (Fig. 3b), but was most impressive at day 21. These results and dilution experiments (data not shown) proved that these results were independent of the titer of IgE antibodies. Using the paired *t*-test, statistical significance at the level of $0.1 > p > 0.001$ was calculated. The absence of the cytoplasmic tail of IgE causes a low-titered response of a poorer quality. The cytoplasmic tail of Ig receptors could thus influence both the efficiency of the somatic mutation process in germinal centers and the efficiency of the recruitment of (high-affinity) B cell clones into the AFC pool.

Therefore, we studied the pattern and extent of somatic diversification in germinal center B cells early and late in the immune response. We compared the occurrence of somatic mutations and the use of DJ elements in the prototype anti-ph-Ox heavy chain variable region, V_HOx1 [14, 18], in IgM, IgG1 and IgE antibodies, both in WT and IgE^{KVKΔtail} mice. A schematic representation of the experimental protocol is given in Fig. 1. Spleen cells of three BALB/c and three IgE^{KVKΔtail} mice that were immunized at days 0, 14 and 43 [13] were isolated on days 7, 14, 21, 43 and 49, and pooled. Germinal center B cells were isolated based on the expression of the surface marker CD45R and the binding of peanut agglutinin (PNA). This procedure excludes plasma cells that do not bind PNA [19]. Germinal center B cells were found with an average frequency of 5%. RNA was prepared from 10⁶ cells, followed by cDNA synthesis. For PCR, primers comple-

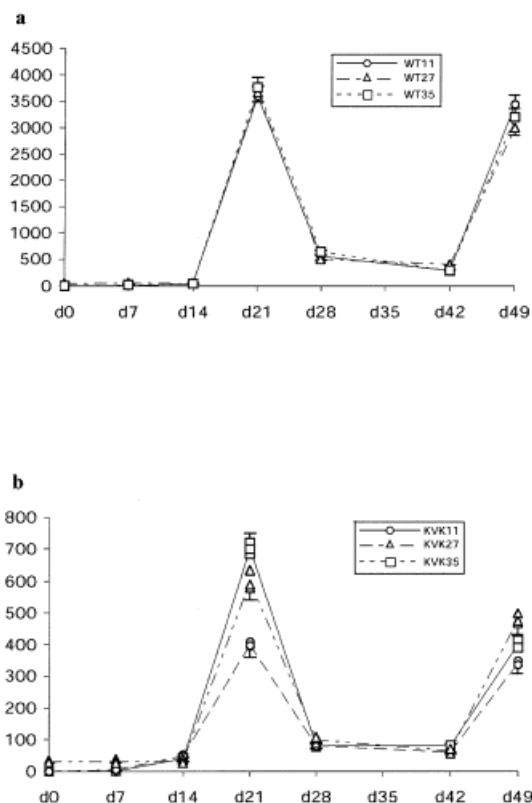


Fig. 3. ph-Ox-specific IgE serum levels in dependence of hapten density. WT (a) and IgE^{KVKΔtail} mice (b) were bled at the indicated time points and the sera of each group were pooled. ELISA plates were coated with BSA coupled with 11, 27 or 35 2-phenyloxazol-5-one groups per BSA molecule (ph-Ox₁₁-, ph-Ox₂₇-, ph-Ox₃₅-BSA). Results are expressed as described in Fig. 2.

mentary to C_μ, C γ1 or C_ε constant regions were combined with a specific primer (Fig. 1) for the leader region of V_HOx1 [4, 20]. A proofreading polymerase was used throughout these experiments to minimize experiment-based mutations.

2.3 Evaluation of somatic diversity of IgM antibodies

To get an impression about the pool of somatic variants used, we first sequenced 36 μ-heavy chain clones from day 7 to day 49 of immunization (Fig. 4) and aligned the sequences with the ph-Ox-specific hybridoma H26 [20], expressing the V_HOx1 germ-line sequence. As expected, V_HOx1 was the preferentially amplified V_H prototype. However, in 20–40% of the primary sequences of all isotypes a repeated pattern of substitutions was found. These V_H regions resembled the MC101 sequence [21, 22] (marked with one asterisk) and most likely represent

	100	110	
H26-IgM	Y Y C A R D S G - - - - - - - - - - D Y M G Q		J2
Wtd7-2	Y Y C A K S S - - - - - - - - - - S Y A M D Y M G Q		J4
Wtd7-4	Y Y C A V I T T - - - - - - - - - - K V D Y M G Q		J2
Wtd7-9	Y Y C A R D H - - - - - - - - - - S M F A Y M G Q		J3
Wtd7-10	Y Y C A R R C Y R - - - - - - - - - - A F D Y M G Q		J2
*Wtd7-16	Y Y C A S F T T V V A Y - - - - - - - - - - Y A M D Y M G Q		J4
Wtd7-20	Y Y C A E - - - - - - - - - - I G F A Y M G Q		J4
Wtd7-21	Y Y C A R E E - - - - - - - - - - L G L L D Y M G Q		J4
*Wtd7-24	Y Y C A R E K G L G R - - - - - - - - - - G G F A Y M G Q		J3
*Wtd7-31	Y Y C A R D Y Y G S S Y V - - - - - - - - - - L L V Y M G Q		J2
*Wtd7-38	Y Y C A R N K G N R Y D - - - - - - - - - - Y F V Y M G Q		J2
Wtd7-49	Y Y C A P - - - - - - - - - - H G G L D Y M G Q		J4
*Wtd7-57	Y Y C A K N G Y G N H A N L - - - - - - - - - - A Y M G Q		J3
Wtd14-13	Y Y C A K - - - - - - - - - - P F A Y Y M G Q		J3
*Wtd14-20	Y Y C A R K D Y R - - - - - - - - - - S P F A Y M G Q		J3
Wtd14-30	Y Y C A Q R M - - - - - - - - - - G G Y Y M G Q		J2
Wtd14-31	Y Y C A G D R G - - - - - - - - - - F D Y M G Q		J2
*Wtd14-34	Y Y C A K N D Y D P L Y - - - - - - - - - - Y A M D Y M G Q		J4
*Wtd14-35	Y Y C A R N S D G Y F Y - - - - - - - - - - Y A M D Y M G Q		J4
Wtd14-44	Y Y C A R D R G G - - - - - - - - - - Y A M D Y M G Q		J4
Wtd14-50	Y Y C A R A F G N Y - - - - - - - - - - Y F D Y M G Q		J2
*Wtd14-51	Y Y C A R N G G Y D G I Y Y - - - - - - - - - - Y A M D Y M G Q		J4
Wtd21-2	Y Y C A K - - - - - - - - - - D W A Y M G Q		J3
Wtd21-4	Y Y C V G S - - - - - - - - - - T M I T Y M G Q		J3
Wtd21-8	Y Y C A R - - - - - - - - - - D W A Y M G Q		J3
Wtd21-9	Y Y C A K S H R S T M I T - - - - - - - - - - T D A M D Y M G Q		J4
Wtd21-12	Y Y C A R E D Y R Y - - - - - - - - - - D P F A Y M G Q		J3
Wtd21-26	Y Y C A R T Y Y G S - - - - - - - - - - S P D Y M G Q		J2
Wtd21-31	Y Y C A K - - - - - - - - - - P F D Y M G Q		J2
Wtd49-2	Y Y C A R - - - - - - - - - - R A A M D Y M G Q		J4
*Wtd49-3	Y Y C A K R I T A L Y - - - - - - - - - - Y A M D Y M G Q		J4
Wtd49-6	Y Y C A E - - - - - - - - - - M I T T Y M G Q		J3
Wtd49-8	Y Y C A R A Y S T D I - - - - - - - - - - P F A Y M G Q		J2
*Wtd49-9	Y Y C A T R G G N - - - - - - - - - - Y A M D Y M G Q		J4
*Wtd49-11	Y Y C A K E D Y D G L Y - - - - - - - - - - Y A M D Y M G Q		J4
*Wtd49-14	Y Y C A K N N L W D - - - - - - - - - - Y A M D Y M G Q		J4
*Wtd49-16	Y Y C A K M I T F P - - - - - - - - - - Y M Y F D Y M G Q		J1

Fig. 4. Sequence alignments, showing the CDR3 region of ph-Ox specific WT μ -heavy chains. Sequence files were analyzed using the DNASTAR package. Amino acid positions are according to Kaartinen et al. [18]. CDR3 starts with amino acid position 96 and varies in length from 7 to 17 amino acids. The J element used is indicated at the end of the sequence. MC101-like sequences are marked with one asterisk. The full sequence alignments can be downloaded on request.

a V_H subset that is closely related to V_H Ox1. As observed before, the CDR3 region displayed a high degree of diversity, generated by VDJ rearrangement. The length differed from 7 to 17 amino acids. All four J elements

were used, with a preference for J3 [7] and J4. Interestingly, during the whole period of immunization not a single identical sequence was found twice (Table 1, Fig. 4). Table 1–4 also summarize the frequency and type of mutations. In these tables, the MC101-like sequences are not represented, because the germ-line sequence of this V_H member has not been established. The number of mutations per sequence did not vary significantly during the course of immunization and replacement mutations were more frequent than silent mutations. In summary, a highly diverse repertoire of V_H Ox1-based antibodies accumulated in the IgM pool, with no indication of one preferred type during the course of immunization.

2.4 Molecular analysis of the somatic diversity following an IgG1 response

A rather different situation was found in γ 1-heavy chains. We analyzed 81 γ 1-specific heavy chain sequences from day 7 up to day 49 of immunization (Fig. 5). In the primary and late (day 43) response, MC101-like sequences as well as the prototype V_H Ox1 sequence were observed. The CDR1 region at position 31–35 showed a higher rate of amino acid exchanges than in μ -heavy chains, particularly late in the immune response (e.g. Ser-31, Tyr-32 and Gly-33). In the CDR2 region, fidelity for amino acid substitutions is comparable to μ -heavy chains, with a few new amino acid substitutions (e.g. Ser at position 56). Highly different sequence motifs were detected in the CDR3 region. During the primary immunization period (days 7 and 14) the sequence motifs are very similar to those in the μ -heavy chains. The length of the CDR3 region varies from 7 to 15 amino acids; however, the total number of different DJ elements was lower

Table 1. Evaluation of the somatic diversity in ph-Ox specific, WT μ -heavy chains

WT (Fig. 4) μ -heavy chains	μ - V_H total no. of sequences	% CDR3 diversity	μ -MC101 total no. of sequences	μ - V_H Ox1 no. of sequences	Total mutations/ sequence	Silent mutations/ sequence	AA exchanges/ sequence	R/S values
Day 7	12	100	5	7	10	3.1	4.7	2.1
Day 14	9	100	4	5	9.6	3	4.8	2.2
Day 21	7	100	0	7	12.1	3.8	6.1	2.1
Day 43	–	–	–	–	–	–	–	–
Day 49	8	100	5	3	13.3	6	5.3	1.2

a) In Tables 1–4, the number of mutations and the type of mutations were counted and analyzed with the hybridoma H26^[20] as reference. The ratio of replacement to silent mutations (R/S values) were calculated only for V_H Ox1 representatives. MC101-like sequences (sequences marked with one asterisk in Figs. 4–7), as well as sequences arising more than once per day (sequences marked with two asterisk in Figs. 4–7) were not considered. For calculation of CDR3 diversity V_H Ox1 and MC101-like sequences were used (AA: amino acid)

Table 2. Evaluation of the somatic diversity in ph-Ox specific WT γ 1-heavy chains

WT (Fig. 5) γ 1-heavy chains	γ 1-V _H total no. of sequences	% CDR3 diversity	γ l-MC101 total no. of sequences	γ 1-V _H Ox1 no. of sequences	Total mutations/ sequence	Silent mutations/ sequence	AA exchanges/ sequence	R/S values
Day 7	13	61	4	9	9.1	3.2	4.8	1.8
Day 14	15	80	6	9	15.7	5.4	8.3	1.9
Day 21	17	52	0	17	4.6	1.1	3.1	3.1
Day 43	10	60	5	5	4.2	1.2	2.8	2.5
Day 49	15	53	1	14	11.2	3.3	6.8	2.3

Table 3. Evaluation of the somatic diversity in ph-Ox specific WT ϵ -heavy chains

WT (Fig. 6) ϵ - heavy chains	ϵ -V _H total no. of sequences	% CDR3 diversity	ϵ -MC101 total no. of sequences	ϵ -V _H Ox1 no. of sequences	Total mutations/ sequence	Silent mutations/ sequence	AA exchanges/ sequence	R/S values
Day 7	13	23	4	9	16.4	4.2	9.8	2.9
Day 14	10	20	0	10	18.3	8.1	8.1	1.2
Day 21	13	38	0	13	13.4	5.9	6.6	1.2
Day 43	13	38	3	10	16.4	4.4	9.3	2.7
Day 49	11	36	2	9	7.3	1.8	5.3	3

Table 4. Evaluation of the somatic diversity in ph-Ox specific, IgE^{KVK Δ tail} ϵ -heavy chains

KVK (Fig. 7) ϵ -heavy chains	ϵ -V _H total no. of sequences	% CDR3 diversity	ϵ -MC101 total no. of sequences	ϵ -V _H Ox1 no. of sequences	Total mutations/ sequence	Silent mutations/ sequence	AA exchanges/ sequence	R/S values
Day 7	6	83	3	3	43	11.3	18.6	2.8
Day 14	10	60	0	10	19.2	6.9	10	1.7
Day 21	6	50	0	6	1.8	1.1	0.6	0.5
Day 43	18	55	6	12	7.5	2.6	4.5	1.8
Day 49	11	91	3	8	9.7	3.8	4.3	1.5

(Table 2, Fig. 5). The length of the CDR3 region decreased during the secondary response. Clones with a short CDR3 most likely represent high-affinity anti-ph-Ox antibodies [21]. Nine different sequence motifs, which varied only by single amino acid substitutions, dominated CDR3 at day 21. Still, no sequence was identical over the entire V_H region, and an immediate interdependence of the sequenced clones is not visible. Late in the secondary response (day 43) a new pool of anti-ph-Ox heavy chains accumulated. The CDR3 region of these resembled the primary set found at days 7 and 14. Also, the MC101-like sequence is found again (marked with

one asterisk). At 7 days after the secondary booster immunization, the immune response is as restricted as at day 21, with a short CDR3 region. However, additional sequence motifs occurred, neither identical nor related. The number of mutations and the ratio between replacement and silent mutations was not significantly different from those found in μ -heavy chain sequences (Table 1). Summarizing, the pool of anti-ph-Ox γ 1-heavy chains is more restricted than that of μ -heavy chains, in particular as to the length and composition of CDR3 early in the secondary and tertiary response.

	100	110	
H26-IgG1	Y Y C A R D S G	Y Y T M D Y W G Q	J2
WTd7-5	Y Y C A R A F Y G N Y D	Y Y T M D Y W G Q	J4
**WTd7-6	Y Y C A R A F Y G N Y D	Y Y T M D Y W G Q	J4
WTd7-7	Y Y C A K H V G D R R	Y Y W F D Y W G A	J1
WTd7-8	Y Y C A R A F Y G N Y D	Y Y T M D Y W G Q	J4
**WTd7-11	Y Y C A R A F Y G N Y D	Y Y T M D Y W G Q	J4
*WTd7-13	Y Y C A K H D D G Y Y G	Y Y S G F A Y W G Q	J3
WTd7-17	Y Y C A R A F Y G N Y D	Y Y T M D Y W G Q	J4
**WTd7-19	Y Y C A R A F Y G N Y D	Y Y T M D Y W G Q	J4
WTd7-21	Y Y C A K P I L Y D G Y	Y Y F W F A Y W G Q	J3
WTd7-56	Y Y C A R R Y G H	Y Y F D Y W G Q	J2
*WTd7-57	Y Y C A R R Y G H	Y Y F D Y W G Q	J2
WTd7-60	Y Y C A R E N D Y E	Y Y D A M D Y W G Q	J4
*WTd7-66	Y Y C A R E N D Y E	Y Y D A M D Y W G Q	J4
*WTd7-73	Y Y C A R R Y G H	Y Y F D Y W G Q	J2
*WTd7-76	Y Y C A R E N D Y E	Y Y D A M D Y W G Q	J4
**WTd7-77	Y Y C A R E N D Y E	Y Y D A M D Y W G Q	J4
**WTd7-79	Y Y C A R E N D Y E	Y Y D A M D Y W G Q	J4
WTd7-81	Y Y C A R E N D Y E	Y Y D A M D Y W G Q	J4
**WTd7-82	Y Y C A R E N D Y E	Y Y D A M D Y W G Q	J4
*WTd7-83	Y Y C A R E N D Y E	Y Y D A M D Y W G Q	J4
WTd7-86	Y Y C A K P H G H	Y Y L A M D Y W G Q	J4
WTd14-2	Y Y C A K H E D S	Y Y S G V D Y W G Q	J4
WTd14-3	Y Y C A K P I F Y D G Y	Y Y F W F A Y W G Q	J3
WTd14-5	Y Y C A R H G G S Y G S	Y Y S S F P Y W G Q	J3
*WTd14-6	Y Y C P T A T	Y Y D A M D Y W G Q	J4
WTd14-7	Y Y C A K T B H G N Y	Y Y V R F G Y W G Q	J3
*WTd14-8	Y Y C P T A T	Y Y D A M D Y W G Q	J4
*WTd14-25	Y Y C A R H W	Y Y D A M D Y W G Q	J4
WTd14-27	Y Y C A K H V G D R R	Y Y W F D Y W G A	J1
*WTd14-94	Y Y C A R H E V N P Y	Y Y F D Y W G Q	J2
WTd14-99	Y Y C A R D Y S S	Y Y S P F A Y W G Q	J3
WTd14-110	Y Y C A R D Y S S	Y Y S P F A Y W G Q	J3
*WTd14-123	Y Y C A K T L T T	Y Y A K T M D Y W G Q	J4
WTd14-125	Y Y C A K H T T	Y Y D A L D Y W G Q	J4
*WTd14-131	Y Y C A K G D Y D	Y Y D D A M D Y W G Q	J4
WTd14-132	Y Y C A K T L T T	Y Y A K T M D Y W G Q	J4
WTd21-1	Y Y C A R D R G	Y Y A Y W G Q	J3
WTd21-3	Y Y C A R D R G	Y Y T Y W G Q	J4
WTd21-4	Y Y C A R D R G	Y Y A D W G Q	J3
WTd21-5	Y Y C A R D R G	Y Y A Y W G Q	J3
WTd21-7	Y Y C A R D R G	Y Y A Y W G Q	J3
WTd21-8	Y Y C A R D Y G	Y Y A Y W G Q	J3
WTd21-34	Y Y C A R D R G	Y Y D Y W G Q	J4
WTd21-36	Y Y C A R D Y G	Y Y A Y W G Q	J3
WTd21-67	Y Y C A R D R G	Y Y D Y W G Q	J2
WTd21-68	Y Y C A R D R G	Y Y D Y W G Q	J2
WTd21-70	Y Y C A R D W G	Y Y D Y W G Q	J4
WTd21-71	Y Y C A R D G G	Y Y D Y W G Q	J4
WTd21-75	Y Y C A R D G G	Y Y D Y W G Q	J4
WTd21-78	Y Y C A R D R G	Y Y D Y W G Q	J4
WTd21-79	Y Y C A R D R G	Y Y D Y W G Q	J4
WTd21-94	Y Y C A R D G G	Y Y D Y W G Q	J4
WTd21-97	Y Y C A R D Y S	Y Y H A M D Y W G Q	J4
WTd43-44	Y Y C A R D R G	Y Y A Y W G Q	J3
WTd43-59	Y Y C A R D R N Y G T	Y Y D A M D Y W G Q	J4
*WTd43-68	Y Y C A R D G G	Y Y D Y W G Q	J2
WTd43-73	Y Y C A R T L Y S N Y	Y Y A M D C W G Q	J4
*WTd43-74	Y Y C A R E C D Y D	Y Y T A M D Y W G Q	J4
*WTd43-79	Y Y C A R D G G	Y Y D Y W G Q	J2
WTd43-80	Y Y C A R D G G	Y Y D Y W G Q	J2
*WTd43-81	Y Y C A R K D G H	Y Y W F F D Y W G A	J1
**WTd43-82	Y Y C A R T L Y S N Y	Y Y A M D C W G Q	J4
**WTd43-83	Y Y C A R T L Y S N Y	Y Y A M D C W G Q	J4
*WTd43-84	Y Y C A R T L Y S N Y	Y Y A M D C W G Q	J4
WTd43-93	Y Y C A R D G G	Y Y D Y W G Q	J2
WTd49-1	Y Y C A R D R D W	Y Y D R F G F W G Q	J3
WTd49-2	Y Y C A R D R D W	Y Y D R F G F W G Q	J3
WTd49-4	Y Y C A R D W G	Y Y D Y W G Q	J4
WTd49-6	Y Y C A R D G G	Y Y D Y W G Q	J3
WTd49-7	Y Y C A R H A D S S Y	Y Y A L D Y W G L	J2
WTd49-8	Y Y C A R D G G	Y Y D Y W G Q	J4
WTd49-9	Y Y C A R D W G	Y Y D Y W G Q	J4
**WTd49-11	Y Y C A R H A D S S Y	Y Y A L D Y W G L	J2
WTd49-25	Y Y C A R D R G	Y Y T H W G Q	J3
WTd49-26	Y Y C A R D R G	Y Y T H W G Q	J3
WTd49-76	Y Y C A R D W G	Y Y A Y W G Q	J3
WTd49-84	Y Y C A R D R G	Y Y T Y W G Q	J3
WTd49-103	Y Y C A R D R G	Y Y T H W G Q	J3
*WTd49-105	Y Y C A T D W G	Y Y T Y W G Q	J3
WTd49-106	Y Y C A R D R G	Y Y T Y W G Q	J3
WTd49-108	Y Y C A T D W G	Y Y T Y W G Q	J3

Fig. 5. Sequence alignments, showing the CDR3 region of ph-Ox-specific WT γ 1-heavy chains. CDR3 starts with amino acid position 96 and varies in length from 7 to 15 amino acids. Nearly 50% of the analyzed sequences use the J4 element. MC101-like sequences are marked with one asterisk. Sequences that were identical at the nucleotide level are marked with two asterisks.

2.5 Evaluation of somatic diversity following an IgE response

We next asked, whether the IgE response follows a maturation pattern similar to the IgG1 response. We analyzed 65 specific ϵ -V_H gene segments (Fig. 6). During the whole course of immunization the IgE response was rather restricted. Again, MC101-like sequences were

	100	110	
H26-IgE	Y Y C A R D S G	Y Y T M D Y W G Q	J2
WTd7-1	Y Y C A R E A Y Y R Y	Y Y G F D Y W G Q	J2
*WTd7-3	Y Y C A R E A Y Y R Y	Y Y G F D Y W G Q	J2
WTd7-7	Y Y C A R E A Y Y R Y	Y Y G F D Y W G Q	J2
*WTd7-8	Y Y C A R E A Y Y R Y	Y Y G F D Y W G Q	J2
WTd7-13	Y Y C A R E A Y Y R Y	Y Y G F D Y W G Q	J2
*WTd7-18	Y Y C A R E A Y Y R Y	Y Y G F D Y W G Q	J2
WTd7-24	Y Y C A R E A Y Y R Y	Y Y G F D Y W G Q	J2
WTd7-56	Y Y C A R E A Y Y R Y	Y Y G F D Y W G Q	J2
WTd7-147	Y Y C A K H V W Y Y E	Y Y G R Y F D Y W G A	J1
*WTd7-148	Y Y C V K	Y Y I G E Y W G Q	J3
WTd7-149	Y Y C A K H V W Y Y E	Y Y G R Y F D Y W G A	J1
WTd7-154	Y Y C A K H V W Y Y E	Y Y G R Y F D Y W G A	J1
WTd7-156	Y Y C A K H V W Y Y E	Y Y G R Y F D Y W G A	J1
WTd14-3	Y Y C A S T G K	Y Y D Y A M D Y W G Q	J4
**WTd14-5	Y Y C A S T G K	Y Y D Y A M D Y W G Q	J4
WTd14-14	Y Y C A S T G K	Y Y D Y A M D Y W G Q	J4
WTd14-15	Y Y C A S T G K	Y Y D Y A M D Y W G Q	J4
WTd14-16	Y Y C A S T G K	Y Y D Y A M D Y W G Q	J4
*WTd14-17	Y Y C A S T G K	Y Y D Y A M D Y W G Q	J4
WTd14-23	Y Y C A S T G E	Y Y D Y A M D Y W G Q	J4
WTd14-95	Y Y C A S T G K	Y Y D Y A M D Y W G Q	J4
*WTd14-96	Y Y C A S T G K	Y Y D Y A M D Y W G Q	J4
WTd14-99	Y Y C A S T G K	Y Y D Y A M D Y W G Q	J4
WTd14-101	Y Y C A S T G K	Y Y D Y A M D Y W G Q	J4
WTd14-102	Y Y C A S T G K	Y Y D Y A M D Y W G Q	J4
WTd14-115	Y Y C A S T G K	Y Y D Y A M D Y W G Q	J4
WTd21-15	Y Y C A R I Y Y D Y P	Y Y A M F A Y W G Q	J3
WTd21-170	Y Y C A R D Q G	Y Y F H W G Q	J3
WTd21-172	Y Y C A R I Y Y D Y P	Y Y A M F A Y W G Q	J3
*WTd21-173	Y Y C A R D Q G	Y Y F H W G Q	J3
WTd21-215	Y Y C A R D R G	Y Y D Y W G Q	J2
WTd21-219	Y Y C A R D Q G	Y Y F H W G Q	J3
WTd21-341	Y Y C A S T G K	Y Y D Y A M D Y W G Q	J4
WTd21-373	Y Y C A S T G K	Y Y D Y A M D Y W G Q	J4
WTd21-471	Y Y C A S T G K	Y Y D Y A M D Y W G Q	J4
WTd21-492	Y Y C A S T G K	Y Y D Y A M D Y W G Q	J4
WTd21-524	Y Y C A S T G K	Y Y D Y A M D Y W G Q	J4
WTd21-620	Y Y C A S T G K	Y Y D Y A M D Y W G Q	J4
WTd21-638	Y Y C A S T G K	Y Y D Y A M D Y W G Q	J4
WTd21-672	Y Y C A S T G K	Y Y D Y A M D Y W G Q	J4
WTd43-302	Y Y C A R D P D Y R Y D G	Y Y S W F A Y W G Q	J3
WTd43-305	Y Y C A K K L H	Y Y S C Y F D Y W G A	J1
WTd43-320	Y Y C A K K L H	Y Y S C Y F D Y W G A	J1
WTd43-321	Y Y C A K K L H	Y Y S C Y F D Y W G A	J1
WTd43-325	Y Y C A K K L H	Y Y S C Y F D Y W G A	J1
WTd43-328	Y Y C A R D P D Y R Y D G	Y Y S W F A Y W G Q	J3
*WTd43-339	Y Y C A K K L H	Y Y S C Y F D Y W G A	J1
WTd43-349	Y Y C A K K L H	Y Y S C Y F D Y W G A	J1
WTd43-359	Y Y C A K K L H	Y Y S C Y F D Y W G A	J1
WTd43-395	Y Y C A R D P D Y R Y D G	Y Y S W F A Y W G Q	J3
WTd43-470	Y Y C A R D P D Y R Y D G	Y Y S W F A Y W G Q	J3
*WTd43-528	Y Y V S G I G	Y Y T E A M D Y W G Q	J4
*WTd43-529	Y Y V S G I G	Y Y T E A M D Y W G Q	J4
*WTd49-2	Y Y C A R D G G	Y Y D Y W G Q	J2
*WTd49-16	Y Y C A R D G G	Y Y D Y W G Q	J2
WTd49-24	Y Y C A R D G G	Y Y D Y W G Q	J2
WTd49-237	Y Y C A R E G S S	Y Y G R F S Y W G Q	J3
WTd49-238	Y Y C A R E G S S	Y Y G R F S Y W G Q	J3
WTd49-240	Y Y C A R E G S S	Y Y G R F S Y W G Q	J3
WTd49-241	Y Y C V R S Q G R	Y Y D L D H W G Q	J2
WTd49-243	Y Y C V R S L G R	Y Y D L D H W G Q	J2
*WTd49-244	Y Y C A R E G S S	Y Y G R F S Y W G Q	J3
WTd49-245	Y Y C A R E G S S	Y Y G R F S Y W G Q	J3
WTd49-246	Y Y C A R E G S S	Y Y G R F S Y W G Q	J3
WTd49-247	Y Y C A R E G S S	Y Y G R F S Y W G Q	J3

Fig. 6. Sequence alignments, showing the CDR3 region of ph-Ox-specific WT ϵ -heavy chains. Comparable to γ 1, CDR3 starts with amino acid position 96 and varies in length from 7 to 15 amino acids. In contrast to μ - and γ 1-heavy chains, the four J elements are distributed equally. Nine MC101-like sequences were identified (marked with one asterisk). Sequences that were identical at the nucleotide level are marked with two asterisks.

found early in the primary and late secondary response. Late in the primary (day 14), early in the secondary (day 21) and in the tertiary response (day 49) a very restricted repertoire was found, with a clear relationship between the sequences. With “a clear relationship” we mean their similarity, in the sense of for instance CDR3 composition, but without a direct “hereditary” relationship. This may reflect the low titer found in the IgE response, with a low number of IgE-specific germinal centers. The reduction in length of the CDR3 region was less pronounced than for $\gamma 1$ and a few dominant motifs characterized CDR3. Diversity returned in the sequences obtained from day 43. At day 49 new CDR3 motifs showed up. None of these variants were found in the sequence motifs at earlier time points. The ratio of replacement to silent mutations was not significantly changed (Table 3).

Summarizing, the IgE response is more restricted than the IgG1 response. The length of the CDR3 region is longer than that found in IgG1 antibodies, suggesting a lower affinity. It differs strongly from the sequence motifs found in the CDR3 regions of IgG1. However, there is no doubt that the IgE response matures. A set of antigen receptors with a distinct accumulation of mutations is selected and preferentially expanded.

2.6 Comparison of somatic diversity of IgE antibodies in IgE^{KVK Δ tail} mice with those of IgM antibodies in WT mice

We finally asked whether the poor affinity maturation seen in IgE^{KVK Δ tail} mice was due to an insufficient somatic

mutation process or a poor recruitment of high-affinity B cells. Analysis of IgM- and IgG1-derived V_HOx1 sequences in IgE^{KVK Δ tail} mice revealed a pattern as found in WT mice (data not shown). Fifty-seven IgE-derived sequences were analyzed (Fig. 7). Again, in the primary and late secondary response MC101-like sequences were found. The primary response was rather different from that in WT mice, particular in the CDR3 region. Only late in the primary response (day 14) did one clone type resemble a dominant clone type seen in WT mice. The secondary response is characterized by a CDR3 pattern that is strongly represented in $\gamma 1$ antibodies. As for the other isotypes, the response was more diverse at day 43, with longer CDR3 regions. A most significant difference to WT BALB/c mice was detected at day 49. Sequences were more diverse than in WT mice, with long CDR3 regions, resembling the pattern seen in μ -heavy chains. The ratio of replacement to silent mutations was not significantly changed (Table 4). These results indicate that the mechanism of somatic mutation in IgE antibodies is not interfered with in IgE^{KVK Δ tail} mice, but that the selection of B cells with high-affinity receptors is impaired.

2.7 Expression of anti-ph-Ox-specific IgE Fab fragments on the surface of phages

Our premise that a short CDR3 region is indicative of a higher affinity anti-ph-Ox antibody is based on the work of Griffiths et al. [21]. To support this interpretation, we expressed anti-ph-Ox-specific IgE Fab fragments on the surface of M13 phages [23] and measured their affinity for ph-Ox with surface plasmon resonance analysis. We first cloned two different ph-Ox-specific light chains,

Table 5. Surface plasmon resonance analysis of anti-ph-Ox specific IgE Fab-fragments, expressed on the surface of phages^{a)}

	pEL 3	pEL 15	pEL 16	pEL 8	pEL 12	pEL 6	pEL 10	pEL 14
	Fc1-Fc2 RelResp	Fc1-Fc2 RelResp	Fc1-Fc2 RelResp	Fc1-Fc2 RelResp	Fc1-Fc2 RelResp	Fc1-Fc2 RelResp	Fc1-Fc2 RelResp	Fc1-Fc2 RelResp
Start of injection	0	0	0	0	0	0	0	0
End of injection	38	2	1	23	125	57	103	181
End of elution	3.9	–2	–4	14	122.6	10	80	171

a) Two anti-ph-Ox-specific light chains were subcloned into pComb 3 (pEL3 low-affinity variant; pEL6 high-affinity variant) and combined with heavy chain WT IgE d7–1 (long CDR3 region; plasmid pEL8 and pEL10), and heavy chain WT IgE d22–170 (short CDR3 region; plasmid pEL12 and pEL14). WT IgE d7–1 and WT IgE d22–170 heavy chains, subcloned in pComb3 without a light chain (pEL15 and pEL16, respectively) serve as controls. The relative response units, expressed as differences (Fc1–Fc2) of ph-Ox OVA and OVA are proportional to binding affinities. “End of injection” expressed the final on-rate value. “End of elution” expresses the final off-rate value.

	100										110												
H26-IgE	Y	Y	C	A	R	D	S	G	-	-	-	-	-	-	-	D	Y	N	G	Q	J2		
*KVDd7-2	Y	Y	C	A	R	D	S	L	Y	D	G	Y	-	-	T	W	F	A	Y	N	G	J3	
*KVDd7-30	Y	Y	C	A	R	N	S	L	Y	D	G	Y	-	-	T	W	F	A	Y	N	G	J3	
KVDd7-31	Y	S	C	A	K	-	-	-	-	-	-	-	-	-	I	R	G	A	Y	N	G	J3	
KVDd7-33	Y	Y	C	A	K	-	-	-	-	-	-	-	-	-	I	R	G	A	Y	N	G	J3	
*KVDd7-34	Y	Y	C	A	R	N	S	L	Y	D	G	Y	-	-	T	W	F	A	Y	N	G	J3	
KVDd7-56	Y	Y	C	V	R	D	F	Y	G	S	-	-	-	-	R	Y	A	M	D	Y	N	G	J4
KVDd14-11	Y	Y	C	A	R	D	P	D	-	-	-	-	-	-	G	P	F	A	Y	N	G	J3	
KVDd14-60	Y	Y	C	A	R	D	P	D	-	-	-	-	-	-	G	P	F	A	Y	N	G	J3	
KVDd14-107	Y	Y	C	A	R	D	P	D	-	-	-	-	-	-	G	P	F	A	Y	N	G	J3	
KVDd14-129	Y	Y	C	A	K	H	Y	D	N	-	-	-	-	-	S	Y	G	M	D	Y	N	G	J4
KVDd14-159	Y	Y	C	A	S	T	G	K	-	-	-	-	-	-	D	Y	A	M	D	Y	N	G	J4
KVDd14-160	Y	Y	C	A	S	T	G	K	-	-	-	-	-	-	D	Y	A	M	D	Y	N	G	J4
KVDd14-167	Y	Y	C	T	S	T	G	K	-	-	-	-	-	-	D	H	A	M	D	Y	N	G	J4
KVDd14-168	Y	Y	C	A	S	T	G	K	-	-	-	-	-	-	D	Y	A	M	D	Y	N	G	J4
KVDd14-178	Y	Y	C	A	S	T	G	K	-	-	-	-	-	-	D	Y	K	L	D	Y	N	G	J4
KVDd14-203	Y	Y	C	A	S	T	G	K	-	-	-	-	-	-	D	Y	A	M	D	Y	N	G	J4
KVDd21-2	Y	Y	C	A	R	D	R	G	-	-	-	-	-	-	A	Y	N	G	Q	J3			
KVDd21-6	Y	Y	C	A	R	D	P	G	-	-	-	-	-	-	A	Y	N	G	Q	J3			
KVDd21-15	Y	Y	C	A	R	D	R	G	-	-	-	-	-	-	A	Y	N	G	Q	J3			
*KVDd21-29	Y	Y	C	A	R	D	R	G	-	-	-	-	-	-	A	Y	N	G	Q	J3			
KVDd21-33	Y	Y	C	A	R	D	R	G	-	-	-	-	-	-	A	Y	N	G	Q	J3			
KVDd21-35	Y	Y	C	A	R	D	Q	G	-	-	-	-	-	-	A	Y	N	G	Q	J3			
*KVDd21-37	Y	Y	C	A	R	D	Q	G	-	-	-	-	-	-	A	Y	N	G	Q	J3			
KVDd21-119	Y	Y	C	A	R	D	R	G	-	-	-	-	-	-	A	Y	N	G	Q	J3			
*KVDd21-121	Y	Y	C	A	R	D	R	G	-	-	-	-	-	-	A	Y	N	G	Q	J3			
*KVDd21-173	Y	Y	C	A	R	D	R	G	-	-	-	-	-	-	A	Y	N	G	Q	J3			
*KVDd21-179	Y	Y	C	A	R	D	R	G	-	-	-	-	-	-	A	Y	N	G	Q	J3			
KVDd43-42	Y	Y	C	A	R	D	G	G	-	-	-	-	-	-	D	Y	N	G	Q	J4			
KVDd43-56	Y	Y	C	A	R	D	G	G	-	-	-	-	-	-	D	Y	N	G	Q	J2			
*KVDd43-59	Y	Y	C	A	K	N	Y	D	-	-	-	-	-	-	Y	L	D	Y	N	G	Q	J2	
*KVDd43-60	Y	Y	C	V	K	N	Y	D	-	-	-	-	-	-	Y	L	G	Y	N	G	Q	J2	
KVDd43-63	Y	Y	C	A	R	D	G	G	-	-	-	-	-	-	D	Y	N	G	Q	J4			
*KVDd43-64	Y	Y	C	A	K	N	Y	D	-	-	-	-	-	-	Y	L	D	Y	N	G	Q	J2	
KVDd43-65	Y	Y	C	A	R	D	G	G	-	-	-	-	-	-	D	Y	N	G	Q	J4			
KVDd43-68	Y	Y	C	A	R	D	R	L	-	-	-	-	-	-	V	G	A	M	D	Y	N	G	J4
KVDd43-69	Y	Y	C	A	R	D	G	G	-	-	-	-	-	-	D	Y	N	G	Q	J4			
*KVDd43-70	Y	Y	C	A	K	N	Y	D	-	-	-	-	-	-	Y	L	D	Y	N	G	Q	J2	
KVDd43-73	Y	Y	C	A	R	N	G	G	N	Y	G	-	-	-	T	W	F	A	Y	N	G	Q	J3
KVDd43-83	Y	Y	C	A	R	D	E	G	T	L	V	V	P	-	S	Y	A	M	D	Y	N	G	J4
KVDd43-85	Y	Y	C	A	R	D	V	G	T	T	V	V	P	-	S	Y	A	M	D	Y	N	G	J4
*KVDd43-87	Y	Y	C	A	K	N	Y	D	-	-	-	-	-	-	Y	L	D	Y	N	G	Q	J2	
KVDd43-165	Y	Y	C	A	T	D	G	Y	Y	G	N	A	-	-	H	Y	S	M	H	Y	N	G	J4
*KVDd43-167	Y	Y	C	A	K	N	Y	D	-	-	-	-	-	-	Y	L	D	Y	N	G	Q	J2	
KVDd43-168	Y	Y	C	A	R	D	W	G	-	-	-	-	-	-	A	Y	N	G	Q	J3			
*KVDd43-170	Y	Y	C	A	R	D	W	G	-	-	-	-	-	-	A	Y	N	G	Q	J3			
KVDd43-175	Y	Y	C	A	R	D	W	G	-	-	-	-	-	-	A	Y	N	G	Q	J3			
*KVDd49-21	Y	Y	C	A	R	N	L	Y	G	Y	-	-	-	-	Y	F	D	Y	N	G	Q	J2	
KVDd49-27	Y	Y	C	A	R	S	F	S	Y	Y	G	S	-	-	S	Y	A	M	D	Y	N	G	J4
KVDd49-28	Y	H	C	A	I	M	K	D	-	-	-	-	-	-	G	P	F	A	Y	N	G	Q	J3
*KVDd49-30	Y	Y	C	A	N	H	K	-	-	-	-	-	-	-	T	R	F	A	Y	N	G	Q	J3
KVDd49-43	Y	Y	C	A	N	H	I	-	-	-	-	-	-	-	T	R	F	A	Y	N	G	Q	J3
KVDd49-49	Y	Y	C	A	R	S	F	S	Y	Y	G	S	-	-	S	Y	A	M	D	Y	N	G	J4
KVDd49-53	Y	Y	C	A	R	E	G	P	R	Y	-	-	-	-	G	P	F	A	Y	N	G	Q	J3
KVDd49-54	Y	Y	C	A	N	H	I	-	-	-	-	-	-	-	T	G	F	A	Y	N	G	Q	J3
*KVDd49-62	Y	F	C	A	R	N	N	-	-	-	-	-	-	-	Y	A	V	D	Y	N	G	Q	J4
KVDd49-63	Y	Y	C	A	N	H	I	-	-	-	-	-	-	-	T	R	F	A	Y	N	G	Q	J3
KVDd49-66	Y	Y	C	A	R	A	D	N	-	-	-	-	-	-	D	K	N	Y	N	G	Q	J2	

Fig. 7 Sequence alignments, showing the CDR3 region of ph-Ox-specific IgE^{KVKΔtail} ε-heavy chains. Comparable to WT ε-heavy chains, CDR3 starts with amino acid position 96 and varies in length from 7 to 15 amino acids. Like in μ-heavy chains, J3 and J4 elements dominate the immune response. Twelve MC101-like sequences were identified (marked with one asterisk). Sequences that were identical at the nucleotide level are marked with two asterisks.

using primer combinations as described by Berek et al. [4]. Light chain variant I comprised V_KOx1 and the J_K5 element. The histidine at position 34 identifies this light chain as a low-affinity anti-ph-Ox variant [4]. This light chain was cloned into phagemid pComb3 [23], resulting in plasmid pEL3. Light chain variant II showed an exchange of histidine to asparagine, resulting in a high-affinity anti-ph-Ox light chain variant. Cloning of this variant into pComb3 resulted in pEL6. Two V_H regions were chosen: WT IgE d7–1 and WT IgE d22–170 as representatives of a long and a short CDR3 region, respectively. They were then re-amplified and subcloned in pComb3, generating plasmids pEL15 and pEL16, respectively, in

pEL3, generating plasmids pEL8 and pEL12, respectively, and in pEL6, generating plasmids pEL10 and pEL14. The four plasmids that functionally expressed anti-ph-Ox-specific Fab fragments (pEL8 and pEL12 as well as pEL 10 and pEL14) and the four control plasmids (pEL3, pEL6, pEL15 and pEL16) were analyzed for their binding affinity using surface plasmon resonance technology. ph-Ox OVA and OVA as reference were immobilized to an appropriate sensorchip. Fab fragments, expressed on the surface of M13 phage, were directly injected onto the sensorchip. The relative response units, expressed as differences (Fc1–Fc2) of ph-Ox OVA (Fc1) and OVA (Fc2) are proportional to binding affinities and are summarized in Table 5. Injection of pEL3 and pEL6, which express only the (low- and high-affinity) light chain does not show binding to ph-Ox. Similarly, injection of the control plasmids pEL15, which expresses the heavy chain segment WT IgE d7–1, and pEL16 which expresses the heavy chain segment WT IgE d22–170 do not show a measurable response. Injection of pEL8, which expresses both the light chain and the WT IgE d7–1 heavy chain segment representing a long CDR3, shows a marginal response. In contrast, injection of pEL12, which expresses the light chain segment together with the WT IgE d22–170 (pEL12) heavy chain segment representing a short CDR3, results in a remarkable increase in binding affinity. Measurements of pEL10 and pEL14 (heavy chains as described before in combination with a high-affinity light chain variant) showed an additional increase in binding affinities if compared to pEL8 and pEL12. This result fully supports our evaluation of the sequence motifs presented in Fig. 4–7, where we interpret short CDR3 regions as representative of high-affinity and long CDR3 regions as representative of low-affinity antibody variants.

3 Discussion

The B cell antigen receptor plays a central role in almost all processes along the developmental pathway of B cells, *i.e.* the generation, maturation, survival and activation of B cells. The cytoplasmic domains of B cell antigen receptors are short and range from only three amino acid residues in the case of mIgM and mIgD (Lys-Val-Lys-COOH in each case) to 28 residues for the other mIg subclasses. B cell antigen receptors exist as a complex between mIg and at least two other transmembrane polypeptides, called Ig-α and Ig-β, which connect the antigen receptor to the signal transduction pathways in the cell. All isotypes of mIg can form a complex with Ig-α and Ig-β [24]. It is now understood that the B cell antigen receptor regulates three central features of the immune system function [25]. First, it acts as a checkpoint regulator in B cell development, controlling both allelic exclu-

sion and early cellular transitions. Second, as predicted by the clonal selection theory, cross-linking the B cell receptor with antigen can lead to clonal expansion and the production of specific, high-affinity antibodies. The third function of the membrane-bound immunoglobulin is as a receptor for antigen capture and presentation.

Upon activation by antigen, B cells follow one of two differentiation pathways [26]: they may differentiate directly into IgM-secreting plasma cells, or they participate in the development of germinal centers. In these specialized structures within lymphoid organs class switch from IgM to other isotypes (IgG, IgA or IgE) takes place together with successive rounds of mutation of the immunoglobulin V region genes. This is followed by expression of the gene products on the cell surface and selection of the cells on the basis of the affinity of the mutated antigen receptors. Selected cells can now expand clonally and form the precursor pool for B memory cells and for short- and long-lived antibody-forming cells.

We have studied the influence of the cytoplasmic domain of Ig receptors on the somatic diversity of the antibody repertoire. The immune response was monitored over a 49-day period after immunization with the T cell-dependent hapten ph-Ox with particular attention for the quality and quantity of serum antibodies, and the generation of diversity at the molecular level in germinal center B cells. We studied these aspects of the immune response also in mutant mice with a truncation of the cytoplasmic tail of IgE (IgE^{KVKΔtail}), rendering this tail similar to that of IgM and IgD, and compared them to WT IgE and IgG1 and IgM responses.

Analysis and comparison of the types of mutations (silent or replacement) and the number of mutations per analyzed sequence in IgM, IgG1 and IgE in WT and IgE^{KVKΔtail} mice showed a mostly comparable mutation type for the different isotypes (Table 1–4), with a slightly higher number of mutations per analyzed sequence for IgE antibodies from WT mice during the whole immunization period. In WT mice sequence motifs found in IgE antibodies differed from those found in IgG1 antibodies. This argues against the notion that consecutive switch recombination via $\gamma 1$ is a major operational mechanism in the generation of IgE-expressing B cells in the mouse *in vivo*. This contrasts with certain data obtained after *in vitro* restimulation of IgG1-expressing B cells with antigen and sufficient Th2 cells, where proof for a consecutive switch was found [27, 28]. The only exception is found in the CDR3-derived sequences revealed in the secondary response (day 21) of IgE^{KVKΔtail} mice; they are strongly related to those found in IgG1 antibodies. This could well reflect a rare event of consecutive switching, revealed through the low number of IgE-expressing cells

generated the “normal” way, and the low affinity of IgE antibodies in IgE^{KVKΔtail} mice. However, mutations found in CDR1- and CDR2-derived sequences of IgG1 antibodies at day 21 in IgE^{KVKΔtail} mice were not reflected in those of IgE antibodies, suggesting either an abrupt stop in affinity maturation of IgE antibodies in IgE^{KVKΔtail} mice, or a serendipitous result. Remarkably, the short CDR3 found in IgE antibodies of IgE^{KVKΔtail} mice at day 21 did not correlate with an improved affinity (Fig. 2 and 3, see also below).

Both IgG1 and IgE showed affinity maturation, as judged by a decrease in the diversity of DJ elements used in CDR3 and the length of CDR3. In contrast, IgM in WT mice and IgE in IgE^{KVKΔtail} mice lacked these signs of affinity maturation, which was substantiated by the measurement of the relative affinities of IgE antibodies in WT and IgE^{KVKΔtail} mice. We corroborated the notion that a short CDR3 correlates with a higher affinity [21] in experiments in which we measured relative affinities of phage-expressed anti-ph-Ox Fab fragments with a short or a long CDR3. Surface plasmon resonance analysis showed a tenfold higher response for Fab fragments with a short CDR3 than for those with a long CDR3. Apparently the truncated cytoplasmic tail precluded an efficient selection of high-affinity AFC.

Serum antibodies are produced by short- and long-lived AFC. The number of short-lived AFC for IgE in spleen after a tertiary booster is 500–1,000-fold lower than for IgG1 [10]. Remarkably, long-lived plasma cells, which are easily found in the bone marrow, are very rare for IgE (unpublished observations). This of course fits well with the observed rapidly waning antigen-specific serum titers of IgE. The decay of IgE antibodies, which have an extremely short half-life, is not compensated by *de novo* synthesis.

Somatic mutations and diversification caused by DJ recombination as found in germinal center B cells are not always representative of those present in AFC [8], but they are clearly indicative for the effectiveness of the mechanism of diversification itself. IgE^{KVKΔtail} mice show a normal type and frequency of somatic mutation of IgE antibodies, but show a defective recruitment of high-affinity IgE-AFC. A similar phenomenon is seen in IgM antibodies of all mice, leading to the conclusion that the truncated tail of mIgM in WT mice and mIgE in IgE^{KVKΔtail} mice is the cause for the poor selection. These results seem to be in disagreement with recently published results of White and Gray [29] who find evidence for affinity maturation in IgM-derived V_HOx1 sequences. White and Gray [29] based their conclusions on expression profiles of mRNA derived from total spleen, bone marrow or blood but did not specify the B cell type that

made mRNA. Plasma cells for instance can severely bias expression profiles. Our studies concentrated on V_H Ox1-specific expression profiles in germinal center B cells. Here we did not find evidence for extensive somatic diversification in IgM-derived sequences, which does not exclude the presence of a few affinity matured IgM-producing plasma cells. The mechanisms leading to selection of high-affinity B cell clones remain unknown. In view of the growing knowledge in the field of the physiology of the B cell receptor, and the low number of IgE-producing cells, we assume that an altered signal transduction in B cells with an Ig receptor with a short cytoplasmic domain (KVK) leads to a decreased survival and, therefore, a poor selection of high-affinity B cell clones.

Our experiments show very clearly that signals generated by the antigen receptor are needed for the selection process and the expansion of antigen-specific cells, and that isotype-specific patterns arise. Therefore, the signals generated by the cytoplasmic tail of IgE are an important target for intervention in allergic patients, in whom the titer and the affinity of the IgE antibodies for the allergen are directly related to disease activity.

4 Materials and methods

4.1 Immunizations

Mice were immunized subcutaneously (s.c.) with 20 μ g ph-Ox₁₁-OVA, precipitated in alum with *Bordetella pertussis* as adjuvant. At days 14 and 43, mice received booster immunizations with 20 μ g ph-Ox₁₁-OVA s.c. and 20 μ g ph-Ox₁₁-OVA intraperitoneally (i.p.). ph-Ox was conjugated to OVA and BSA according to Mäkelä et al. [13].

4.2 ELISA assays

Serum was taken at different times (days 0, 7, 14, 21, 28, 43 and 49) after immunization. Sera were depleted for IgG as described by Yu et al. [10, 16]. For measuring relative affinities of ph-Ox-specific IgE and IgG1, ELISA plates were coated with BSA coupled to varying densities of the hapten ph-Ox (ph-Ox₁₁, ph-Ox₂₇ or ph-Ox₃₅)-BSA. Specific IgG1 and IgE antibodies to ph-Ox were detected with alkaline phosphatase-conjugated isotype-specific goat anti-mouse antibodies (Southern Biotechnology Associates) or rat anti-mouse IgE monoclonal antibody (EM-95-3). Titers are expressed as the reciprocal serial dilution, at which a half-maximal absorbance value was measured.

Because of the low titer of specific IgE in the serum, sera of three mice per data point were pooled. Measurements of individual mice were made to validate the use of pooled sera. The measurements in individual sera were in full agreement with those obtained in pooled sera.

4.3 Flow cytometry

Mouse spleens were homogenized and erythrocytes were lysed isotonicity. Subsequently, the obtained single-cell suspension was prepared for fluorescence activated cell sorting using a FACS-Vantage flow cytometer (Becton Dickinson). Phycoerythrin-labeled antibody to CD45R (clone 6B₂), a B cell marker and the lectin PNA (peanut agglutinin) coupled with FITC were used to stain the germinal center B cells [4]. Double-positive cells were sorted. For further RNA extraction, 10⁶ double-positive B cells were used.

4.4 RNA extraction

B cells (10⁶) were homogenized with QIAshredder according to manufacturer's instructions. The cell lysate was used for further total RNA extraction using RNeasy (Qiagen). About 20 μ g total RNA per 10⁶ B cells was isolated; 3 μ g total RNA was used for further cDNA construction.

4.5 Reverse transcription-PCR

cDNA was synthesized using oligo(dT) priming. After cDNA synthesis, antigen specific μ -, γ 1- and ϵ -heavy chain variable regions were amplified by nested PCR [30]. The primers bind specifically to the leader sequence of the V_H Ox1 heavy chain variable region and to the constant region (CH1 or CH2 or CH4) of μ -, γ 1- and ϵ -heavy chains, respectively. PCR products were subcloned into the pCR-Script cloning vector (Stratagene) and sequenced. Cloning of an anti-ph-Ox-specific light chain was achieved with a semi-nested approach: nested 5' primers (leader V_L OXA and leader V_L OXB), complementary to the leader region of the ph-Ox-specific V element V_K Ox1 were used; for cloning purposes, V_L OXB contains an internal Sac 1 restriction site. As 3' primer L_K -reverse2, complementary to the 3' end of the constant region of the κ -locus with an internal Xba 1 restriction site was used. PCR products were subcloned into the pCR-Script cloning vector (Stratagene) and sequenced. For all amplifications a proof-reading DNA polymerase was used (Pfu polymerase, Stratagene).

Nested PCR conditions were: 2 min at 95°C (primary denaturation); 45 s at 95°C; 45 s at 59°C; 1 min at 72°C; (35 cycles); 3 min 72°C (final extension).

4.6 Primers

The following primers were used: oligo(dT): 5'-GAGA-GAGAGAGAGAGAGAGAACTAGTCTCGAGTTTTTTTTTTT-3'; leader V_H OXA: 5'-TCCTCTCTAGAGCCCCCA-TCAGAGCATGGC-3'; leader V_H OXB: 5'-GCCTGGTTGA-ATTCCAAGCTGTGTC-3'; CH4 ϵ -reverse: 5'-GATATTGTTTT-CTCCAGTTTC-3'; CH2 ϵ -reverse: 5'-CAGTTTGTGCAAGT-

GTATCAG-3'; CM-reverse: 5'-CAGGGGGCTCCTGCAGG-AGACGAGG-3'; CG1-reverse: 5'-CAGCAGCTGCAGGG-GCCAGTGGATAG-3'; CG2-reverse: 5'-GGGAAATAGCTC-TAGACCAGGC-3'; leader V_LOXA: 5'-CCTGCTAATCAGT-GCCTCAGTCATAATA-3'; leader V_LOXB: 5'-TCCAGAG-GAGAGCTCCAAATTGTTCTC-3'; L_K-reverse2: 5'-ACCTTT-GTCTTCTAGACTAACACTCATTC-3'.

4.7 pComb3 cloning

The anti-ph-Ox-specific light chain was subcloned in pComb3 [23] by digesting the PCR product with Sac 1 and Xba 1 followed by direct insertion into the pComb3 plasmid. Anti-ph-Ox specific heavy chains were re-amplified with a 5' primer (leaderV_H OXC), with an internal Xho 1 restriction site and a 3' primer (CH1Comb) with an internal Spe 1 restriction site. Internal restriction sites were designed to guarantee in frame fusion of the light chain with the pelB-leader, and the heavy chain with the pelB-leader and the gIII phage surface protein, respectively; leader V_H OXC: 5'-GTGTCCTGTCCCTCGAGCAGGTGCAGC-3'; CH1Comb: 5'-GACAGGTCGAACACTAGTTAGGATAGTCC-3'. PCR-conditions were as described before.

4.8 Generation of phages

pComb3 plasmids, containing in frame fusions with anti-ph-Ox-specific light and heavy chains were transformed to *E. coli* strain XL1-blue. *E. coli* were grown to an optical density of OD₆₀₀ ≥ 0.5. Helper phage VCS-M13 (10¹¹/ml) was added. After incubation (1 h at 37°C) kanamycin was added (final concentration 35 µg/ml) and incubated overnight at 30°C. After centrifugation (20 min, 4,000×g), phage were precipitated with 4% PEG 8000 and 3% NaCl for 2 h on ice. After centrifugation (20 min, 11,000×g) the phage pellet was dissolved in 1 ml TBS (10 mM Tris-HCl pH 7.5, 150 mM NaCl). Titer was determined by diluting the phage from 10⁻⁹ to 10⁻¹¹ and re-infection of 100 µl *E. coli* XL1-blue (OD₆₀₀ ≥ 0.5) (10 min, room temperature). Infected XL1-blue were plated on LB-amp plates. The generated phage were diluted to a titer of 10¹¹ plaque-forming units. The phages were directly used for surface plasmon resonance analysis.

4.9 Surface plasmon resonance analysis

Using Biacore-J, the sensorchip CM5 (Biacore) was activated and coupled with ph-Ox-OVA and OVA as reference, respectively, according to the manufacturers specifications. 9,000 resonance units (RU) were coupled for each. Analysis was performed on the Biacore J. Phage (10¹¹) were injected for 3 min. Phages were eluted for 2 min. Binding curves were calculated using the BIA-viewer software program. Relative response units after the elution step are indicative for binding affinities.

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