Analysis of Immunoglobulin (Ig) Isotype Diversity and IgM/D Memory in the Response to Phenyl-Oxazolone

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Abstract

The distribution of immunoglobulin (Ig) isotypes within specific B cell clones in vivo after immunization is not well defined. Using an IgV_H/CDR3- and isotype-specific reverse transcription polymerase chain reaction method, we have carried out a survey of the diversification of the isotype in a splenic response to phenyl-oxazolone (phOx) on a chicken serum albumin carrier. The phOx-specific V_H (V_HOx-1 with specific CDR3 motif) is associated with all of the heavy chains (μ, δ, α, γ, and ϵ) after simple immunization with antigen in alum. The kinetics of expression of each isotype are distinct and reproducible. Focusing mainly on the expression of secretory Ig transcripts, IgM, IgG1, and IgE are found after priming, whereas IgD and IgA appear after boosting. Secretory IgD transcripts are found reproducibly at moderate levels and may, therefore, contribute significantly to the secreted Ig response in mice. Most crucially, we find enhanced levels of secretory IgM/V_HOx-1 transcripts (with 'phOx-specific' CDR3) after boosting, strongly indicating the existence of IgM memory cells that give rise to an enhanced specific IgM secretion in the secondary response.

Key words: B cell memory • immunoglobulin isotype • repertoire • IgD • oxazolone

Introduction

Upon exposure to antigen, specific B cell clones are activated and expanded and their Ig receptors are often somatically mutated (1–3) to allow selection for enhanced memory and effector functions. This clonal maturation also involves Ig class switch recombination, which profoundly alters the location and effector function of the secreted antibodies as well as the signaling function of those that are membrane bound (4). Although we know much about the factors (mainly cytokines) that regulate switching at a molecular level (5–7), relatively little attention has been paid to the evolution of isotype usage within specific B cell clones in vivo. Analysis of serum antibody responses shows that IgM expression/secretion is followed by switching to IgG (and IgA in the gut) but delivers only a crude measure of what is happening to the dominant populations across the broad range of the response, while ignoring the subtlety of V gene/isotype combination diversity. A higher resolution is required to reveal how particular V genes are brought into opposition with the variety of heavy chain isotypes during the course of an immune response in vivo. This will throw light on issues such as the functional diversity of antibodies and regulation of switching in vivo, sequential switching, and the importance and fate of B cells that do not class switch, i.e., putative IgM^{+} memory cells.

Although IgM is generally viewed as an isotype expressed on naive B cells and secreted after primary activation, a number of studies in recent years point to the fact that some B cells with a memory phenotype and V region somatic mutations still express IgM (8–11). IgM^{+} cells in the marginal zone of the mouse spleen have been shown to class switch upon secondary challenge, although it was not clear whether these were virgin or memory responses or were somatically mutated (12). Studies involving oral administration of DNP-KLH have shown an increased number of antigen-specific IgM^{+} cells after in vitro restimulation but no evidence for enhanced antigen-specific IgM in serum after secondary challenge in vivo, suggesting that the fate of any IgM^{+} memory cells is to class switch after secondary challenge in vivo (13). The clonal relationship between secondary IgM and class-switched responses is also obscure. The data derived from this study address directly the relevance of an IgM memory population and specific IgM secretion into the serum in secondary responses.

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The drawback of methods that exploit antibody function (i.e., measuring serum antibodies, making hybridomas, probing tissues with antigen or antiidiotypes) is that they place limits on the parts of the response analyzed; rare cells, cells not secreting antibody, or those with low-affinity antibody may be missed. To overcome some of these problems, we have studied different stages of the immune response to phenyl-oxazolone (phOx)[1] by reverse transcription (RT)-PCR. A significant part of the high-affinity response to the hapten carrier phOx–CSA (chicken serum albumin) is comprised of Igs formed from the V_HOx-1 gene with a particular, short CDR3 (six amino acids) that has the general sequence DXGXXW (2, 14, 15). We define DXGXXW as the Ox motif. Using a primer specific for this gene and another specific for heavy chain constant regions (both membrane and secretory forms), we have carried out isotype-specific RT-PCR to detect oxazolone-specific V_HOx-1 gene expression in vivo (16).

In situations where PCR primers span the CDR3 and the target is the pool of V_HOx-1 containing Ig RNA in the spleen, the PCR product contains a population of molecules varying in length by codon units according to the natural CDR3 length variation caused by VDJ recombination. Such length differences are easily resolved on polyacrylamide gels to give a ladder of PCR products. Mouse V region CDR3 lengths are distributed over the range of 3–18 amino acids (9–54 bp), with a modal value of 10 (16, 17). The phOx-specific V_HOx-1 segment CDR3 is six amino acids long, so transcripts of this Ig give an RT-PCR signal four codon units down from the modal value (referred to as the 'C6' band to reflect the length of the encoded CDR3). This method delivers the specificity and resolution to detect V_HOx-1–expressing, phOx-specific B cell clonal expansions from whole spleen RNA.

We present a survey of isotype profiles of the phOx-specific, V_HOx-1–expressing B cell repertoire early and late after primary immunization and after secondary immunization. All isotypes are generated after immunization with distinct and highly reproducible kinetics. We find clear evidence of IgM memory and enhanced expression of specific serum IgM in secondary responses.

Materials and Methods

RT-PCR. Total RNA extraction was performed using the RNAzol B reagent (Biogenesis) according to the manufacturer's instructions using 1 ml of reagent for the pelleted cells from 10% of a whole spleen suspension. 3.5 μg of RNA was denatured at 96°C for 3 min with 0.5 μg of oligo-dT (see Figs. 2 and 3) or pdN6 (see Fig. 4) and placed on ice before RT in a 50-μl reaction for 1 h at 42°C (oligo-dT) or 37°C (pdN6) with 1× RT buffer, 0.2 mM dNTPs, 1 mM dithiothreitol, 800 U/ml RNAsin (Promega), and 400 U of MoMuLV reverse transcriptase (all from Life Technologies except where indicated).

First round PCR (see Fig. 2) was done with 2 μl of cDNA reaction with standard reaction conditions using manufacturers' supplied buffer with Pfu-turbo polymerase (Stratagene) and 20 pmol of the appropriate primer, OXC2 and membrane or secretory isotype-specific primer for Figs. 2 and 3, OXC2 and a 3′ C_H1 or hinge sequence isotype-specific primer for Fig. 4, in a 50-μl reaction for 30 cycles with the following cycling: 96°C, 30 s; 59°C, 30 s; 72°C, 2 min; preceded by an incubation at 96°C for 3 min before addition of polymerase.

For each single run-off reaction equivalent, 2 pmol of nested isotype-specific primer was end labeled with 3 μCi of γ-[33P]dATP (2,500 Ci/mmol; Amersham Pharmacia Biotech) using the User Friendly T4 polynucleotide kinase labeling system (Amersham Pharmacia Biotech). The reaction was NaOAc/EtOH precipitated and washed with 75% EtOH before use. Run-off reactions were performed for 12 cycles in a 20-μl volume with Pfu-turbo polymerase and buffer as before using 2 μl of the first-round PCR product with the following cycling: 96°C, 30 s; 60°C, 30 s; 72°C, 1 min. After cycling, reactions were treated as for standard sequencing gel electrophoresis and run on 5% acrylamide/urea gels, dried, and exposed to x-ray film (Kodak XAR and Biomax films). The maximum theoretical yield from the first round PCR is 20 pmol, a level probably reached by the secretory IgA and IgG1 amplifications from boosted mice. 2/50 of this = 0.8 pmol would be used in the run-off reactions, which contain 2 pmol of primer, and so these reactions would exhaust labeled primer after only a few cycles. The conditions were designed like this to bring up the signal from rarer transcripts for Fig. 3, but give a semiquantitative read out of total V_HOx-1 expression levels in Fig. 2, which shows the signal after one cycle of run-off.

CDR3 Sequence Determination. For PAGE purification of C6 transcripts, quadruple-sized run-off samples from the pooled PCR product of all three mice in a cohort were loaded on triple-width wells, electrophoresed, and located by autoradiography. Bands were cut out from the dried gel with fine scissors, soaked for 10 min in 100 μl of water, heated to 100°C for 15 min, and centrifuged at 15,000 rpm for 2 min. DNA was recovered from the supernatant by addition of 10 μg of linear polyacrylamide and NaOAc/EtOH precipitation on dry ice. After centrifugation, the pellet was washed with 75% EtOH, air dried, and redissolved in 10 μl of water. 4 μl of this was used in a 50-μl, 15-cycle PCR with Taq polymerase and manufacturers' supplied buffer with conditions as above and the OXC2 and appropriate nested isotype-specific primer that was also used in the run-off, with the following cycling: 94°C, 30 s; 59°C, 30 s; 72°C, 1 min. 2 μl of this product was TOPO-TA cloned, transformed into *Escherichia coli* TOP-10, and plated out on selective media according to manufacturer's instructions (Invitrogen). Individual colonies were picked and directly amplified for 25 cycles with Pfu-turbo (Stratagene), conditions as above, using the OXC2 and appropriate nested isotype-specific primer. 1 μl of this product was sequenced using the ABI Big-Dye kit (PerkinElmer) and analyzed on an ABI 377 DNA sequencer.

Oligonucleotide Primers. OXC2, GGTGGAAGCACAAAT-TATAATTCG; Dmem, ACACGAGTGTTGGATGGTGT-TGAC; Dsec, ACCGTCTGACTCAGGCAGGAGGTG; DCR2, TCTGGGGCTTTGCACTCTGAGAGG; Mmem, GCCTTC-CTCCTCAGCATTCACCTC; Msec, CATGATCAGGGAGA-CATTGTACAG; MCR1, CAGATCTCTGTTTTTGCCTC-CGTA; MCR2, GGCCACCAGATTCTTATCAGACAG; Amem, TAGCACATAGGAAAGTGGCTCTTG; Asec, CAT-GATCACAGACACGCTGACATT; ACR2, ATCAGGCAGC-

CGATTATCACTGGG; G1mem, TGGGCCTCAGCACAGG-
TCTCGTCC; G1sec, ACCAGAGGGCTCCAAGGACACTGG;
G1CR1, ATGCAAGGCTTACAACCACAATCC; G1CR2,
TCACCATGGAGTTAGTTTGGGCAG; Emem, CACCTCT-
TCAATACATAGGTCCTG; Esec, GGAGGGACGGAGGGAG-
GTGTTACC; and ECR2, TTACTAGGCAGCCTAGGGTCA-
TGG. CR1 primers were used in place of membrane or secretory
primers for the amplifications in Fig. 4.

Polymerase Error. For the full RT-PCR/sequencing proce-
dure—first round PCR (30 cycles, Pfu), linear run-off (12 cycles,
Pfu), gel band amplification/cloning (15 cycles, Taq), and direct
colony PCR (25 cycles, Pfu)—only the first round PCR and the
gel band amplification have the potential to fix polymerase errors
in the bulk of the to-be-sequenced PCR product. The other ma-
nipulations are either linear amplifications or the direct colony
PCR, which is then directly sequenced. As 30 of these 45 cycles
are done with Pfu-based polymerase (claimed error rate, 1.3×10^{-6}) and only 15 with Taq (claimed error rate, 7.7×10^{-6}), and
the important target region—the CDR3—is only 18 bp, we ig-
nored the possible effects of polymerase error. The CDR3 DNA
sequences determined were very diverse, but those in the adja-
cent C_H should give a reflection of the error rate by their diver-
gence from germline. In the 23 clones sequenced for Table III,
we find a divergence from germline in the first 42 bp of IgM C_H1
of 1/966 bp (0.1%), confirming the low PCR mutation rates in
the small region under analysis.

Immunization. The hapten carrier phOx–CSA was made as
described previously (18). All mice immunized were 6-wk-old
female BALB/c. For primary immunizations (day 0), mice were
injected with either 30 μg of alum-precipitated phOx–CSA with
10^9 heat-killed pertussis intraperitoneally or PBS (nonimmu-
nized). For boosting, (day 84) mice were injected with 30 μg of
soluble phOx–CSA intraperitoneally in PBS.

Results

Protocol. We carried out RT-PCR analysis of V_HOx-1
RNA expression from whole spleen cell populations. Spec-
ificity was provided by two primers and subsequent CDR3
sequence determination. The forward primer OXC2 was
specific for V_HOx-1, the member of the Q52 mouse V
gene family that forms a significant part of the response to
phOx. It would be expected to amplify on the order of 1%
of all Ig RNA expressed in the spleen. The reverse primers
amplify from the membrane (mem) or secretory (sec) forms
of IgD, IgM, IgA, IgG1, and IgE. Further control for iso-
type-specific amplification is provided by the use of a
nested constant region primer run-off.

Fig. 1 shows a summary of the methods used in this
study. It shows a map of a typical rearranged Ig heavy
chain cDNA, the position of the primers used in this study,
and a scheme for the PCR and ^{33}P-labeled primer run-off
analysis. The method differs little, in general, from previ-
ous methods for TCR 'spectratyping' (19). Radioactive
rather than fluorescent labeling is used to facilitate PAGE
purification of phOx-specific transcripts for CDR3 se-
quencing. Because the primers span the CDR3, as de-
scribed in the Introduction, the amplified cDNA popula-
tion forms a ladder of transcript lengths, of which the
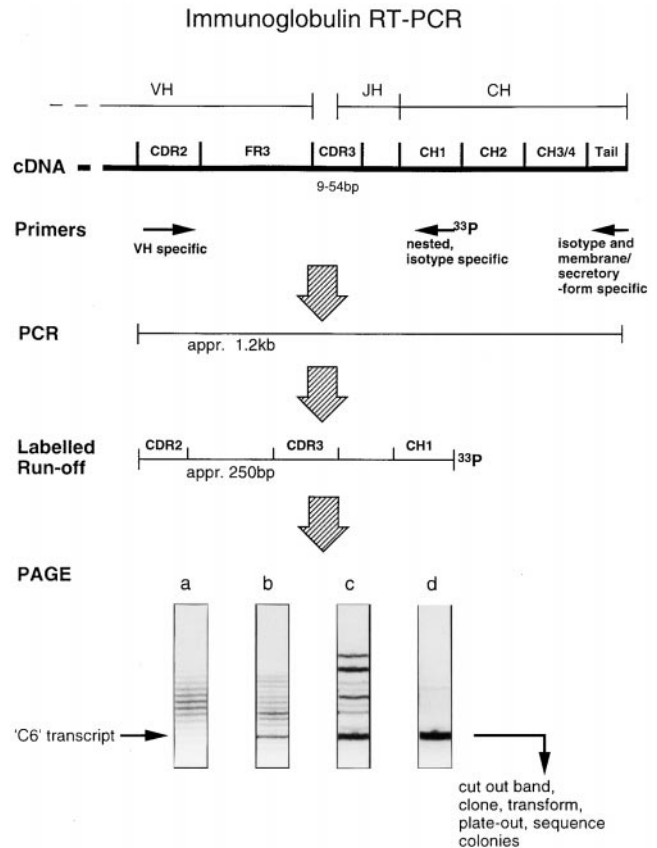phOx-specific heavy chain transcript is one 'rung.' This

Figure 1. Scheme of Ig heavy-chain RT-PCR. Map of Ig heavy chain
cDNA (not to scale) shows position of PCR primers used in the study and
relative location of isotype-specific run-off primer for generating ^{33}P-labeled
fragments for PAGE. Autoradiograph images show typical outcomes ob-
tained when repertoires of differing complexity are amplified by primers
of different isotypes: (a) memIgD unimmunized mouse; (b) secIgM
phOx-boosted mouse; (c) secIgG1 phOx-primed mouse; (d) secIgG1
phOx-boosted mouse. This study has focused on phOx-specific transcripts
using V_HOx-1 with a six–amino acid CDR3 that migrates in the position
indicated by the arrow. These can be gel purified for further analysis.

heavy chain CDR3 is six amino acids long and so is re-
ferred to as the C6 band.

Four typical outcomes obtained with this analysis are
shown in Fig. 1. (a) Smooth: when the target cDNA pool
is complex, in this case $OXC2 \times$ memIgD, and individual
clones do not reach a large relative size, the amplified PCR
product appears as a ladder with a smooth change in band
densities. This reflects the Gaussian frequency distribution
for in-frame transcript lengths. (b) Rough: when the am-
plified Ig pool is less complex and/or contains larger clones,
then clonal expansions are detectable against the back-
ground of other specificities, as in this case, which is the
product of PCR with $OXC2 \times$ secIgM. (c) Oligoclonal:
class-switched repertoires show less diversity. They also
contain larger clones that express higher levels of Ig. Con-
sequently, the PCR product appears oligoclonal, with sev-
eral easily detectable clones superimposed on a greatly di-
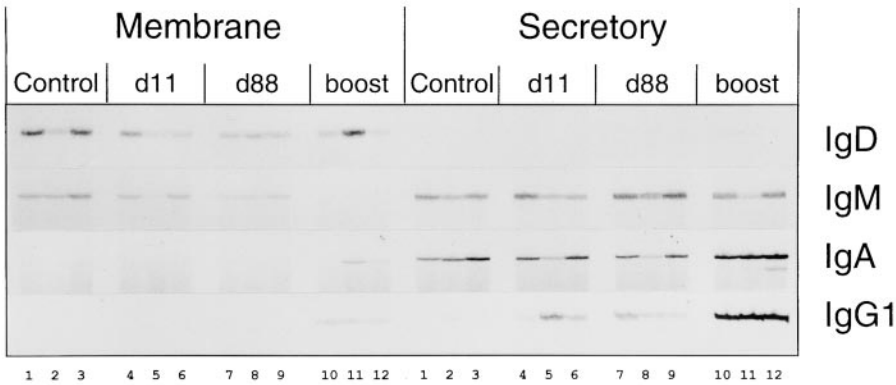minished or zero background; $OXC2 \times$ secIgG1 from

a

Figure 2. (a) Isotype-specific RT-PCR analysis of total splenic V_HOx-1 expression in immunized mice. After the primary membrane- or secretory-specific PCR, samples were subject to one cycle of ^{33}P-labeled nested primer run-off and electrophoresed 1 cm into a 5% sequencing gel and exposed to x-ray film. This gives a standard RT-PCR estimation of total V_HOx-1 gene expression on different isotypes with the added specificity of a nested isotype-specific run-off. Control, PBS-immunized mice; d11, 11 d after phOx–CSA immunization; d88, 88 d after phOx–CSA immunization; boost, 4 d after secondary phOx–CSA immunization at day 84. Numbers along the bottom label samples from individual mice, so samples running vertically up the figure are from the same mouse. (b) Control PCR for HPRT from same cDNA used for V_HOx-1 amplification. Samples are from individuals 1–12 running left to right.
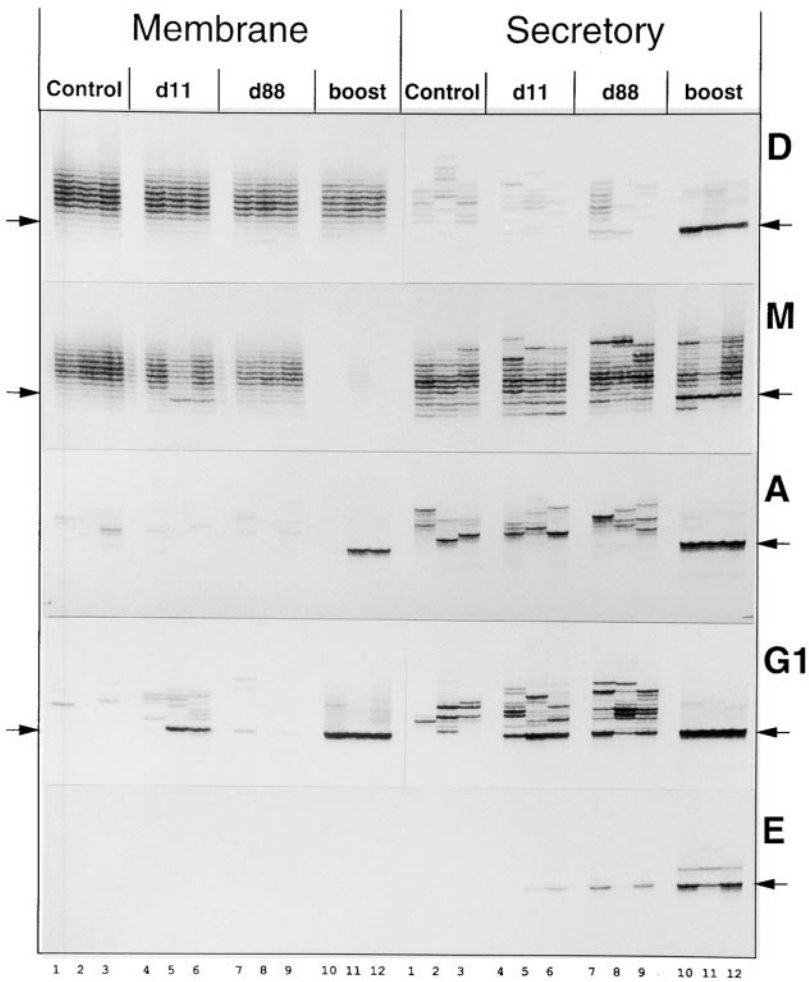
b

Figure 3. PAGE-resolved RT-PCR samples showing phOx-specific V_H transcripts. After 12 cycles of ^{33}P-labeled primer run-off, samples were fully resolved on 5% sequencing PAGE and exposed to x-ray film. Column labels are as for Fig. 2. D, IgD; M, IgM; A, IgA; G1, IgG1; E, IgE. Arrows indicate positions of V_HOx-1 transcripts with six–amino acid CDR3s. From Table I, all upregulated transcripts contain the Ox-motif CDR3, DXGXXW. Numbers along the bottom refer to individual mice; samples running vertically up the figure are from the same mouse.

phOx-primed mouse. (d) 'Nearly monoclonal': in particular circumstances, for instance here in the PCR product from an OXC2 × secIgG1 amplification from a phOx-boosted mouse, the level of antigen-specific Ig is so high that the relative contribution of other clones is greatly diminished and the bulk of the PCR product is derived from V_HOx-1/C6 Igs.

For the core of this study, nine mice were immunized in groups of three by intraperitoneal injection with 30 μg of phOx-20–CSA with alum/pertussis adjuvant. Three were analyzed at day 11 (d11), three were analyzed at day 88 (d88), and three were boosted at day 84 and analyzed at day 88. We take these groups to be primed (d11), long-term postprimed (d88), and boosted, respectively. Three age-matched, unimmunized mice were also analyzed.

Dynamics of Specific Isotype Responses. The primary RT-PCR product from these samples indicates the total level of RNA coding for all of the Igs using the V_HOx-1 gene, of a particular isotype. Fig. 2 a shows the RT-PCR analysis from the phOx-immunized cohort of mice. Rather than observing the PCR product directly on an agarose gel, it was subject to one cycle of run-off labeling with a nested primer and analyzed on PAGE without running the gel far enough to resolve the different CDR3 lengths. This gives a normal RT-PCR analysis of Ig expression with the added isotype specificity of a nested run-off primer. Fig. 2 b shows amplification of the cDNA from the same samples with primers for HPRT (hypoxanthine phospho-ribosyl transferase). After 11 further cycles of run-off and 5% PAGE, differing transcript lengths are resolved, and this is shown in Fig 3. Ig transcripts with the DXGXXW Ox motif migrate at the C6 position, indicated with arrows. C6 bands were cut from gels when appropriate, cloned, and sequenced to confirm the presence of the Ox motif, and these sequences are shown in Table I. This table shows that in every case where the C6 band is upregulated or appears for the first time in response to immunization, it is associated with DXGXXW containing CDR3s.

Fig. 2 gives the overall level of V_HOx-1 V segment expression in the spleen, and Fig. 3 and Table I show the proportion of the transcript that is phOx specific. PhOx-specific transcript levels are defined, therefore, as the product of total V_HOx-1 levels from Fig. 2 and the proportion of these that are C6 transcripts (Fig. 3) carrying the Ox motif (Table I). For ease of interpretation, the data from each of these analyses is described in detail for each isotype in turn.

IgD. Whole spleen levels of V_HOx-1 memIgD transcript vary within and between groups of mice (Fig. 2); however, on average, immunized mice seem to have less memIgD transcript than controls. As the bulk of memIgD is carried by mature but naive recirculating (M^+D^+) B cells, this might be due to the loss of IgD expression that accompanies specific and bystander (by pertussis) activation of cells after immunization. The memIgD CDR3 length profile is smooth (Fig. 3), as expected, in control mice. The memIgD-expressing cell population is complex, and sequences were not obtained for this study from memIgD C6 transcripts.

Total V_HOx-1 secIgD was only just detectable in two boosted mice by RT-PCR (Fig. 2), but after further run-off and PAGE, all mice show an oligoclonal secIgD profile (Fig. 3). On top of this, the upregulation of secIgD after boosting, just visible in Fig. 2, appears to be entirely associated with production of phOx-specific secIgD in all boosted mice (Fig. 3 and Table I). This Ig is absent at d11 or d88 after priming. The expression of phOx-specific IgD after boosting is consistent with the presence of a long-lived/memory pool created by priming that is or can become IgD positive. There are other possible sources of the increased IgD (and IgM) seen after boosting, and these are investigated for IgM in the following section.

IgM. Levels of V_HOx-1 memIgM remain similar between the groups of mice during the primary response, but these drop off after boosting with phOx–CSA (Fig. 2). Many splenic B cells express memIgM including the mature, naive recirculating IgM/D^+ double-positive B cells and IgM single-positive (marginal zone) B cells. The decrease of memIgM RNA in boosted mouse spleen, which is reproduced by the three individuals boosted here and has been observed subsequently in further experiments, could be due to a number of factors. Competition for space or

Table I. *CDR3 Sequences of Secretory Form C6 Transcripts*

	Day 11	JH	Day 88	JH	Boost	JH
IgD					DRGDYW	2
					DRGDYW	2
					DRGDYW	2
					DRGAYW	3
					DRGDYW	2
					DGGDYW	4
IgA					DSGVYW	2
					DLGDYW	4
					DRGISW	3
					DRGISW	3
					DRGDYW	4
IgG1	DGGVFW	3	DRGASW	3	DRGAYW	3
	DRGAYW	3	DRGDSW	3	DRGDYW	4
	DRGAYW	3	DRGDYW	2	DRGDYW	4
	DRGAYW	3	DRGASW	3	DRGVYW	3
	DRGAYW	3	DRGAYW	3	DYGAYW	3
IgE	DRGNPW	3	DGGDYW	4	DRGDYW	2
	DRGDYW	4	DRGDYW	2	DLGDYW	2
	DWGAYW	3	DRGDYW	4	DQGDFW	2
	DRGYDW	3	DPGNHW	2	DRGVLW	4
					DRGVYW	3

Column headings are as for Fig. 2.

2213   White and Gray

Table II. *CDR3 Sequences of MemIgM C6 Transcripts*

Control	JH	Day 11	JH	Day 88	JH	Boost	JH
DDGTYW	3	DGGAYW	3	GLFAYW	3		
NAMDYW	4	DGGAYW	3	ELEDYW	4		
ALYAYW	3	DGGAYW	3	EGFAYW	3		
MGFAYW	3	DGGAYW	3	VEGDYW	4	Low	
EGFAYW	3	DWGDYW	2	TGLAYW	3	signal	
DYDAYW	2	DGGNSW	4				
DRFAYW	3	DRGAYW	3				
		AWFAYW	3				
		NLFDYW	2				
		NYGAYW	3				

Column headings are as for Fig. 2. Ox motif sequences are underlined.

niches in the antigen-boosted spleen could exclude some surface IgM^hi cells and reduce the proportion in the sample. Modulation of the IgD/M expression ratio, selective exit of IgM^hi single-positives, or a bias in RNA splicing toward the secretory form could also all have this effect. It is possible to amplify more memIgM product from boosted mice using a more efficient polymerase and/or more cycles, but under these conditions many of the PCR reactions for other isotypes would become saturated and not allow comparisons between primed and boosted samples. For this whole panel of RNA/cDNA samples, a standard polymerase, number of cycles, and master mix was used, and all samples for particular isotypes were processed at the same time.

The memIgM C6 transcript is significantly upregulated in two of three primed (d11) mice (Fig. 3), with this signal becoming less pronounced by day 88. All upregulation of memIgM/C6 in response to phOx is attributable to DXGXXW Ox motif–containing Ig (Table II).

Overall levels of V_HOx-1 secIgM expression remain similar over time and between groups of mice and always appear greater than levels of memIgM (Fig. 2). In contrast to expression of the membrane form, levels of secIgM C6 transcript do not appear enhanced after priming (Fig. 3), although 4/18 of these transcripts contain the Ox motif at day11 (Table III). At this stage, therefore, the high-affinity response is present but at a low level. Interestingly, the C6 secIgM transcript is overrepresented in all unimmunized

Table III. *CDR3 Sequences of SecIgM C6 Transcripts and FR3 Mutations in Ox Motif Clones*

Control	JH	Day 4/S	JH	M	R	Day 4/A	JH	Day 11/A	JH	M	R	Day 88	JH	Boost	JH	M	R
DGFAYW	3	DDGPYW	4	0	0	WSFAYW	3	DRGAYW	3	0	0	TGFDYW	2	DGGDYW	4	1	0
DGFAYW	3	TGFDYW	2			DGFAYW	3	DGGNSW	4	0	0	WGFAYW	3	DWGAYW	3	2	1
DQFAYW	3	TVVDYW	2			DTFAYW	3	DRGDYW	2	na	na	WGFAYW	3	DGGDYW	4	1	0
TGFDYW	2	IAMDYW	4			DRRDYW	4	DGGDYW	2	0	0	GGYHYW	2	DGGDYW	4	1	0
GTLDYW	2	DGNYYW	2			TGFDYW	2	NGFDYW	2			WGFAYW	3	DGGDYW	4	3	2
WAFAYW	3	WRFAYW	3			AWDNYW	2	DGSAYW	4			RGFAYW	3	DGGDYW	4	1	0
WGFAYW	3	WGFAYW	3			WGFAYW	3	TGFDYW	2					DRGDYW	2	2	1
DGFAYW	3	EGFAYW	3			EGIAYW	3	DGFAYW	3					DGGDYW	4	1	0
		NAMDYW	4			EGIAYW	3	GPFDYW	2					DGGAYW	3	0	0
		DGFAYW	3			DGFAYW	3	TGFDYW	2					DGGDYW	4	1	0
		DGNYYW	2			DGFAYW	3	AGFDYW	2					DRGVYW	3	0	0
		WGFAYW	3			GNLAYW	3	TGFDYW	2					DGGDYW	4	1	0
		LGFDYW	2			WAMDYW	4	TGGIYW	2					DGGDYW	4	1	0
		WLLDYW	2			DGFAYW	3	TGFDYW	2					DGGDYW	4	0	0
		WGFAYW	3			YAMDYW	4	LYYGNW	2					DRGVYW	2	2	1
		YAMDYW	4			TGFDYW	2	WAFAYW	3					DGGDYW	4	1	0
								WDVDYW	2					DGGDYW	4	1	0
								TGGIYW	2					WGFAYW	3		
														DTFAYW	3		
														WGFAYW	3		
														WGFAYW	3		

Column headings are as for Figs. 3 and 5. Ox motif sequences are underlined. M, total number of mutations found in the FR3 region of the corresponding Ox motif clone; R, number of mutations causing an amino acid replacement; na, FR3 sequence not analyzable; S, soluble antigen; A, alum/pertussis adjuvant.

mice (Fig. 3), but none of the sequences obtained from this contain the Ox motif (Table III). At day 88, there is no obvious difference in C6 transcript levels, and we find no Ox motif CDR3s in six sequenced transcripts. After boosting, the level of the C6 secIgM band increases significantly in all mice by over twofold on average, as determined by PhosphorImaging of the gel (Fig. 3 and Table III). Correlated with this, we find the Ox motif in 17/21 transcripts sequenced from the C6 band (Table III). Thus, all of the increase in secIgM C6 transcript levels in boosted mice is attributable to expression of high-affinity secIgM V_H. The level of phOx-specific IgM after boosting is, therefore, approximately eightfold higher than that after priming. This figure is arrived at by multiplying the increase in average band density by the increase in proportion of Ox motif clones. See Fig. 5 for the band intensity data and an explanation of the rationale behind it.

If the enhanced expression of IgM is derived from a memory cell population, these cells may have somatically mutated V genes, as seen in memory cells that contribute to class-switched secondary responses. We have analyzed the V_HOx-1 framework-3 (FR3) sequences from the Ox motif–containing secIgM clones, and the numbers of point mutations and amino acid replacements found in this region are shown in Table III as well. The V_HOx-1 FR3 is 96 bp long. Of the four analyzable Ox motif clones from primary immunizations, three from day 11 and one from day 4, we find no mutations in 384 bp of FR3. Of the 17 Ox motif clones derived from boosted mice, we find that 14/17 have at least one mutation, and the average level is 19/1,632 bp = 1.2%, which is ~10-fold higher than that seen as a result of PCR (0.1%; see Materials and Methods). We find a significant level of somatic mutation, therefore, in the V_HOx-1 IgM expressed after boosting, and this is consistent with a memory cell origin. As a comparison, we

have detected FR3 mutation levels of up to 3% in class-switched V_HOx-1 FR3s (data not shown).

As well as an IgM$^+$ memory cell population, other factors could cause an enhanced secondary IgM response. Selective recruitment of antigen-specific cells from other tissues after boosting could have this effect. Fig. 4 shows the RT-PCR/run-off for IgM in the bone marrow and peripheral blood, as well as the spleen, in the same cohort of mice. This figure shows total rather than membrane and secretory forms of IgM expression. Avoiding use of the membrane/secretory-specific primers allows much more sensitive detection of specific isotype transcripts from the small and variable peripheral blood and bone marrow samples because of the use of random hexamer cDNA priming and much shorter primary PCR targets.

It is clear from this figure that the enhanced secondary secIgM response seen in the spleen for V_HOx-1/C6 transcripts, and reproduced here for total IgM, is also observed in the bone marrow and peripheral blood, where there is no obvious primary response, and this argues against recruitment of antigen-specific IgM$^+$ cells from these tissues to the spleen on secondary challenge.

It is possible that IgM$^+$ plasma cells could be generated early in a primary focus reaction that has decayed by day 11, when we take our primary immunization samples. In this case the secIgM expression we detect 4 d after boosting is simply a new, naive, primary focus reaction sampled at the appropriate time. We have immunized mice with phOx–CSA, with and without alum/pertussis adjuvant, and sampled them at day 4 after priming to see if a V_HOx-1/Ox motif–based primary focus response is detectable at this time, and the results are shown in Fig. 5 and CDR3 sequences are shown in Table III. There is no evidence for an Ox motif–type primary focus response in these day 4 samples that would explain the response seen after boost-
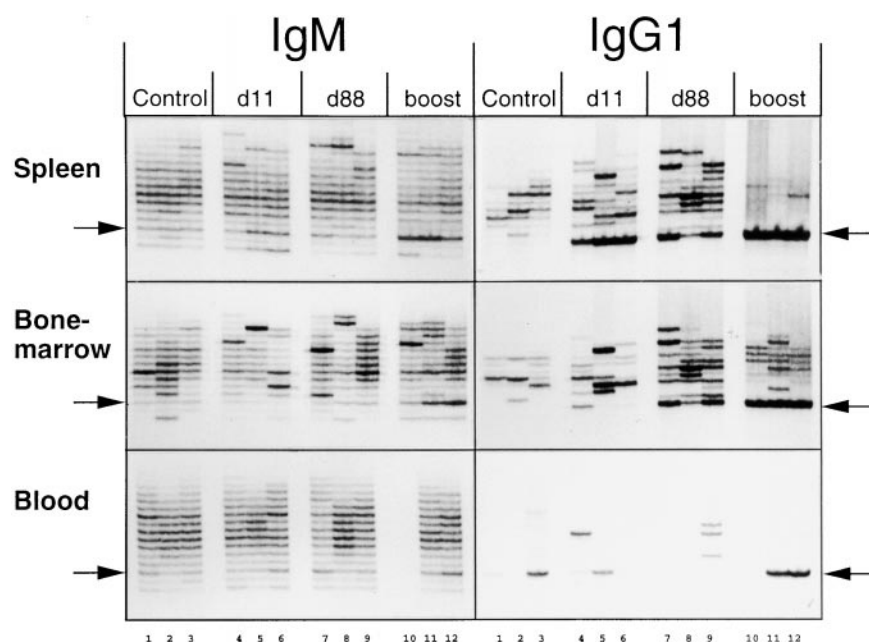

Figure 4. Tissue specificity of phOx-specific IgM and IgG1 transcripts. Ig RT-PCR of RNA samples from spleen, bone marrow, and peripheral blood. Column labels as before. Arrows indicate position of C6 transcripts. Peripheral blood sample 10 failed. First round RT-PCR was done with a reverse primer specific for the hinge region (IgG1) or the 3' end of the C_H1 exon (IgM) rather than for the membrane- or secretory-specific sequences at the far 3' end of the cDNA for reasons given in Results. Numbers along the bottom refer to individual mice; samples running vertically up the figure are from the same mouse.
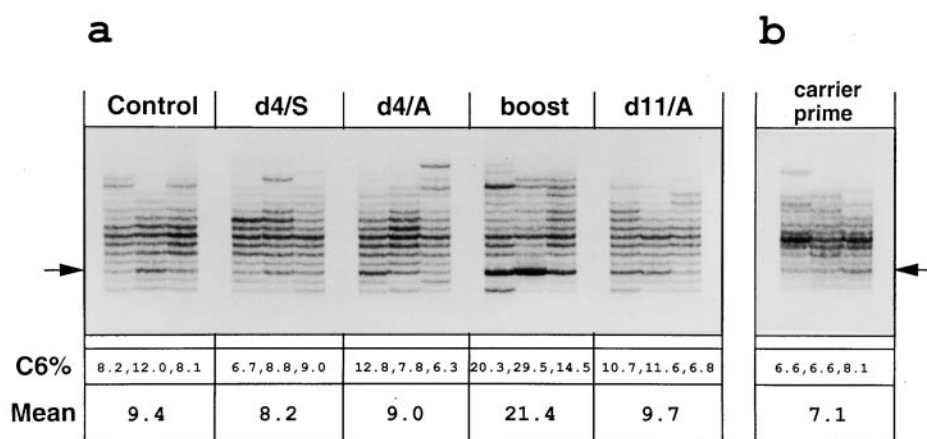
Figure 5. PAGE-resolved RT-PCR samples showing phOx-specific secIgM V_H transcripts. (a) Control, unimmunized mice; d4/S, day 4 samples from mice primed with soluble antigen; d4/A, day 4 samples from mice primed with antigen and alum/pertussis adjuvant; boost, samples from boosted mice 4 d after boosting; d11/A, day 11 samples from mice primed with antigen and alum/pertussis adjuvant. (b) Carrier prime, day 4 after boosting samples from mice carrier-primed and boosted with phOx–CSA 31 d later; C6%, the amount of the C6 band expressed as a fraction of the total V_HOx-1 ladder. We have used this measure because it should be independent of any PCR bias and sample size variation. When the C6 band is a minor fraction of total V_HOx-1 expression, and total V_HOx-1 expression does not vary systematically between different groups of mice (for example see secIgM, Fig. 2), it represents an accurate, semilinear measure of the level of expression of the C6 band. Band densities were measured directly from the gel with a Fuji PhosphorImager. Mean, the mean of the C6% figures for each group.

ing. The V_HOx-1/C6 band is not increased, and there was only one Ox motif CDR3 detected in the total of 32 day 4 clones, a level lower than at day 11.

It has been reported in hapten-carrier systems that increased carrier-specific T cell help, induced by priming, can contribute to an enhanced response to hapten on secondary exposure (20, 21). Fig. 5 b shows the V_HOx-1/secIgM spectratype 4 d after phOx boosting of carrier-primed mice. Mice were boosted 31 d after carrier priming, a time when CSA-specific T cell help would be high. These samples show no increase in V_HOx-1/C6 levels and, therefore, imply that increased carrier-specific help is not responsible for the enhanced secIgM expression we observe on secondary challenge. Taken together, these observations are strong evidence for an IgM$^+$ memory cell population that produces an enhanced secondary response containing IgM V_H with a significant level of somatic mutation. The possible nature of these cells is dealt with in the Discussion.

IgA. Total levels of V_HOx-1 memIgA transcript are very low in all except two of the boosted mice (Fig. 2). Levels of secIgA are consistently high, even in controls, but increase further after phOx boosting (Fig. 2). This is associated with the appearance, at high levels, of phOx-specific secIgA transcript (Fig. 3 and Table I). This may have arisen by switching from another isotype immediately after boosting.

IgG1. Studies of hybridomas derived from mice undergoing responses to phOx–CSA suggest that IgG1 is the dominant isotype in this response (15). In this study, total V_HOx-1 memIgG1 is detectable by RT-PCR (Fig. 2) after boosting. V_HOx-1 secIgG1 increases significantly after priming, remains at approximately these levels after 88 d, and increases dramatically again after boosting (Fig. 2). Fig. 3 and Table I show that the bulk of this increase is associated with production of Ox motif–containing C6 transcripts. PhOx-specific memIgG1 appears after priming, falls of by day 84, and rises again after boosting but by several times the level induced by priming. PhOx-specific secIgG1

rises after priming, declines a little by day 88, and then rises to very high levels after boosting. Fig. 4 shows that the decline of antigen-specific IgG1 at day 88 in the spleen is associated with an increase in the bone marrow. This is consistent with migration of IgG1$^+$ plasma cells to this tissue.

IgE. Although not detected by V_HOx-1 isotype–specific RT-PCR (not shown), after 12 cycles of run-off, phOx-specific (C6 band and Ox motif) secIgE is detectable in two out of three primed mice. It continues to be produced until day 88 and then increases after boosting (Fig. 3 and Table I). The pattern of this is similar to that of secIgG1. The use of alum as the priming adjuvant may be important in achieving the phOx-specific IgE.

Discussion

The data presented here demonstrates that V gene–specific, isotype-specific RT-PCR analysis is a powerful tool for studying Ig expression during an immune response, revealing insights that are not readily available to analysis at the protein level. All major isotypes of antibody, including IgD and IgA, using the same V_H with closely related CDR3s, are produced in response to a simple primary or secondary challenge with phOx. The pattern of expression of particular isotypes is very reproducible between mice, and each isotype is expressed with characteristic kinetics. Findings of particular note are the routine appearance of secIgD in the secondary response and enhanced expression of IgM in the secondary response, indicative of IgM$^+$ memory cells.

We have focused mainly on secretory rather than membrane-bound Igs in this study. MemIg is expressed on cells from a variety of developmental stages, and full interpretation of results will require fractionation of subpopulations (studies underway). In contrast to this situation, PCR of unfractionated spleen cells for secIg gives a measure of the antibody output of the spleen, on the assumption that levels of secIg mRNA map to levels of secreted Ig protein. Par-

ticularly for the class-switched antibodies, we would expect the secretory product to be derived largely from plasma cells. This approach allows us to directly detect all isotypes of secretory antibody expression by related B cell clones in the spleen as a whole and, therefore, study a humoral response in vivo with the sensitivity of PCR.

PhOx-specific secIgG1 is detected in the expected pattern. It is expressed after priming, maintained perhaps with a slight average reduction in levels to d88, consistent with migration of IgG1$^+$ cells to the bone marrow, and increases severalfold after boosting. Expression of secIgE follows a similar pattern, although average levels may increase slightly between 11 and 88 d after priming. A priming dose of phOx–CSA, therefore, induces long-term, persisting, antigen-specific IgE expression.

In the spleen, the phOx-specific secV$_H$Ox-1–IgA expression is found only after boosting; no upregulation of mem- or secIgA is detected in any of the six primed mice. Reproducible (in three mice) expression of secIgA suggests that IgA plasma cell development in the spleen is a normal feature of a recall response. Given the lack of mem- or secIgA in the spleen before boosting, the IgA recall response may be derived from cells that have recently switched and were expressing another isotype before the boost.

PhOx-specific secV$_H$Ox-1–IgD is readily detected in all three boosted mice, and for this reason IgD must be considered a part of normal antibody responses in mice. Levels of expression appear higher than levels of IgE transcripts but lower than those for IgA and IgG1 (Figs. 2 and 3), although we cannot control for differences in primer efficiency between isotypes. The detection of secIgD only after boosting suggests the existence of a memory cell population that is committed to secreting this isotype upon rechallenge. The phenotype of these memory cells, however, is not obvious from this data. They will not be class switched, but it is not possible to state whether they express IgD, with or without IgM, or express IgM alone. This IgD secondary response might be explained by migration of secIgD-expressing cells to the spleen from elsewhere, but if this were the entire explanation we would observe some expression after primary immunization.

The phenotype of the cells secreting IgD is not clear either. We have observed that FACS®-sorted IgM$^+$/IgD$^+$ cells from phOx-boosted mice show no secV$_H$Ox-1/IgD C6 transcript expression (our unpublished observation), and so it is likely they are IgD single-positive cells. IgD has previously been shown to be involved in normal immune responses in IgM knockout mice (22).

A clear observation to arise from this study is the detection of eightfold enhanced antigen-specific secIgM that is somatically mutated after secondary exposure in the spleen. This suggests the existence of IgM$^+$ memory cells that provide for IgM secondary responses. The kinetics of expression of this secIgM are similar to those of IgG1 and contrast with those seen when polyclonal serum IgM is analyzed in primary and secondary responses. The C6 secIgM band is not increased after priming (Fig. 3), and sequence analysis shows the presence of only 0/16 or 1/16 (day 4) or 4/18 (day 11) Ox motifs (Table III). In contrast, after boosting, not only do the levels of the C6 secIgM band increase significantly in all three mice, but sequence analysis shows the presence of 17/21 Ox motifs in these bands.

We have excluded carrier-primed T cell help and an early primary focus response as an explanation of the secIgM transcripts we detect after boosting. We conclude that we have detected an enhanced secondary IgM response produced by a long-lived (84 d) population of IgM$^+$ cells that express somatically mutated Ig. This is strong evidence for an IgM$^+$ memory cell population that produces an IgM secondary response. It remains to be determined whether these cells have properties similar to those of the memory cells that contribute to class-switched secondary responses.

Long-lived class-switched plasma cells have recently been described in the mouse (23–25). It is possible that there could be long-lived IgM$^+$ plasma cells expressing low levels of surface IgM that could mediate an enhanced secondary response. This point raises an important issue about the very definition of memory cells. There is no data to imply that memory is the sole privilege of a particular type of B cell of a single developmental stage or phenotype. It is more likely that memory, as it is functionally defined, is distributed across different phenotypes, isotypes, and indeed developmental stages; it is the property of a diverse compartment, the composition of which may change over time after antigenic exposure. The conventional definition of immune memory is that it facilitates a faster, greater, and usually higher affinity antibody response to secondary exposure. We have documented a faster and greater IgM response and find somatic mutation consistent with affinity maturation.

If some of the secIgM we detect after boosting is derived from a type of restimulatable 'plasma cell' that expresses surface IgM, then these cells are a type of memory cell. The long-lived plasma cells that have been described as a source of long-lasting protective immunity are class switched, have no/low surface Ig, appear antigen independent, and do not respond to antigenic challenge (23–25). We think it is unlikely, therefore, that this particular type of cell is a part of the IgM-producing IgM$^+$ memory cell compartment.

As discussed for IgD, the IgM secondary response is unlikely to result from migration of these cells from elsewhere, as it would be seen after priming. Also, the enhanced expression of IgM seen on boosting is detected in the bone marrow and peripheral blood as well as the spleen (Fig. 4), and this argues against recruitment of IgM-expressing cells from these tissues. The data presented here cannot resolve the question of whether these IgM-secreting cells are, or were derived from, IgM single-positive or IgM/IgD double-positive memory cells.

A significant upregulation of the memIgM C6 transcript is observed in two of three day 11 mice. The memIgM transcripts from these mice were sequenced (Table II), and this data confirms that elevated levels of the C6 transcript are attributable to expression of Ig with Ox motif CDR3s. These elevated levels of membrane-bound phOx-specific

V_H/IgM are consistent with the establishment of a memory cell pool.

We have not designed this study as an in depth analysis of somatic mutation or CDR3 sequence diversity. We have used CDR3 sequencing simply to confirm the presence of the Ox motif. From Tables I and III, however, it does appear that the D<u>G</u>GXXW V_HOx-1 CDR3 motif is more popular in IgM as compared with the other isotypes. We find D<u>G</u>GXXW in only 2/38 non-IgM secreted Ox motifs overall (Table I). It appears in 13/17 Ox motif secIgM sequences from pooled samples after boosting (Table III). To check the generality of this effect, we sequenced clones from the secIgM C6 band from the three individual boosted mice. Of the secIgM Ox motifs in these mice, 3/7, 9/9, and 1/4 were D<u>G</u>GXXW. This shows that D<u>G</u>GXXW is present in the secIgM CDR3 of all three mice, being popular in one and dominant in another. This observation suggests that although the kinetics of expression of secV_HOx-1/IgM is comparable to other isotypes, the receptor diversity is different. This bias could be explained by reduced levels of somatic mutation in IgM$^+$ cells, affinity-based selection out of the IgM compartment, or both. It is difficult to draw firm conclusions on this point because of the lack of information about light chain pairing.

It is often argued that the serum IgM repertoire is largely made up of 'natural' antibodies selected by endogenous ligands (26, 27), many of which are synthesized by CD5 B cells (28, 29). Our data would suggest that a significant proportion of serum IgM is the result of a specific immune response. That we observe an enhanced secIgM response 84 d after priming in a mouse suggests that the longer a mouse lives, the more significant and diverse the memory component of the secreted IgM response would become. The upregulation of phOx-specific secIgM upon boosting is not sufficient to affect the overall levels of V_HOx-1 transcripts (Fig. 2), and so the amount of RNA detected appears less than that seen for IgG and IgA. It seems likely to us that these specific IgM clonal expansions would be masked by polyclonal serum antibody in in vitro assays and emphasizes the sensitivity of the PCR approach.

All three unimmunized mice show 'overrepresentation' of the secIgM C6 band: it is darker than it would be if it followed the Gaussian distribution of CDR3 lengths seen with random VDJ joining in unselected antigen receptor repertoires (16, 19). As shown in Table III, the sequences present in this band in the control mice are diverse and so do not represent a particular dominant clonal expansion. This suggests that the six–amino acid CDR3 is generally popular in the V_HOx-1 gene repertoire of secIgM-expressing cells. This effect is not apparent in the memIgD tracks from the same three mice, which are completely smooth, nor is it obvious in the levels of memIgM expression. Thus, the cells expressing this diverse six–amino acid CDR3/V_HOx-1 appear to be IgM^{hi}IgD^{lo} or IgM-only cells. These cells are less frequent in the mouse spleen than IgM$^+$IgD^{hi} cells and express higher levels of sec- than memIgM (data not shown). These may be the 'natural' antibody-secreting B1 cells or possibly the result of previous IgM memory ex-

pansions, a hypothesis consistent with the observation of V_HOx-1 IgG1 C6 transcripts in the peripheral blood of unimmunized mice.

The immune system is arithmetically as well as biologically complex. We have found that focusing too early on particular parts of the phOx response using marker-defined cell populations as well as replica experiments and different tissues and time points leads to an exponentiating experimental throughput, particularly when large numbers of CDR3 sequences are needed to generate statistical significance for individual data points. In these circumstances, it is necessary to start with a coarse-grained study to redefine phenomena, recall responses for example, as observed with PCR. More focused studies on particular observations can then be designed within the context of these.

The interpretation of this type of data has some restrictions. It is not possible to define the specificity of expressed antibodies by their function, and there is no information on light chain pairing. We assume we are detecting antigen-specific antibody responses for two reasons. First, we are studying an immune response known to involve reproducibly a particular V_H gene/CDR3 combination, which we identify, and define, with a V gene–specific primer and sequence determination. Second, the levels of expression of this defined heavy chain increase from zero in most compartments in response to immunization. With IgM, the PCR target population is more complex, the signal/noise ratio (after versus before immunization) becomes noninfinite, and the justification for detection of a V_H response and its association with antigen is statistical. Not knowing light chain pairing is not a problem unless it is important to study the progeny of a single naive B cell. We are observing an immune response by a family of related clones defined by expression of a particular heavy chain, and it is within this context that our data is to be interpreted. In previous studies (2), 21/23 anti-Ox, V_HOx-1–expressing hybridomas obtained from different stages of immunization used the VκOx-1 light chain, so it is likely that the bulk of the heavy chains we analyze are paired with this. Irrespective of this, the response we are studying is obviously not monoclonal. Sequences obtained from individual mice for secIgM show the Ox motif linked to more than one J_H region, implying the existence of more than one founder clone (data not shown).

We have shown that all major heavy chain isotypes, attached to the same V_H and using variants of the same CDR3, are involved in the oxazolone response in vivo and are expressed and probably secreted in reproducible patterns at the organ level. We have also directly detected an antigen-specific memory response derived from the IgM$^+$ and, perhaps, IgD$^+$ populations that contribute to enhanced expression of these isotypes on secondary challenge.

The reproducibility of this system and its potential to measure specific B cell responses from early developmental compartments makes RT-PCR of phOx, or other V-restricted responses, a promising approach to analyzing the effects of cytokines and manipulated genotypes on the immune response in vivo.

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