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# THE ORIGIN OF IMMUNE REPERTOIRES BY MEANS OF NATURAL SELECTION

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## INTRODUCTION

Biological systems are the result of opposing forces between conservation and flexibility. Both evolutive choices share their submission to the very same natural selection constraints, together to random genetic drift of nearly neutral mutations (*Allen*, 1991). In the immune system, between the presumed genetic potentiality to generate variable (V) regions (its random element) and individual V region repertoires, there are discriminating filters and driving forces. They should focus our interest if we want to get some insight of the "immunological topography" (*Jerne*, 1960), physical substrate of what has recently been called the "intentionality" of the system (*Cohen*, 1992). Interspecies evolutive selection from bony fishes to man (although not necessarily moving in an ascending way) results in a Darwinian pressure to increase, through gene duplication, variability of immune receptors, acting specifically over their (hypervariable) CDR regions (*Tanaka* and *Nei*, 1989; *Schroeder* et al., 1990; *Tutter* and *Riblet*, 1989; *Schwager* et al., 1989; *Ghaffari* and *Lobb*, 1991). Throughout the ontogeny of the individual, there are germline-encoded, developmentally controlled patterns of expression of particular V genes (*Yancopoulos* et al., 1984). In vertebrate development, and in the critically initial conditions, the cellular fates use to be consequence of continuing cell-to-cell interactions in defined times and micro-environments, more than the result of intrinsically programmed cell lineages developing in mosaic patterns (*Wilkins*, 1986; *Raff* et al., 1991). They adjust the genome potentiality to basic levels of variability (its norm of reaction) fitted to the requirements of each moment, their early products, however, frequently persisting for the whole lifespan of the individual as it is the case for perinatal lymphoid subsets in mouse. In a third level, ligand-dependent selection (superantigens, epitopic fine specificities) is required for the full development (*Era* et al., 1991) and unique picture of each V-repertoire, at its most unpredictable component. To summarise, between what is possible and the real V-region repertoire, there are: 1) the evolutionary filter, 2) the constraints of tightly regulated gene expression in development, and 3) mechanisms of somatic selection imposed by both self and non-self environments. To know what repertoire modifications take place at each level of selection (evolutive, developmental, somatic Ag-dependent) is relevant to elucidate the pathogenic mechanisms underlying immune-mediated diseases, as well as the eventual possibilities of immunomodulation. We

also consider that this is the only way of having some insight into the physiological function of this biological complex. And, although not definitive, these analyses might provide (semi-quantitative) information about the antigenic world which "interests" more the immune system, be self, foreign or any other unclassified pattern.

## EVOLUTION, GENOMIC ORGANISATION AND STRUCTURE OF THE V-REGION SYSTEM

Immunity (that is, resistance to pathogenic encounters) and self-non-self discrimination exists both in invertebrates and in vertebrates (*Theodor*, 1970). The structural bases of these properties, however, completely differ between the two subphyla, and vertebrates utilise the diversity of variable receptors (Ig, TCRs), built from rearranging DNA fragments, as well as they rely on the very polymorphic MHC locus. V receptors are multidomain proteins, evolving through gene duplication (the Ig superfamily) from primordial receptors of intercellular adhesion (CAMs), which are characterised by homophilic binding and auto-affinity (*Edelman*, 1987). Each one of these domains, being encoded by a different exon, is supported by intra-segment interactions defining its local functionality, and it is able of autonomous selection (*Simon* and *Rajewsky*, 1990). The phenotype emerging from these "supergenes" is somatically impinged by the products of the polymorphic MHC locus, genetic substrate of individuality (*Holmberg* et al., 1984; *von Boehmer* et al., 1978). V-regions represent the compromise between evolutive forces of positive selection for variability (*Tanaka* and *Nei*, 1989) *versus* the conservation of basic requirements for binding to the ligand. These two alternatives are distributed inside the same Ig V region (hypervariable CDR 1-3 *versus* framework regions) and they also distinguish different $V_H$ families, some of which are highly conserved inter-species (*Ghaffari* and *Lobb*, 1991; *Meek* et al., 1991), some others represent evolutive newcomers. While the same VL is frequently used to form Abs with different specificities, this is rarely true of $V_H$, suggesting that the Ab-forming system rely much more on $V_H$ than on $V_L$ (*Kabat* and *Wu*, 1991). This could also apply to TCR Vβ versus Vα, but formal data are lacking. It is suggestive for this point to note that both Ig $V_H$ and TCR Vβ are first rearranged, and expressed alone in cellular stages, which support very high turnovers and stringent selection mechanisms. V genes are classified in families, defined by the internal homology of their components; they display very different complexity sizes between them. Also, on the basis of the conservation of solvent-exposed, FR1 intervals, $V_H$ families have been clustered in clans, which are maintained through evolutionary barriers (*Kirkham* et al., 1992). The loop region of FR3 determinants differentiates the families within a clan. As it has been revealed for Vβ-homologous determinants (*Choi* et al., 1990; *Cazenave* et al., 1990), these conserved residues could be involved in the initial recognition of clan/family-specific ligands in H-chain expressing precursors, previously to the full development of H-L Ig clonal specificity. These genes are distributed throughout the H chain locus either in clusters (mice) or more interspersed (humans) (*Meek* et al., 1990; *Schroeder* et al., 1990; *Walter* et al., 1990; *Shamblott* and *Litman*, 1989; *Amemiya* and

*Litman*, 1990; *Schwager* et al., 1989)

The selection of V genes (evolutive, developmental, somatic) passes in all cases through their products, by means of encounters with complementary ligands. However, V genes only account for the less variable CDR1 and CDR2 regions, and some residues of CDR3, which is the most relevant site to define Ag binding. Concerning the dominant H, β and γ chains, this CDR3 region is completed by combinatorial rearrangement to D and J segments, by exonuclease-dependent nucleotide nibbling, and the addition of non-templated P (complementary to the last two bases at the end of the coding joints) and N nucleotides (these latter only in H, β and γ, but no or very little in L, α and δ chains). All together, these mechanisms are probably more relevant in the building of target determinants for the ongoing selection of Ig repertoires. The former ones (use of particular V gene families, conservation of framework determinants, etc.), are based on stringent genetical constraints, and are probably devoted to assure the best starting of the system. Besides the described junctional diversity, V-D-J rearrangements in H and β chains give potentially rise to three DH reading frames. In most of adult B cells, only one, RF1, is evolutive and somatically selected; early B cells show, however, a more diversified usage of RFs (*Gu* et al., 1990), and no preferences are evident among TCR β chains (*Prochnicka-Chalufour* et al., 1991). In chicken Igs, whereas there is no DH RF selection in spleen and bone marrow, this is very strong when B cells home the bursa (*Reynaud* et al., 1991). The preferential generation of aminoacid residues (Gly, Ser, Pro, Tyr) implicated in loop formation within the CDR3 has been advanced as responsible factor for these DH RF biases (*Abergel* and *Claverie*, 1991).

Finally, the most recently evolved mechanism for diversity generation is Ag-triggered, somatic hypermutation, acting over rearranged Ig V segments and their flanking sequences ($10^{-3}$/bp/cell generation, $10^3$-$10^4$-fold the basal levels taking place in the rest of the genome) (*Lebecque* and *Gearhart*, 1990). In the course of immune responses, it multiplies the offering of V regions for the best Ag fit (*Rajewsky* et al., 1987; *Milstein*, 1990; *French* et al., 1989). Besides their unbiased usage of DH RFs, TCR genes also differ from Ig genes in their lack of somatic hypermutation. Significant biases from the neutral replacement/silent (R/S) ratio of mutations (*Shlomchik* et al, 1987) towards the R component demonstrate the positive selection of these clonal specificities. Negative selection for aminoacid changes, either in order to maintain global Ig structure (in FR regions) or because germline Ig sequences already displayed ideal binding for certain Ags (e.g. bacterial polysaccharides) reduces R/S ratios. Also, there is the possibility that early B-cell populations and most of selected natural antibodies disregard this mechanism of diversity (*Forster* et al., 1988; *Rajewsky* et al., 1989). Amphibia, for instance, although able to hypermutate, do not select these mutants. Consequently, they do not have significant affinity maturation in ongoing responses, either because the long lifespan and advantage of initial cells, or due to the lack of germinal centres where intraclonal competition of Ab mutants happens in mouse (*Wilson* et al., 1992; *Jacob* et al., 1991). In selected situations and in species as chicken, rabbits and probably humans, gene conversion mechanisms also diversify rearranged Ig sequences (*Reynaud* et al., 1989; *Becker* et al., 1990; *Sanz*, 1991).

These mechanisms of V-region diversity generation plus the unrestricted

pairing of H and L chains (*De Lau* et al., 1991), are all devoted to construct Ig molecules which will bind ligands through energetic interactions, at least able to displace water molecules. From this basic constraint to the high-affinity, highly specific, Ag-Ab complex, a dynamic process of physical selection will take place between the two components of the complex. The optimal interaction is not necessarily the strongest one, if only because this might disturb complex dissociation, reduce its half-live by increasing elimination and, consequently, limit the global efficacy. Crystalographic work has revealed that all the possible human Igs ascribe to a reduced number of canonical structures, defining the CDR fold (mostly, CDR1 and CDR2) on the nature of a small number of conserved residues and the length of the loop (*Chothia* and *Lesk*, 1987). They can provide a certain insight of the presumed ligands for the Ab, as e.g. protein-binding Abs tend to have flat surfaces, while hapten and other Ag-specific Abs show deep grooves or pocket-like binding sites. It is intriguing to note that these canonical structures contain very different sequences from several unrelated $V_H$ families (*Chothia* et al., 1992), posing a question to the analysis of $V_H$ distribution as representative of Ig selection for fine Ag specificity. These restrictions in Ab conformation further underline the value of additional (junctional) mechanisms of diversity, and eventually, reduce previous calculations about repertoire sizes based in maximal genetic potentialities.

This genetic potential has a tissue-specific expression in haematopoietic cell lineages that, in contrast to other systems, conserve a germline pool of immature stem cells (HSCs). This means that V-region repertoires can be (more or less) continuously renewed from this source of novelty, what generates the necessity for stringent mechanisms of somatic selection throughout the lifespan of the individual. The rules (cellular and genetic) governing the entry of new V-region specificities in early ontogeny versus those displayed in the adult are probably rather distinctive. For example, many more TCRs emerge from the very active postnatal thymus, while adult T lymphocytes are mostly maintained by clonal expansion of mature lymphocytes in the periphery.

## WHY V-REGION REPERTOIRES OF EARLY ONTOGENY DIFFER SO MUCH FROM THOSE USED IN THE ADULT

The expression of the mature Ig $V_H$ repertoire in the adult mouse usually does reflect the genomic complexities of $V_H$ families, with small mouse strain-dependent variations (*Wu* and *Paige*, 1986; *Zouali* and *Theze*, 1991). Very soon, however, it was observed that there is a preferential rearrangement and expression of the most J-proximal, 3' $V_H$ genes early in ontogeny, in pre-B cells and in continuously, newly-emerging lymphocytes (*Yancopoulos* et al., 1984; *Perlmutter* et al., 1985; *Schroeder* et al., 1987; *Schroeder* and *Wang*, 1990; *Malynn* et al., 1990; *Freitas* et al., 1990; *Decker* et al., 1991; *Berman* et al., 1991). These genes are little polymorphic (*Sanz* et al., 1989) and highly conserved elements between species (*Ghaffari* and *Lobb*, 1991; *Meek* et al., 1991), classical features attributed to biologically relevant molecules. This ontogenic pattern does not completely apply to VL genes, which shows non-stochastic biases early in development, that are not related with chromosomal position (*Kaushik* et al., 1989; *Teale* and *Morris*, 1989; *Kalled*

and *Brodeur*, 1990; *Gulgou* et al., 1990). Both H and L chains family preferences result in the fact that nearly half of all mature, resting B cells in perinatal stages are accounted by only 6 $V_H$ + Vκ family pairs (*Kaushik* et al., 1990). Concerning the TCR-forming chains, Vγ genes also appeared in a developmentally-controlled series of waves, probably due to targeted rearrangements, which are selected and home particular environments (mostly, epithelial layers) (*Haas* and *Tonegawa*, 1992). There is also experimental support that 3' Vα and 5' Jα segments predominate in foetal and neonatal mice (*Roth* et al., 1991), and we lack of clearly positive evidences concerning Vβ genes. Even in the case that more data appear about these V family distributions, it seems that developmental biases linked to chromosomal position are more remarkable among early $V_H$, and Vγ genes. They remind of the genetic organisation of enzymes responsible for integrated metabolic pathways, which are also codified in gene clusters, or the regulated expression of developmentally crucial genes on the basis on their order in broad units of transcription in the chromosome (*Edelman* and *Jones*, 1992).

The change from D-proximal to global $V_H$ family usage is clearly influenced by somatic selection pressures, be external encounters (*Bos* and *Meeuwsen*, 1989) and/or natural Igs (*Freitas* et al., 1991). It is surprising that, while the usage of $V_H$ families in the mature compartments is well defined in each mouse strain and roughly related to their size, individual $V_H$ genes are very strongly selected in each individual, from the primary organs to the periphery (*Gu* et al., 1991). It seems an open question to us whether the mechanisms implicated in family usage (or normalisation?) follow different rules (*Kirkham* et al., 1992) from the Ag-dependent selection of individual genes.

As exposed above, junctional diversity is a relevant component of the paratopic specificity at the CDR3. The length of this domain increases progressively with time, substantially expanding the diversity of a single V(D)J rearranged gene. While exonuclease nibbling and P nucleotides addition are constant throughout life, there is a progressive increase in the addition of non-templated N nucleotides to H, β, and γ D-J and V-D junctions, probably due (but not only) to TdT enzymatic activity (*Feeney*, 1990, 1991; *Gu* et al., 1990; *Itohara* and *Tonegawa*, 1990; *Bogue* et al., 1991; *Bangs* et al., 1991; *Rellahan* et al., 1991; *Meek*, 1990). Perinatal repertoires, already restricted at the level of V family utilisation, are further limited in junctional variability, and this restriction is tightly controlled by both developmental and somatic selection forces (*Ikuta* and *Weissman*, 1991; *Bogue* et al., 1991; *Carlsson* et al., 1992). Finally, most of early Ig repertoires do not experiment the diversity potential of somatic hypermutation, and maintain their germline character. In mouse, but not so clearly in humans, this is also true of the majority of ligand-selected peripheral B cells (*Gu* et al., 1991). It thus seems that somatic mutation is only triggered by a particular signalling pattern whose constraints (Ags, T-cell stimuli, germinal centre microenvironments, etc.) are not fully elucidated.

From the point of view of the molecular components of early V-region repertoires, it can be concluded that they are few -in terms of diversity- (much less than in adult times), they are very conserved, and they do not utilise further diversification mechanisms appearing in the adult. Although it can be argued that this design is due to system "immaturity", the starting V-repertoires are fully functional in terms of recogni-

tion and response, and they actually persist for the whole lifespan of the individual. Their pattern of recognition is devoted to self-binding (autobodies), V-to-V region connectivity, self-Ag recognition and multireactivity (*Kearney* and *Vakil*, 1986; *Holmberg* et al., 1986; *Carlsson* and *Holmberg*, 1990; *Lehuen* et al., 1992). Subsequent to this, they connect apparently unrelated immune pathways, and modifications in their dynamics induce dramatic changes in adult immune behaviour (*Vakil* et al., 1986). Probably due to both expanding environments and driving internal complementarities, these initial cell compartments are highly activated. They will be quantitatively dominated in adult times by clones prepared to react with non-self new encounters. Concerning the biological value of these findings, our current experimental tools should allow us to analyse: 1) the implication of each one of the selective levels defined before (evolutive, developmental, somatic) in the building of these very different repertoires, and 2) the eventual dispensability of each component and mechanism, due to system redundancy or use of parallel pathways, for the global integration and physiology of the immune system.

## B CELL POPULATIONS, COMPARTMENTS, LINEAGES, REPERTOIRES. WHAT IS THE MATTER?

Mouse B lymphocytes are divided, on the basis of surface Ag patterns, in clearly distinguishable cell groups (*Herzenberg* et al., 1992). As it was shown for erythrocyte and macrophage populations, haematopoietic stem cells (HSC) from different sources and/or timings tend to differentiate *in vivo* to particular sets of B lymphocytes (*Kantor* et al., 1992). The earliest HSCs detected in the embryo produce, upon cell transfer/graft, perinatal B cells (B1a, B1b), while bone marrow-derived HSCs differentiate preferentially to adult (B-2) cells. These differentiation events happen in a sort of wave-like fashion, as, when advancing in time, the maturation to previous cell subsets goes down or is even exhausted, while the next one expands and dominates (*Marcos* et al., 1991). Although it was postulated the existence of intrinsically different progenitors (*Herzenberg* and *Herzenberg*, 1989), we consider more plausible the view that, while maintaining their totipotentiality, HSCs undergo selected fates after distinctive intercellular regulation in development. Alternatively (and not in an exclusive way), a sort of HSC clock counting the past number of cell divisions versus the timing where the HSC is thrown in a pathway of cell commitment by asymmetric division, might also be considered (*Holliday*, 1991). Emerging from the same HSC pool, various experimental set-ups (*in vitro* culture, cell transfer, graft) reveal different mature populations, in some cases, preferentially revealing HSC potentiality (*in vitro*), in others, more close to their actual cell fates (grafts). Our current experiments and views support that branching decisions for different B-cell populations are developmental, although a lot of work remains to be done, in order to elucidate the variables implicated in these processes (Table 1).

Together to the disappearance of embryonic haematopoietic potentials, there is an arrest in the entry of novel B cell and V-region specifities in the primordial cell populations, which, however, persist throughout life. They need to be maintained by clonal survival and/or expansion, and they are submitted to the

**Table 1**: Generation of B lymphocytes throughout mouse ontogeny

| PROGENITOR CELLS | | B-CELL PRODUCTION | | |
|---|---|---|---|---|
| Sites | Timing | *In vitro* | Cell transfer | Graft |
| Yolk sac | 8-10 d | ++ | +/- | - - |
| Emb. (below diaphragm) | 8-10 d | n.d. | ++ | n.d. |
| Embryo | 9,5 d | ++ | n.d. | n.d. |
| Para-aortic Splanchnopleura | 8,5-9 d (10-18 som.) | ++ | ?? | ++ (B1a) |
| Omentum | 13 d | ++ | n.d. | ++ (B1a>B1b) |
| Liver | 11-19d | ++ | ++ | n.d. (B1b>B1a>B2) |
| Spleen | 15-5 d post-birth | ++ | ++ | n.d. (B1b>B2>B1a) |
| Bone marrow | adult | ++ | ++ | n.d. (B2>B1b>>B1a) |

POTENTIALITY → CELL FATE
Microenvironment
Cell interactions
Short-range growth factors
Genetic programs

Earlier HSC asymmetrical divisions
More germline constrained
Lower repertoire size; less open to novelty

Latter HSC asymmetrical divisions
Full display of genetic diversity

The data summarised in this Table came from: *Paige* et al., 1979; *Tyan* and *Herzenberg*, 1968; *Ogawa* et al., 1988; *Godin* et al., 1992; *Solvason* et al., 1992; *Velardi* and *Cooper*, 1984; *Kantor* et al., 1992; and our unpublished material.
n.d.: not done, to our knowledge.
??: ongoing experiments.

same kind of selective pressures than other peripheral B cells. This long-term Ag selection obviously can result in oligoclonality (*Stall* et al., 1988). Although perinatal B lymphocytes are relatively "diluted" throughout life by adult populations, this is probably not so much the case for one of their most relevant aspects, that is, Ab secretion by plasma cells: Experimental findings from different sources all agree with the fact that a big fraction of adult plasma cells and natural Abs in the serum came from these early waves of differentiation (*Forster* and *Rajewsky*, 1987; *Kroese* et al., 1989; *Marcos* et al., 1992; *Godin* et al., 1992). And it is clear that, in a cell-per-cell basis, the initial B cells are more prone to plasma cell differentiation than the latter ones. It can be concluded that they are not only selected to persist as available components of the system, but also that they are positively driven to "occupy" a very central role in the B-cell compartment. In this way, critical information about the primordial conditions of immune starting is maintained throughout life in the functional core of the system. Recent studies of *in situ* hybridisation for $V_H$ family usage in Ig-congenic mice, and in PBL cDNA from adult human beings (*Viale* et al., 1992; *Braun* et al., 1992) extend that notion to adult V-region repertoires. Together to any other kind of Ag pattern, the products of these long-lived B lymphocytes could impinge definitively the selection of novel specificities arising from the H chain-expressing, late pre-B cells in the adult bone marrow (*Marcos* et al, 1991; *Freitas* et al., 1991; *Sundblad* et al., 1991). Besides the implication of early-born, long-lived B-1 cells to "remember" perinatal repertoire patterns (*Jeong* and *Teale*, 1990; *Gu* et al., 1990), these

lymphocytes also go through great life periods of cellular selection. This (and not preferential rearrangements) results in the increased expression of certain $V_H$ /$V_L$ chain combinations and specificities only inside these populations, and not in the others, even if located at the same sites (coelomic cavities) (*Andrade* et al., 1989; *Carmack* et al., 1990; *Conger* et al., 1991; *Hayakawa* et al., 1990). The distinctive significance of each one of these parameters (time and selective microenvironment of emergence, cell lifespan and turnover, somatic selection by certain Ags, etc.) in the maintenance of perinatal B-1 cells in the adult is still a matter of debate and current research.

## SOME REMARKS ABOUT IMMUNE DISEASE

Behind any abnormal immune functionality, there are disturbed processes at one or several of the selection levels that we have discussed above. Although many accompany the disease, probably, the control of a few critical ones is really relevant to re-establish physiology. We need to distinguish between what necessarily determines a phenotype, and what only reinforces it, or is merely an epiphenomenum. Thus, most of current debates in present-day autoimmunity and lymphoproliferative diseases need to be approached from these perspectives. Several representative examples can be mentioned:

- If we want to understand how BM-uncommitted, lymphoid progenitors can either transfer or cure autoimmune diseases (*Kawamura* et al., 1990), we need to know what epigenetic decisions they have already passed through, in order to be already settled as either normal or diseased.
- Even considering the former statement, autoimmune disease is Ag triggered and Ag-dependent (*Shlomchik* et al., 1987).
- Many Ab specificities, which accompany disease development, reveal the markers of strong Ag selection, and it has been speculated that they could represent a pathogenic component. However, and in the very same individuals, people frequently detect self-reacting germline Abs, preferentially codified by those genes dominant in early ontogeny (*Möller*, 1992). What is the real meaning for pathology of both types of clonal anti-self specifities? The latter ones, are they pathogenic, are they accompanying and irrelevant epiphenomena, or are implicated in some kind of homeostatic, regulatory behaviour? It is possible, for instance, to genetically segregate many of these traits, as it is the case with the increased levels of serum Igs and autoimmune pathology, with recombinant inbred mouse lines (*Datta* et al., 1982). Depending of the answer, one can imagine how much different will be the therapeutical approach to the patient.
- More and more, it has been confirmed the preferential expansion of perinatal lymphocytes in the context of immune disease (*Marcos* et al., 1988). We only have some guesses of the putatively disrupted filters, which drive these populations out of their ontogenical timing.
- Although with contradictory data, there are clear descriptions of TCR Vβ restricted usage in some autoimmune conditions. Is this because the stringent immune response, either to some kind of "superantigens" or to fine MHC-peptide specificities, or are there some more basic restrictions to the whole display of TCR diversity?
- Depending of the Ig V-repertoire they

express, the spectrum of B-cell neoplasias extends from clinically indolent, early germline Ig-expressors (B-chronic lymphocytic leukaemia, monoclonal gammapathy of undefined significance, Waldenström's macroglobulinaemia, etc.) to more aggressive tumours, bearing a representation of all Igs, which are frequently mutated (follicular lymphoma, multiple myeloma, etc.). We consider that these patterns of B-cell neoplasia's clonotypes can provide relevant information about the lymphoproliferative origin, and their future evolution under somatic selection pressures (*Friedman* et al., 1991; *Marcos* et al., 1991).

This list is obviously unfinished, and we only wanted to underline with it how important we consider the study of basic principles of V-region repertoire building for the better approach to clinical problems. The behaviour of our immune system is simply the result of complementary solutions (*Coutinho*, 1989) to the challenge of natural selection, interspecies, in the developmental uncoiling of DNA information, and confronted with a world of distinctive patterns and novelty.

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