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63. We thank the many colleagues in the immunology department of the Institute for Genetics who contributed to this work: D. Allen, C. Berek, R. Dildrop, C. Kappen, C. Kocks, U. Krawinkel, W. Müller, A. Radbruch, J. Roes, F. Sablitzky, M. Siekevitz, T. Simon, P. Vieira, and U. Weiss. We are grateful to U. Ringeisen for the artwork and to Å. Böhm, K. Gladisch, and E. Siegmund for typing the manuscript. Supported by the Deutsche Forschungsgemeinschaft through SFB74, the Bundesministerium für Forschung und Technologie, and the Fazit Foundation. I.F. was supported by the Fritz Thyssen Foundation.

The Chicken B Cell Compartment

JEAN-CLAUDE WEILL AND CLAUDE-AGNÈS REYNAUD

A very unusual molecular mechanism is involved in generating the preimmune repertoire in the chicken bursa of Fabricius. A unique rearranged V gene is diversified through a program of segmental gene conversion with a pool of noncoding pseudogenes being used as donors. A specifically committed progenitor that originates in the embryonic bursa is responsible for long-term maintenance of the B cell population. Both these properties and the characteristics of the peripheral B cell compartment are discussed in terms of the evolution of the T and B immune systems.

A VAST AMOUNT OF INFORMATION CONCERNING THE MAMmalian immunoglobulin (Ig) superfamily has been collected, but several areas of cellular regulation have remained unexplained. For the mouse B cell system, data have pictured a multiple gene family organization comprising several hundred functional variable (V) genes [for both heavy (H) and light (L) chain loci] in association with a massive daily production of B cells. This production, which occurs in the bone marrow, probably involves continuous rearrangement that results in the random expression in each

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newly formed B cell of any member of this V gene family (*1*).

A description of the immune systems of species below mammals has recently emerged (*2*). It appears from these models that, whereas the immune systems have the same goal to generate enough internal diversity to cope with the outside world, the means of achieving this goal are very diverse. In this review we describe the strategy used by birds to generate a diversified B cell compartment. The specific properties that characterize this cellular population may give some insight into the evolution of the T and B immune systems.

The Bursa Is a Primary Organ of Differentiation for the B Cell Lineage

The bursa is a unique organ, found only in birds, that arises at day 5 of embryonic life as a dorsal diverticulum of the cloaca. Its removal during early embryonic development (up to day 17 of incubation) induces severe agammaglobulinemia and prevents the animal from mounting an immune response to any immunizing antigen (*3*). The stem cells (prebursal stem cells) that give rise to B lymphocytes colonize the bursa from the general circulation (days 8 to 14 of incubation) (*4*) (Fig. 1). They migrate through the bursal mesenchyme where they may give rise to granulocytes. When they reach the bursal epithelium, they induce the formation of bursal follicles (*5*). Inside each follicle the stem cells (bursal stem cells) divide and give rise to a population of cells bearing immunoglobulin M (IgM) at their surface, 90 percent of bursal cells being B lymphocytes at day 20 of embryonic development. Bursal development involves two phases: (i) the intraembryonic phase, which includes the colonization and the growth of about 10^4 bursal follicles by expansion of their B cell clones and (ii) the posthatching period, which includes the seeding of bursal cells to the periphery and the continuous expansion of the bursal follicles; by 4 weeks of age a sufficient number of stem cells has migrated out of the bursa as postbursal stem cells, thus installing the mature chicken B cell immune system in the periphery (*6*). At this stage, B cells cannot be generated from an early multipotent stem cell because the bursa has involuted and can no longer provide the environment for this early step to occur.

Structure of the Heavy and Light Chain Loci

Chicken light chains are mostly of the λ type (*7*). The genomic locus includes one constant region (C_λ), one junctional region (J_λ), and almost adjacent to it (2 kb on the 5′ side), a functional variable gene ($V_\lambda 1$) (*8*). Upstream from $V_\lambda 1$ lies the V_λ subgroup, which contains 25 pseudogenes in a 19-kb cluster with both transcriptional orientations (Fig. 2). These pseudogenes cannot code for a functional variable domain. They either have truncated 5′ or 3′ coding regions or present an incorrect heptamer-nonamer recombination signal; moreover, they lack leader sequences (*9*). The single functional $V_\lambda 1$ gene is rearranged to J_λ in probably every chicken B cell (*8*).

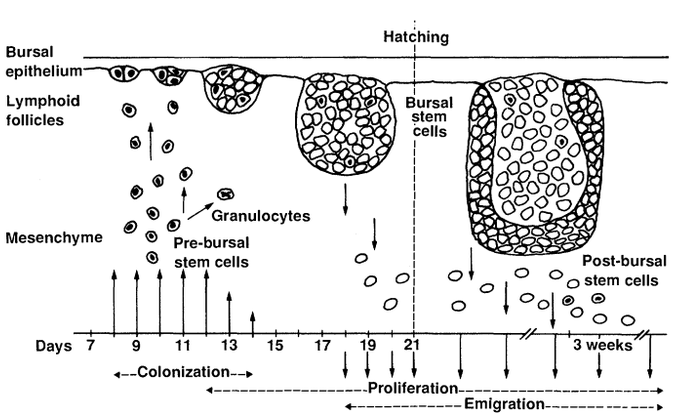

Fig. 1. Schematic representation of bursal B cell morphogenesis.

As for the heavy chain, we have not been able to characterize the complete μ locus because this genomic region undergoes large deletions during isolation. However, based on sequence analysis of isolated partial genomic regions and from Southern blot data, it appears that there is one Cμ gene, one junctional J_H region approximately 15 kb to the 5′ side of Cμ, one D region, and a cluster of V_H genes closely linked to the D-J-C region at approximately 15 kb to the 5′ side of J_H. When looking at the μ heavy chain gene configuration in normal chicken lymphoid tissues, we always observe a major rearrangement event, as we do for the light chain. However, in this case, a few minor bands are present, implying that for this locus other minor events may also occur (*10*).

The Rearrangement of Chicken Ig Genes Occurs Only During Embryonic Development

The follicles of the bursa can be easily isolated, giving access to pure segregated populations of B cells at different stages of development (1×10^5 to 3×10^5 cells per follicle at 3 to 6 weeks). We wanted to determine the rearrangement pattern of Ig genes in individual follicles and compare it to the corresponding pattern obtained from total bursal DNA. We hoped to determine whether Ig gene rearrangement is a continuous process within each bursal follicle. Such analysis revealed that the average number of rearrangement events taking place in a bursal follicle is two (*10*). This corresponds to the number of precursors populating a follicle, as estimated when parabiosis is performed between two embryos differing at the bursal antigen locus, Bu-1 (*11*). This implies that the progenitors populating a follicle and giving rise to B lymphocytes are already committed to a particular Ig gene rearrangement and do not continuously rearrange their genes during the embryonic maturation of this organ. It also agrees with the fact that in normal birds heterozygous for the IgM molecule, about 30 percent of the bursal follicles express only one of the two relevant alleles (*12*).

This surprising result, showing that the chicken B cell progenitor is a surface IgM-positive cell, was previously suggested on the basis of cellular data. Long-lasting Ig suppression can be obtained in the chicken after in vivo injection of an antiserum to IgM at day 13 of incubation, implying that the target of this immunosuppression is an embryonic surface IgM-positive progenitor cell (*13*). Moreover, in classical restoration experiments in which the lymphoid system of B cell–depleted 4-day-old chicks is replaced with bursal cells of the same age, the reconstitution can be abolished by prior incubation of the donor cells with an antiserum to IgM, suggesting that the cells that restore depleted follicles 4 days after hatching are already committed to IgM production (*14*).

When we looked at a large series of B cell lymphomas induced by virus infection at early stages of development, unlike virus-immortalized pre–B cell lines in the mouse, we never found a cell that had rearranged only the heavy chain and not the light chain locus or even an uncommitted cell not yet having carried out any of these events.

In summary, cellular and molecular data are in favor of a model in which B cells are derived only from committed bursal stem cells that have already rearranged their Ig genes and can no longer be generated from an uncommitted progenitor after the colonization of the embryonic bursa has ended. If 10^4 follicles contain an average of two bursal stem cells each, 2×10^4 productive rearrangements will

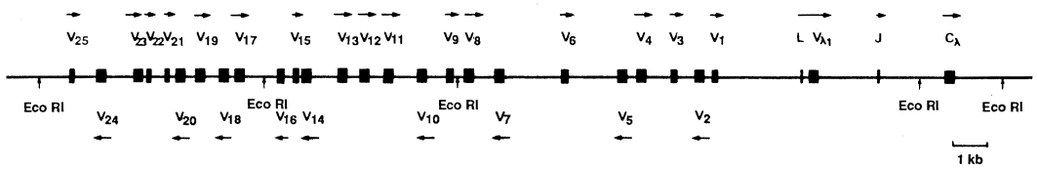

Fig. 2. Organization of the chicken λ light chain locus. The locus comprises a single J-C unit, a single functional V gene ($V_\lambda 1$) with a leader sequence (L), and an adjacent pool of 25 pseudogenes (V1 to V25) with both possible transcriptional orientations (indicated by an arrow for each coding element) (*9*). Rearrangement involves the joining of $V_\lambda 1$ and J sequences with the deletion of 2 kb of intervening DNA (*8*).

occur in the life of the chicken B cell system. In such a model the rearrangement would be essentially to produce a population of progenitor cells for the B cell lineage. It is not clear whether the rearrangement of Ig genes occurs before or during entry of the circulating stem cells in the embryonic bursa. Whatever the answer, the bursal step is an obligate requirement, possibly allowing for the clustering and eventual proliferation of these progenitor cells.

In the mouse immune system the massive daily production of B cells (2×10^7 to 3×10^7 cells) during the life of the animal is most probably ensured by precursor cells with their Ig genes in an unrearranged configuration. These cells give rise to B cells through a precisely regulated program of hierarchical rearrangement from the heavy to the light chain locus (*1*). Moreover, the large number of different coding elements (V, D, J) allows a multiplicity of coding assortments and alternate use of these elements (*15*), thus providing the system with an opportunity to maximize diversity and numerical performance through the rearrangement process.

Generation of Diversity

It has been shown that different specificities arise successively in the embryonic bursal follicles (*16*). Such diversification is necessary for the B cell compartment to develop fully. It occurs at the IgM level and, as a preimmune repertoire, it gives to each clone of B cells seeding the periphery the opportunity to be expanded by a wide variety of circulating antigens. How diversity can be generated from a restricted number of rearrangements then becomes a central issue in this system.

We have sequenced several rearranged $V_\lambda 1$ sequences isolated from bursal cells at both day 18 of embryonic development (3 days before hatching) and 3 weeks after hatching, at which time ablation of the bursa has no consequence on the adult chicken immune system. This work was carried out on an inbred chicken strain whose V subgroup members, the $V_\lambda 1$ functional gene and the 25 V pseudogenes, have all been sequenced. Because of the unique rearrangement event that can be isolated from the whole bursal B cell population, the generation of diversity can be analyzed without the bias of experimental selection introduced by an arbitrary antigen or procedures for cell line establishment. Such randomly cloned rearranged $V_\lambda 1$ sequences are representative of the normal physiological pattern of the chicken immune system during its development (*9*).

Comparing the rearranged $V_\lambda 1$ sequences with the sequences of the V pseudogenes, we confirmed our preliminary observations that the pool of pseudogenes can provide donor sequences to diversify the rearranged $V_\lambda 1$ gene (*8*). The essential argument in favor of a gene conversion mechanism is the observation that no reshuffling of sequences among the pseudogenes could be found either in the bursal B cell population or in isolated lymphoma cell lines (*9*). However, a double recombination event between sister chromatids cannot be formally excluded.

The molecular basis for such a mechanism is the extensive homology between donor and acceptor genes. The sequence of the 25 pseudogenes has confirmed this homology, which is located mainly in the framework region. The amount of DNA transferred varies from several nucleotides to a stretch of 100 bp. The number of events necessary to account for the modifications observed increases with time, four to six events having occurred in some cases at 3 weeks after hatching (Fig. 3). This is consistent with the idea that gene conversion occurs in a stochastic manner, with more events accumulating as the number of cell divisions increases. It can then be estimated that one successful conversion event should occur every 10 to 15 cell divisions.

Combinatorial possibilities provided by such a mechanism are very large. For example, if ten donors can diversify a recipient gene in four different segments, 10^4 different sequences can be created. Further diversification may be added by a variability sometimes observed at the border of the conversion, introducing single nucleotide substitutions not present in any of the pseudogenes (Fig. 3).

Modifications of the chicken $V_\lambda 1$ rearranged sequences appear to be quite different from the somatic mutations that have been described in the mouse's secondary response. They affect an IgM molecule and, probably in most cases, prior to any antigenic stimulation. They also appear highly localized within the V coding region and do not extend outside the V gene (*17*). However, both gene conversion in the chicken and point mutations in the mouse appear to be triggered by the rearrangement process. Conversion occurs only in the rearranged allele, whereas the unrearranged $V_\lambda 1$

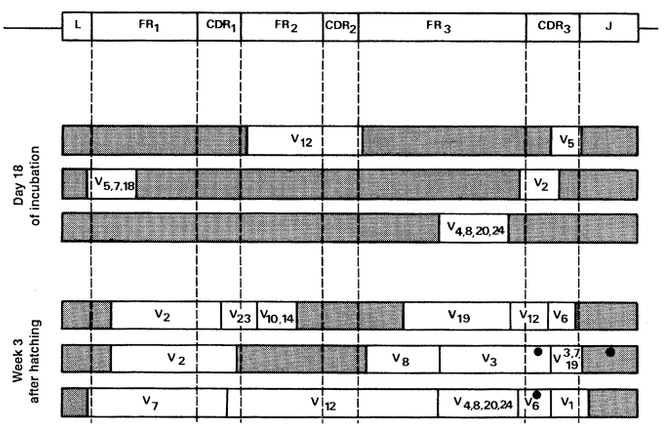

Fig. 3. A schematic proposal for the generation of six rearranged bursal $V_\lambda 1$ sequences from day 18 embryos or from a 3-week-old chicken through multiple gene conversion events (*9*). Sequences are aligned and the different regions of the variable domain delineated: leader (L), framework (FR), complementarity determining regions (CDR), and J segment (J). Gray boxes indicate sectors of nonmodified $V_\lambda 1$ sequence; white boxes represent approximate borders of converted segments with the name of the putative pseudogene donor (several numbers in a box refer to multiple equivalent donors). A heavy dot represents an isolated modified nucleotide with no possible donor.

sequence, 2 kb to the 5′ side of the J region of the other allele, remains unmodified (*9, 18*). Moreover, an abortively rearranged $V_\lambda 1$ sequence, as in the mouse (*19*), shows a large number of modifications.

Data on the generation of diversity of the heavy chain variable genes suggest that a similar type of strategy must occur. The rearranged sequences that we have isolated at different times of development show a gradual increase of modifications similar to that observed for the light chain locus (*20*). The fact that germline V_H or V_L sequences are never found 3 weeks after hatching confirms that there is no de novo rearrangement of Ig genes during B cell production in the bursa after hatching.

Many questions remain unanswered concerning this model. (i) What could be the specific signals initiating and propagating the gene conversion mechanism at such a high rate? The unique organization of this locus, contained in 30 kb and presenting 25 genes encoded on both DNA strands with an almost systematic alternation of polarities, could be the basis of the mechanism. It is also possible that a specific enzymatic machinery could be induced in B cells during their transit in the developing bursa. The introduction of the light chain locus along with pseudogenes into transgenic mice should enable us to address this question (*21*). (ii) What is the accuracy of the gene conversion mechanism? According to the high proliferation rate of B cells in the developing bursa [8 to 12 hours generation time (*16*)], the bursa should contain theoretically over 10^{20} cells at 3 weeks. The 3-week bursa in fact contains 1×10^9 to 3×10^9 cells, and it has been estimated on the basis of the animal's weight that there are approximately 6×10^9 B cells in the periphery (*22*). Only 1 to 10 percent of the cells produced leave the bursa every day, and the remaining cells probably die in situ (*23*). These figures may accommodate a very high number of abortive events, probably due to the imprecision of the gene conversion mechanism, assuming that a B cell has to present an IgM molecule at its surface to be maintained in the actively dividing population. We would then expect that the "freezing" of bursal cells in vivo upon transformation by the avian leukosis virus should allow us to rescue such events. We are currently analyzing such a cell line, which may provide information on the mechanism involved.

A Mini-Peptide Model

How can an immune repertoire be defined in terms of nucleic and amino acid sequences, and why would it be different in species having to interact with the same universe of epitopes? Complete sequences of mouse and human V genes will certainly be determined soon, but whether they will give such information remains questionable. For the chicken light chain locus we have all the coding information. When the pseudogene sequences are compared with the $V_\lambda 1$ gene at the amino acid level, framework regions (FR) are conserved and usually only single amino acid substitutions are found, with a low variability level. However, the variations in the complementarity determining regions (CDRs) generally appear as short peptides that are more diversified (Fig. 4). In somatic $V_\lambda 1$ sequences, gene conversion involves FR and CDR segments equally (Fig. 3). However, because of the conservation pattern of the pseudogenes, these CDRs appear preferentially modified when analyzed at the amino acid level. The basis of the generation of the repertoire consists of inserting a family of mini-peptides from the CDRs of the pseudogenes into the CDR of the unique rearranged functional V gene (*9*). How have these mini-peptides been selected, and do they have any significance by themselves regarding their recognition potential?

It has been elegantly shown that an antibody combining site can

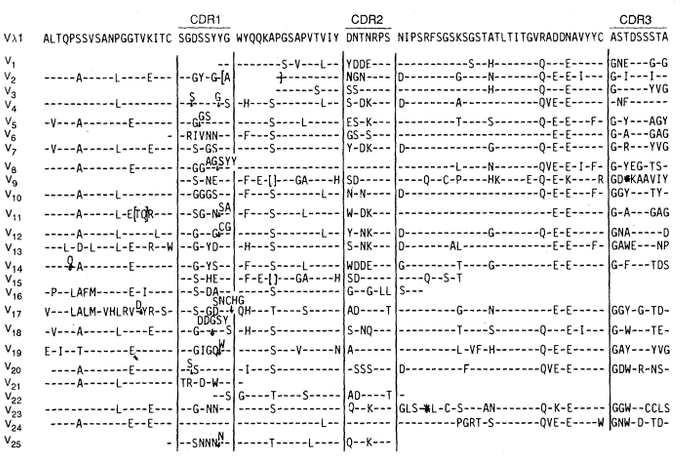

Fig. 4. Amino acid sequence comparison between the single functional V gene and the pool of pseudogenes. Sequences of the 25 pseudogenes (V1 to V25) are compared at the amino acid level to the functional $V_\lambda 1$ sequence with a single letter code (* stands for stop codon) (*9*). Sequences are interrupted at the end of their V homologous domain. Position of CDR is indicated. Arrows indicate insertions in the pseudogenes. Brackets represent introduced gaps.

be artificially transferred by grafting the CDRs from one framework to another at the nucleic acid level (*24*). Such CDR replacements are performed physiologically in the developing bursa. These in vitro and in vivo examples give some support to the controversial view of framework regions being represented as a β sheet scaffold on which CDR regions can be grafted almost independently (*25*). If this model could be generalized, the chicken immune system, with one functional V_H and V_L sequence, could generate as many different combining sites as the mouse, which has evolved many different V gene families.

Antigenic profiles recognized by T and B cell receptors can now be deduced at the amino acid level by using synthetic peptides (*26*). With the catalogue of stored and utilized peptides in the chicken immune system, the project of designing peptides that could mimic antibody combining sites may eventually become more feasible (*27*).

A Unique B Cell Compartment

Precise kinetic studies allowing one to follow the fate of specific components of the B and T cell compartments have not been done with the chicken. With the mouse, such studies have shown that B and T cells behave differently during the life of the animal. Mature B cells are constantly renewed from bone marrow precursors, and most of them die after 2 to 4 days. Peripheral mature T cells, on the contrary, are able to expand and maintain population size by continuous cell renewal even in the absence of any cellular contribution from the thymus (*28, 29*). In the chicken, B cell precursors are segregated in the bursa where they give rise during ontogeny to a large population of B cells. At later stages the bursa involutes, but the peripheral B cell population is maintained and is able to restore long-term humoral immunity when transferred to a B cell–deficient recipient. It is not known whether the bursal stem cells go on giving rise to B cells after they leave the bursa or whether any B cell possesses this self-renewing capacity. The large number of peripheral B cells necessary to restore a depleted host argues in favor of the first hypothesis (*6*).

How these expanded clones are maintained at the periphery, through constant birth and death or as long-lived B cells, is not known. When tolerance is induced within 2 weeks after hatching

Table 1. Essential features of the chicken B cell compartment.

1	Extensive cellular proliferation during bursal development
2	All specificities present at a very early stage
3	Involution of the bursa in the adult
4	Self-renewing capacity of differentiated cells
5	Persistence of expanded clones at the periphery
6	Lower adaptability of the system (compared with the mouse)

and followed by bursectomy, recovery is extremely slow, whereas in the unoperated animal it starts after 5 to 8 weeks (*30*). These observations imply that peripheral stem cells may not be able to generate somatic variants in a fashion comparable to their bursal equivalent, thus imposing on the system a very low adaptive capacity. When the main characteristics of the chicken B cell compartment are aligned, they clearly evoke the properties of the mouse thymic population (Table 1). However, it has been shown that new migrants from the adult mouse thymus still have considerable expansion capacity, implying that this system remains in a dynamic state during the life of the animal (*29*).

How the chicken B cell system can provide the overall immune surveillance and adaptability necessary throughout the life of the animal remains paradoxical. It would be essential to have more data on the diversity of this B cell repertoire particularly at the heavy chain level.

Immune systems seem to have arisen in primitive species from a cellular recognition device mediating self/non-self discrimination (*31*). One may envisage primitive B cell systems evolving from this preexisting cellular compartment. The chicken B cell population may in some ways provide a picture of this ancient event. It will obviously be important to understand the properties of B cell lineages below the avian species to obtain a clearer picture of this evolution.

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Redesigning Nature's Poisons to Create Anti-Tumor Reagents

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Immunotoxins are conjugates of cell-reactive antibodies and toxins or their subunits. In this report, the chemistry, biology, pharmacokinetics, and anti-tumor effects of first generation immunotoxins; the preparation of improved second generation immunotoxins that display greater anti-tumor efficacy; and the role of genetic engineering in creating third-generation immunotoxins are discussed.

THE MOST REMARKABLE FEATURE OF THE MAMMALIAN IMmune system is the virtually unlimited repertoire of antibody molecules of different specificities that can be generated by a single individual. The advent of monoclonal antibody technology (*1*) has made it possible to "tap" this repertoire by immortalizing single B cells. The resultant hybridoma cells produce large amounts of homogeneous antibody of a single desired specificity, such as

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