Rearrangement of chicken immunoglobulin genes is not an ongoing process in the embryonic bursa of Fabricius

(B lymphocyte/stem cell)

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ABSTRACT   We report a molecular analysis of the chicken Ig loci in single bursal follicles from 3- to 7-week-old chickens. Each follicle contained between 10^5 and 3×10^5 cells. The Ig gene rearrangement patterns obtained were compared to the pattern observed with the corresponding total bursal DNA. The results obtained for the light chain locus imply that a very small number (two on average) of rearrangement events takes place in each follicle. For the heavy chain locus similar results were obtained, each follicle showing a more restricted pattern than the total bursa. These data favor a model in which each follicle is colonized by a very few prebursal stem cells that are committed to a particular Ig gene rearrangement at the very beginning of the development of the embryonic bursa. The role of the bursa as the organ in which such a committed stem cell population for the B-cell lineage arises is discussed.

Commitment to the B-lymphoid lineage during development in mammals is a multistep process involving a hierarchy of cells, which, starting from a hematopoietic multipotent stem cell, vary in their differentiation potential as well as in their proliferative and self-renewing capacity. In adults, a hematopoietic multipotent stem cell has been described, but it has not yet been established whether it is this cell or some more restricted progenitor that contributes to the daily massive production of B cells (1–5).

The avian bursa of Fabricius offers a unique anatomical model to study the entry into the B-cell lineage and the physiological behavior of this cellular population during the first months of development. The bursa is composed of $\approx 10^4$ lymphoid follicles (6) that can be easily isolated, giving access at different stages of development to pure, segregated populations of B cells. Prebursal stem cells, which can be detected at day 7 in the embryonic mesenchyme (7), colonize the bursal rudiment between day 8 and day 14 of incubation in the chicken embryo (8). From this period on, these bursal stem cells divide and give rise in each follicle to a population of cells progressively expressing IgM at their surface, 90% of bursal cells bearing cell-surface IgM at day 20 of incubation (9). After hatching (day 21) the bursal follicles continue to expand while seeding B cells to the periphery. By about 5 weeks of age, the stem cells responsible for this continuous B-cell production have left the bursa and, as postbursal stem cells, ensure the renewal of the B-cell pool during the life of the animal (10). Many data are in support of this schematic picture, but the associated molecular events remain to be characterized.

We report in this paper a molecular analysis of the Ig loci in single follicles containing $1–3 \times 10^5$ cells a few weeks after hatching. The restriction patterns obtained imply that very few rearrangement events take place in each normal bursal follicle. This result favors a model in which each follicle is

colonized by a small number of prebursal stem cells that are committed to a particular Ig gene rearrangement at the very beginning of the development of the embryonic bursa.

MATERIALS AND METHODS

Chickens. Chickens used in these experiments were maintained at Roche (Gipf-Oberfrick, Switzerland). The H.B21 strains were derived from Hyline B15/21(FP) stock by six generations of selection for birds carrying the B21 major histocompatibility complex haplotype (11). The H.B21 birds were chosen because they often display a polymorphic *Eco*RI or *Bam*HI site on one allele of their Ig light chain locus. Heterozygous birds were selected by analysis of their erythrocyte (RBC) DNA. For birds used as donors in the cell transfer experiments, this screening was performed with RBCs from 18-day embryos.

Construction of Chimeras. Cyclophosphamide-treated bursa-reconstituted birds were produced as described (10, 12). Each recipient was given an intraperitoneal injection of 4 mg of cyclophosphamide (Farmos, Turku, Finland) on the day of hatching and on three subsequent days, followed by an intravenous injection of bursal cells (about 20×10^6) from a normal 4-day-old donor chicken 1 day after the last injection of cyclophosphamide. The reconstituted bursal follicles were isolated and analyzed at 6–7 weeks after the transfer.

Isolation of Follicles and DNA Preparation. Single bursal follicles from 3-week and 6- to 7-week-old normal birds and 6- to 7-week reconstituted birds were isolated as described (12). After the crushing of each follicle with the plunger of a 1-ml plastic syringe in round-bottomed wells of a microtitration plate, the cells were suspended in 400 μl of 10 mM Tris·HCl, pH 8.0/0.15 M NaCl/10 mM EDTA in an Eppendorf tube. The yield of cells per follicle was between 10^5 and 3×10^5, depending on the age of the chicken. Their viability as measured by trypan blue exclusion was around 70–85%. After addition of 5 μg of carrier DNA (nonsonicated salmon sperm DNA), the cell lysate was incubated overnight at 37°C with 100 μg of protease K per ml 0.2% sodium dodecyl sulfate. After two phenol and two chloroform extractions the DNA was precipitated in ethanol, washed three times with 80% ethanol, and redissolved in the appropriate buffer for restriction enzyme digestion.

Southern Blots. Southern blot analysis was performed according to Southern (13), with the modifications of Wahl *et al.* (14). Probes (50–100 ng) were nick-translated (15) using 2 $\times$ 300 μCi (1 Ci = 37 GBq) of two deoxy[α-^{32}P]nucleotides (Amersham, 3000 Ci/mmol) to a specific activity of 2–5 $\times 10^9$

cpm/μg. Films were preflashed and exposed 2–3 weeks at −70°C using an intensifying screen.

Probes. Probes used for the blot hybridization were (i) a subfragment J of the genomic λ-Ch 5 phage (16); (ii) a subfragment U located between the V_λ and J_λ regions (where V = variable and J = joining) of the genomic λ-Ch 5 phage (16) (see Fig. 1); (iii) a 500-base-pair (bp) C_μ1–C_μ2 subfragment (where C = constant) of a V_H cDNA plasmid. This plasmid was isolated from a selected cDNA library constructed from chicken spleen mRNA as described (17). For the synthesis of the first cDNA strand we used as primer the C_μ2 domain prepared from a C_μ cDNA plasmid (18).

RESULTS

Analysis of the Light Chain Locus in Single Follicles of Normal and Reconstituted Birds. *Experimental strategy.* Previously we have described the organization of the chicken Ig light chain locus and reported that the same rearrangement, involving a unique V_λ functional gene, occurred in most B cells of this animal (16). To evaluate the number of rearrangements in isolated follicles, we had to distinguish between identical (involving the same *V* gene) but independent rearrangement events. We therefore took advantage of the presence of a polymorphic *Eco*RI or *Bam*HI site located in the light chain locus and used H.B21 birds heterozygous for these sites. The general outline of this approach is shown in Fig. 1. When isolated from a bird heterozygous for the *Eco*RI site located in the J_λ–C_λ intron, RBC DNA (used as a reference for germline DNA), gave two *Eco*RI fragments at 16.5 kilobases (kb) and 14 kb hybridizing with a J_λ probe: G_1 and G_2 (Fig. 1 *Top Left*). When total bursal DNA was digested with *Eco*RI and hybridized under the same conditions, we detected the two germline bands G_1 and G_2 and two rearranged fragments R_1 and R_2 at 14.5 and 12 kb. These two restriction fragments correspond to the deletion of the V–J intervening sequence (2 kb) that occurs during the rearrangement of the most-proximal V_λ gene in chicken B cells (16).

Since rearrangement generally occurs at a single allele at the light chain locus (16) and since both alleles are used at similar frequencies (19), these four bands—G_1, R_1, G_2, R_2—will have approximately the same intensity in a mixed population of B cells. On the other hand, a monoclonal population of B cells from such heterozygous birds will show either one or the other rearrangement and we shall then obtain a G_1R_2 or G_2R_1 pattern, depending on the allele that is rearranged (Fig. 1 *Top Right*). (In practice, the G_2 and R_1 fragments have very similar mobilities and may appear as a single band rather than a doublet.)

In experiments with birds heterozygous for the polymorphic *Bam*HI site, we used as a probe a segment of DNA ("U") that is deleted during the V_λ–J_λ joining process (Fig. 1 *Bottom*). Such blot hybridization experiments allow detection of the allele remaining in the germline configuration and will show two germline fragments with a *Bam*HI digest of RBC DNA. These same two fragments will be detected at half of their germline intensity when hybridization is made with total bursal DNA. A monoclonal population of B cells will show only one germline band, the rearranged fragment not being detected by the U probe.

Single follicles from reconstituted birds show one rearrangement event. Normal bursal cells from 4-day-old H.B21 chickens heterozygous for the *Bam*HI or the *Eco*RI site on the light chain locus were transferred into cyclophosphamide-treated recipients of the same age and strain. Six to 7 weeks later the recipients' bursae were removed and DNA was prepared from single follicles containing ≈2 × 10⁵ B cells. In blot hybridization experiments using an *Eco*RI (Fig. 2 *Left*) or a *Bam*HI (Fig. 3 *Lower*) digest and probing with a J or a U DNA fragment, almost all of the restored follicles
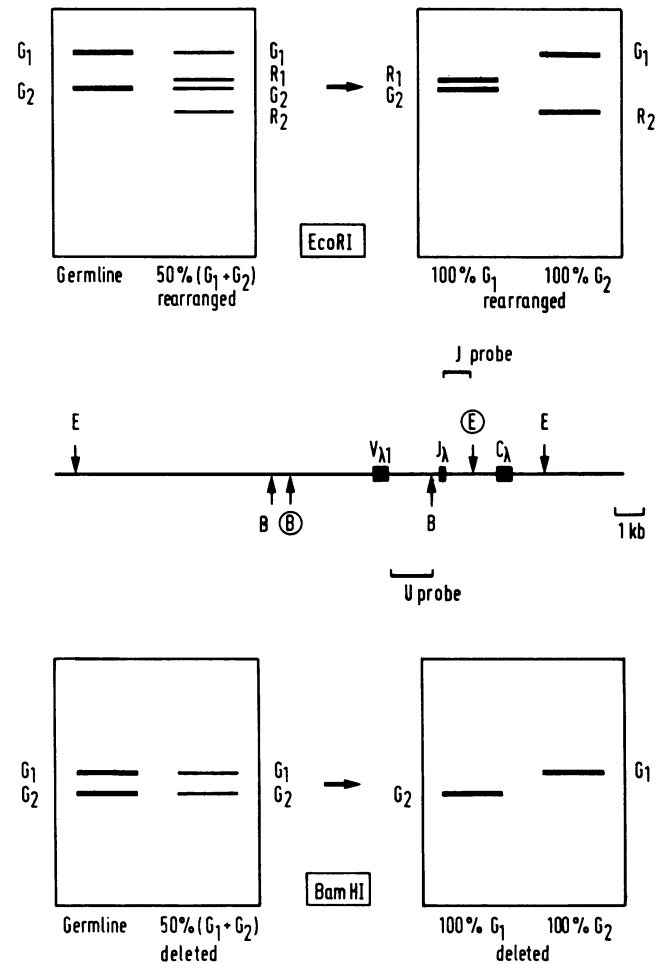

FIG. 1. Schematic representation of the different configurations of the light chain locus in heterozygous birds. (*Middle*) The C_λ genomic locus with C_λ, J_λ, and $V_\lambda 1$ exons. *Eco*RI (E) and *Bam*HI (B) restriction sites are indicated. The polymorphic sites are in circles. (*Top*) Hybridization patterns of *Eco*RI digests probed with a J region fragment. (*Top Left*) Cartoon of *Eco*RI digests of germline (RBC) and rearranged (bursal) DNA. The germline configuration shows the two alleles G_1 and G_2. The rearranged configuration shows G_1 and G_2 together with two rearranged fragments R_1 and R_2. If both alleles are rearranged in a random fashion, these four fragments should have the same intensity (half of the germline intensity G_1 and G_2). (*Top Right*) Cartoon of the same experiment on two different monoclonal populations of B cells. In this case all cells have rearranged either G_1 or G_2 and show an R_1G_2 or a G_1R_2 pattern. (*Bottom*) Hybridization patterns of *Bam*HI digests probed with a U region fragment that is deleted during the joining process. (*Bottom Left*) Cartoon of *Bam*HI digests of germline (RBC) and rearranged (bursal) DNA. The germline configuration shows the two alleles G_1 and G_2. The rearranged configuration shows that each of the two alleles has lost, in a statistical manner, half of its intensity. (*Bottom Right*) Cartoon of the same experiment on two different monoclonal populations of B cells. The U probe hybridizes only with the unrearranged allele and does not detect the rearranged fragment. A monoclonal B-cell population shows only either G_1 or G_2.

(>50) showed a single pattern of rearrangement. In some rare cases (<10%), a mixture of the two rearrangements could be detected in a single reconstituted follicle (not shown).

Single follicles from normal birds show a very small number of rearrangement events. We have performed the same blot hybridization on DNA extracted from single follicles of 6- to 7-week-old birds heterozygous for the *Eco*RI or the *Bam*HI site. A typical mixed pattern was observed for total bursal DNA, but very few single follicles presented a similar diversified configuration after digestion with either *Eco*RI or *Bam*HI. Moreover, the number of follicles showing

a typical monoclonal pattern was surprisingly large (27/53) (Fig. 2 *Right*; lanes N1, N2, N5, N8, N12). The remaining follicles showed a mixed pattern more easily explained by an unequal contribution of the two types of rearrangement (Fig. 2 *Right*; lanes N3, N6, N9). In one of these follicles (lane N9) one of the germline fragments was lost, probably as a result of a nonproductive rearrangement, but in all of the follicles analyzed, in several series of experiments, the same type of translocation involving the proximal V_λ gene was always observed. These data allow us to estimate the number "n" of rearrangement events occurring in each normal follicle from the equation $(Fa)^{1/n} + (Fb)^{1/n} = 1$, where Fa and Fb are the frequencies of monoclonal follicles showing a G_2R_1 or a G_1R_2 configuration, respectively. This formula is derived from the binomial distribution. The results we have obtained give an average number of two rearrangement events in each single follicle.

We have performed a similar series of experiments on normal bursal follicles from 3-week-old birds, each follicle containing $\approx 10^5$ B cells. The proportion of monoclonal patterns observed was very similar to that obtained with older animals (Fig. 3 *Upper*).

Analysis of the Heavy Chain Locus in Normal and Reconstituted Birds. *A major rearrangement event in total bursa.* Previously we have reported the isolation and characterization of a cDNA clone coding for three domains ($C_\mu 2$–$C_\mu 3$–$C_\mu 4$) of the chicken μ gene (18). We have constructed a partial cDNA library from chicken spleen mRNA using as a primer the $C_\mu 2$ domain of the C region. One clone coding for a V_H region, the $C_\mu 1$, and 18 bp of the $C_\mu 2$ domain was isolated and characterized by sequencing (unpublished data). A subfragment of 500 bp containing the $C_\mu 1$ and part of the $C_\mu 2$ domain was prepared and used as a probe for analysis of the heavy chain locus. In such hybridization experiments with a *Pvu* II digest of RBC DNA, we could detect one band at 25 kb (Fig. 4). A single fragment was also detected with other restriction enzymes (not shown), implying the presence of a single μ gene in chicken DNA. When total bursal DNA digested with *Pvu* II was examined and compared to reference (RBC) DNA, most of the germline band had disappeared and a major rearrangement event was detected at 18 kb, together with some minor bands that appeared as a smear between this band and the germline position (Fig. 4). When bursal DNA was digested with several different restriction enzymes a similar pattern of rearrangement was obtained, the additional minor rearrangement events giving bands very similar in size to the major rearranged fragment (not shown).

Single follicles from normal and reconstituted birds present a very similar pattern. In each of the follicles from the reconstituted birds we detected a different combination of rearranged bands localized in the 18- to 25-kb region, but the typical diversified pattern seen in total bursal DNA was never observed (Fig. 4 *Left* and *Center*). With single follicles from normal birds the patterns were very similar to the ones obtained with the reconstituted animals (Fig. 4 *Right*). In most cases the major rearranged fragment at 18 kb was present either alone or together with another band of the same intensity. In some rare follicles this band appeared to be the conserved germline fragment (lane N11; lanes R6 and R8 for the restored follicles). In some other cases a doublet was detected in the 18- to 25-kb region, but again a typical mixed pattern was never observed in these experiments.

DISCUSSION

Gene Rearrangements in Individual Bursal Follicles. The rationale of this study was to analyze the rearrangement of Ig genes in isolated bursal follicles of normal birds and to compare the pattern observed with that obtained from the corresponding total bursal DNA. Such an analysis will demonstrate whether each follicle shows a monoclonal or a diversified rearrangement pattern and should then allow us to estimate the average number of rearrangement events taking place in a single follicle. A similar study was conducted on cyclophosphamide-treated chickens reconstituted with normal bursal cells. Such bursal follicles have been shown previously to be of monoclonal origin at the phenotypic (IgM allotype) level (12, 20). We wanted to see whether these reconstituted follicles display a monoclonal rearrangement of their Ig genes. In this sense, they should serve as a control for the analysis of the follicles from normal birds.

In the light chain experiments we used a polymorphic restriction site (*Bam*HI or *Eco*RI) that allows us to differentiate, in heterozygous birds, between polyclonal and monoclonal cell populations. This analysis showed that a large number of normal single follicles (27/53) presented a monoclonal type of light chain rearrangement. The translocation event involved the most proximal V_λ gene in each follicle analyzed so far (>100). These data are in agreement with our previous quantification of the light chain rearrangement in total bursal DNA (16). This analysis also implied that most of the rearrangement events involve only one allele, the other allele remaining in the germline configuration. In fact, among all of the follicles that we have examined, only one was
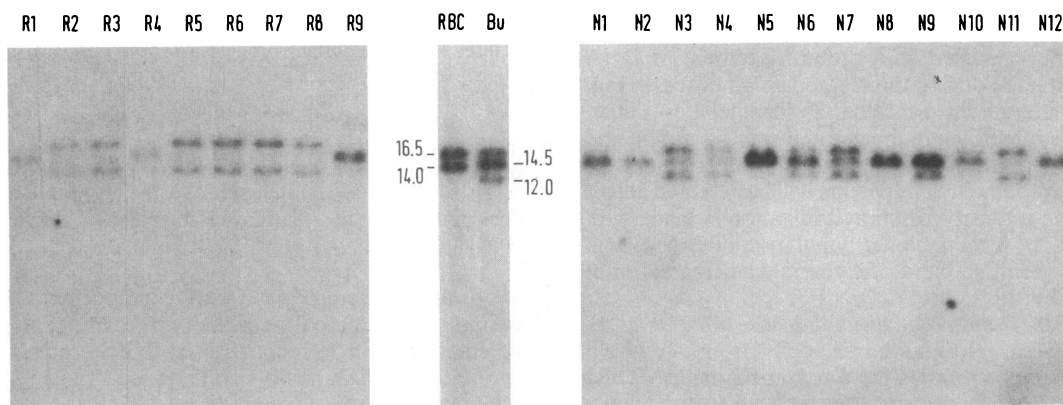

FIG. 2.   Light chain gene configuration in isolated follicles from normal and reconstituted H.B21 birds heterozygous for the *Eco*RI site. (*Left*) Lanes R1–R9: *Eco*RI digest of DNA from single follicles isolated from a 6-week-old bird treated with cyclophosphamide and reconstituted with bursal cells. (*Center*) *Eco*RI digest of RBC DNA (lane RBC) and bursal DNA (lane Bu) from a normal 6-week-old heterozygous animal. The two germline bands are shown at 16.5 kb and 14 kb, and the two rearranged fragments are shown at 14.5 kb and 12 kb, respectively. The same pattern is obtained with an *Eco*RI digest of bursal DNA from a reconstituted bird (not shown). (*Right*) Lanes N1–N12: *Eco*RI digest of DNA from single follicles isolated from a normal 6-week-old heterozygous bird. All of the blots are hybridized with the J probe (see Fig. 1).
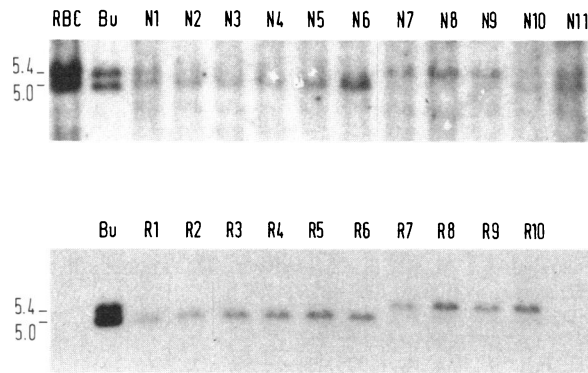
FIG. 3. Light chain gene configuration in isolated follicles from normal and reconstituted birds heterozygous for the *Bam*HI site. (*Upper*) *Bam*HI digest of RBC DNA (lane RBC), bursal DNA (lane Bu), and single follicle DNA (lanes N1–N11) from a normal 3-week-old heterozygous bird. (*Lower*) *Bam*HI digest of bursal DNA and single follicle DNA (lanes R1–R10) from a reconstituted 6-week-old animal. The two germline bands are shown at 5.4 and 5.0 kb. All of the blots are hybridized with the U probe (see Fig. 1).

rearranged on both chromosomes (see Fig. 2, follicle N9, showing the loss of the upper 16.5-kb germline fragment).

When analyzing total bursal DNA with a C_μ probe, we always observed a major rearrangement. Moreover, in contrast to the light chain situation, the germline band disappeared almost completely, a situation very similar to the mouse μ rearrangement in splenic B cells (21, 22). When different restriction enzymes were used, a few minor bands of very similar size to the major rearranged fragment were always observed. They could be due to alternate usage of different diversity (D) or J_H segments or to incomplete D–J_H or nonproductive V_H–D–J_H translocations. A precise characterization of the μ genomic region will allow a better definition of these different events. When the μ locus of single follicles was examined, most follicles showed a simplified pattern as compared to the total bursa. In some rare cases, the germline fragment appeared to be conserved and it is not yet clear whether this event is frequent enough to suggest a different order of rearrangement or a different genomic organization of the heavy chain locus in this species (23).

The same hybridization experiments were performed on single follicles isolated from reconstituted birds. Our data showed that most of these follicles were also monoclonal at the DNA level, each of them showing a rearrangement on one allele of the light chain locus. In a few cases, we observed two rearrangement events in one restored follicle, presumably due to population of some follicles by more than one precursor cell (12, 20). In the heavy chain analysis each reconstituted follicle showed a unique configuration very

similar to the pattern observed with the normal follicles. Our results are thus in agreement with previous observations showing that the cell that can reconstitute a depleted follicle is already committed to expression of a particular IgM allotype (20, 24).

Early Commitment of B-Cell Precursors. Our data on the heavy and light chain gene rearrangement in single follicles of normal birds are in agreement with data on IgM allotype expression in normal follicles (24) and allow us to estimate, on the basis of the light chain results, the number of independent rearrangements to be, on average, two events per follicle. However, two caveats to this quantitative argument should be made. First, the bursa is colonized by circulating cells during $\approx$6 days of embryonic development (day 8 to day 14), with the peak of this migration around day 10 to day 12. We cannot exclude that some cells that colonize a follicle 24–48 hr later would represent <5% of its B-cell content and thus not be detectable in our hybridization procedure. However, experiments involving quail–chicken chimeras have suggested that bursal colonization occurred in a rather synchronous fashion and that each follicle, once populated, was not further seeded by additional stem cells (25). An additional criticism is that clonal dominance could occur inside a follicle, so that an originally heterogeneous population of B cells would progressively become more homogeneous. The very similar results obtained with normal follicles at 3 and 7 weeks of age tend to minimize the possible importance of this process.

Thus, our data do not indicate the number of cells that migrate into each follicle during the embryonic period. They only imply that this number is very small and cannot discriminate precisely between two different possibilities. (*i*) One stem cell migrates into a follicle and divides several times before being committed to an Ig gene rearrangement. (*ii*) Several stem cells give rise to a rearranged configuration as soon as they colonize a bursal follicle, or perhaps are already rearranged even before entering the bursa.

Following parabiosis between embryos whose B cells could be distinguished by the presence or absence of an alloantigen (Bu-1a), the number of precursor cells populating a follicle was estimated previously to be approximately three or four (12). These results and those obtained in the quail–chicken chimeras (25), in which mixed populations could be detected in single follicles, are strongly in favor of the second interpretation. Our conclusion is that B-cell precursors entering the bursa and giving rise to clones of B cells are committed to a particular Ig gene rearrangement at the first stage of colonization and do not continuously rearrange their Ig genes during the embryonic maturation of this organ.

In previous experiments the reconstitution of cyclophosphamide-depleted bursal follicles could be abolished by prior incubation of the donor cells with anti-μ allotype antiserum
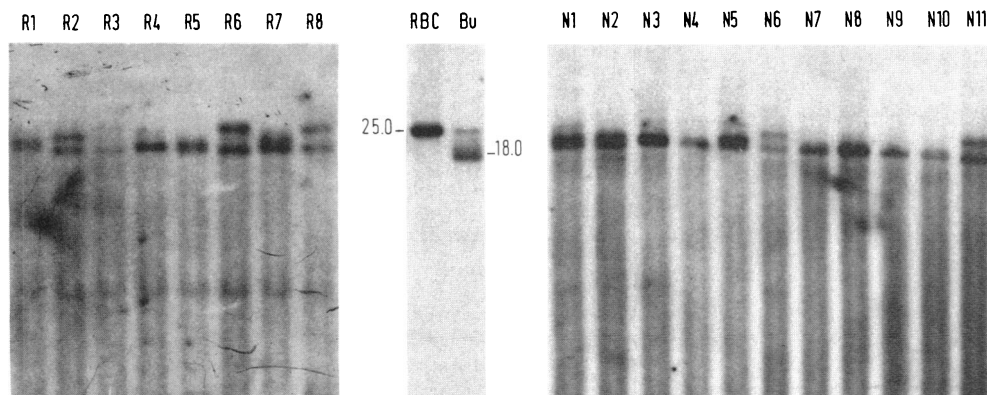

FIG. 4. Heavy chain gene configuration in isolated follicles from normal and reconstituted birds. (*Left*) Lanes R1–R8: *Pvu* II digest of DNA from single follicles isolated from a reconstituted 6-week-old bird. (*Center*) *Pvu* II digest of normal RBC DNA (lane RBC) and bursal DNA (lane Bu). The 25-kb germline band and the major rearranged fragment at 18 kb are shown. (*Right*) Lanes N1–N11: *Pvu* II digest of DNA from single follicles isolated from a normal 6-week-old bird. All of the blots are hybridized with a 500-bp $C_\mu 1$–$C_\mu 2$ subfragment.

(20). Moreover, long-lasting Ig allotype suppression could be obtained in chickens after *in vivo* injection at day 13 of incubation of an anti-μ allotype serum (26). Our results are in agreement with these observations, suggesting that the stem cell that can reconstitute a depleted follicle 4 days after hatching is already committed to a particular Ig rearrangement. The surface IgM-positive progenitor cell that is the target for anti-μ suppression would then appear in the embryonic bursa during the period of colonization (1–4 × 10⁴ stem cells at day 8 to day 14 of incubation).

Previously we have suggested that the generation of the chicken light chain repertoire may rely chiefly on somatic diversification mechanisms (16). An identical picture seems to emerge for the heavy chain locus. The bursal step would then be an obligate requirement for these stem cells to accumulate and give rise to an amplified repertoire of B-cell specificities. Each bursal follicle would be able, starting from a very small number of rearrangement events, to accumulate a large array of different combining sites. Several weeks after hatching these bursal stem cells reach the postbursal stage, where they can restore a depleted animal without homing to the bursa (10). This pool of extrabursal committed progenitor cells would then assume the renewal of the B-cell population. At this point, they may have already diversified their Ig receptors to a large extent in the bursa or their progeny may be able to do so in extrabursal sites in the adult animal. Apparently under no circumstances could they be newly regenerated from an early multipotent stem cell, since the bursa, having involuted at this stage, can no longer provide the necessary environment for this early step to occur.

There could be similarities between the ontogenic pathway that we wish to propose for the chicken B-cell lineage and the corresponding process in mammals. It has been suggested that there could be formation at a very early stage in the mouse of a population of committed progenitor cells that would contribute to a large extent to the generation of B cells during the life of the animal (27–30). These committed progenitor cells derived from the multipotent stem cells would be, if confirmed, equivalent to the bursal stem cells. One main difference would emerge between these two models. In the mouse the committed stem cells have probably, with the possible exception of some B-cell subpopulations (31), not rearranged their V genes and will give rise to rearranged progeny in the fetal liver and later on in the bone marrow (32, 33). In the chicken, however, these stem cells are committed to IgM production and will first be present in the bursa from the embryonic period until about 5 weeks after hatching and then in peripheral lymphoid tissues. These cell populations, both in the mouse and chicken, will be capable of long-term restoration of humoral immunity. Such a different choice of phenotype for these progenitor cells could be explained at the molecular level. The mouse's large repertoire of germline V genes will permit the random expression at each division of a new V region. In the chicken, the paucity of the V gene pool could favor a model in which somatic mutations would accumulate at the stem cell level in order to increase the magnitude of the B-cell repertoire.

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