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Regeneration of Natural Antibody Repertoire After Massive Ablation of Lymphoid System: Robust Selection Mechanisms Preserve Antigen Binding Specificities¹

Alberto Nobrega,²✉ Beatriz Stransky, † Nathalie Nicolas,* and Antonio Coutinho*‡

Natural Abs (NAbs) are Igs present in the serum and body fluids of healthy vertebrate animals, without any previous intentional immunization. NAbs often exhibit autoreactivity but also play an essential role in immunity, being a first line of defense against infectious microorganisms. We have previously analyzed the natural serum IgM Ab repertoire of normal mice, characterizing their reactivity with hundreds/thousands of self Ags; a significant similarity among different individuals was observed, and it was found that many reactivities of NAbs stably kept during adulthood were established early in life, implicating that period as a critical time window in the physiology of NAb repertoire selection. In the work reported here, experiments were conducted to address the role of normal lymphocyte ontogeny to the formation and stability of adult NAb repertoire. The massive destruction of the lymphoid system was promoted in adult mice with gamma-irradiation, and regeneration of hemopoietic tissues was granted by bone marrow or fetal liver inoculum. NAb repertoire regeneration was followed for 60 days after gamma-irradiation in bone marrow or fetal liver chimeric animals. The analysis of serum IgM reactivity with hundreds/thousands of self Ags showed that the NAb repertoire regenerated most of its original format after massive destruction of lymphoid compartments, characterizing autoreactive repertoire selection as a robust biological process. The data also show that regeneration of the NAb repertoire occurred similarly in fetal liver and bone marrow chimeras, although the latter animals poorly reconstituted their CD5⁺ B1 cell compartment, suggesting that B1 cells are not essential for natural Ab regeneration. The Journal of Immunology, 2002, 169: 2971–2978.

The physiological role of autoreactive NAbs is elusive, but regulatory functions on tolerance and autoimmunity have been proposed (7–9). In contrast, a critical role for NAbs in defense against infections has been evidenced in different experimental models (10, 11). These apparently antagonic behaviors depend on the overall diversity of circulating Ig molecules, the repertoire of NAbs, and it is important to understand how those properties relate to the original antigenic stimuli that generate them. NAbs were suggested to be mainly produced by CD5⁺ B1 lymphocytes and would preferentially exhibit germline-encoded VDJ rearrangements (7, 12). It has been proposed that the absence of N nucleotides in VDJ joinings would endow NAbs with the property of multireactivity, which could account for their ability to react with both self and non-self Ags (4, 7, 12). In support of the latter, it was recently demonstrated that TdT-transgenic mice showed increased susceptibility to bacterial infections, suggesting that germline-encoded NAbs play a critical role in immunity that cannot be performed by Igs with extensive N-additions (13).

Our previous results and data from others groups showed that serum IgM NAbs from both normal humans and normal mice exhibit specific binding to defined Ags when assayed for reactivity with hundreds/thousands of self or non-self molecules, demonstrating that NAb repertoire is nondegenerate and well defined (3, 5, 14–16). A significant similarity of the NAb repertoire among normal individuals was observed; both mice and humans, however, exhibiting a distinct repertoire. The study of NAb repertoire ontogeny showed that the main NAb binding specificities were established early in life and preserved during adulthood (14, 15, 17), implicating that period as a critical time window in the physiology of NAb repertoire selection. The immune system during this period is characterized by singular conditions, both developmental and environmental; low microbiological loads and transfer of infections has been evidenced in different experimental models

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3Abbreviations used in this paper: NAb, natural Ab; PCA, principal component analysis; BMC, bone marrow chimera; FLC, fetal liver chimera; MANOVA, multivariate ANOVA; BCR, B cell receptor.

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maternal IgGs predominate until weaning, whereas CD5+ B1 lymphocytes are abundant and constitute the majority of the population of activated B cells, which are enriched for VDJ rearrangements with short or absent N sequences (18–21).

It has been argued that the privileged conditions prevailing during early ontogeny would be critical for the generation of the NAb repertoire (1, 7, 18, 19, 21). Here, to evaluate the specific relevance of the normal ontogeny and early postnatal period on NAb repertoire formation, we used the experimental system of lethally irradiated mice reconstituted with adult bone marrow, which regenerate their immune system in microbiological conditions totally different from the almost sterile environment of the neonatal and weaning period, and with a profound reduction of CD5+ B1 cells. The regeneration of the NAb repertoire after the massive destruction of the lymphoid system was compared with its normal formation; the results showed that NAb repertoire is essentially established during the first 2 weeks after birth and regenerates most of its original format after destruction of the lymphoid compartments, evidencing a robust dynamic equilibrium and homeostasis. The significance and possible mechanisms underlying these phenomena are discussed.

Materials and Methods

Animals and sera

Specific pathogen-free C57BL/6 and BALB/c mice were obtained from IFFA CREDO (Saint Germain sur Arbrisseau, France). Adult mice were bled by retro-orbital puncture. Animals 7 and 15 days of age were bled by intracardiac puncture. IgM concentrations in serum were determined by ELISA, using anti-mouse IgM-specific reagents (Southern Biotechnology Associates, Birmingham, AL).

Cells

Bone marrow chimera (BMCs) were obtained from the femurs of the mice (8 wk old) and resuspended in 4°C cold medium (RPMI 1640 without FCS, 2 mM l-glutamine, 10 mM HEPES, 100 U/100 mg/ml penicillin/streptomycin at 4°C). Cells were washed, counted, and suspended at 50 × 10^6/ml. Fetal liver cell suspensions were prepared from E14 fetuses, washed, and treated as previous. Peritoneal cavity cells were obtained after vigorous flushing of the cavity with 10 ml of cold medium as above.

BMCs and fetal liver chimera (FLC)

Male animals, 8 wk old, were irradiated with 750 rad and immediately received an i.v. injection of 5 × 10^8 bone marrow or fetal liver cells from syngeneic donors. Mice were treated for 15 days before and 15 days after irradiation with antibiotics in drinking water (ampicillin 0.2 mg/ml, polymyxin 500 IU/ml). Animal food was sterilized during the entire experiment, and mice were not fed the day before irradiation.

Organ extracts and electrophoresis

Organ and tissues from C57BL/6 mice were mechanically disrupted and homogenized with an electrical Potter homogenizer (Wheaton, Millville, NJ) in homogenizing buffer (2% SDS, 100 mM DTT, 60 mM Tris (pH 6.8), 1 mg/ml aprotinin, 1 mg/ml pepstatin, 50 mg/ml N,N,N′,N′-tetraacylcholomethyl ketone, 1 mm EDTA) on ice. Protein concentration in the extract was determined by the Lowry assay. Electrophoretic separation of the proteins was performed by SDS-PAGE. The composition of the separating gel was 10% acrylamide, 0.27% bisacrylamide, 0.375 M Tris (pH 8.8), 0.1% SDS, 0.1% \( N,N,N′,N′′ \)-tetramethylethylene diamine, 0.25% ammonium per sulfate. The composition of the stacking gel was 4% acrylamide 0.1% bisacrylamide, 0.125 M Tris (pH 6.8), 0.1% SDS, 0.08% \( N,N,N′,N′′ \)-tetramethylethylene diamine, 0.05% ammonium per sulfate. All reagents used in the electrophoresis were purchased from Bio-Rad (Richmond, CA). The gels were run in a Mighty Small II SE 250 apparatus ( Hoefer Scientific Instruments, San Francisco, CA) at 45 mA until the front dye reached the bottom of the gel. Gels were 1.0 mm thick, and 0.4 to 0.5 mg of protein was applied in preparative gels, without combs.

Immunoblot

After electrophoresis, proteins were transferred from the gel to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) by semidyrid electrophoret transfer, during 75 min at 0.8 mA/cm² using a Semi Dry Electrob lott er B (Ancos, Hoejby, Denmark). After the transfer the nitrocellulose membrane was blocked with PBS, 0.2% Tween 20 overnight at room temperature. Incubation of different sera with the membrane was performed using the Cassette Miniblot System (Immunetics, Cambridge, MA) with 28 channels. The sera were incubated for 4 h at room temperature. When possible, sera were adjusted to 20 µg/ml for the assay. After incubation, the sera were washed with PBS, 0.1% Tween 20. Secondary anti-IgM Ab coupled to alkaline phosphatase (Southern Biotechnology) was incubated for 2–3 h and then washed as before. The immunoreactivities were revealed with nitroblue tetraclo-romochlorinidyl phosphate substrate (Promega, Madison, WI) in substrate buffer (100 mM Tris (pH 9.5), 100 mM NaCl, 5 mM MgCl₂). The reaction was stopped after 3–5 min by rinsing the membrane with distilled water.

Protein staining

After revelation of the immunoblot, proteins blotted onto the nitrocellulose were stained with colloidal gold (Protagol; BioCell, Cardiff, U.K.) for 3–8 h with agitation until a suitable coloration was obtained and then was washed three times for 5 min with distilled water.

Image acquisition

The pictures of immunoblots before and after protein staining were acquired by scanning (Silverscanner II, 500 dpi resolution, 256 gray levels; LaCie, Hillsboro, OR), generating computer fles of the corresponding images. Pictures showed in the paper have been edited with Adobe Photoshop software (Adobe Photoshop, Mountain View, CA) to enhance contrasts for better observation; images of reactivity profiles were calibrated to have similar gray levels on background. Densitometric quantitation was performed in original, nonedited images, as described below.

Rescaling of the immunoblots and data analysis

Before protein staining, the densitometric profiles of immunoreactivities were quantituated in the original, nonedited immunoblot images. Densitometric trace of a immunoreactivity profile consisted of 600 numerical values of 8-bit gray levels intensities (0–255), corresponding to a resolution of ~0.15 mm. The immunoblot membrane was then stained with colloidal gold, revealing the migration position of the proteins. The densitometric profiles of the protein staining of original images were subsequently quantitated in the spaces between the lanes where the sera were tested, such that the immunoreactivities do not interfere with the colloidal gold coloration. The comparison between any two immunoreactivity profiles of different lanes could thereafter be done by referring to their corresponding protein profiles. Correction for distortions during electrophoretic migration can be made comparing the homologous peaks of protein profiles of each lane as described (14). Data analysis was performed on a Macintosh computer (Apple Computer, Cupertino, CA) using IGOR Pro software (Wave metrics, Lake Oswego, OR). Special software packages were developed for the correction of electrophoretic migration defects.

Multivariate statistical treatment of the data

The multivariate analysis of the data follows the treatment presented in our previous works (3, 14, 15). Briefly, the reactivity profiles were quantitated by densitometry as explained above. Each profile resulted in a densitometric trace of 600 numerical values, encoded as an array, or vector, of 600 numbers of intensity of immunoreactivity. The collection of vectors (38 profiles) was submitted to principal component analysis (PCA), a multivariate treatement that reduces the dimensionality of the data while preserving most of its variance. Each vector is plotted on factorial planes, according to their coordinates in the principal axis. The relative positions of the different vectors are indicated with points in the factorial planes. Similar profiles corresponded to points lying close to each other, whereas distinct profiles corresponded to points apart. The amount of variance contained in each factorial plane is indicated in the legend of Fig. 5. A PCA algorithm was implemented using MATHEMATICA software (Wolfram Research, Champaign, IL). Statistical significance for multivariate comparison of repertoire profiles was evaluated using multivariate ANOVA (MANOVA), applied to the first two factors obtained with PCA. Theoretical aspects of multivariate statistics can be found in the work of Rencher (22).

Results

To investigate the repertoire of serum IgGs, we adopted the functional approach based on the Ag binding of the Ig molecule, instead of the description of gene sequence or gene family to which it belongs. A large collection of Abs from different autologous
Ontogeny of serum IgM reactivity with muscle Ags in the method, with adequate modifications, allows extraction of a large diverse Ag array for the binding assay of immunoblot. This then blotted onto nitrocellulose membrane, was used as a highly organs extracts, previously fractionated by electrophoresis and then blotted onto nitrocellulose membrane, was used as a highly diverse Ag array for the binding assay of immunoblot. This method, with adequate modifications, allows extraction of a large amount of information on Ig repertoire that can be further analyzed with multivariate statistics (14, 15).

Ontogeny of NAb repertoire

As a basis for comparison with the studies on NAb repertoire regeneration further described in this paper, we analyzed the serum IgM NAb repertoire during ontogeny in normal C57BL/6 mice ages 7 days to 5 mo. Serum IgM was assayed for reactivity on immunoblots of different self tissues/organ extracts (muscle, liver, lungs, and spleen). The results of serum IgM reactivity on immunoblots of muscle extract are shown in Fig. 1, showing that a significant part of NAb IgM repertoire is established early in life, as we previously reported (15, 16) during the first 2 wk of age. The main conserved reactivities are indicated with arrows on the left of the figures and are present since the first week of age. The same main reactivities were scored in BALB/c mice (Fig. 2). Analogous results were obtained on liver, lungs, and spleen extracts (not shown).

Serum IgM concentration varies considerable during ontogeny; for the B6 mice used in this work, concentrations rose from 7 μg/ml at 1 wk of age to 50 μg/ml at 30 days and 200 μg/ml at 60 days of age, on average, resulting in an ~28-fold increase. Body weight and volume multiply at least 10-fold from birth to adulthood, such that the mass of serum IgM is minimally 100-fold larger in adult animals, providing full opportunity for repertoire alteration and renewal, which is further augmented by the short half-life of serum IgM in the mouse, 12–40 h. These observations place in appropriate perspective the remarkable conservation of the main NAb reactivities throughout life. Some variability in the NAb repertoire of C57BL/6 and BALB/c mice is present when comparing animals 2 or 3 mo of age and up and can be pronounced in older B6 animals (12 mo of age and older), a fact we did not observed in BALB/c mice (our unpublished observations).

FIGURE 1. Ontogeny of serum IgM reactivity with muscle Ags in the C57BL/6 mouse. Serum IgM reactivity profile from 20 C57BL/6 mice, with age varying from 7 days to 5 mo, assayed for binding in immunoblot assay of syngeneic muscle Ags. Individual animals are indicated with lower case letters above each reactivity profile. All lanes represent individual animals, except for the profile of 7 days which is a pool of six animals, and 15 days, which are a pool of two animals each. 2ndAb indicates the background reactivity of secondary anti-IgM Abs alone. The estimated molecular masses (MW) of relevant reactivities are indicated on the left ordinate.

FIGURE 2. Ontogeny of serum IgM reactivity with muscle Ags in the BALB/c mouse. Serum IgM of 16 BALB/c mice, with age varying from 7 days to 2 mo, assayed for binding in immunoblot assay of C57BL/6 muscle Ags. Individual animals are indicated with lower case letters above each reactivity profile, except for the profile of 7 days which represents a pool of six animals. In this experiment, 15 days sera represent individual animals. A B6 reactivity profile, animal (b) from Fig. 1, was included for comparison. 2ndAb indicates the background reactivity of secondary anti-IgM polyclonal Abs alone. The estimated molecular masses (MW) of relevant reactivities are indicated on the left ordinate.

Natural Ab repertoire regeneration in BMCs

The conservation of NAb repertoire during ontogeny is the result of homeostatic mechanisms actively operating in the selection and activation of B lymphocytes. Self-renewing CD5+ B1 lymphocytes may constitute the basis of this stability, but other biological principles could be operating, e.g., dynamic equilibrium in competition between lymphocytes for triggering and survival (23). To investigate the nature and robustness of this control, we promoted the extensive destruction of the adult lymphoid system and then followed its regeneration in time. For that purpose, 8-wk-old B6 male animals were irradiated with 750 rad and immediately reconstituted with 5 × 10⁶ BMCs from a pool of syngeneic male donors. The NAb repertoire was followed for 60 days during regeneration. Studies with IgH allotype congenic, bone marrow irradiation chimeras, have shown that 2 mo after grafting, the majority of serum IgM (95% or more) derives from donor cells (23, 24).

Fig. 3A shows the evolution of serum IgM repertoire reactivity of individual animals, on immunoblots of autologous muscle at day −1, 7, 15, 30 and 60 days after irradiation and bone marrow reconstitution. It can be seen that a very significant regeneration of NAb repertoire is achieved in all eight chimeras, especially in what concerns the main reactivities preserved during ontogeny. It is interesting that before irradiation some mice differed from others in intense immunoreactivities, e.g., BMC6 (Ag μ7, left ordinate) and BMC7 (Ag μ8), that were very diminished or even disappeared along regeneration, rendering the reactivity profile of those animals more similar to their counterparts. The same observation also applies to moderate, less intense reactivities, which also diminish or disappear from serum (BMC3 μ2, BMC6 μ3). The progressive augmentation in time of few reactivities (BMC1 μ4) were also noted, as well as strong fluctuations in the intensity of the immunoreaction (BMC4 μ5, BMC5 μ6). Quantitative analysis of the densitometric traces of immunoreactivity profiles on muscle extract shows that the reactivity indicated in Fig. 3A as μ5 exhibits 300% augmentation when comparing BMC4 on day 60, to BMC4 either on day 30, 15, 7 or (−1) (Fig. 3B); μ6, in BMC5, is >200% augmented at day 30 compared with day (−1), 15, or 60 (densitometric traces not shown). In contrast, one can also identify some
reactivities unique to a single animal, not observed or weak in others, that are nonetheless preserved during regeneration in that animal (Fig. 3A, BMC1 μ1). The pronounced variations/ fluctuations of the intensity of reactivity are reproducible in replicate immunoblots and thus are not due to technical aspects of the method and probably reflect variation in clonal sizes and affinities in the actual IgM repertoire of different animals. It is important to observe that control animals injected only with saline maintained their respective reactivity profiles essentially identical with themselves during the duration of the experiment, 60 days (one control is shown in Figs. 3A and 4, indicated as CTR), such that significant variations of repertoire observed in BMCs are due to the repertoire destruction/regeneration processes.

Fig. 4 shows the reactivity profiles of BMC serum IgM on immunoblots of autologous liver extracts. As for muscle Ags, a significant regeneration of NAb repertoire is achieved in all eight chimeras. Again, some variability in the reactivities profiles of different animals was observed before irradiation, being more pronounced in BMC1 and BMC5. Most conserved reactivities in ontogeny are present during regeneration. However, as for muscle extract, strong fluctuations in the intensity of reactivities (BMC1 λ1, BMC2 λ2, BMC3 λ3, BMC8 λ4 and λ6) were observed, whereas others were diminished or vanished (BMC8 λ7, BMC6 λ8). Preservation of rare reactivities (BMC5 λ5) was also observed. Control animals essentially maintained their repertoire (see CTR in Fig. 5). Analogous results were obtained on immunoblots of spleen and lung extracts (not shown).

We seek independent confirmation of the conclusions we made above using a comprehensive and unbiased analysis of the reactivity profiles, not concentrating the analysis on any defined reactivity a priori. For that purpose, the densitometric quantification of all reactivity profiles shown in Figs. 3A and 4 were collected into a matrix of data, 38 × 600 (number of reactivity profiles × number of days × number of treatments).
of densitometric values per profile), which was submitted to PCA, a classical multivariate statistical treatment that reduces the dimensionality of the data while preserving most of its variance and structure (see Materials and Methods). When the major part of the variance is concentrated in the first two principal factors, as was the case here, the result of the PCA is adequately represented as a plot in the principal factorial plane, where each reactivity profile is indicated by a point on that plane; similar profiles corresponded to points lying close to each other, divergent profiles to points far from each other. Fig. 5 summarizes the results of PCA for the data on muscle and liver immunoblots (75% of total variance for muscle data and 81% for liver data), showing the distribution of the profiles on the principal factorial plane. For the sake of simplicity, only animals BMCs on day (−1), before irradiation, and 60 days after reconstitution are indicated in the figures. The following conclusions could be drawn from this analysis: 1) the majority of reactivity profiles of animals before and after irradiation clustered not far from each other, indicating their overall similarity; 2) deviant animals before irradiation became more similar to their counterparts after regeneration, as indicated by the relative position of the points corresponding to these profiles in the factorial plane; 3) the deviant profiles revealed by the PCA on muscle and liver data are the ones we pointed out in our previous empirical, visual analysis of the blot pictures. Statistical equivalence of repertoires was obtained applying the MANOVA test to the two groups, before irradiation and after regeneration. The values of \( p = 0.95 \) and 0.83 were obtained for muscle and liver immunoblots, clearly evidencing the overall equivalence of reactivity profiles in both groups.

**NAb regeneration in FLCs**

There have been conflicting results concerning the capacity of adult BM precursors to generate CD5\(^+\) B1 lymphocytes. Studies with irradiation bone chimeras showed that B1 did not recover when the donor BMCs were derived from adult mice, but this interpretation was challenged by others that could obtain partial reconstitution of this population (24, 25). We investigated this point in BMCs studied here, and 60 days after reconstitution a profound reduction of B1 CD5\(^+\) cells in the peritoneal cavity was found (Fig. 6). Because it has been attributed to CD5\(^+\) B cells, a major role in the NAb repertoire in the mouse, we also prepared irradiation FLC, which regenerate the B1 compartment, and studied NAb repertoire recovery, as was done for BMCs. Figs. 7 and 8 show the evolution of serum IgM repertoire reactivity on immunoblots of muscle and liver extracts, respectively, at day (−1), 15, 30, and 60 days after irradiation and hemopoietic reconstitution with fetal liver precursors. Analogous to what was observed with BMCs, it can be seen that regeneration of the NAb repertoire is essentially achieved in all five chimeras. In BMCs, some reactivities diminish or disappear (e.g., in muscle: FLC2 \( \eta_2 \); in liver: \( \eta_2 \)).

![FIGURE 5](image-url)  
**FIGURE 5.** PCA of serum IgM reactivity with muscle and liver Ags in BMCs. The data obtained from the densitometric quantification of reactivity profiles of muscle and liver immunoblots shown in Figs. 4 and 5 from all eight chimeras on days (−1) and 60 after irradiation were collected in matrices and submitted to PCA (see Materials and Methods). Results are of each reactivity profile represented by a point on the principal factorial plane (71% of total data variance for muscle immunoblot is represented in the factorial plane; 80% for liver data). Similar profiles corresponded to points lying close to each other, divergent profiles to points far from each other. Filled symbols correspond to day (−1), open symbols to day 60 after irradiation.

![FIGURE 6](image-url)  
**FIGURE 6.** Phenotypic characterization by flow cytometry of CD5\(^+\) B cells in the peritoneal cavity. Two-color flow cytometry analysis of resident peritoneal cells from one control animal and from one irradiated, bone marrow reconstituted chimeric animal is shown. All five controls and eight chimeras gave similar results. Stainings are for the membrane expression of IgM and CD5. The numbers indicate the percentage of cell population in the depicted gates.
FLC1 κ1, FLC5 κ3 and κ4, FLC2 κ6), and new and strong reactivities appears (FLC2 γ1, FLC5 γ4, and FLC5 κ2), whereas others fluctuate significantly (FLC1 γ3 and FLC4 κ5), suggesting vigorous selection processes operating during repertoire regeneration. Statistical equivalence of repertoires was demonstrated applying the MANOVA test to the two groups, before irradiation and after regeneration. The values of \( p = 0.93 \) and 0.78 were obtained for muscle and liver immunoblots, again evidencing the overall equivalence of reactivity profiles.

**Discussion**

The analysis of NAb repertoire reported in this paper is based on the evaluation of serum IgM reactivity with a large array of self Ags, using the immunoblot assay as we previously described (14, 15). We emphasize that the IgM reactivities scored with the immunoblot method are specific for muscle and liver immunoblots, again evidencing the overall equivalence of reactivity profiles.

The homogeneity of the serum IgM NAb repertoire was evidenced in normal young adult mice despite the high discriminatory power of the method used for the analysis of Ig reactivities (14). The presence and conservation since the neonatal period of many NAb specificities (15, 17) were again confirmed in the present study. Taking into consideration the large accumulation of Ig molecules during ontogeny (100-fold expansion) and their normal turnover, there is room for complete modification of the NAb repertoire, and its stability must involve efficient homeostatic mechanisms to ensure dynamical equilibrium. What could be the main elements of these homeostatic mechanisms, and how do they operate? A first possibility to explain the stability of repertoire formation would be to have a stable plasma cell compartment; plasmacytes produced in the neonatal period could have a long life span or self-renewal capacity. The permanence of the clonal component of this cell compartment would automatically guarantee the stability of the NAb repertoire. This hypothesis finds some support on data about experimental reconstitution of RAG-2 knockout mice, where the plasma cell compartment, once filled, admits little renovation from a new lymphocyte population (27, 28). Considering that plasmacytes are abundant in the neonatal period and rapidly achieve numbers equivalent to those of adult mice, it is possible to explain NAb repertoire conservation in ontogeny within this framework. This interpretation is also consistent with the suggestion that plasma cells producing NAbs would derive from CD5+ B1 cells, which are predominant in the neonatal period, and because TdT expression is low at that time, those plasmacytes would mainly secrete germline encoded IgGs (1, 7, 18, 21).

The above depicted scenario, however, heavily depends on a privileged environment supporting a stable population of cells. The elimination of this population should in principle result in severe disturbance of NAb repertoire, but the results we obtained here after massive destruction and regeneration of the lymphoid system do not support that notion. The irradiated, bone marrow-reconstituted chimeric mice (BMC) that we have analyzed here undergo an almost complete replacement of lymphoid cells and serum IgGs but nevertheless regenerate essentially their original NAb repertoire (23, 24). In addition, BMCs reconstituted poorly the CD5+ B cell compartment in the peritoneum, and do not suggest a major role for CD5+ B1 cells in NAb regeneration, although their involvement cannot be formally excluded because of the presence of residual numbers of B1 cells in BMCs. In all three experimental systems studied, normal ontogeny, BMCs, and FLCs, the NAb repertoire appeared largely equivalent. The results suggest that a possible privilege of NAb-secreting plasmacytes, or plasmacyte precursors, for recruitment and survival would not be linked to cell lineage but to B cell receptor (BCR) idiotype. In agreement with this interpretation, a critical role for BCR idiotype in determining the cell phenotype, including CD5 expression, and its localization in lymphoid compartments was demonstrated in transgenic mice expressing defined BCR specificities (29, 30). Thus, even if long lived plasmacytes or a particular lymphoid lineage/population may contribute to repertoire stability during normal ontogeny, the data we obtained with chimeric mice strongly suggest that expression and control of the NAb repertoire stem on variable region selection mechanisms that are mostly independent of developmental genetic programs operating in B lymphocyte ontogeny and maturation.

**References**


Our results characterize NAb regeneration as a robust biological phenomenon, a general and mostly quantitative reconstitution of highly diverse molecular structures in serum and body fluids. The homeostatic mechanisms underlying this reconstitution are poorly understood and should be the subject of further investigations. The possibility that mature B cells of donor origin present in the bone marrow graft could extensively contribute to the reconstitution of the NAb repertoire in chimeras seems to us unlikely. Mature bone marrow B cells are typically B2 CD5+ lymphocytes and are considered to be recirculating follicular B cells of splenic origin. Most splenic B cells, 95%, are eliminated 1 wk after transfer into irradiated hosts, so that at most 15,000 B cells would be present in chimeric mice 7 days after grafting, and would probably continue to decay because they have little proliferative capacity (28, 31). We favor the notion of positive clonal selection, by self ligands, of newly formed B cell clones as the basic mechanism for NAb repertoire formation. Evidences for positive selection of B lymphocytes by self ligands have been presented in other experimental systems (6, 32, 33).

Quantitative aspects of lymphocyte population dynamics can contribute for repertoire stability and regeneration. In young adult mouse, bone marrow precursors produce nearly $20 \times 10^9$ new B cells daily, which then interact with variable affinity/avidity with self ligands, leading to deletion, survival, and/or Ig secretion (34–36). During reconstitution of chimeric animals or normal ontogeny, because of the random nature of variable region generation, uneven clonal representations in different animals may occur, resulting in heterogeneous repertoires, a fact not supported by the data we obtained. However, heterogeneity among different individuals could be overcome by a high rate input of new clones, providing ubiquitous distribution and clonal completeness for the organism, thus resulting in homogeneous repertoires in different individuals. Indeed, the daily production of $20 \times 10^9$ B lymphocytes constitute a highly diverse collection of lymphocytes providing V region complementarity to most Ags, if one considers that a typical Ag have a frequency of reactivity of $1/10^4$ among naive B cells. Breaking of homogeneity in repertoire formation could eventually be tested using the experimental model of lymphopelic reconstitution of recombination-activating gene-deficient animals (27). If the above argument holds, one would expect higher heterogeneity of repertoire among lymphopelic animals because of statistical fluctuations, which should decrease/increase according to bone marrow B cell output. It may be possible to identify the minimum rate of B cell production necessary to guarantee homogeneous regeneration of NAb repertoire. This information could be of interest for clinical applications (37). Progressive normalization of NAb IgM repertoire was observed in patients following myeloablative therapy (38). The results presented here shows that even after total ablation regeneration of NAb repertoire can occur, provided the inoculum of hematopoietic precursors is sufficient. Although defined specificities have been shown to disappear in chimeric animals (39), all chimeras studied shared the major fraction of repertoire specificities with normal animals, characterizing a common, universal equilibrium. Given the huge diversity of clones and Ags present in the organism, from the point of view of a dynamic system, this equilibrium is not a trivial result and needs appropriate explanation from theoretical models.

NAb often exhibit autoreactivity (1–5). A bias for self-reactivity of T lymphocytes has been explained by the nature of TCR-MHC+ peptide interaction and the need of constant signaling through TCR for survival in the periphery (40–42). Evidence for an analogous requirement for B cell selection and survival has been produced (43), and the identification of self Ags involved in this process is of great interest. Although in transgenic models self-reactive B lymphocytes are often eliminated by clonal deletion during B cell maturation, others have produced evidence of their specific selection for survival and stable inclusion in the peripheral lymphoid compartments (5, 6, 31, 32, 44). A critical role for the autoreactivity of NAb has not yet been unraveled, but they have the potential to interfere in many aspects of immune system physiology. One interesting possibility consists of specifically targeting self Ags to be efficiently presented by APCs; considering that the dominant phenotype of splenic activated T cells in normal mice is regulatory (45), a physiological role for APCs could be to recruit regulatory T cells for those main Ags ubiquitously recognized by NAb. This would be a highly useful property to restrict autoimmunity while preserving the defense functions of NAb. In contrast, NAb may have a strong impact in the selection of regulatory T cell repertoire, a point that can now be studied with genetically engineered B cell-deficient mice.

The autoreactivity of natural Abs is not indiscriminate, but why some self Ags elicit natural autoantibodies and others do not is a key question currently unanswered (1, 44). Our present knowledge on B cell repertoire selection by self Ags does not allow formulation of a criterion to predict which Ags would trigger lymphocytes to deletion, survival, activation, or plasmacyte differentiation and Ig secretion. Differences in tissue expression and body fluid concentrations, intracellular localization, and Ag presentation are factors that should certainly have an impact and have not been appropriately studied, but even if most self Ags could induce autoantibodies, the overall homeostatic control of total lymphocyte numbers would impose limits. Here, as previously described (3, 14), many self Ags abundantly present in the blot as verified by colloidal gold staining of the nitrocellulose membrane were not recognized by any of the sera tested (not shown). The main Ags that constitute what we call the common, universal reactivity profile and that are recognized by all sera tested are for the moment characterized only by their estimated molecular mass. Muscle Ags with approximate molecular masses of 200 kDa and 42 kDa are likely to be myosin and actin, and reactivity of NAb with these purified Ags has been demonstrated by ELISA (1). Much experimentation will be needed to ascertain that the Ags being recognized in vitro are the same that triggered lymphocytes in vivo, and their molecular identification would be necessary to elucidate this critical point (6, 16).

Specificities present in the NAb repertoire are the end result of selection processes operating among the lymphocyte population. In the competition paradigm of lymphocyte repertoire selection, where each clone would struggle for his own survival, repertoire dynamics would be expected to be sensitive to variation of the antigenic environment and to the input/renew/output rate of lymphocytes, resulting in many alternative equilibria between the competing clones (36). However, the similarity of NAb repertoire observed in significant different conditions we have studied, early normal ontogeny, normal young adults, and adult repertoire regeneration, and also germfree and Ag-free animals (3) shows little evidence for alternative equilibrium states, a result that should have important theoretical consequences. The robust, dominant equilibrium state evidenced here suggests strong regulation coordinating the expression of the NAb repertoire, raising the question of its necessity and utility. These regulatory mechanisms are powerful enough to rebuild the “molecular shapes” (46) of the natural Ab repertoire, endowing the organism with an autoregeneration property at the level of variable region idiotypic.

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References


