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# Genetically Programmed Temporal Ordered Activation of Neonatal B-Cell Clones

## 1. INTRODUCTION

Several molecular factors contribute to the restricted neonatal repertoire. Among them, the most important are position-dependent usage of a few *V* gene families, shorter length of CDR3, lack of region diversity, and low frequency of somatic mutation. The ordered emergence of B-cell clones after birth is an additional, important factor. This may be because of the fact that some B-cell clones appear or mature at different rates rather than they do in concert. Studies using inbred animal strains demonstrated that the temporal pattern of B-cell clone emergence is similar in all individuals, indicating that a heritable genetic program determines the development of clones producing antibodies with various antigen specificities.

Two approaches were used to study sequential activation of B-cell clones after birth: (a) the study of the antibody response after immunization of the fetus at various periods of gestation or of the newborn at various intervals after birth, and (b) the study of the antibody response in lethally irradiated adult mice reconstituted with syngeneic fetal or neonatal cells and immunized with foreign antigens at various intervals after reconstitution.

Hierarchical B-cell activation during fetal life and after birth was initially characterized in lambs. This study was facilitated by three factors: (a) early B-cell maturation during fetal life (mature B cells are detected at d 40 of the 150-d gestation period), (b) lack of maternal antibody influence because the placenta of a pregnant sheep does not allow IgG transfer from mother to fetus, and (c) the relative ease of surgical procedures for intrafetal immunization.

Fetal lambs immunized by d 60 with ϕX phage or at 65–70 or 80–101 d of gestation with ovalbumin and ferritin produced specific antibodies detected 6–52 d after immunization. The majority of antiphage antibodies produced by the fetus were IgM. In sharp contrast, immunization with BCE failed to induce antibody production. Antibody responses against these three antigens were observed only after immunization of 40-d-old lambs.

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The sequential activation of B-cell clones also was described in the opossum. At birth, the opossum enters the maternal pouch, newborns can be immunized directly, and antibody responses can be studied at various intervals after immunization. Antibodies to the following antigens appeared in a temporal order: bovine serum albumin-dinitrophenol (BSA-DNP) or hemocyanin-DNP conjugates in embryos age 8 to 19 d; bacteriophage F2 in embryos age 15 d: ϕX-174 and T4 phage in embryos age 29 d: RNAase in embryos age 40 to 49 d; and lysozyme in embryos age 50 d. It is interesting to note that the response to ϕX-174 bacteriophage was first seen at day 15 when the thymus had defined cortical and medullary zones, whereas the response to T2 was found in 20-d-old embryos immunized when the spleen had begun to acquire perivascular lymphoid elements.

Although a distinct hierarchy of responsiveness against various antigens was clearly demonstrated in these pioneering studies in the lamb and opossum, an analysis of antigen specificity of clones was not possible because these animal models lack adequate methodology to study responses at the clonal level.

## 2. SEQUENTIAL ACTIVATION OF B-CELL CLONES IN LETHALLY IRRADIATED MICE RECONSTITUTED WITH FETAL LIVER CELLS

The study of B-cell ontogeny in mice was precluded by the difficulty of immunizing the early fetus as was done in fetal lambs. This difficulty was overcome by grafting fetal liver cells into syngeneic lethally irradiated adult mice. Grafted cells proliferate in the irradiated host and give rise to distinct spleen colonies. Subsequent transfer of a spleen colony into lethally irradiated mice is capable of repopulating lymphatic tissue with cells derived from a single progenitor cell. These mice were immunized at various times after reconstitution with various antigens, and antibody responses were measured at various intervals after antigen injection. This experimental model allowed for the determination of the time course of occurrence of B-cell clones that produce antibodies specific for various antigens.

Such a model was used to establish the onset of immunocompetence against and sheep red blood cells in 12-d-old fetal cells.This study showed that the ability to recognize foreign antigens was established between 19 and 20 d, reaching the level of adults by 25–32 d of development. In contrast, recognition of the erythrocyte antigen became significant over a period of 26 to 33 d, corresponding to 7 to 14 d after birth. The results of this experiment were the first to indicate sequential acquisition of immune reactivity during B-cell development in fetal liver precursors.

In another set of experiments, the hierarchy of antigen responsiveness was determined in lethally irradiated mice reconstituted with cells obtained from

the livers of 18- to 19-d-old fetal or newborn mice. After reconstitution, the recipient mice were immunized at various times. In this experiment, the response against F2 and ϕX-174 phages occurred between 3 and 7 d after reconstitution. Meanwhile, the T4 phage-specific response occurred after 14 d, anti-DNP and antilysozyme responses occurred after 21 d, antifluorescein and anti-RNase responses were detected after 28 d, and antibodies specific for myoglobin occurred 6 wk after reconstitution. The ordered maturation of these responses was independent of major histocompatibility complex (MHC) and allotype because a similar pattern was observed in C3H/HeJ, AKR, and Balb/c mice. The sequential acquisition of responses against these antigens cannot be explained by the immunogenicity of the antigens because different patterns were observed for different haptens coupled to the same carrier, such as DNP-BSA and FTC-BSA. The response pattern also cannot be explained by the lack of T-cell maturation because the responses induced by phages, lysozyme, RNase, and myoglobin are all T-cell-dependent responses. The most likely explanation of these results is that there is a genetically programmed maturation of B-cell clones derived from fetal or neonatal liver precursors. This is in spite of the fact that environmental factors may affect the circulation and migration of fetal cells in irradiated hosts.

The absence of environmental influences and the genetic control of sequential acquisition of fetal cell responsiveness were demonstrated in another experiment. Fetal tissues from 14- to 19-d-old embryos were cultured in vitro for 4–5 d and then injected into KLH-primed irradiated recipients. The recipients were scarified 14–16 d later, and the frequency of hapten-reactive B cells was determined by splenic focus assays after stimulation of cultures with KLH-hapten conjugates at a concentration of $10^{-6}$ of hapten. In this experiment, it was shown that DNP-specific B cells were found in the fetal liver at d 16 of gestation, whereas fluorescein-specific B cells were found at 16–18 d of gestation. NP or phosphoryl choline-reactive B cells were detected in the fetal liver at any age. This experiment demonstrated that the maturation of B-cell precursors from fetal livers followed a genetically controlled temporal order.

## 3. FREQUENCY OF HAPTEN-SPECIFIC B CELLS IN NEONATAL MICE DETERMINED BY SPLENIC FOCUS ASSAY

The frequency of hapten-specific B cells can be enumerated accurately by combining adoptive transfer of a limited number of cells in carrier-primed irradiated mice with in vitro culture of spleen fragments with antigen or hapten-carrier protein conjugates. This technique, called the splenic focus assay, permits assessment of the reactivity pattern of monoclonal antibodies determined by isoelectric spectrum or idiotype expression as markers of V region genes encoding antibody specificity.

This method was used to determine the frequency of neonatal clonotypes for several haptens such as DNP 2,4,6-trinitrophenol (TNP), fluorescein, p-azophenylarsonate dimethylaminoapthalene-sulfonyl (Dansyl), and PR8 influenza virus.

The frequency of DNP clonotypes from 1-d-old mice ranged from 1 to 2 foci/$10^6$ spleen cells. The number was somewhat higher from 3- to 5-d-old donors (2.3/$10^6$), indicating that in the neonatal spleen the frequency of B cells producing anti-DNP antibodies remained constant during the first week after birth. The same frequency was observed for B cells producing anti-TNP antibodies, whereas the frequency of fluorescein-specific B cells was five- to six-fold lower. Whereas the frequency of DNP- and TNP-specific precursors at birth is almost identical to the frequency in adults, the analysis of reactivity patterns of DNP and TNP antibodies by isoelectric focusing showed that during the first 4 d of neonatal life, three clonotypes were dominant with three distinct pH isoforms (pIs) (5.05, 5.25, and 5.55 for DNP-specific antibodies and 5.00, 5.15, and 5.40 for TNP-specific antibodies). The frequency of these clonotypes decreased by half by d 6 and represented a small minority by d 9, having been replaced with new clonotypes. Dansyl-specific precursors were found at a high frequency at birth, reaching the level of adults during the first week of life, whereas the frequency of *p*-azophenylarsonate-specific prescursors appeared to decrease during the first week of life.

In adult mice, the frequency of influenza virus hemagglutinin (HA)-reactive B cells was substantially lower than the frequency of hapten-specific B cells. Analysis of the reactivity pattern of clonotypes in adult mice showed that it contains a minimum 100–200 unique specificities. Analysis of the HA-specific B-cell repertoire of 12- to 14-d-old mice showed that the clonotype reactivity pattern was considerably less diverse and that some neonatal clonotypes were rapidly replaced by others during postnatal life. These results argue again for a temporally ordered diversification during postnatal life. From these observations, several conclusions can be drawn:

a. The precursors for hapten-specific B cells are present soon after birth in peripheral lymphoid organs.
b. The clonotype pattern constantly and rapidly changes during postnatal life.
c. A genetic mechanism may account for early expression of some clonotypes and late expression of others.
d. The scoring of hapten-specific precursor frequency can be determined more accurately in focus spleen assay, which maximizes T-cell help even in the absence of fully mature antigen-presenting and T helper cells.

## 4. TEMPORALLY ORDERED ACTIVATION OF B-CELL CLONES AFTER IMMUNIZATION OF NEWBORN MICE WITH VARIOUS ANTIGENS

The T-cell dependence of the immune responses elicited by polysaccharide antigens represent the best model to investigate sequential activation of B-cell clones for several reasons. First, the majority of polysaccharide antigens are T-cell independent and, therefore, B cells expressing a BCR specific for these antigens are stimulated after interaction with the BCR. Second, in contrast to responses induced by T-cell-dependent antigens (hapten-protein conjugates or proteins) the responses elicited by polysaccharides are generally oligo- or pauciclonal. Third, as illustrated in Table 19, antipolysaccharide antibodies are encoded by a limited number of *V* genes and may exhibit a particular $V_{H-}$-$V_L$ gene pairing. Fourth, *V* genes that encode antipolysaccharide antibodies expressed cross-reactive idiotypes (IdX) that are phenotypic markers of V germ line genes.

### *4.1. Antibody Responses Induced in 1-d-Old Mice*

One-day-old Balb/c mice immunized with gum ghatti, grass or bacterial levan, or lipopolysaccharide (LPS) can develop antibodies specific for β2-6-D-galactan, β 2-6 fructosan, and α methyl-D-galactoside, respectively. Antigalactan antibodies produced by Balb/c mice in response to immunization with gum ghatti share the X24 IdX with the XRPC24 galactan-binding myeloma protein. The immunization of 1-, 7-, 14-, or 21-d-old mice showed development of a significant immune response as assessed by scoring the number of plaque-forming cells (PFCs). PFCs are reduced by approx 30% on addition of rabbit anti-X24 IdX antibodies to the assay. The magnitude of PFC response did not vary substantially in 1- to 21-d-old mice.

The antilevan antibody response is induced after immunization with polyfructosans such as grass and bacterial levan. Grass levan consists of a linear backbone of β 2-6 fructosan, whereas bacterial levan consists of a backbone of β 2-6 fructosan with β 2-1 branch points. The immune response against β 2-6 fructosan is T-independent, because it can be induced in nude mice. Anti-β 2-6 fructosan antibodies do not share IdX of ABPC48 and UPC10 myeloma proteins that bind to levan. This indicates that the B cells able to produce anti-β 2-6 fructosan antibodies bearing ABPC48 or UPC10 IdX are silent in Balb/c mice. Anti-β 2-6 fructosan antibodies can be elicited following immunization of 1-d-old Balb/c mice with bacterial levan, as assessed by both plague-form-

**Table 20**
**Anti-Polysaacharide Responses Elicited by the Immunization of 1-d-Old mice**

Anti-galactan response

| Age of mice | anti-galactan PFC/$10^6$ | % expressing X24IdX |
|---|---|---|
| 1 d-old | 130+/–24 | 44 |
| 7 d-old | 150+/–12 | 42 |
| 14 d-old | 148+/–12 | 31 |
| 21 d-old | 115+/–42 | 51 |

PFC response was measured 5 d after the immunization with 50 μg gum ghatti

Anti-α-methyl-D-galactoside response

| Age of mice | immunization with | HA titer* of anti LPS Ab | HI titer:* of 348Id |
|---|---|---|---|
| 1-d-old | saline | 1.2+/–0.3 | 0 |
| 1-d-old | 10 μg S.tranaroa LPS | 4.1+/–1.8 | 2.0+/–0 |
| 32-d-old | saline | 2.8+/–0.5 | 1.2+/–0.3 |
| 32-d-old | 10 μg S.tranaroa LPS | 8.7+/–1.2 | 4.4+/–0.3 |

*expressed as $\log_2$ units

Antibody response was tested 5 d after immunization.

Anti-β 2-6 fructoasan antibody response

| Age of mice | immunization with | HA titer* | PFC/$10^6$ |
|---|---|---|---|
| 1-d-old | saline | 0.3+/–0.3 | 5+/–2 |
| 1-d-old | 10 μg levan | 1.8+/–0.4 | 46+/–7 |
| 9-d-old | saline | 2.0+/–0.3 | 14+/–7 |
| 9-d-old | 10 μg levan | 5.1+/–0.8 | 89+/–13 |
| 56-d-old | saline | 3.8+/–0.4 | 21+/–5 |
| 56-d-old | 10 μg levan | 9.5+/–0.9 | 156+/–25 |

Adapted from ref. *596*.

ing cell (PFC) and hemmaglutination assays. The magnitude of the response gradually increases during postnatal life reaching the adult level by 8 weeks.

LPS from Salmonella tranaroa, Salmonella tel-aviv and Proteus mirabilis has D-methyl D-galactoside as the immunodominant sugar. The myeloma protein MOPC384 binds to this dominant sugar, providing a tool to investigate the expression of its idiotype (i.e., 384 IdX) on the response induced by LPS. We showed that the immunization of 1-d-old Balb/c mice induced an anti-methyl-galactoside response expressing 348 IdX. The magnitude of the response induced in 1-d-old mice was about half of that observed in 8-wk-old mice.

The experimental results illustrating the observations described earlier are depicted in Table 20. These results clearly demonstrate that the precursors of

B-cell clones that produce antibodies specific for some polysaccharides are present early after birth.

### *4.2. Antibody Responses Induced During the First Week After Birth*

The sequential activation of neonatal B cells implies that antibody responses specific for other antigens may be induced by the immunization of young mice rather than that of newborns.

Several reports showed that the B cells stimulated by phosphoryl choline (PC), phenyl arsonate, and DNP or TNP conjugates can be induced only in 1-wk-old mice.

Balb/c mice immunized with microbes containing PC in their cell wall, such as Streptococcus pneumonia or Proteus morganii, develop T-independent anti-PC responses, whereas those immunized with PC that is conjugated with carrier proteins develop T-dependent responses. In both cases, the majority of anti-PC antibodies express a germ line gene-encoded idioype called T15, which is shared with PC-binding myeloma proteins such as TEPC15 or S107.

Using splenic focus assay, analysis of the frequency of T15 IdX-dominant PC-specific precursors showed that they appear quite late in neonatal development. Although no PC-specific foci were detected in the spleens of 1- to 5-d-old mice, a significant number were detected in 6- to 7-d-old mice. Sixty-six percent of monoclonal antibodies produced by PC-specific foci expressed T15 IdX, and the percentage of B cells producing T15 $IdX^+$ antibodies increased further by d 9 after birth. Whereas the T15 IdX-dominant precursors occur late after birth, an anti-PC antibody response lacking T15 IdX can be elicited in 1-d-old mice immunized with PC-LPS conjugate. These results showed that during the first week of postnatal life, the mice acquire a sequential responsiveness to different PC antigens. Only TI1 antigens such as PC-LPS induced the earliest response. This response consists of the activation of B cells producing T15 $IdX^-$ anti-PC antibodies, whereas the dominant T15 $IdX^+$-producing B cells are activated later, by the end of first week after birth. It is noteworthy that the *xid* gene plays an important role in the activation of B cells that produce anti-PC antibodies. For instance, CBA/N females exhibiting a defect in B-lymphocyte maturation are unable to respond to PC that is conjugated with various carriers.

The immunization of mice with phenyl arsonate conjugate induces antiphenyl arsonate antibodies of which 30–50% expressed an IdX. The expression of IdX is allotype linked. Transfer experiments to study the neonatal development of B cells that produce antiphenyl arsonate antibodies showed that B cells that produce dominant IdX antiphenyl arsonate antibodies are present by d 9 after birth.

**Table 21**
**Ontogeny of Anti-TNP Antibody Response and the Expression of 460Id**

| Age of mice | Immunization | PFC/$10^6$ | % of 460 Id |
|---|---|---|---|
| 1-d-old | Saline | 2±2 | 0 |
| 1-d-old | TNP-LPS | 112±21 | 0 |
| 1-d-old | TNP- Ficoll | 7±4 | 0 |
| 3-d-old | Saline | 4±3 | 0 |
| 3-d-old | TNP-LPS | 179±11 | 0 |
| 3-d-old | TNP-Ficoll | 16±7 | 0 |
| 7-d old | Saline | 24±6 | 0 |
| 7-d-old | TNP-LPS | 211±44 | 16±3 |
| 7-d-old | TNP-Ficoll | 18±5 | 0 |
| 14-d-old | Saline | 26±9 | 0 |
| 14-d-old | TNP-LPS | 280±31 | 20±7 |
| 14-d-old | TNP-Ficoll | 345±26 | 8±2 |
| 28-d-old | Saline | 82±34 | 0 |
| 28-d-old | TNP-LPS | 506±124 | 35±6 |
| 28-d-old | TNP-Ficoll | 613±16 | 6±2 |

Adapted from ref. *601*.
Abbreviations: TNP, trinitrophenyl; LPS, lipopolysaccharide, PFC, plague-forming cells.

In contrast to the responses elicited by PC, which are pauciclonal, the responses induced by DNP and TNP conjugates are polyclonal and are encoded by V genes belonging to various $V_H$ and $V_L$ gene families. Precursors of B cells producing anti-DNP or anti-TNP antibodies were identified in fetal and neonatal livers in adoptive transfer experiments in irradiated mice or by spleen focus assay. The results of these experiments suggested that newborn mice must be able to mount a response against TNP or DNP haptens. A fraction of anti-TNP antibody-producing cells share 460IdX expressed on MOPC460 and MOPC315 DNP and TNP-binding myeloma proteins. Whereas Ig molecules expressing 460IdX are not detected in naïve mice, anti-TNP antibodies bearing 460IdX were noted in mice immunized with T-dependent or -independent antigens. Based on this information, we have studied the anti-TNP response and the expression of 460IdX in mice immunized with TI1 (TNP-LPS and TNP-B.abortus conjugates) and TI2 (e.g., TNP-Ficoll) antigen. Immunization of 1-d-old mice with TNP-LPS or TNP-B.abortus conjugates elicited a substantial anti-TNP response, but B cells producing $460IdX^+$ anti-TNP antibodies were detected only in 7-d-old Balb/c mice. These results clearly demonstrate the sequential activation of B-cell clones producing anti-TNP antibodies, because the clones producing antibodies and expressing 460IdX occurred 1 wk after the clones producing anti-TNP antibodies lacking 460IdX. The experimental results supporting this conclusion are presented in Table 21.

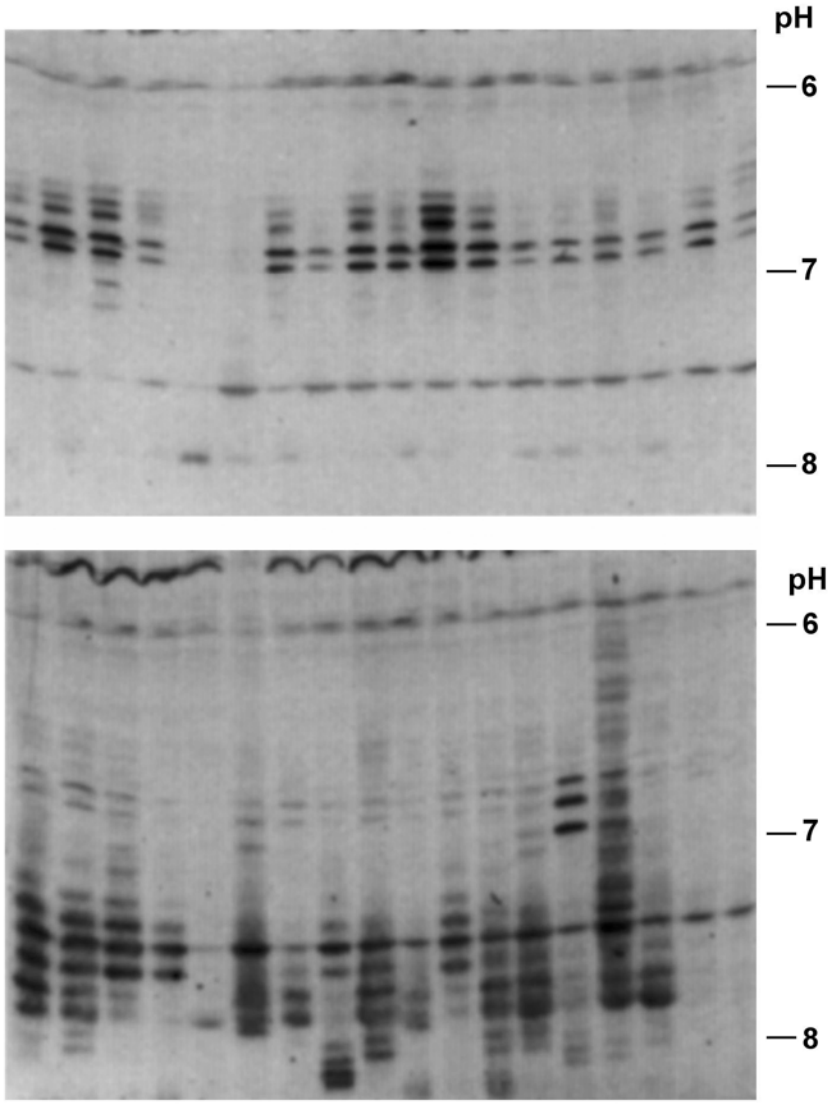


Fig. 30. Isoelectrofocusing (IEF) pattern of Balb/c antibacterial levan antibodies. Sera obtained from 18 individual Balb/c mice 10 d after immunization with bacterial levan. The upper panel shows anti-β2, 1)-fructosan antibodies reactive with $^{125}$I-inulin-BSA and the lower panel shows anti-β(2,6)-antibodies reactive with $^{125}$I-bacterial levan. (From Stein K, et al. J Exp Med 1980;151:1088–1102.)

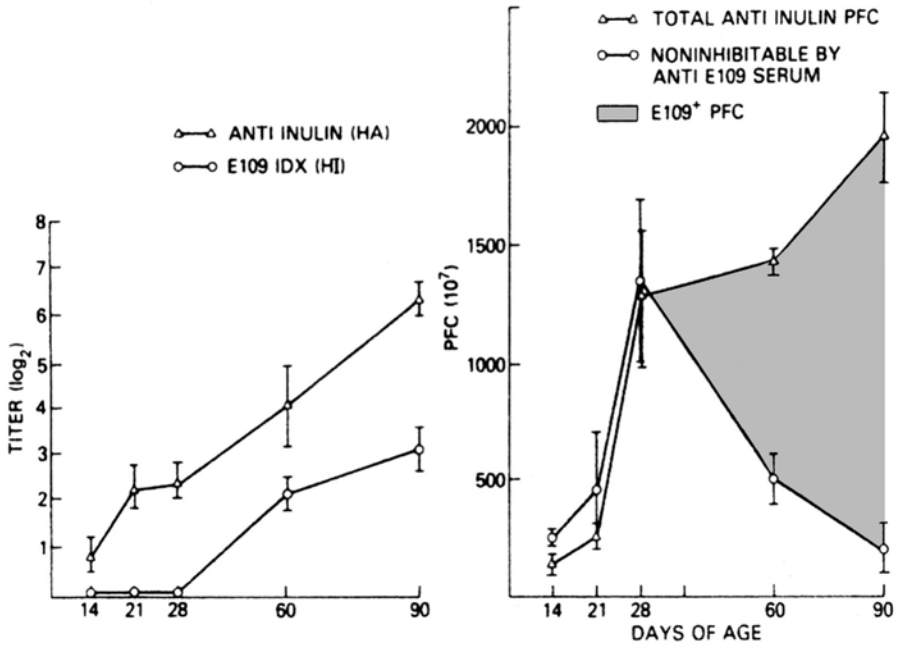


Fig. 31. Age dependence of anti-inulin response in Balb/c mice. Left panel, serum antibody response 5 d after immunization of BALB/c mice of various ages with bacterial levan. Right panel, anti-inulin PFC response and expression of E109 IdX 5 d after immunization of Balb/c of various ages with bacterial levan. (From Bona C, et al. J Immunol 1979;123:1484–1490.)

### *4.3. Antibody Responses Induced Late in Postnatal Life*

Study of the ontogenic development of anti-β 2-1 fructosan and anti-α 1-6 dextran antibody responses showed that they could be induced late in postnatal life.The β 2-1-linked fructose linear polymer is found in inulin, but β 2-1 fructosan also represents the branch points of the β 2-6 fructosan backbone of bacterial levans. Although levans can induce a T-independent antibody response, inulin by itself is not immunogenic without being coupled with a protein carrier.

The anti-β 2-1 fructosan antibody response induced by inulin-BSA conjugate is very restricted compared to that induced by bacterial levan. This was demonstrated by analysis of the isoelectrofocusing (IEF) pattern. In Balb/c mice, anti-inulin antibodies display a characteristic IEF pattern that is essentially identical in all individuals, consisting of a single spectrotype comprised of five bands, of which three predominate and focus in the pH 6.3 to 6.8 range. Figure 30 illustrates the IEF pattern of anti-inulin and antibacterial levan antibodies in adult Balb/c mice. Anti-inulin antibodies share three distinct IdXs (IdX G, IdX A, and

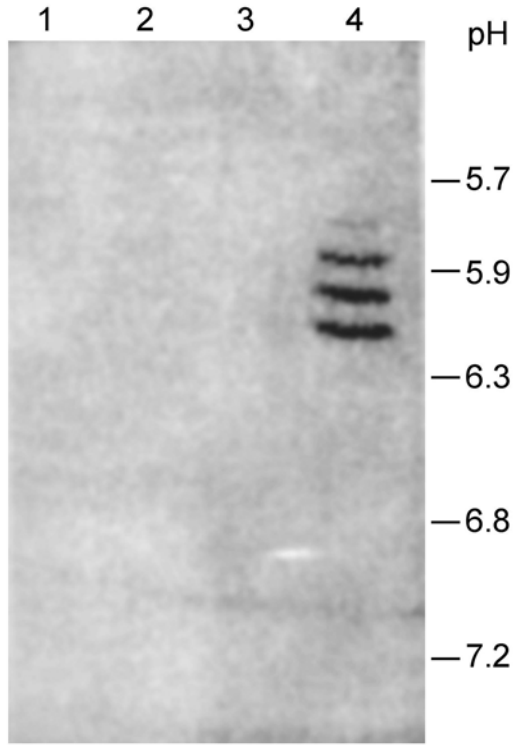


Fig. 32. IEF pattern of anti-inulin antibodies of various ages. Serum from Balb/c mice of various ages was electrophoresed and the blots were incubated with $^{125}$I-inulin-BSA. Lane 1 serum from mice immunized at 7-d of age; lane 2 serum from mice immunized at 14 d of age; lane 3 serum from animals immunized at 21 of age; lane 4 serum from mice immunized at 12 wk of age. (From Bona C et al. J Immunol 1979;123:1484–1490.)

IdX B) with 11 β 2-1 fructosan-binding myeloma proteins. Study of the ontogeny of anti-β 2-1 fructosan antibodies by hemagglutination, PFC, and IEF assays indicated that they occur long after birth. A weak PFC response was first observed in 21-d-old mice, followed by a substantial increase at 28 d (Figs. 31 and 32). As can be seen in Fig. 31, the dominant anti-inulin clones expressing IdX D-E109 were detected only in mice older than 4 wk. Several experimental findings support the concept that the late occurrence of anti-β 2-1 fructosan is related to a genuine ontogenic delay. First, it was found that polyclonal activation by a B-cell mitogen, NWSM, which is known to induce the proliferation of B cells in their early stages, induced an increased in 3H-thymidine incorporation in spleen cells of 1-wk-old Balb/c mice. The cells producing anti-β 2-1 fructosan antibodies stimulated by NWSM were not detected until the donors were age 4

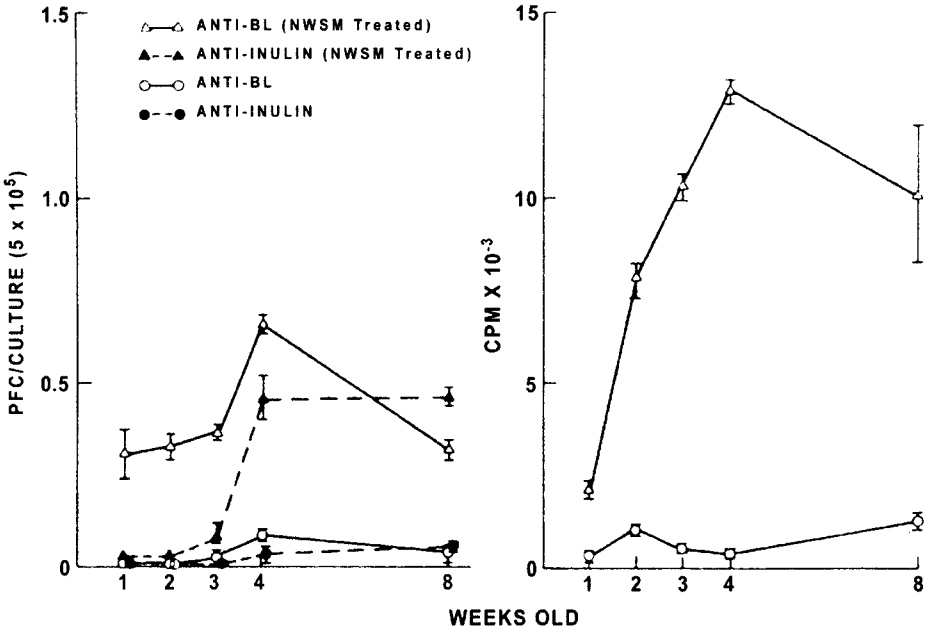


Fig. 33. Age dependence of PFC response and proliferation induced by NWSM. Left panel, anti-inulin and antilevan PFC response after 3 d of stimulation of splenic cells from Balb/c mice of various ages. Right panel, 3H-thymidine incorporation of spleen cells from Balb/c mice stimulated for 3 d with NWSM. (From Bona C, et al. J Immunol 1979;123:1484–1490.)

wk (Fig. 33). Second, we have shown that the ontogenic delay was not caused by the absence of environmental antigens because bacterial levan is produced by various bacterial species present in normal flora. In adoptive transfer experiments, it was shown that the infusion of B cells from 1-wk-old Balb/c mice into irradiated CAL.20 adult mice developed a response 7–14 d after the transfer, thereby corresponding to the time required for the maturation of the response in Balb/c mice (600). Third, the ontogenic delay of the antibody response correlates with a very low frequency ($1–2/10^6$ B cells) in the spleens of 3-wk-old mice. Taken together, these observations clearly demonstrated a significant ontogenic delay of the anti-inulin antibody response.

A longer delay was observed in the case of the anti-α-(1,6)-dextran antibody response. In Balb/c mice, the response induced by B512 dextran is characterized by a predominant idiotype-QUPC52. Howard and Hale reported that α-(1,6)-dextran-specific antibodies were observed particularly late. For example, at age 55 d, they represented only 10% of the amount of antibodies produced by 3-mo-

old mice. This observation is supported by other observations in which the response for α-(1,6)-dextran was studied following immunization with B512 dextran and a six-sugar hapten, isomaltohexose, in which structure is homologous with the antigenic determinant of α-(1,6)-dextran. Immunization of mice at various ages from 1 d to 12 wk after birth showed that antidextran antibodies after immunization with B512 dextran or isomamaltohexose-KLH conjugate were not detected until the mice were 12 wk old.

The data reviewed in this chapter clearly demonstrate that there is a temporally ordered and sequential activation of the antigen-specific B-cell precursors during fetal development and postnatal life that appears to be antigen-independent. Several explanations can be put forth to support this concept.

First, the position-dependent rearrangement of the most proximal $V_H$ and $V\lambda$ gene families during B-cell development restricts the fetal and neonatal repertoire. Although this possibility should be considered for lack of antibody responses during the first week after birth, it cannot explain entirely the late responses because the preferential expression of proximal $V_H$ gene families after birth switch and normalize by d 7–14. In addition, this cannot explain the usage of $V_K$ families, which does not follow the paradigm of position-dependent expression of $V_H$ families, because in newborn mice the $V_K$ families located in the center of the $V_K$ locus are highly expressed.

Second, the delayed ontogenic response can not be the result of induction of tolerance because the precursors of B cells activated late after birth were detected in fetal livers and in neonatal spleens by very sensitive methods such as splenic focus assay. In addition, adoptive transfer experiments of cells from newborns into irradiated adult mice showed, in the case of inulin-specific B cells, that they can respond to antigen stimulus only several weeks after transfer, a time corresponding to natural maturation in normal mice.

Third, the long ontogenic delay may be related to tolerization of immature B cells by environmental antigens. This explanation is not likely, because polysaccharide antigens that were present early in life did not induce tolerance except when they were administered in very high doses. Furthermore, this cannot explain the delayed response to haptens or to inulin, which are not naturally present in environment.

Fourth, the delayed response cannot be related to maternal antibodies, because the vast majority of antibodies specific for polysaccharides are IgM, which does not cross the placenta.

Finally, the most plausible explanation is that an "internal clock" genetically programs the sequential acquisition of the neonatal repertoire by mechanisms that are not yet understood.

It is of importance that these mechanisms be elucidated in the future, because it would help to produce better vaccines for newborns and young children.