IDIOTYPE-ANTI-IDIOTYPE NETWORK

II. Activation of Silent Clones by Treatment at Birth with Idiotypes Is Associated with the Expansion of Idiotype-specific Helper T Cells*

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The immune system has been envisaged as a web of variable region domains in which the connection between clones is based on fragile idiotype links (1). The delicate balance that is established between clones as well as the various subsets of cells that regulate their expression can be easily upset by the influence of antigen, idiotypes, and anti-idiotypes (2).

We have studied the consequences of perturbing the immune network formed by the clones that comprise the bacterial levan (BL)1 idiotype (Id) system. This system displays certain characteristics that render it particularly suitable for studies of regulation of the immune response: first, immunization with BL, which is a β2→6 polyfructosan with β2→1 branch points, leads to a vigorous T cell-independent antibody response in adult mice. This response in IghC+ mice is composed of two distinct families of antibodies: the first binds to β2→6 fructosan, whereas the second binds to β2→6 as well as β2→1 epitopes. This latter group expresses the EPC109 (E109) dominant cross-reactive idiotype (IdX), E109IdX, shared with inulin-binding myeloma proteins (3). Second, there is a substantial ontogenic delay in the activation of genes that encode for E109IdX β2→6 and β2→1 fructosan response. However, the β2→6 fructosan-reactive clones can be expanded at birth and their expression can be studied independently of the anti-β2→1 fructosan-reactive clones in 1-mo-old mice (4). Third, antibodies bearing the idiotopes expressed on APBC48 (A48) and UPC10 (U10), two independent β2→6 fructosan-binding myeloma proteins, cannot be identified in the sera of BL-immune mice, suggesting that A48Id or U10Id are markers of silent clones (3).

In previous studies we have shown that A48Id silent clones can be activated by an anti-idiotype induced perturbation of the steady state network in three experimental conditions as follows: (a) an alteration in the balance of E109IdX dominant clones vs.

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1 Abbreviations used in this paper: A48, ABPC48; BL, bacterial levan; BSA, bovine serum albumin; DNP, dinitrophenyl; E109, EPC109; ELISA, enzyme-linked immunosorbent assay; HA, hemagglutination assay; HI, hemagglutination-inhibition assay; Id, idiotype; IdX, cross-reactive idiotype; In, inulin; In-BA, In-Brucelea abortus; KLH, keyhole limpet hemocyanin; M384, MOPC384; M460, MOPC460; NP, 4-hydroxy-3-nitrophenyl)acetyl; PFC, plaque-forming cell; RIA, radioimmunoassay; SRBC, sheep erythrocytes; TNP, trinitrophenyl; U10, UPC10.
A48Id⁺ clones was achieved in nude mice after the administration of anti-E109IdX antibodies. This treatment led to the appearance of A48Id⁺ anti-β2→6 fructosan antibodies (5); (b) mice were injected at birth with minute amounts of anti-A48Id antibodies. About 40% of the anti-BL antibodies produced in these animals bore the A48Id (6); (c) silent clones were activated in adult mice hyperimmunized with anti-A48Id antibodies in Freund’s complete adjuvant. These animals, which produced anti-(anti-A48Id) antibodies, developed substantial A48Id⁺ anti-BL antibodies in response to immunization with BL (7).

In this paper, we present results that demonstrate the activation of A48Id silent clones by the administration at birth of minute amounts of immunoglobulin bearing the A48 idiotype. This activation is associated with the expansion of A48Id-specific helper T cells, which presumably function in the fine tuning of the expression of the bacterial levan idiotype repertoire.

Materials and Methods

Animals. Normal and nu/nu BALB/c mice, 8–12 wk old, were purchased from the Charles River Breeding Laboratory, Wilmington, MA. 1-d-old BALB/c mice were obtained from breeding in our colony at the Mount Sinai School of Medicine, New York.

Antigens. BL from *Aerobacter laevenicum* was prepared according to a previously described technique (3). Trinitrophenyl (TNP)-aminoethylcarbamylmethyl-Ficoll (TNP-FicolI) was prepared as described previously (8). Inulin (In) obtained from Calbiochem-Behring Corp., American Hoechst Corp., La Jolla, CA, was coupled to *Brucella abortus* (In-BA) according to a described method (8). TNP₁₈-BSA, TNP₁₄-A48, and TNP₁₄-M384 were labeled according to the method described by Bikoff (9).

Myeloma Proteins and Monoclonal Antibodies. The properties of all myeloma proteins and monoclonal antibodies used in this study are presented in Table I.

Preparation of Anti-Idiotype Antibody. Syngeneic BALB/c anti-A48Id and anti-MOPC460Id (M460Id) antibodies were prepared by immunization with monoclonal protein-keyhole limpet hemocyanin (KLH) conjugates. Antibodies were purified on Sepharose 4B Id columns. Coupling of antibodies to KLH and immunization schedules are as previously described (10).

Labeling of Sheep Erythrocytes (SRBC) with BL, In, and TNP. α-stearoyl derivatives of BL and In were prepared according to the method of Hämmerling and Westphal (11) and were used to coat SRBC as previously described (3). TNP-SRBC were prepared by the method of Rittenberg and Pratt (12). SRBC were coated with myeloma proteins by the chromic chloride method of using a concentration of 1 mg/ml of the appropriate protein (3).

Labeling of Antibodies. A48 monoclonal protein was labeled with 1²⁵I by the chloramine T method (13). M460 and A48 myeloma protein was tritiated according to the method of Wilder

| Designation | Antigen specificity | Bearing idiotype | Class |
|-------------|---------------------|------------------|-------|
| ABPC48⁺     | β2→6               | A48              | IgAk  |
| W3082⁺      | β2→1               | IdX A, B         | IgAk  |
| UPC10⁺      | β2→6               | U10              | IgG2a  |
| 76BL(Aba)36 | β2→6               | A48              | IgM   |
| 76BL(Aba)42 | β2→6               | A48              | IgM   |
| MPOC460⁺    | DNP                | M460             | IgAk  |
| MOPC384⁺    | *Salmonella typhosa* lipopolysaccharide | M384 | IgAk  |
| CD5.3       | M460Id             | Undefined        | IgG2a  |
| LPC1        | Unknown            | Unknown          | IgG2a  |

*These myeloma proteins were a kind gift of Dr. Michael Potter, National Cancer Institute, National Institutes of Health, Bethesda, MD.
et al. (14), and affinity-purified anti-A48Id was labeled with alkaline phosphatase by a method previously described (15). 125I-goat anti-mouse was obtained from New England Nuclear (Boston, MA).

**Determination of Antibody Titers.** Hemagglutination assay (HA) titers of antibody specific for $\beta_2\rightarrow6$, $\beta_2\rightarrow1$, and TNP were determined in microtiter plates using SRBC coated with o-stearoyl BL, o-stearoyl In, and TNP, respectively. The titer recorded is log2 of the highest dilution of antisera giving agglutination.

**Radioimmunoassay (RIA).** The anti-$\beta_2\rightarrow6$, anti-$\beta_2\rightarrow1$, and anti-TNP titers were determined by an RIA on microtiter plates coated with BL (50 $\mu$g/ml), In-bovine serum albumin (BSA) (30 $\mu$g/ml) or TNP-BSA (30 $\mu$g/ml), respectively, by incubating on the plates for 18 h at 4°C. This was followed by three washings and then the plates were incubated with 50% fetal calf serum for 1 h at 4°C. Radioactivity was measured in a gamma counter after extensive washing. The concentration of anti-$\beta_2\rightarrow6$, $\beta_2\rightarrow1$ fructosan, and anti-TNP antibodies in the sera of immunized animals was determined from the linear part of a standard inhibition curve obtained with known amounts of unlabeled UPC10 (IgG2a), W3082 (IgA), and IgM anti-dinitrophenyl (DNP) monoclonal antibody (Fig. 1). The amount of $\beta_2\rightarrow1$ fructosan antibodies obtained from the standard curve can be over-estimated because of the low binding affinity of W3082 myeloma protein, which is an IgA.

**Determination of Idiotype.** A hemagglutination-inhibition assay (HI), described previously (2), was used to test sera for the presence of antibodies bearing either A48Id or M460Id. The titer recorded is given as log2 of the highest dilution of sera giving inhibition. The RIA serum level of A48Id was determined by using microtiter plates coated with affinity-purified anti-A48Id antibodies (50 $\mu$g/ml) and 125I-A48 as ligand. The precise concentration of antibody expressing A48Id was determined from a standard inhibition curve obtained with A48 monoclonal protein. The serum level of antibodies bearing the M460Id was determined by using microtiter plates coated with CD5.3 monoclonal antibody (3 $\mu$g/ml) and [3H]M460 as ligand. The precise concentration of M460Id-bearing molecules was determined from a standard inhibition curve obtained with unlabeled M460. This method was described in detail elsewhere (7, 10).

**Determination of Shared A48 Idiotypes by Enzyme-linked Immunosorbent Assay (ELISA).** Microtiter plates (Dynatech Cap., Alexandria, VA) were coated for 18 h at 4°C with A48, U10, 76BL(AB3)36, or 76BL(AB3)42 (50 $\mu$g/ml) followed by three washings with phosphate-buffered saline. The plates were then incubated for 3 h at 4°C with alkaline phosphatase-labeled, affinity-purified syngeneic anti-A48Id antibodies (5 $\mu$g/50 $\mu$l) to establish the extent of binding of the anti-A48Id antibodies to individual monoclonal antibodies. To confirm the sharing of idiotypes, the alkaline phosphatase-labeled anti-A48Id antibodies were preabsorbed for 30 min at 23°C with equal amounts of purified A48, U10, 76BL(AB3)36, or 76BL(AB3)42 monoclonal antibodies. After extensive washing, the plates were incubated with substrate for 1 h at 37°C, and the reaction was measured at 405 nm on a microelisa spectrophotometer.

**Detection of Plaque-forming Cells (PFC).** The number of cells secreting antibody specific for
BL or TNP was determined by a modification of the Jerne plaquing technique as previously described (10). Briefly, 50 μl of a suspension of immune spleen cells was added to 0.4 ml of 0.5% agarose that contained 0.1 ml of sensitized erythrocytes (10%). The slides were incubated initially for 2 h at 37°C and then they were reincubated for an additional hour in the presence of guinea pig complement (1:20). Control slides were prepared using a mixture of the same immune spleen cells and unsensitized SRBC. Anti-BL PFC carrying the A48Id were enumerated by incorporating BALB/c anti-A48 antiseraum into the agarose at a final concentration of 1:100 and scoring the difference in the number of PFC obtained in the presence and absence of the inhibitor (anti-A48). Similarly, the number of anti-TNP PFC expressing the M460Id was determined by the addition to the agarose of BALB/c anti-M460 antiseraum (1:500) as inhibitor.

**Purification of T and B Cells.** Splenic T lymphocytes were purified by passage over nylon wool columns. Purified splenic B lymphocytes were obtained from BALB/c mice injected intraperitoneally with 0.4 ml rabbit anti-mouse thymocyte serum (M.A. Bioproducts, Walkersville, MD) that were killed 2 d later. The splenic lymphocytes were harvested and treated with anti-Thy-1.2 plus complement to remove any residual T cells.

**Results**

**Occurrence of A48Id-bearing Immunoglobulins in BALB/c Mice Treated at Birth with A48 Monoclonal Protein.** We are extending our previous observation, which showed that A48Id silent clones can be activated by injection of anti-A48 antibodies, by studying the effect of the administration of BL and A48 monoclonal protein on the expression of these clones at birth. Injection of 10 μg of A48 monoclonal protein at birth, followed 1 mo later by immunization with BL, led to the appearance of A48Id-bearing molecules in the serum of these mice, whereas injection of 10 μg of BL alone did not activate the A48Id response. The appearance of A48Id+ molecules in the serum required antigenic stimulation, because mice treated at birth only with A48 monoclonal protein—but not immunized with BL—do not show significant increases in the levels of anti-BL antibodies and A48Id-bearing molecules (Table II). These results indicated that administration of A48 monoclonal protein at birth led to the activation of clones expressing the A48Id; this activation requires antigenic stimulation.

**Occurrence of A48Id Molecules Was Not Related to Persistence of A48 Monoclonal Protein Injected at Birth.** The presence of A48Id-bearing immunoglobulins in the serum of mice treated at birth with A48 monoclonal protein can originate in principle from the persistence of protein injected at birth. To investigate this possibility, we injected mice with 13 × 10^6 cpm of [3H]A48 and studied the clearance of radioactivity from the serum for 34 d by collecting blood samples at various intervals. As can be seen in Fig. 2, the amount of radioactivity measured at 34 d would correspond to <8 × 10^-4 μg

| Treatment at birth | Immunization 1 mo later | Number of mice studied | Titer log2 units |
|--------------------|------------------------|------------------------|-----------------|
| Nil                | Nil                    | 6                      | 1.5 ± 0.8       | 0.0 ± 0.0       |
| Nil                | BL                     | 2                      | 5.5 ± 0.5       | 0.5 ± 0.5       |
| 10 μg BL           | BL                     | 3                      | 5.0 ± 0.6       | 0.0 ± 0.0       |
| 10 μg A48          | Nil                    | 5                      | 0.8 ± 0.2       | 0.4 ± 0.2       |
| 0.1 μg A48         | BL                     | 4                      | 6.0 ± 0.6       | 3.2 ± 0.2       |
| 10 μg A48          | BL                     | 7                      | 5.7 ± 0.6       | 2.7 ± 0.7       |

Anti-BL and A48Id titers were determined 5 d after immunization with 20 μg BL. The titers recorded are the mean ± SEM.
Fig. 2. Kinetics of the clearance of $[^{3}H]$A48 injected intraperitoneally at day 0.

Fig. 3. Dose-effect relationship of treatment of newborn mice with various doses of A48 monoclonal protein. Mice were 1 d old at the time of treatment and challenged 1 mo later with 20 µg BL. (○) PFC/spleen (■) PFC/spleen detected when syngeneic anti-A48Id antisera had been added to the agarose. Shaded area shows the number of PFC bearing A48Id. Each point represents the mean ± SEM of determinations performed on five mice.

of A48 monoclonal protein remaining in the circulation from the original injection of 10 µg at birth. Furthermore, we studied the PFC response of mice treated at birth with various amounts of A48 monoclonal protein and immunized 1 mo later with BL. The results depicted in Fig. 3 showed that the magnitude of the total direct anti-BL PFC response was not significantly different in the mice whether or not they were treated at birth. However, the A48 component of this response was significantly increased. The combined results regarding the complete clearance of labeled A48 protein 1 mo after injection and the possibility of detecting an A48Id$^+$ PFC response demonstrate a genuine activation of A48Id$^+$ clones. It should be mentioned that the PFC results indicate that A48Id silent clones dominate the anti-BL response in animals treated at birth with A48 monoclonal protein.

Ontogeny of the A48Id Response. The ontogeny of the A48Id response was studied in two groups of animals. The first group was injected at birth with A48 monoclonal protein and immunized either 1, 2, or 3 wk later with BL. The second group was
injected at various intervals after birth with A48 monoclonal protein and immunized 1 mo later with BL. The results of this experiment, presented in Table III, showed that 1-d-old A48-pretreated BALB/c mice immunized 2 or 3 wk later with BL developed an A48Id response. Similar results were obtained by using a PFC assay (data not shown). In contrast, the animals injected with A48 at various intervals after birth (7–28 d) and immunized 1 mo later with BL failed to develop an A48Id response despite a strong anti-β2→6 fructosan antibody response (data not shown). These results show that treatment at birth with A48 monoclonal protein is critical for the activation of A48Id component of the anti-BL response.

**Antigenic Specificity of Activation of A48Id Clones.** The specificity of the antigen-dependent activation of A48Id component of the anti-BL response was studied in animals treated at birth with A48 and immunized 1 mo later with either BL, In-BA, or TNP-Ficoll as well as in animals treated at birth with M460 and immunized 1 mo later with BL and TNP-Ficoll (Table IV). The animals immunized with BL at 1 mo of age have a significant increase in the titer of only anti-β2→6 fructosan antibodies and of A48Id component response compared with the mice treated at birth with A48Id monoclonal protein. Anti-β2→6 and anti-β2→1 fructosan antibody levels were slightly increased in mice immunized with In-BA, however, a significant increase of anti-β2→6 and β2→1, and a slight increase of A48Id component was observed in animals treated at birth with A48Id monoclonal protein. These results show that, despite the ontogenic delay of the anti-β2→1 clones that we previously described (4), the administration at birth of A48 monoclonal protein probably stimulated a set of clones that express at least some A48 idiotopes and that secrete antibody able to bind both β2→6 and β2→1 fructosan epitopes. TNP-Ficoll immunization does not affect the activation of A48Id+ clones and, conversely, the M460Id component of the anti-TNP response was not altered by A48 treatment at birth. Furthermore, treatment at birth with M460Id did not activate A48Id component of the anti-BL response. Therefore, our results suggest that the activation of A48Id silent clones is antigen, as

| Table III |
| --- |
| **Ontogeny of the A48Id Response** |

| Age of mice | Antibody response: Anti β2→6 fructosan | Idiotype response: A48Id |
| --- | --- | --- |
| A48 treatment | BL immunization | Assay of response | | |
| | d | d | | |
| Nil | 7 | 12 | ND | <0.1 | ND | ND |
| 1 d | 8 | 12 | ND | 1.1 ± 0.1 | ND | ND |
| Nil | 14 | 19 | 4.3 ± 0.3 | 1.6 ± 0.2 | 0.3 ± 0.3 | 1.7 ± 0.7 |
| 1 d | 14 | 19 | 5.7 ± 0.3 | 1.2 ± 0.8 | 1.7 ± 0.3 | 14.2 ± 3.3 |
| Nil | 21 | 26 | 4.3 ± 0.7 | 1.1 ± 0.6 | 0.0 ± 0.0 | 1.7 ± 0.1 |
| 1 d | 21 | 26 | 4.7 ± 0.3 | 1.3 ± 0.7 | 1.0 ± 0.6 | 0.4 ± 0.1 |

Immunization of mice was performed with 20 μg BL intravenously, whereas the treatment at birth was carried out with 10 μg A48 monoclonal protein intraperitoneally.

* Titer recorded is mean ± SEM of logs of the highest dilution of antisera giving agglutination.
† Titer recorded is mean ± SEM for of protein in sera (μg/ml).
§ Titer recorded is mean ± SEM of logs of the highest dilution of sera giving inhibition.
¶ Not determined.
### Table IV

Specificity of Activation of A48Id Silent Clones

| Treatment at birth | Immunization | Antibody response | Idiotype response |
|-------------------|--------------|-------------------|-------------------|
|                   |              | Anti-β2→6         | Anti-β2→1         | Anti-TNP         | A48Id | M460Id |
|                   |              | HA | RIA | HA | RIA | HA | RIA | HA | RIA |
| Nil               | BL           | 5.7 ± 0.3 | 5.0 ± 2.6 | 1.3 ± 0.3 | 47.7 ± 22.3 | 2.7 ± 0.3 | <3 | 0 | <1 |
| A48               | Nil          | 3.3 ± 0.8 | <0.1 | 1.3 ± 0.7 | <3 | 2.2 ± 0.5 | ND* | 0.3 | <1 |
| A48               | BL           | 5.3 ± 0.4 | 4.6 ± 0.2 | 0.9 ± 0.4 | 26.8 ± 16.6 | 1.0 ± 0.5 | <3 | 2.6 ± 0.4 | 82.4 ± 47.6 |
| Nil               | In-BA        | 3.3 ± 0.3 | <0.1 | 2.7 ± 0.3 | 4.0 ± 0.5 | 2.7 ± 0.3 | <3 | 0 | 1.7 ± 0.1 |
| A48               | In-BA        | 6.0 ± 0.0 | 1.5 ± 0.7 | 5.0 ± 1.0 | 83.0 ± 41.6 | 1.7 ± 0.9 | <3 | 1.3 ± 0.3 | 65.0 ± 59.5 |
| Nil               | TNP-Ficoll   | 1.3 ± 0.7 | 0.7 ± 0.3 | ND | ND | 5.7 ± 0.3 | >100 | 0 | 2.5 ± 1.6 | 2.3 ± 1.2 |
| A48               | TNP-Ficoll   | 4.3 ± 0.7 | 2.2 ± 0.1 | ND | ND | 6.7 ± 0.3 | >100 | 0 | 3.6 ± 1.4 | 3.0 ± 1.0 |
| A48               | BL + TNP-Ficoll | 7.3 ± 0.3 | 37.1 ± 21.7 | ND | ND | 7.3 ± 0.3 | >100 | 3.7 ± 0.3 | 179.3 ± 63.3 |
| M460              | BL + TNP-Ficoll | 3.2 ± 0.5 | 3.3 ± 0.7 | ND | ND | 4.0 ± 0.8 | 42.2 ± 17.5 | 0.2 ± 0.2 | <1 |

HA and HI titers expressed as mean log2 units ± SEM; RIA expressed as mean μg/ml ± SEM. Immunization of 1-mo-old mice treated or not with 10 μg A48 or M460 monoclonal proteins at birth were immunized with 20 μg BL intravenously, 0.1 ml of 1% In-BA intraperitoneally, or TNP-Ficoll (20 μg intraperitoneally), respectively. The response was tested 5 d later.

* Not determined.
as well as idiotype, specific. Interestingly, the activation of a set of clones expressing the A48Id reactive to both β2→6 and β2→1 fructosan epitopes was observed in these animals. The existence of these clones was confirmed by analysis of monoclonal antibodies obtained by fusion of SP2/0 myeloma cells with spleen cells from BALB/c mice treated at birth with A48 and immunized with BL. Among eight monoclonal antibodies, we found five that bound to β2→6 and β2→1 fructosan epitopes. (B. Goldberg and C. Bona, manuscript in preparation).

Effect of BL-binding Monoclonal Antibodies Sharing the A48Id. There are data that suggest that the A48Id of A48 myeloma protein is composed of several idiotopes and that some of them are shared with UPC10, another β2→6 fructosan-binding myeloma (16). Recently, we have obtained four monoclonal antibodies from BALB/c mice producing anti-(anti-A48Id) antibodies that were immunized with BL. These antibodies also express some of the idiotopes of the A48 family, as was assessed by cross-adsorption experiments of anti-A48Id antibodies in the ELISA assay. The results presented in Table V showed that only a few idiotopes of A48Id are shared by UPC10, 76BL(Abs)36, and 76BL(Abs)42 because: (a) the binding of alkaline phosphatase-labeled anti-A48Id antibody to U10, 76BL(Abs)36, and 76BL(Abs)42 microtiter plates is significantly lower than that of anti-A48 to A48; (b) the binding of anti-A48 antibodies to microtiter plates coated with A48 is only partially adsorbed by equal amounts of U10 and 76BL(Abs)42; (c) the binding of anti-A48 alkaline phosphatase-labeled antibody to U10 and 76BL(Abs)42 was completely adsorbed by A48 and only partially by 76BL(Abs)42. These results indicate that some of the A48 idiotopes are shared by U10, 76BL(Abs)42, and 76BL(Abs)36 monoclonal antibodies. It should be mentioned that 76BL(Abs)36 monoclonal antibodies probably share some idiotopes of U10 because the adsorption with U10 completely inhibited the binding of labeled anti-A48 to 76BL(Abs)36-coated plates, whereas the adsorption with A48 caused only 60% inhibition of this binding. Based on these results, we investigated the ability of the three monoclonal antibodies that share A48 idiotopes to activate A48Id clones. The results presented in Table VI show that treatment at birth with U10 and 76BL(Abs)42 followed by immunization with BL 1 mo later elicited an anti-BL response whose magnitude was comparable with that of normal animals. The pretreatment with U10 and 76BL(Abs)42 caused a discrete activation of A48Id clones, whereas the pretreatment with 76BL(Abs)36 did not. This difference can be related to the fact that 76BL(Abs)36 monoclonal antibody shares only one or

### Table V

| Microtiter plates coated with | Alkaline phosphatase-labeled anti-A48Id antibodies adsorbed with |
|-----------------------------|---------------------------------------------------------------|
|                             | Nil | M384 | A48 | U10 | 76BL(Abs)36 | 76BL(Abs)42 |
| A48                         | 1.85 | 1.86 | 0.05 | 1.15 | ND | 0.57 |
| UPC10                       | 0.55 | 0.53 | 0.01 | 0.12 | ND | 0.08 |
| 76BL(Abs)36                 | 0.35 | 0.36 | 0.11 | 0.01 | 0.0 | ND |
| 76BL(Abs)42                 | 0.39 | 0.38 | 0.02 | 0.05 | ND | 0.01 |

Microtiter plates were coated with 50 μg antibodies. Adsorption of labeled anti-A48Id antibodies (5 μg/ml) was performed by preincubation for 30 min at 23°C with 10 μg of affinity-purified monoclonal proteins. Optical density is at 405 nm.
TABLE VI

| Treatment at birth | Immunization at 1 mo | Antibody response | Idiotype response |
|--------------------|---------------------|-------------------|------------------|
|                    |                     | BL | TNP |                  | A48 | M460 |
| Nil                | BL                  | 5.0 ± 2.6 | <3 | <1 | 0.2 ± 0.2 |
| Nil                | TNP-Ficoll          | 0.7 ± 0.3 | >100 | 2.5 ± 1.6 | 0.3 ± 0.0 |
| A48                | BL + TNP-Ficoll     | 37.1 ± 21.7 | >100 | 173.3 ± 63.3 | 1.8 ± 0.8 |
| U10                | BL + TNP-Ficoll     | 1.1 ± 0.4 | ND* | 12.0 ± 6.4 | 1.8 ± 0.7 |
| 76-36              | BL + TNP-Ficoll     | 2.0 ± 0.8 | 84.9 ± 39.2 | <1 | 0.3 ± 0.1 |
| 76-42              | BL + TNP-Ficoll     | 3.6 ± 1.8 | 37.0 ± 17.0 | 27.7 ± 5.0 | 0.9 ± 0.6 |

1-d-old BALB/c mice were injected with 10 μg monoclonal antibodies. 1-mo-old BALB/c mice were injected with 20 μg BL or 20 μg TNP-Ficoll. Antibody and idiotype concentrations were determined in RIA and expressed as mean ± SEM (μg/ml).

* Not determined.

TABLE VII

| Lethally irradiated BALB/c mice infused with | Anti-BL PFC/spleen* | Number of mice studied | A48Id* % |
|---------------------------------------------|---------------------|------------------------|---------|
| 50 × 10⁶ normal B cells                     | 1,195 ± 225         | 2                      | 0       |
| 50 × 10⁶ A48 B cells                        | 873 ± 335           | 4                      | 0       |
| 25 × 10⁶ normal B cells + 25 × 10⁶ A48 B cells | 850 ± 120         | 2                      | 0       |

* Expressed as the mean ± SEM.

few A48 idiotopes that are not regulatory idiotopes, as was assessed by partial inhibition of binding of labeled anti-A48 to 76BL(Ab3)36 and subsequent absorption with A48 monoclonal protein (Table V).

The Inability of Purified B Cells to Transfer the A48Id Response. The ability of B cells originating from animals that were treated at birth with A48 to confer A48 clonal dominance on the BL response in naive recipients was studied in transfer experiments. Purified B cells obtained from animals either injected with A48 at birth or not were prepared as described before (Materials and Methods). 5 × 10⁷ B cells obtained from normal animals, A48-treated animals, or an equal mixture of both, were infused in lethally irradiated adult BALB/c mice (4–6 mo old), which were subsequently immunized with BL. The anti-BL response was estimated by the PFC method 5 d after challenge. As can be seen in Table VII, no A48Id response was detected in these animals. These results clearly indicate that A48 treatment at birth does not directly stimulate A48Id B cell precursors, and that the B cells from A48-treated animals had no effect on B cells from the normal animals.

Expansion of A48Id-specific Helper T Cells. In further experiments, we investigated the effect of T cells from animals treated at birth with A48 on the activation of silent clones. In the first set of experiments, a mixture of 5 × 10⁷ B cells plus 2.5 × 10⁷ T cells from A48-treated animals was infused into lethally irradiated adult BALB/c mice, which were immunized with BL. An anti-BL PFC response comparable in magnitude with that of the animals infused with B cells alone was obtained. However,
only the animals infused with B and T cells together developed an anti-BL PFC response, of which 90% expressed the A48Id (Table VIII). An A48Id+ response was not obtained in animals infused with B cells from A48-treated mice mixed with T cells from normal animals, or T cells from A48 mice treated with anti-Lyt-1.2 plus complement. The results of this experiment indicated that the expression of A48Id+ BL-specific clones is dependent upon the presence of Lyt-1.2+ helper cells that are lacking in the animals not treated at birth with A48. It should be mentioned that an infusion of equal numbers of T cells from normal animals together with A48-treated

**Table VIII**
*Ability to Transfer A48Id+ Response in Lethally Irradiated BALB/c Mice with Syngeneic T Cells from Mice Treated at Birth with A48 Monoclonal Protein*

| Mixture of cells infused into irradiated BALB/c mice | Anti-BL response (%) | Total PFC/spleen | A48Id+  
|----------------------------------------------------|----------------------|-----------------|------------
| B cells A48 T cells                                  |                       |                 |            |
| Normal Treatment                                    | A48 Treatment        |                 |            |
| Nil Nil                                             | 10                    | 873 ± 335       | 0          |
| 50 × 10^6 Nil Nil                                   | 25 × 10^6 Nil        | 380 ± 10        | 91         |
| 50 × 10^6 Nil 25 × 10^6 Anti-Lyt-1.2 + C’           | 140 ± 60              | 8               |
| 50 × 10^6 25 × 10^6 Nil                             | 25 × 10^6 Nil        | 310 ± 10        | 91         |
| 50 × 10^6 25 × 10^6 Anti-Lyt-1.2 + C’              | 190 ± 40              | 24              |

B cells A48 and T cells A48 originate from 1-mo-old BALB/c mice injected at birth with 10 μg A48. Lethally irradiated mice after infusion of lymphocytes were injected intravenously with 20 μg BL and the anti-BL PFC response was studied 5 d later. The anti-BL PFC/spleen response is expressed as the mean ± SEM.

* C’, complement.

**Table IX**
*Demonstration of A48Id-specific Helper T Cells in BALB/c Mice Treated at Birth with 10 μg A48 Monoclonal Protein: Helper Effect on Anti-TNP Antibody Response in Nude Mice*

| Nude BALB/c mice infused with | Treatment of T cells | Immunization with | Number of mice studied | Anti-TNP PFC/spleen |
|------------------------------|----------------------|-------------------|------------------------|---------------------|
| Nil                          | Nil                  | TNP-Ficoll        | 3                      | 492 ± 79            |
| Nil                          | TNP-A48              | 2                 | 275 ± 275              |
| Nil                          | TNP-M384             | 2                 | 378 ± 298              |
| 25 × 10^6 normal T cells     | TNP-A48              | 4                 | 490 ± 437              |
| 25 × 10^6 normal T cells     | TNP-M384             | 4                 | 212 ± 116              |
| 25 × 10^6 A48 T cells        | TNP-A48              | 7                 | 1,674 ± 653            |
| 25 × 10^6 A48 T cells        | TNP-M384             | 2                 | 5 ± 5                  |
| 25 × 10^6 A48 T cells        | Anti-Thy-1.2 + C’    | 2                 | 50 ± 0                 |
| 25 × 10^6 A48 T cells        | Anti-Lyt-1.2 + C’    | 6                 | 524 ± 190              |
| 25 × 10^6 A48 T cells        | Anti-Lyt-2.2 + C’    | 4                 | 935 ± 343              |

T cells were purified on nylon wool columns from spleens of 1-mo-old BALB/c mice treated at birth with 10 μg A48 monoclonal protein. The anti-TNP response was studied 7 d after infusion of T lymphocytes and immunization with TNP conjugates. All mice were immunized with 50 μg of the appropriate TNP conjugate. The anti-TNP PFC/spleen response is expressed as the mean ± SEM.

* C’, complement.
animals did not alter the A48Id response. These results show that the activity of these cells was not altered by putative, naturally occurring A48Id-specific suppressor cells (5), at least on a one-to-one cell basis ratio, as was assessed by the appearance of A48Id+ cells in animals infused with a mixture of T cells purified from normal as well as mice treated with A48 at birth. To study the specificity of these cells, we tried to enrich them on petri dishes coated with A48 monoclonal protein. Because we failed to enrich for or deplete whole splenic populations of these T cells by the plating method, we studied the specificity of these cells in nu/nu BALB/c mice infused with T cells and immunized with an A48-TNP conjugate. We studied this response by measuring the anti-TNP PFC response. The rationale of this experiment was that if A48-treated mice generate A48Id-specific helper T cells, such cells will provide a helper effect to anti-TNP B cell precursors through an A48-TNP antigen bridge. Indeed, the infusion of purified T cells from mice treated with A48 at birth enabled nu/nu BALB/c mice immunized with A48-TNP, but not with MOPC384 (M384)-TNP conjugates, to mount an anti-TNP response. The helper effect of these T cells was ablated by treatment with anti-Thy-1.2 and anti-Lyt-1.2 plus complement and was not altered by anti-Lyt-2.2 plus complement (Table IX). In another set of experiments aimed to determine the specificity of helper T cells, we studied the effects of treatment of these cells with A48 monoclonal protein and complement. The rationale of this experiment follows from previous findings in which it was shown that murine IgA-antigen complex can activate the complement (17) and that its binding to cellular antigen can cause the lysis of cells in presence of complement (18). Kohler has succeeded in eliminating the T15Id-specific helper T cells by treatment with T15 and complement (personal communication). Therefore, T cells from BALB/c mice injected with 10 μg A48 at birth were incubated for 1 h at 37°C with 1 mg A48 or M384 proteins and guinea pig complement diluted 1:3. After washing, 25 × 10⁶ T cells were infused into four nude mice that were challenged with 50 μg TNP-A48 conjugate. Mice infused with T cells that were pretreated with M384 plus complement developed 3,538 ± 512 anti-TNP PFC/spleen, whereas the mice infused with T cells treated with A48 plus complement showed a significant decrease in the anti-TNP PFC response (i.e., 883 ± 478). In this experiment, the control nude mice infused with 25 × 10⁶ T cells from normal mice and subsequently challenged with TNP-A48 developed only 490 anti-TNP PFC/spleen. These results confirmed the presence of A48Id-specific T cells in mice treated at birth with A48 monoclonal protein.

Discussion

The balance between clones as well as communications which is established between various subsets of regulatory T cells based on fragile idiotypic links can be easily perturbed by antigens, idiotypes, and anti-idiotype antibodies. In this paper, we present new results that show that the administration of A48Id monoclonal protein at birth has a profound effect on the expression of the A48Id+ anti-β2→6 fructosan precursor of antibody-forming cells. Neonatal treatment as well as the treatment of mice during the first weeks after birth with monoclonal protein led to the A48Id dominance of the anti-β2→6 fructosan antibody response. Once the dominant β2→1 fructosan reactive clones emerge 28 d after birth, one can no longer activate A48Id clones. Indeed, no A48Id-bearing antibodies were detected in 4-wk-old BALB/c mice treated with A48 monoclonal protein and immunized 1 mo later with BL.
Two groups of findings demonstrate that this activation is idiotype as well as antigen dependent and specific: (a) the administration at birth of M460 monoclonal protein led to an increase of M460Id component of anti-TNP antibodies, but not of the A48Id component of anti-β2→6 fructosan response; (b) only the administration with BL of 1-mo-old BALB/c mice treated at birth with A48 protein elicited an A48Id+ BL-specific response, whereas immunization with TNP-Ficoll does not. These results are in agreement with those reported by Reth et al. (19), which showed that the increase of (4-hydroxy-3-nitrophenyl)acetyl(NP) VHId component after injection of NP-binding monoclonal antibodies in adult mice requires specific antigenic stimulation. A small but significant stimulation of the β2→1 fructosan-reactive clones was observed in BALB/c mice that were injected at birth with A48 monoclonal protein and challenged 1 mo later with In-Ba. This observation is quite surprising as we have previously shown a significant ontogenic delay of the expression of these clones in that they only become dominant in 6-wk-old mice (4). However, the premature activation of these clones can be exclusively related to a more profound alteration of the steady-state balance at birth maintained between the clones by the administration of A48 monoclonal protein. We previously described an “indirect idiotype phenomenon” based on the observation, made in nude mice, that treatment with anti-E109IdX antibodies resulted in a long-lasting suppression of anti-β2→1 fructosan response that was paralleled by an increase in the total anti-β2→6 fructosan response in addition to the appearance of A48Id+ antibodies (20). Preliminary data regarding the analysis of antigen-binding specificity of several monoclonal antibodies obtained from mice treated at birth with A48 monoclonal protein show that they exhibit a high binding activity to β2→6 fructosan epitopes, but they also bound β2→1 fructosan. These results would suggest that the products of clones that were activated by manipulation of the immune network differ from those activated in adult mice after immunization with BL as well as from In-binding myeloma proteins that exhibit a higher binding activity for β2→1 fructosan epitopes than for β2→6 fructosan epitopes (3).

The A48 idiotype expressed on A48 myeloma protein is composed of several idiotopes (16); some of them are shared by U10 monoclonal protein and β2→6 fructosan-binding monoclonal antibodies that we have recently obtained. The treatment at birth with U10 and 76BL(Ab3)42, but not with 76BL(Ab3)36, monoclonal antibodies led to the activation of A48Id. These results support our previous concept concerning the regulatory idiotype network in that only few idiotopes—that is, regulatory idiotopes—function in an autologous system. We proposed that regulatory idiotypes might be a feature only of those idiotypes that are capable of becoming dominant idiotypes, possibly because it is these determinants that call for the T cell regulatory responses (2, 7).

Our results show that the activation of A48Id silent clones depends on the expansion of A48Id-specific helper T cells. The infusion of B cells from A48-treated mice into irradiated mice at birth does not cause the production of A48Id-bearing antibodies in response to immunization with BL. This clearly demonstrated that A48Id-bearing immunoglobulins have no effect on B cells that carry the A48 immunoglobulin receptor. By contrast, the infusion of irradiated animals with a mixture of T and B cells from A48-treated mice induced the occurrence of A48Id-bearing antibodies. These T cells express Lyt-1.2 alloantigen and their activity cannot be overcome by putative naturally occurring A48Id-specific suppressor T cells (5), at least at a 1:1 cell
ratio. Because we failed to enrich for the putative A48Id-specific helper T cells on A48-coated dishes, we studied the specificity of the receptor of these cells in nude mice. In these experiments we found that nude mice infused with T cells from animals treated at birth with A48 contained a discrete subset of cells that recognize the A48 idiotypes. When these cells were infused into nude BALB/c mice, they rendered them capable of mounting an anti-TNP response upon immunization with A48-TNP conjugate. This subset of cells that exert their helper effect through an antigen bridge were also sensitive to A48 and anti-Lyt-1.2 antiserum plus complement treatment. It is conceivable that the antigen bridge through which these T cells exert their helper effects in A48-treated animals can be composed of bacterial levan-A48Id+ antibody complex. A precedent for T cells that exert their effect through an antigen bridge in which the antigen is bound to an immunoglobulin receptor which is simultaneously recognized by T cell receptor were previously described in the MOPC315 system. In this system, it was shown that BALB/c splenocytes specific for TNP-modified syngeneic cells could induce the lysis of MOPC315 cells in the presence of soluble TNP conjugates (21). The possibility remains, however, that they may be distinct from the subset of cells observed to help in the expression of A48Id+ BL-specific response in lethally irradiated mice. Therefore, our present results suggest that the treatment with A48 monoclonal protein at birth activates A48Id-specific helper T cells that select for expansion subsequent antigenic stimulation those clones that express A48Id on their immunoglobulin receptors. Attempts to clone these helper T cells in our laboratory will provide information on their fine idiotype specificity, target, and phenotype, as well as major histocompatibility complex restriction.

One may ask whether clonal activation by parenteral administration of idiotype at birth has any physiological significance. It is well known that significant amounts of IdX-bearing antibodies specific for environmental antigens can be detected in the sera of nonimmunized adult mice (22). Furthermore, the precursors responsible for mounting these antibody responses can be detected in newborn mice by precursor frequency analysis (23). In addition, various investigators have observed that a vigorous immune response for environmental antigens (e.g., phosphocholine, β2→6 fructosan, α1→3 dextran, galactan, arsonate, TNP coupled to TI-1 antigens) can be elicited in 1–7-d-old mice (24). Therefore, the passive influx of maternal idiotypes in the immune system of the embryo or newborn, by placenta or colostrum transfer, can influence the idiotype distribution by favoring the expression of those clones that were dominant in the maternal immune system. In the rabbit, Wikler et al. (25) have also shown that progeny can learn to make antibodies bearing the idiotype of another rabbit by maternal transfer of anti-(anti-Id) antibodies. In physiological terms it is extremely advantageous to have the immune experience of the mother transmitted to the next generation with the resulting expansion of precursors that are specific for a response against commonly encountered pathogens prevalent in the species. This is particularly important in a given period of the postnatal life when the concentration of antibodies provided from the mother starts to decline. Absence of an idiotype-induced expansion can clearly explain the observation that exposure of the human population to new pathogens that had not been encountered in previous generations, frequently results in widespread epidemics (e.g., influenza pandemics after a major antigenic shift of the influenza virus hemagglutinins) (26).

In theoretical terms, this concept would redefine the notion of inheritance of
immunity as not only a passive acquisition of maternal antibodies, but also a priming of the immune system for specific immune responses. This would suggest that once an immune response is initiated by antigenic stimulation, the actual antigen-response clones that will proliferate can be determined by the idiotypic repertoire of the mother that has been established by an earlier encounter with the same antigen.

Summary

BALB/c mice immunized with bacterial levan (BL) produce a vigorous antibody response that fails to include antibodies expressing the idiotype of the $\beta_{2\to6}$ fructosan-binding myeloma protein ABPC48 (A48). Treatment of newborn BALB/c mice at 1 d of age with 0.1–10 $\mu$g of either the A48 myeloma protein or monoclonal proteins that share idiotopes with the A48 family, followed by immunization with BL 2–4 wk later, produces an anti-BL response that is dominated by the A48Id. Various degrees of activation of the A48Id BL response were observed by injecting mice with A48 monoclonal protein only up until 3 wk of age. Activation of the A48Id clones by treating with A48 monoclonal protein was ineffective in mice who were older than 4 wk. Elicitation of an A48Id BL response required specific antigenic stimulation with either $\beta_{2\to6}$ or $\beta_{2\to1}$ fructosan epitopes, because it does not occur after injection with TNP-Ficoll in spite of the A48 treatment. The expansion of A48Id clones in mice treated at birth with A48 monoclonal protein is associated with an increase in A48Id-specific helper T cells. The binding specificity of these cells was demonstrated by infusing them into $nu/nu$ BALB/c mice and observing that they rendered help that enabled the animal to mount an anti-TNP response after immunization only with A48-TNP, but not with MOPC384-TNP conjugates. The helper activity of these cells is sensitive to the effects of treatment with anti-Lyt-1.2 antibodies plus complement. A predominantly A48Id BL-specific response can be transferred into lethally irradiated mice by infusing them with purified T and B cells from A48-treated mice. The transfer of this response can be ablated by treating the T cells with anti-Lyt-1.2 antibodies plus complement.

These results indicate that A48Id-specific helper cells possess the ability to select the A48Id-bearing B cell precursors for expression, thus exerting a fine-tuning effect on the idiotypic expression of the anti-BL repertoire. We propose that this idiotype-induced idiotype response, which can be, in principal, induced by idiotypes provided by the mother, plays an important role in the expansion of precursors of antibody-forming cells during embryonic as well as postnatal life.

References

1. Jerne, N. K. 1974. Towards a network theory of the immune response. *Ann. Immunol. (Paris).* 125C:373.
2. Paul, W. E., and C. Bona. 1982. Regulatory idiotypes and immune networks: a hypothesis. *Immunol. Today.* In press.
3. Lieberman, R., M. Potter, W. Humphrey, Jr., E. B. Mushinski, and M. Vrana. 1975. Multiple individual and cross-specific idiotypes on 13 levan-binding myeloma proteins of BALB/c mice. *J. Exp. Med.* 142:106.
4. Bona, C., R. Lieberman, C. C. Chien, J. Mond, S. House, I. Green, and W. E. Paul. 1978.
Immune response to levan. I. Kinetics and ontogeny of anti-levan and anti-insulin antibody response and expression of cross-reactive idiotype. J. Immunol. 120:1436.

5. Lieberman, R., C. Bona, C. C. Chien, K. E. Stein, and W. E. Paul. 1979. Genetic and cellular regulation of the expression of specific antibody idiotypes in the anti-polyfructosan immune response. Ann. Immunol. (Paris). 130C:247.

6. Hiernaux, J., C. Bona, and P. J. Baker. 1981. Neonatal treatment with low doses of anti-idiotype antibody leads to the expression of a silent clone. J. Exp. Med. 153:1004.

7. Bona, C., E. Heber-Katz, and W. E. Paul. 1981. Idiotype-anti-idiotype regulation. I. Immunization with a levan-binding myeloma protein leads to the appearance of auto-anti-(anti-idiotype) antibodies and to the activation of silent clones. J. Exp. Med. 153:951.

8. Inman, J. K. 1975. Thymus-independent antigens: the preparation of covalent, hapten-FicolI conjugates. J. Immunol. 114:704.

9. Bikoff, E. 1982. T-cell proliferative response to hapten-modified self immunoglobulins: recognition of conjugate-specific determinants. Proc. Natl. Acad. Sci. U. S. A. In press.

10. Bona, C., R. Hooghe, P. A. Cazenave, C. Leguern, and W. E. Paul. 1979. Cellular basis of regulation of expression of idiotype. II. Immunity to anti-MOPC-460 idiotype antibodies increases the level of anti-trinitrophenyl antibodies bearing 460 idiotypes. J. Exp. Med. 149:815.

11. Hammerling, U., and O. Westphal. 1977. Synthesis and use of o-steroyl polysaccharides in passive hemagglutination and hemolysis. Eur. J. Biochem. 1:46.

12. Rittenberg, M. B., and K. L. Pratt. 1969. Anti-trinitrophenyl (TNP) plaque assay. Primary response of BALB/c mice to soluble and particulate immunogen. Proc. Soc. Exp. Biol. Med. 132:575.

13. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of 131I-labelled human growth hormone of high specific radioactivity. Biochem. J. 89:114.

14. Wilder, R., C. C. Yuen, B. Subbarao, V. L. Woods, C. B. Alexander, and R. G. Mage. 1979. Tritium (H) radiolabeling of protein A and antibody to high specific activity. Application to cell surface antigen radioimmunoassays. J. Immunol. Methods. 28:255.

15. Hiernaux, J., and C. A. Bona. 1982. Shared idiotypes among monoclonal antibodies specific for different immunodominant sugars of lipopolysaccharide of different Gram-negative bacteria. Proc. Natl. Acad. Sci. U. S. A. 79:1616.

16. Legrain, P., D. Voegtle, G. Buttin, and P. A. Cazenave. 1981. Idiotype-anti-idiotype interactions and the control of the anti-β(2-→6) poly-fructosan response in the mouse: specificity and idiotypic antibodies. Eur. J. Immunol. 11:578.

17. Pfaffenbach, G., M. E. Lamm, and I. Gigli. 1982. Activation of the guinea pig alternative complement pathway by mouse IgA immune complexes. J. Exp. Med. 155:213.

18. Adinolfi, M., A. A. Glynn, M. Lindsay, and C. M. Milne. 1966. Serological properties of γA to Escherichia coli. Immunology. 10:517.

19. Reth, M., G. Kelsoe, and K. Rajewsky. 1981. Idiotypic regulation by isologous monoclonal anti-idiotype antibodies. Nature. (Lond.). 290:257.

20. Bona, C., K. E. Stein, R. Lieberman, and W. E. Paul. 1979. Direct and indirect suppression induced by anti-idiotype antibody in the inulin-bacterial levan antigenic system. Mol. Immunol. 16:1093.

21. Abbas, A. K., S. E. Ratnofsky, and S. J. Burakoff. 1980. T lymphocyte-mediated suppression of myeloma function in vitro. II. Evidence for regulation of hapten-binding myelomas by syngeneic hapten-specific cytolytic T lymphocytes. J. Exp. Med. 152:306.

22. Porter, M. 1979. Mouse myeloma proteins. Adv. Immunol. 25:141.

23. Klinman, N. R., A.R. Pickard, N. H. Sigal, P. J. Gearhard, E. S. Metcal, and S. K. Pierce.
1976. Assessing B cell diversification by antigen receptor and precursor cell analysis. *Ann. Immunol. (Paris)*. 127C:489.

24. Bona, C. 1981. Idiotypes and Lymphocytes. Academic Press, Inc., New York.

25. Wikler, M., C. Demeur, G. Dewasme, and J. Urbain. 1980. Immunoregulatory role of maternal idiotypes. Ontogeny of immune networks. *J. Exp. Med.* 152:1024.

26. Kilbourne, E. D. 1978. Influenza as a problem in immunology. *J. Immunol.* 120:1447.