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MATERNAL IMMUNOLOGICAL EXPERIENCE GUIDES THE EDUCATION OF THE NEONATAL IMMUNE SYSTEM

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SUMMARY

Immune responses to thymus-dependent antigens undergo immune maturation through somatic hypermutations which accumulate with repeated immunisations and comprise the memory B cell as well as the memory antibody pool. Consequently, it can be concluded that the B lymphocyte compartment, as a whole, stepwise acquires a knowledge of the external antigenic world with increasing quality and precision. This progression may be regarded as an ontogenetic learning process the development of which, although strictly based on and starting from a genetic basis, is *entirely* dependent on the interaction with the immunologically relevant environment and is only driven by adequate stimuli. Since early ontogeny is an exceptionally sensitive phase for the development of the immune system, we asked whether the immunological experience of the mother, represented by IgG antibodies from different stages of immune maturation, might influence a particular immune response in the offspring. The following results were obtained: 1. An immunisation of female CBA mice with phospholipase A_2 (the main allergen of bee venom) as well as the transfer to the offspring of a mixture or one single monoclonal anti-PLA_2 antibody suppressed the IgE immune responsiveness to PLA_2 in F1 animals for a long period of time. 2. The maternal influence on the quality of immune response was tested in the well explored response to the hapten 2-phenyloxazolone (phOx) in BALB/c mice. BALB/c dams received a primary, secondary or tertiary immunisation with phOx-coupled chicken serum albumin and the primary anti-phOx response was investigated in offspring of the F1 or F2 generation at an age when no maternally derived anti-phOx serum antibodies could be detected. (a) The kinetics of the primary response were altered. (b) About half of the F2 mice developed maximal antibody levels as in a secondary response. (c) The expression of the normally dominant Ox1 idiotype (Id_{Ox1}) was rendered exceedingly variable. (d) Half of the non-Id_{Ox1} anti-phOx antibodies exhibited strongly enhanced affinities, being either identical to or even 7-25 times higher than those of Id_{Ox1} antibodies which are normally of highest affinity. Hence, maternal antibodies of the late stages of immune maturation, which may be perceived as acquired immunological phenotypic characters, have the capacity to interfere with isotype regulation and improve the quality of a considerable proportion of antigen-reactive antibodies in the available repertoire and thus are suspected to give the offspring a benefit in handling of external antigens.

INTRODUCTION

The immune system is endowed with the capacity to develop a state of memory of former events. This memory is only induced in cells which generate adaptive immune responses, namely T- and B lymphocytes. In contrast, those cells which are responsible for the first line of defence and form the innate immune system, i.e. granulocytes and monocytes/macrophages, are not able to develop memory. The formation of immunological memory depends on the activation of T lymphocytes and thus can only be induced by thymus-dependent (TD) antigens. Consequently, memory itself is thymus-dependent. However, whereas antigen recognition of T memory cells is of the same quality as that of virgin T cells, can B cells develop an improved quality = affinity of their antigen receptors during the primary and the following antigen-induced immune responses. This is demonstrated by the fact that TD antibody responses show immune maturation (*Eisen* and *Siskind*, 1964) which is brought about by somatic hypermutations (*Rodwell* et al., 1983; *Griffiths* et al., 1984; *Wysocki* et al., 1986; *Cumano* and *Rajewsky*, 1986; *Berek* and *Milstein*, 1987). Hence, mutated antibodies may be perceived as acquired immunological phenotypic characters. Since these somatic mutations accumulate with repeated immunisations and comprise the memory B cell pool (*Wysocki* et al, 1986; *Berek* and *Milstein*, 1987; *Weiss* and *Rajewsky*, 1990), it can be concluded that the B lymphocyte compartment, as a whole, stepwise acquires a knowledge of the external antigenic world with increasing quality and precision. Hence, this progression can be regarded as an ontogenetic learning process the development of which, although strictly based on and starting from a genetic basis, is *entirely* dependent on the interaction with the immunologically relevant environment and is only driven by adequate stimuli.

The induction of an immune response with a thymus-dependent antigen, e.g. with proteins from normal commensal or pathogenic microorganisms, activates B lymphocytes which, after differentiation to plasma cells, secrete large amounts of immunoglobulins. Their variable domains of the H and the L chain harbour individual antigenic determinants termed *idiotopes* which are not only immunogenic in the xenogeneic and allogeneic, but also in the syngeneic and the autologous host. A particular antibody expresses in its V regions a collection of idiotopes which form the idiotype (Id). The antigen-induced increased synthesis of antibodies (= Id = Ab1) can lead to activation or suppression of B cells expressing antigen receptors reacting with idiotopes of Ab1. Those antibodies activated in the second step are designated anti-idiotypic (aId) or Ab2 and their variable region idiotopes are recognised again by a third set of B cell receptors, named anti-(anti-idiotypic) or Ab3 and so forth. Each of these activation steps leads to a quite heterogeneous population of antibodies. This holds true for the Id population which is activated by antigen as well as for the populations of aId (Ab2) and anti-(anti-Id) (Ab3). The extrapolation of this chain of reactions led to the formulation of the idiotypic network theory (*Jerne*, 1974, 1985; *Jerne* et al., 1982).

Although interactions between an idiotype with its anti-idiotype may, depending on the immunising dose, result in stimulation or suppression of the reactive partner (*Takemori* and *Rajewsky*, 1984a, 1984b), the suppressive effects of anti-idiotypic antibodies have been in the foreground of investigations since they were regarded as important regulatory compounds to prevent overshooting of immune responses. While the

suppression of a particular idiotype by anti-idiotypic antibodies in adult animals is transient in nature, it may especially be long-lasting when the anti-idiotypic response is induced in new-borns shortly after birth (*Strayer* et al, 1975; *Augustin* and *Cosenza*, 1976; *Hiernaux* et al., 1981) or the corresponding idiotype may even be permanently lost (*Kearney* et al., 1983; *Vakil* et al., 1986). When anti-idiotypic antibodies are actively induced or injected into pregnant mothers and reach the foetus via the maternal route before and/or after birth, they also suppress the corresponding idiotype in the offspring (*Weiler* et al., 1977; *Victor* et al., 1983). Interestingly, if an anti-idiotypic manipulation either by direct immunisation of the neonate or via the mother is directed towards a highly connected idiotype expressed by multispecific, cross-reactive IgM antibodies, a long-term or permanent severe disturbance of a larger part or even the *entire* antibody repertoire of that animal may occur (*Vakil* et al., 1986; *Bernabe* et al., 1981). Moreover, it has been shown that the transfer of idiotypes or anti-idiotypes solely with the colostrum and milk after birth is sufficient to induce idiotypic interaction and / or protection against microbial infection from the mother to the offspring (*Weiler* et al., 1977; *Rothstein* and *Vastola*, 1984; *Stein*, 1985; *Ali* et al., 1988; *Heiman* and *Weisman*, 1989). This clearly emphasises the importance of natural postnatal rearing and supports the idea that not only the experimentally induced but also naturally-occurring antibodies of the mother can influence the development of the new-borns´ immune system and the generation of the antibody repertoire (*Wikler* et al., 1980; *Bernabe* et al., 1981; *Stein*, 1985; *Kearney* et al., 1986; *Martinez* et al., 1986; *Vakil* et al., 1986; *Andrade* et al., 1990).

Based on such idiotypic interactions in the immune system, we assumed: (a) Highly maturated antibodies which are transferred before and/or after birth from the dams to the offspring will not only confer passive protection, but are expected to influence a particular immune response in the offspring, probably induced by idiotypic-anti-idiotypic interactions. (b) Furthermore, maternal antibodies will supposedly not only induce a clonal alteration of unknown functional relevance for an antigen-induced immune response, but might influence the quality of immune responses in the offspring in a biologically meaningful fashion. These assumptions were tested by determining the IgE-regulatory potency of maternally derived monoclonal IgG antibodies and by analysing the primary immune response to the hapten 2-phenyl-oxazolone in F1 and F2 offspring of dams which had been immunised or injected with anti-phOx monoclonal antibodies.

METHODS

Animals

The immune response to 2-phenyl-oxazolone (phOx) was studied in BALB/c (H-2^d) mice and that to phospholipase A_2 (PLA_2) in CBA/J (H-2^k) mice, both of which were obtained from Harlan/Winkelmann (Borken, Germany) or Bomholtgaard (Ry, Denmark) and reared under conventional conditions in the animal house of the university.

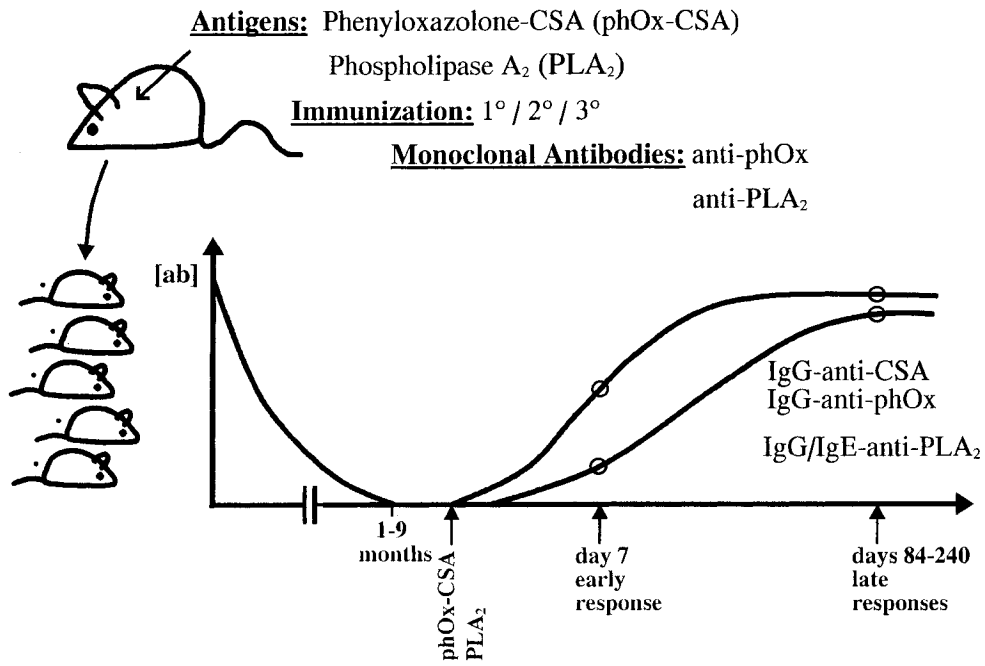

Figure 1: Experimental design (for explanation see text).

Immunisations, production of monoclonal antibodies and analysis of immune responses

Anti-PLA_2 immune response

Immunisation of CBA/J mice was performed as described by *Kolbe* and co-workers (1991) who have shown that minimal doses (MD) of PLA_2 (0.1 μg per injection) leads to a strong IgG-response and small but consistent production of IgE antibodies, while large doses (LD) of PLA_2 induce a strong IgG but no IgE antibody response which can not be reactivated by minimal doses. The determination of IgG and IgE antibody titres in the sera of immunised mice was performed with solid-phase bound sandwich ELISAs as described by *Kolbe* et al. (1991).

Anti-phOx immune response

The immunisations with the thymus-dependent antigen phOx-coupled chicken serum albumin (CSA) have been described recently (*Lemke* et al., 1994; *Lange* et al., 1999). For an analysis of the phOx-reactive primary repertoire, spleen cells of BALB/c mice were fused on day 7 after primary immunisation with the non-secretor X63-Ag8.653 myeloma cells (*Kearney* et al., 1979) by the conventional polyethylene glycol fusion technique. The idiotypic analysis of anti-phOx immune sera and monoclonal antibodies and the sequencing of their variable regions of the H and L chains have been described in (*Lange* and *Lemke*, 1996; *Lange* et al., 1999).

RESULTS

Experimental design

The experimental design is depicted in Figure 1. Female BALB/c mice were immunised 1-3-times (primary = 1°mo,

secondary = 2°mo or tertiary = 3°mo) with the hapten-carrier conjugate phOx-CSA (*Lemke* et al., 1994) or CBA/J females were immunised with the IgE-inducing phospholipase A_2 (PLA_2), the main allergic component of bee venom (*Seeger* et al., 1998). Alternatively, females of both strains were injected with monoclonal anti-phOx or anti-PLA_2 antibodies, respectively. Depending on the maternally-derived anti-phOx, anti-CSA or anti-PLA_2 serum titres, the F1 and F2 generation offspring were allowed to rest until the maternally derived antibodies had vanished from the circulation. Four weeks later, the BALB/c offspring received a primary immunisation with phOx-CSA and the kinetics of the immune response were followed (*Lemke* et al., 1994). Moreover, the quality of the early response on day 7 was analysed with respect to the idiotypic composition of the antisera, i.e. the proportion of Id_{Ox1} antibodies which dominate the early anti-phOx response was determined with Id_{Ox1}-specific monoclonal antibodies. Hybridoma antibodies were also produced on day 7 after primary immunisation and their affinities and the expression of the Id_{Ox1} were analysed and the V(D)J gene sequences of non-Id_{Ox1} antibodies were determined (*Lange* et al., 1999). The offspring of CBA/J dams was immunised with IgE-inducing minimal doses of PLA_2 [primary immunisation with 10 μg and the following injections with 0.1 μg, according to *Kolbe* et al. (1991)] and the IgE and IgG immune responses to PLA_2 were followed (*Seeger* et al., 1998).

Suppression of IgE responsiveness by maternally derived monoclonal IgG antibodies

Twenty years ago, it has already been demonstrated that the IgE immune response to ovalbumin (OVA) in rats can effectively be suppressed by a pre-immunisation of the dams (*Jarrett* and *Hall*, 1979). A variety of experimental conditions indicated that this IgE suppression was, with all likelihood, mediated by IgG-anti-OVA antibodies transferred from the dams to the offspring and that antigen was not involved in the process (*Jarrett* and *Hall*, 1979, 1983, 1986; *Jarrett*, 1984). We have repeated such experiments in mice with another antigen, namely bee venom PLA_2 (*Seeger* et al., 1998), and the results of Jarrett and co-workers could be confirmed and extended: (a) The IgE suppression lasted much longer than IgG-anti-PLA_2 could be detected in the sera of the F1 animals. (b) An IgE response could not be reactivated by repeated immunisations with IgE-inducing minimal doses of PLA_2 for up to half a year. (c) Moreover, we could provide convincing evidence that the IgE suppression by maternally derived IgG works independently of antigen: We prepared anti-PLA_2 monoclonal antibodies which were purified from the culture supernatants by affinity chromatography on Sepharose-immobilised recombinant Protein A or Protein G (rProtein-A- and rProtein-G-Sepharose; Pharmacia, Freiburg i.Br., Germany). These purified IgG-anti-PLA_2 were injected into pregnant CBA/J females 10 days before and 7 days after giving birth to their offspring. It could be demonstrated (Figure 2) that a mixture of ten (nine IgG1 and 1 IgG2b) or even *one* single monoclonal maternally derived IgG-anti-PLA_2 (IgG1 antibody MS613) were equally effective in mediating an IgE-suppression. This suppression of IgE responsiveness was detectable when immunisation with minimal doses of PLA_2 was started at an age of 4 months when low levels of IgE antibodies to PLA_2 were still detectable in the sera (data not shown) as well as when immunisation was started at an age of 8 months when no mater-

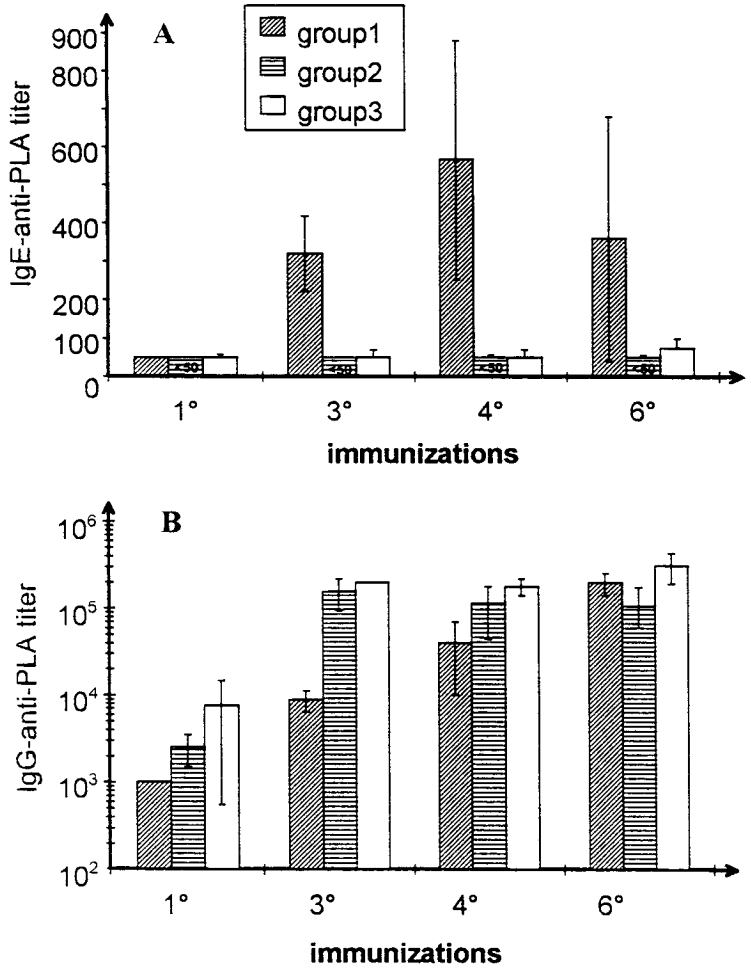

Figure 2: IgE (A) and IgG (B) immune responses of CBA/J mice (n=5-7) born to dams which had received different monoclonal antibodies 10 days before and 7 days after delivery of their offspring. With each injection, the dams received 0.6 mg of one purified antibody or a mixture of antibodies. Dams of group 1 animals had received the monoclonal IgG1-anti-2-phenyloxazolon antibody NQ2/16.2 (*Kaartinen* et al., 1983), dams of group 2 mice had received mAb MS613 as the only IgG1-anti-PLA_2 antibody and dams of group 3 mice had received a mixture of nine IgG1- (including mAb MS.613) and one IgG2b-anti-PLA_2 antibodies. All F1 offspring mice were started to be immunized at an age of *eight* weeks when maternally-derived monoclonal anti-phOx or anti-PLA_2 antibodies could not be detected in their sera. The primary immunization was done with 10 μg PLA_2 while all following immunizations were performed with the IgE-inducing minimal doses of 0.1 μg PLA_2. Two weeks after each immunization, the IgE and IgG titers were determined. The bars indicate the mean ± standard deviation. [Reprinted from *Seeger* et al. (1998) with permission].

nally derived IgG-anti-PLA_2 could be detected, as shown in Figure 2. (d) These experiments demonstrated that one IgG1 antibody was effective to induce IgE suppression. The isotype-dependence of IgE suppression with respect to the other IgG subclasses is currently under detailed investigation with isotype switch variants of the effective monoclonal antibody MS613. Hence,

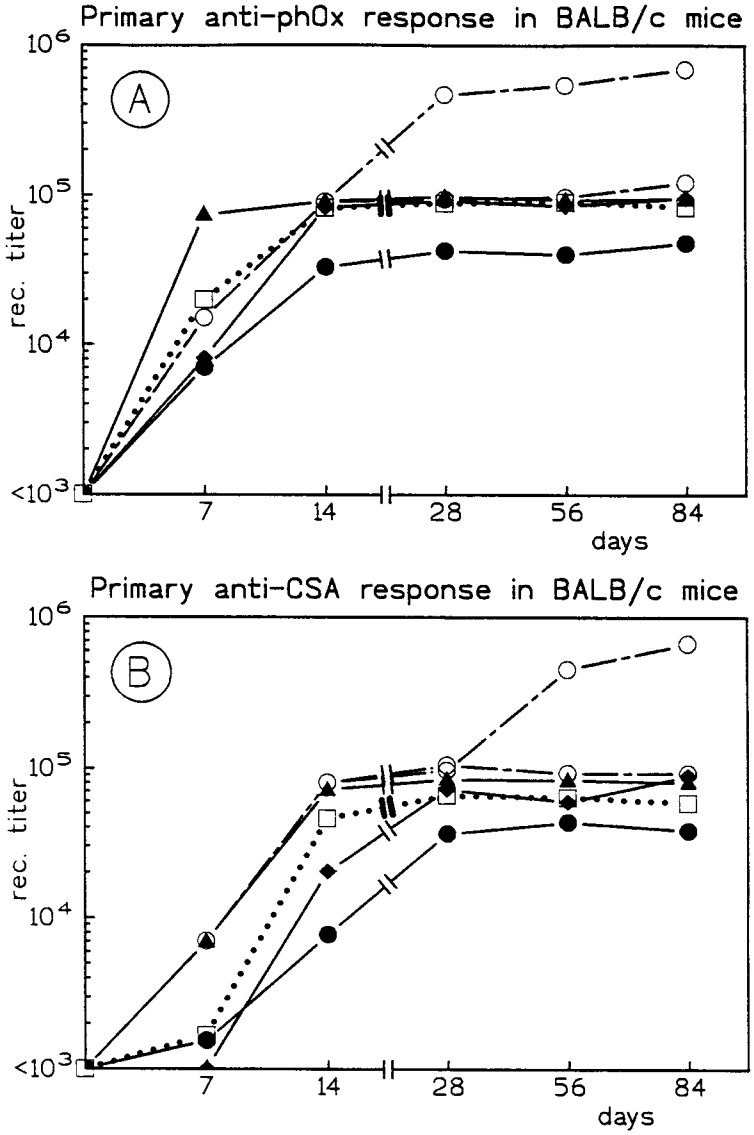

Figure 3: Primary anti-phOx (A) and anti-CSA (B) response in normal BALB/c mice (o·····o), F1-generation offspring born to primarily (s—s), secondarily (u—u) or tertiary (l—l) immunised mothers and F2-generation mice descending from tertiary immunised grandmothers, but non-immunised dams (m—·—m). The primary response was induced with phOx-CSA 4 weeks after complete disappearance of maternal or grandmaternal antibodies from the circulation. Each value indicates the mean titre of a group of mice which consisted of at least 5 and maximally of 17 animals. For the sake of clarity, the standard deviations are omitted here, but included in the comparison for the titres on day 7 and day 84 in Figure 5. For statistical significance see text. The titres were calculated as that serum dilution corresponding to three times cpm background values without antiserum. [Reprinted from *Lemke* et al. (1994), with permission].

our experiments formally prove that antigen is *not* involved in the process and provide an experimental basis to investigate the underlying mechanisms.

Maternal immunisation modulates the primary response to phOx-CSA

For an elucidation of the principle possibility that the immunological experience of the mother may be beneficial for the development of immune responsiveness in the offspring, the kinetics of the primary anti-phOx-CSA immune response were investigated in the offspring of dams which had received a primary (1°mo), secondary (2°mo) or tertiary (3°mo) immunisation. To exclude or at least drastically reduce the possibility that maternally derived antibodies might directly influence this response, the primary immunisation in the offspring was induced 4 weeks after maternal antibodies had declined to background levels of normal mice. The development of anti-phOx and anti-CSA responses in mice born to 1°mo, 2°mo or 3°mo is depicted in Figure 3. In comparison to normal mice, it is striking to see that the early primary response on day 7 of mice born to 1°mo was enhanced ($p<0.001$) whereas it was reduced in mice born to 2°mo ($p<0.001$) or 3°mo ($p<0.001$). Hence, F1 animals born to 1°mo seemed to react with an accelerated response and maximal antibody levels were already reached by day 7 after primary immunisation. In offspring of 2°mo and 3°mo the response was delayed, but mice born to 2°mo reached nearly maximal titres by day 14. In offspring of 3°mo, however, the level of anti-phOx and anti-CSA antibodies was significantly reduced ($p<0.05$) up to 84 days post immunisation.

The development of both antibody specificities was also tested in animals of the F2 generation which received a small amount of antibodies from their grandmothers (*Lemke* et al., 1994). In about half of these F2 mice (45%), the anti-phOx production developed like in normal mice whereas in the other half (55%) of the animals, these grandmaternal antibodies had dramatic effects on the kinetics of the immune response, in that anti-phOx titres continued rising beyond 4 weeks and reached titres of $6x10^5$ at 12 weeks after immunisation (compared to normal mice: $p<0.001$). Such high titres have never been observed before during a primary antibody response to phOx. The anti-CSA response in this subpopulation of F2 mice showed a significant ($p<0.001$) earlier onset as did those animals born to 1°mo. Although the response seemed to reach plateau levels 14 days after immunisation, the anti-CSA titres started to rise again in those 55% of animals and reached, like the anti-phOx response, titres of about $6x10^5$ by day 84.

Maternal tertiary immunisation induces a clonal alteration of the primary day 7 anti-phOx repertoire

Next, it was tested whether the maternal immunisation or the transfer of monoclonal antibodies from the dams to F1 and F2 mice could not only modulate the early day 7 primary anti-phOx antibody response in quantitative terms, but might also induce an alteration of the clonal composition in these early immune sera (*Lange* et al., 1999). For this purpose, the dominant Ox1 idiotype (Id_{Ox1}) was used as a marker. In BALB/c mice, the Id_{Ox1} is quantitatively stable expressed by about 75% of the anti-phOx antibodies during the early primary response (*Kaartinen* et al., 1983a; *Lange* and *Lemke*, 1996; *Lange* et al., 1999). After its early expression, the Id_{Ox1} is lost during the following 2-3 weeks of immune maturation through somatic mutations (*Lange* and *Lemke*, 1996). This is also depicted in Figure 4 (lanes 1 and 2). When BALB/c females were immunised with the IgM-Id_{Ox1} H11.5 two months before mating, IgG-anti-Id_{Ox1} could be detected in the offspring up to an age of 2 months. When these F1 mice were immunised with phOx-CSA after two more months, the

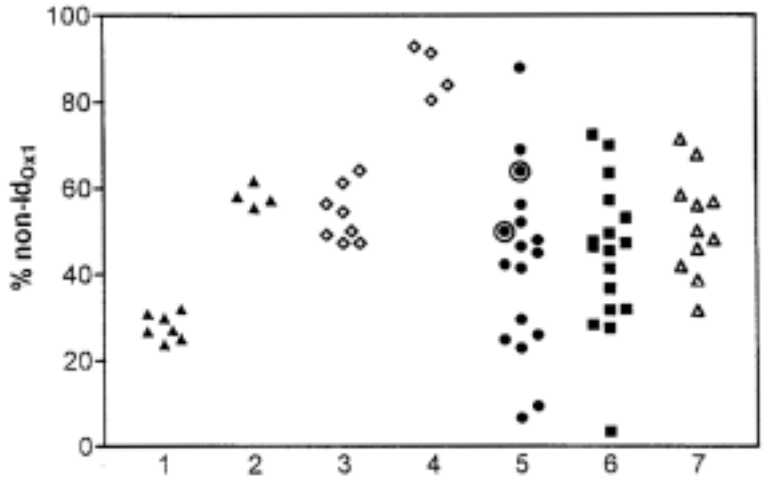

Figure 4: Expression of non-Id_{Ox1} in primary anti-phOx antisera. Normal BALB/c or offspring mice born to mothers or descending from grandmothers immunised in different ways received a primary immunisation with phOx-CSA. The percentage of non-Id_{Ox1} or Id_{Ox1}, respectively, was determined by inhibition with Id_{Ox1}-specific monoclonal antibodies (see section Methods). The percentage of *non*-Id_{Ox1}, is shown for a) primary antisera of normal mice on day 7 (lane 1) and day 14 (lane 2); b) primary day 7 (lane 3) and day 14 (lane 4) antisera of mice born to mothers which had been immunised with the IgM-Id_{Ox1} H11.5; c) primary day 7 antisera of mice born to tertiary phOx-CSA-immunised mothers (lane 5) or descending from tertiary immunised grandmothers, but non-immunised F1 dams (lane 6); d) primary day 7 antisera of mice born to mothers which were injected 2 weeks after mating with a mixture of three highly mutated quaternary anti-phOx antibodies (lane 7). In lane 5 the dots surrounded by circles represent the two mice from which monoclonal day 7 antibodies were produced and whose V(D)J gene sequences were determined. [Reprinted from *Lange* et al. (1999), with permission].

expression of Id_{Ox1}^{+} anti-phOx antibodies in day 7 immune sera was suppressed to about 40% and this proportion decreased to about 10% by day 14 (Figure 4, lanes 3 and 4). Remarkably, as in normal mice (Figure 4, lanes 1 and 2), the expression of the Id_{Ox1} showed little variation between different animals. When the F1 offspring of tertiary immunised dams was analysed in this respect, the pattern of IdOx1 expression was quite different (Figure 4, lane 5). Only 4 out of 17 immune sera showed an Id_{Ox1} expression like those of normal mice (comparison with lane 1). In the majority of 11 mice, the expression of the Id_{Ox1} was suppressed to values of 10-60%. However, 2 mice were observed which even exhibited an enhanced Id_{Ox1} expression to 90-95%. Hence, tertiary immunisation of the dams rendered the Id_{Ox1} expression exceedingly variable.

The proportion of the IdOx1 in day 7 primary antisera was also determined in F2 mice born to non-immunised F1 dams which again descended from females which had undergone a tertiary immune response to phOx-CSA. The content of Id_{Ox1} antibodies in these primary day 7 immune sera (Figure 4, lane 6) varied between individual mice nearly to the same extent as it did in the direct F1 offspring of those tertiary immunised dams (Figure 4, lane 5).

High affinity antibodies derived from late stages of the maturation process represent the highest quality of the mother's immunological experience with regard to a particular immune re-

sponse. It was suspected that they also might influence the outcome of the primary immunisation in the F1 generation. Therefore, the late stages of immune maturation were tried to be imitated by injecting pregnant BALB/c females 2 weeks after mating with a mixture of three anti-phOx monoclonal antibodies (mAb-mo) which were generated from a quaternary immune response. In comparison to the primary Id_{Ox1}^{+} IgG-anti-phOx antibody NQ2/16.2 ($\gamma1,\kappa$), the affinities of these antibodies HL4°/2-16, HL4°/18-22, HL4°/21-3 (all $\gamma1,\kappa$) were enhanced by factors of 1100, 120 and 550, respectively, and their V/D/J gene expression have been described (*Lange* et al., 1999). Four weeks after the disappearance of these maternally derived antibodies from the circulation, the F1 animals received a primary immunisation with phOx-CSA and again, the proportion of the Id_{Ox1} among the anti-phOx humoral antibodies in day 7 antisera was determined. Strikingly, the expression of the Id_{Ox1} varied between individual mice (Figure 4, lane 7) nearly to the same extent as in the two former groups, i.e. 3°mo/1°Fl animals (lane 5) and 3°mo/1°F2 mice (lane 6). Moreover, in comparison to normal mice (lanes 1 and 2) and to the offspring born to IgM-Id_{Ox1}-immunised dams (lane 3 and 4), it was evident that the proportion of Id_{Ox1} varied to a much greater extent in the F1 and F2 offspring of tertiary immunised dams as well as in F1 mice which received the mixture of high-affinity quaternary anti-phOx antibodies.

The pre-immune anti-phOx repertoire of mice born to tertiary immunised dams contains antibodies with enhanced affinities

For an analysis of the quality of the primary antibody repertoire of mice born to tertiary immunised dams, the F1 offspring were allowed to rest until their maternal anti-phOx humoral antibody titres had vanished. Eight weeks later, the mice received a primary immunisation with phOx-CSA. Hybrid cell lines secreting anti-phOx antibodies were produced on day 7, a time point at which in normal mice practically no somatic mutations have been observed (*Berek*, 1992). From two of these 3°mo/1°F1 mice, born to two dams, 55 (23+32) anti-phOx antibody-secreting hybridomas were produced. Forty-two (14+28) of them secreted sufficient amounts of antibody for further analysis. Typing with specific monoclonal anti-IdOx1 antibodies (*Lange* et al., 1999) revealed that 23 (9+14) of them, corresponding to about 55% (64% and 50%), were non-Id_{Ox1} (Table 1). This proportion corresponded to the content of non-Id_{Ox1} observed in the sera of these mice (Figure 4, lane 5, circled dots). The relative affinities of these antibodies were measured with a hapten binding inhibition test (Table 1) and compared with that of the prototype IgG-Id_{Ox1} NQ2/16.2 (Kaartinen et al., 1983b). Two randomly chosen Id_{Ox1}^{+} antibodies from one of the two mice (group 1 antibodies JL2/24-22 and JL2/26-14) had nearly identical affinities as mAb NQ2/16.2 (Table 1). Twelve (52%) of the 23 non-Id_{Ox1} antibodies from our 3°mo1°F1 mice exhibited enhanced affinities in comparison to non-Id_{Ox1} antibodies in normal mice (*Pelkonen* et al., 1986). Compared to the IgG-Id_{Ox1} NQ2/16.2 (Table 1), the relative affinities of group 2 antibodies (n=5, ~22%, JL1/1-1, JL1/1-10, JL1/13-8, JL2/18-7 and JL2/19-3) were even 7-25-times higher, and those of group 3 antibodies (n=7, ~30%; JL1/1-17, JL1/3-10, JL1/11-7, JL2/19-6, JL2/27-13, JL2/28-18, JL2/29-3) were in the same range as the IgG-Id_{Ox1} NQ2/16.2 (factors of 0.9-1.9). Another group of non-Id_{Ox1} antibodies (n=11, ~48%) exhibited considerably lower affinities than the IgG-Id_{Ox1} NQ2/16.2. Group 4 antibodies JL1/14-22, JL2/18-

1 and JL2/25-5 (Table 1) belong to this group and exhibited 4-10-times lower affinities (factors of 0.152 to 0.28) than mAb NQ2/16.2. In normal mice, the Id_{Ox1}-negative antibodies of the primary anti-phOx response are of 10-100-times lower affinity than the dominant Id_{Ox1} antibodies (*Pelkonen* et al., 1986). Hence, the enhanced affinities of primary non-Id_{Ox1} antibodies in the offspring of tertiary immunised dams seem to indicate that this maternal pre-immunisation is able to select a hitherto unknown B cell population to the pre-immune repertoire which can, in contrast to normal mice, be activated by the thymus-dependent hapten-carrier complex phOx-CSA.

Variable region sequences of non-Id_{Ox1} antibodies in the offspring of tertiary immunised dams

Since such high affinities of primary day 7 anti-phOx antibodies have so far not been observed for Id_{Ox1}-negative antibodies, the V region sequences of these antibodies were determined. They are depicted in Table 1 and have been described in Lange et al. (1999). The key features of the expressed genes can be summarised as follows:

(1) *None of the non-Id_{Ox1} antibodies derived from Id_{Ox1} antibodies by somatic mutations.*

Although some antibodies of groups 2 and 3 in Table 1 are encoded by the Id_{Ox1} genes V_κOxl (JL1/13-8, JL2/18-7, JL2/19-3) and/or the V_HOxl gene (JL2/19-6, JL2/18-7, JL2/27-13, JL2/28-18) they were idiotypically Id_{Ox1}-negative and the coding variable genes contained no somatic mutations which were identical to known affinity-increasing substitutions. We take this as an indication that maternally derived antigen phOx-CSA is not the driving force for the early activation of these antibodies. This view is corroborated by the fact that these antibodies were established from a suppressed primary response (see Figure 2) which showed in no way typical secondary kinetics.

(2) *Non-Id_{Ox1} of the early primary response in maternally-manipulated F1 mice may be encoded by V_H and V_L genes and combinations thereof which are typical of memory responses in normal mice.*

Some of the non-Id_{Ox1} from the early primary response of 3°mo/F1 mice were encoded by variable region genes which previously have been found in memory responses of normal mice:

a. The V_H of JL2/19-3 has previously been observed in the secondary antibody NQ10/12.5 (*Berek* et al., 1985).
b. The V_H of JL1/13-8 was also expressed in the tertiary anti-phOx antibody NQ22/56.1 (*Berek* and *Milstein*, 1987; *Berek* et al., 1987).
c. and d. Both antibodies JL1/11-7 and JL2/29-3 were encoded by V_H6 U21563 which has already been observed in the secondary antibody NQ10/2.12.4 (*Berek* et al., 1985).
e. Group 3 antibody JL2/28-18 is encoded by unmutated V_κ8 and V_HOxl genes. The V_κ8 gene was also expressed by one of our quaternary anti-phOx antibody, however, in connection with a highly mutated V_HOxl (Table 1). Hence, the unmutated V_HOx1/V_κ8 germline configuration is the fifth example of an early appearance of a gene combination which in normal mice was first observed during a memory response.

(3) *Non-Id_{Ox1} may be encoded by new germline genes.*

The V_κ1 gene coding for antibody JL2/19-6 probably represents a new germline gene.

(4) *Early primary non-I_{Ox1} may be encoded by known germline genes hitherto unknown in the anti-phOx response of normal BALB/c mice.*

Some of the primary day 7 antibodies in the offspring of tertiary immunised

dams expressed V_L genes and V_H/V_L gene combinations which have so far not been described for any phase of the immune maturation in normal mice. The V_κ45.1 of the V_κ1 family has so far only been found in an essentially unmutated form in normal mice during the anti-phOx response. In contrast, three non-Id_{Ox1} of group 3 are encoded by V_L germline genes of the V_κ1 family which are new for the anti-phOx response, i.e. JL1/1-17 and JL2/27-13 by the V_κ1 gene M28131 and, as mentioned above, the V_κ1 gene of antibodies JL2/ 19-6 probably represents a new germline gene. Moreover, the V_κ2 gene coding for the high affinity antibody JL1/1-10 has also not been observed during any phase of the immune maturation in normal mice. In contrast, group 4 non-Id_{Ox1} antibodies exhibited affinities in the range of non-Id_{Ox1} antibodies from normal mice (*Pelkonen* et al., 1986). Three members of this group have been sequenced (JL1/14-22, JL2/18-1 and JL2/25-5 in Table 1) and were found to be encoded by the V_L genes $V_\kappa$ars, V_κ45.1 and V_κ8 respectively which have also been found in normal BALB/c mice 7 days after primary immunisation (*Kaartinen* et al., 1986).

(5) *Non-Id_{Ox1} may be encoded new V_H/V_L combinations from different gene families*.

Moreover, in the offspring of tertiary immunised dams (3°mo/1°F1), some of the early primary non-Id_{Ox1} with higher (group 2) and equal (group 3) affinities as Id_{Ox1} antibodies were encoded by new V_H/V_L gene combinations. In normal mice, the V_HOxl (V_H2 gene family) is associated either with V_κOxl (V_L4/5 family) or $V_\kappa$ars (V_L10 family) (*Berek* et al., 1987), whereas in 3°mo/1°F1 mice two new combinations were found: In antibody JL2/27-13 the V_HOxl gene is associated with a member of the V_κ1 family (M28131) and in antibody JL2/28-18 with a member of the V_κ8 family (M34616) (Table 1). Id_{Ox1}-negative antibodies in normal BALB/c which express the V_HOxl/$V_\kappa$ars gene combination have lower affinities than the Id_{Ox1} NQ2/16.2 (V_HOx1 / V_κOxl). In contrast, the two antibodies from 3°mo/1°F1 mice have either an equal (JL2/27-13 with V_HOxl/V_κ1) or even a slightly higher affinity (JL2/28-18 with V_HOxl/V_κ8) than the IgG-Id_{Ox1} antibody NQ2/16.2 (Table 1). The Id_{Ox1}-negative antibodies JL2/25-5 (Table 1, group 4) and JL1/1-10 (Table 1, group 2) provide even more impressive examples for an affinity enhancement by new gene combinations. Antibody JL2/25-5 is encoded by a V_H1 gene in association with a V_κ8 gene which is also known from the anti-phOx response in normal mice, while the V_H1 of mAb JL1/1-10 is associated with a V_κ2 gene. This gene combination confers a 56-times higher affinity than the "normal" combination V_H1/V_κ8 in JL2/25-5. Hence, among other reasons, the higher affinities of a considerable proportion of non-Id_{Ox1} antibodies in the offspring of tertiary immunised dams may also be due to a selection of new V_H/V_L gene combinations.

Does maternally mediated immunomodulation activate the natural IgM pool?

In the offspring of secondarily immunised BALB/c dams (2°mo), maternal IgG antibodies to the hapten phOx and the carrier CSA could be detected up to an age of about 4 months (*Lemke* et al., 1994). In the sera of these F1 mice (n=20), phOx-specific antibodies were further tested for up to 8 months and it became evident that anti-phOx titres increased again. At an age of 8 months, all animals exhibited titres which were higher than in a control group of normal mice born to non-im-

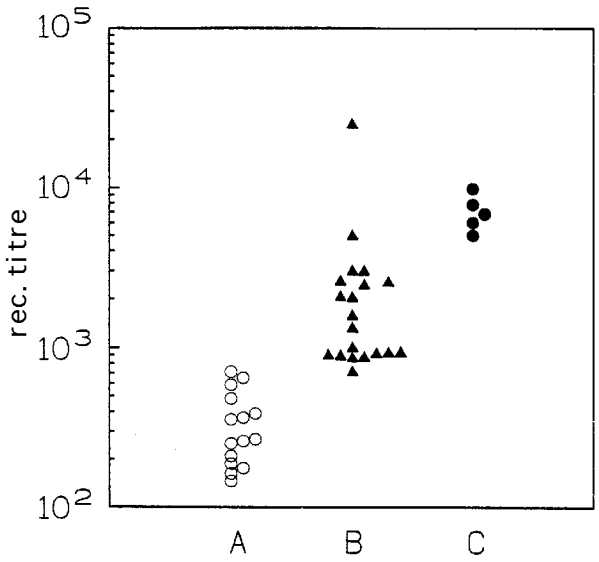

Figure 5: Comparison of anti-phOx IgM serum titers in (A) normal non-immunized BALB/c mice (8 months old, n = 15), (B) non-immunized F1-generation offspring (8 months old, n = 20) born to secondarily immunized dams and (C) normal BALB/c mice (n = 5) on day 78 after primary immunization with phOx-CSA. [Reprinted from *Lemke* et al. (1994), with permission].

munised dams (Figure 5A, n=15, $p<0.001$) and these anti-phOx antibodies turned out to be of the IgM class (Figure 5B) . As a control, the level of IgM anti-phOx serum titres in a group of normal BALB/c mice (n=5) 7 days after primary immunisation with phOx-CSA is also shown (Figure 5C). Since the production of IgM anti-phOx in the offspring of secondarily immunised dams was not followed by switch to IgG antibodies, it may be asked whether the enhanced level of IgM indicate an activation of natural antibodies.

DISCUSSION

Isotype regulation by maternal IgG antibodies

Earlier experiments in the rat had already provided solid evidence that maternally derived IgG antibodies have the capacity to suppress an IgE response in the offspring (*Jarrett* and *Hall*, 1983) and our own data confirm this conclusion (see above and *Lemke* et al., 1994). Moreover, it could be demonstrated that maternally-derived exogenous monoclonal IgG antibodies, i.e. without participation of antigen, suppressed the IgE response to PLA_2 in CBA mice to the same extent as does an active immunisation of the dams. Strikingly, the transfer of a *one* single IgG-anti-PLA_2 was as effective as a mixture of ten of these antibodies or an active immunisation of the mothers. It is highly unlikely that one IgG mAb can cover the whole range of antigenic determinants on PLA_2. Moreover, it has been shown that IgE- and IgG-anti-PLA_2 antibodies recognise non-cross-reactive determinants when induced by immunisation with MD doses of PLA_2 (*Kolbe* et al., 1995). Therefore, it can be concluded that maternally derived IgG antibodies do not act by simply masking antigenic determinants of PLA_2. This is supported by experiments

which demonstrated that mAb MS.613 did *not* inhibit the binding of a monoclonal IgE-anti-PLA_2 to its plastic-bound antigen (data not shown). Moreover, it was tested whether PLA_2 which was injected at high concentration (100 μg) into pregnant CBA/J female mice together with a IgG-anti-PLA_2 mAb (300 μg) could be transferred to the offspring. However, neither free PLA_2 nor a primary anti-PLA_2 immune response could be detected in F1-generation mice, thus allowing the conclusion that neither free nor antibody-bound PLA_2 antigen was transferred from the dams to the offspring. These results are in line (a) with those of *Jarrett* and *Hall* (1979) who observed that treatment of the dams with ovalbumin alone (in the absence of adjuvant could in no way influence a subsequent IgE response of the offspring, and (b) with the finding that also immune complexes are not transferred to the foetus via the placenta (*Wood*, 1994). The data obtained in rats (*Jarrett* and *Hall*, 1986) and in the mouse (*Seeger* et al., 1998) demand for an investigation of possibility to suppress IgE responsiveness by maternally derived IgG antibodies in humans (*Jarrett*, 1984).

Since it has been assumed that decisive events for the development of allergy or asthma in man occur early in life (*Holt*, 1994, 1995; *de Weck* et al., 1995), the conclusion may be valid that a quantitatively or qualitatively insufficient transfer of maternal IgG antibodies to the babies may be at least one possible cause. This in turn would favour the idea that mothers should experience as many environmental allergens as possible and at concentrations which stimulate strong IgG responses. This view is in line with the conception that deficits in immunological stimulations, in particular by T_H1-stimulating microbial infections, is one possible cause for the increase in atopic allergies in highly developed countries (*Rook* and *Stanford*, 1998) and may as well have been decisive for the differential development in allergic and asthmatic diseases in East and West Germany (*Wichmann*, 1996).

Maternally modulated kinetics of primary immune responses

Our investigations started from the idea that maternal high-quality immunological experience would induce beneficial effects in the immune system of the new-borns. However, it was not clear as to how such effects could be detected. Therefore, we chose the well-studied anti-hapten immune response of BALB/c mice to the hapten-carrier conjugate phOx-CSA and expected to see alterations of any of the characteristic features of this response which have been summarised in *Lange* et al. (1999). The analysis of the primary response to phOx-CSA at an age when no maternal antibodies could be detected above normal background levels seemed to support our hypothesis: (a) In the F1 offspring of primarily immunised dams, the primary response to phOx as well as to CSA developed faster and this also held true for the anti-CSA response in F2-generation mice (Figures 3A and B). (b) In the offspring of 2°mo, the primary responses to phOx and CSA were delayed, but reached maximal levels of normal mice. In 3°mo/1°F1 mice, however, both responses were not only delayed, but remained at lower levels. (c) A striking results was the observation that about half of the F2-generation mice born to non-immunised F1 mice developed a continuing or a second increase during the late phases of the primary anti-phOx as well as anti-CSA responses. Especially the latter two observations that the maternal immunisation even showed early and late immuno-modulatory effects in the F2 generation argue against the possibility that antigen transferred from the grandmothers via the F1 females to the F2 offspring might be responsible.

A particularly interesting point is the difference in primary responses of F1 mice born to 3°mo and their F2-generation offspring. We are inclined to regard the suppressed response in the F1 animals to indicate a *supra*optimal stimulation, whereas the F2 mice may have received a nearly optimal stimulation after an appropriate dilution of the relevant antibodies in the F1 mice. This may be related to the observation that anti-idiotypic antibodies may enhance the corresponding idiotype at low doses, but suppress it at higher doses (*Kelsoe* et al., 1981).

Maternally induced alteration of the primary repertoire

The idiotypic analysis of the early day 7 primary immune response in F1- and F2-generation mice indicated that high-affinity antibodies of tertiary or quaternary anti-phOx responses are able to alter the clonal composition of the available phOx-reactive repertoire (Figure 4) (*Lange* et al., 1999). Since the tertiary immune and the quaternary monoclonal anti-phOx antibodies which were transferred from the dams to the offspring, exhibited an extremely weak cross-reactivity with the dominant Id_{Ox1} of the primary response (*Lange* et al., 1999), the question arose as to how antibodies from the late stages of immune maturation could interfere with the establishment of the primary repertoire. The genetic analysis revealed that the maternal influence caused a strong affinity enhancement in the non-Ox1 idiotypic antibody repertoire (Table 1) and it seems unquestionable that this affinity increase is *not* induced by antigen which might have been transferred from the dams to the offspring. This view is based on the fact that no known affinity increasing somatic mutations could be observed in the primary anti-phOx antibodies of 3°mo/1°F1 mice. This finding seems to contrast with the idea that idiotype suppression might be important for the selection of "*somatic antibody mutants . . . through suppression of the wild type*" which might operate already "*in the pre-immune repertoire, generating mutants before contact with antigen*" (*Cumano* and *Rajewsky*, 1986). If this mechanism is not responsible for the altered expression of the major Id_{Ox1} in the offspring of maternally manipulated dams, it has to be asked how else this outcome may be induced by the extremely weak cross-reactivity of high-affinity quaternary anti-phOx antibodies with the Id_{Ox1}. Two possible mechanism may be envisaged. The first rests on investigations performed by Klinman and co-workers (*Linton* et al., 1989; *Klinman*, 1996) who observed that naive and memory B cell progenitors participating either in the primary or memory responses respectively, can have different requirements for activation. Hence, it is conceivable that maternal antibodies might induce a pre-activation of the B cell clones in the memory compartment so enabling them to participate already in the primary response. This possibility may be indicated by our finding that some of our non-Id_{Ox1} antibodies from 3°mo/1°F1 mice were encoded by V(D)J gene combinations which in normal mice have been found during secondary to quaternary memory responses. Secondly, it has been demonstrated that the specificity of the membrane-bound immunoglobulin may be altered by receptor editing via successive recombinations (*Levy* et al., 1989; *Radic* and *Zouali*, 1996). It is conceivable that maternal antibodies might influence the establishment of the B cell repertoire by interference with the association of productive V_L and V_H gene recombinations. In the present context it is especially intriguing that an editing of the B cell antigen receptor can be induced by anti-idiotypic antibodies (*Levy* et al., 1989, 1998; *Hertz* and *Nemazee*, 1997) and that this process may be induced by

Table 1: Idiotype, affinity and VL- and VH-gene expression of anti-phenyloxazolone antibodies from maternally manipulated mice.

Clone[a]	Group[b]	Id_{Ox1}[c]	relative affinity[d]	affinity factor[e]	V_L-gene[f]	J_κ[g]	V_v-gene[f]	J_H[g]
NQ2/16.2	1	+	7.0×10^{-3}	1	$V_\kappa4$ (V_κOx1) Z22128*[h]	5*	V_H2 (V_HOx1) K00724*	3*
JL2/24-22	1	+	8.6×10^{-3}	0,81	$V_\kappa4$ (V_κOx1) Z22128*	5*	V_H2 (V_HOx1) K00724*	3*
JL2/26-14	1	+	8.3×10^{-3}	0,84	$V_\kappa4$ (V_κOx1) Z22128*	5*	V_H2 (V_HOx1) K00724*	3*
JL1/1-1	2	–	1.0×10^{-3}	7	n.d.	n.d.	n.d.	n.d.
JL1/1-10	2	–	4.8×10^{-4}	14.6	$V_\kappa2$ M34622*	5*	V_H1 S73918*	2*
JL1/13-8	2	–	2.8×10^{-4}	25	$V_\kappa4$ (V_κOx1) Z22128	5*	V_H1 M36225	4*
JL2/18-7	2	–	4.8×10^{-4}	14.6	$V_\kappa4$ (V_κOx1) Z22128*	5*	V_H2 (V_HOx1) K00724*	4*
JL2/19-3	2	–	3.8×10^{-4}	18	$V_\kappa4$ (V_κOx1) Z22128*	5*	V_H5 U04229*	3
JL1/1-17	3	–	7.7×10^{-3}	0.9	$V_\kappa1$ M28131*	1*	V_H1 M15224*	4
JL1/3-10	3	–	5.3×10^{-3}	1.3	$V_\kappa1$ (V_κ45.1) X66640	2	V_H1 Z73349*	4*
JL1/11-7	3	–	7.4×10^{-3}	0.9	$V_\kappa1$ (V_κ45.1) X66640*	5*	V_H6 U21563*	3*
JL2/19-6	3	–	6.6×10^{-3}	1.06	$V_\kappa1$ M28131, new germline gene[i]	2	V_H2 (V_HOx1) K00724*	3*
JL2/27-13	3	–	6.6×10^{-3}	1.06	$V_\kappa1$ M28131*	1*	V_H2 (V_HOx1) K00724*	3*
JL2/28-18	3	–	3.6×10^{-3}	1.94	$V_\kappa8$ M34616*	5*	V_H2 (V_HOx1) K00724*	4*
JL2/29-3	3	–	4.5×10^{-3}	1.55	$V_\kappa1$ (V_κ45.1) X66640	1*	V_H6 U21563*	3*
JL1/14-22	4	–	4.6×10^{-2}	0.152	$V_\kappa10$ M54905*	1	V_H1 M15224*	4
JL2/18-1	4	–	4.3×10^{-2}	0.163	$V_\kappa1$ (V_κ45.1) X66640	2*	V_H1 S73918*	3
JL2/25-5	4	–	2.5×10^{-2}	0.28	$V_\kappa8$ M34616*	5*	V_H1 S73918*	3

ultralow affinity ligands (*Lang* et al., 1996). Hence, it seems possible that our high affinity tertiary or quaternary antibodies which are extremely weakly cross-reactive with Id_{Ox1} antibodies might activate network components which in turn interfere with the normal regulation of the major Id_{Ox1}. Moreover, an activation of the receptor editing can be recognised by L-chain rearrangements (*Hertz* and *Nemazee*, 1997), an observation which may well be related to our finding that the L-chain repertoire of the anti-phOx response is considerably increased in F1 mice born to immunised dams (see above). Thirdly, another striking observation was the induction of IgM anti-phOx antibodies in non-immunised offspring of secondarily immunised dams. Since the production of these IgM antibodies was not followed by a switch to IgG, it is tempting to speculate that those IgM belong to the pool of natural antibodies. This possibility is under current investigation by analysing the phOx-reactive repertoire of $CD5^+$ B lymphocytes. The induction of IgM antibodies by maternal influence has been confirmed in the anti-arsonate response by showing that maternal Ab1 induced IgM anti-arsonate antibodies in the offspring without immunisation of the F1 animals (*Ismaili* et al., 1995).

Finally, it may be questioned whether the observed beneficial effects of a maternal immunisation for the development of immune responsiveness in the offspring may also be of relevance for far back dating immunological experiences of the mother and not only for immune responses which happen shortly before or during pregnancy. In this respect is important to consider recent investigations which have demonstrated that memory of the B cell compartment does not only depend on memory cells, but also on long-lived plasma cells in the bone marrow (*Manz* et al., 1997). Importantly, the survival of these long lived plasma cells is independent of antigen (*Manz* et al., 1998). Hence, it

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a: All antibodies from the JL1- and JL2-fusions were of the γ1/κ isotype.

b: The antibodies have been arranged into groups according to their Id_{Ox1} expression and affinity compared to the reference antibody NQ2/16.2 (6, 39, 41).
Group 1: Id_{Ox1}^+ with affinities as the NQ2/16.2; group 2: *non*-Id_{Ox1} with higher affinities; group 3: *non*-Id_{Ox1} with affinities as NQ2/16.2; group 4: *non*-Id_{Ox1} with lower affinities.

c: The idiotyping of the antibodies has been performed with Id_{Ox1}-specific monoclonal anti-bodies 8-21/W18 and 6-8/R20 as described (64); see also legend to Figure 1.

d: The indicated values represent the dilutions of a 0,0035 M stock solution of phOx-cap which inhibited the binding of pre-determined identical amounts of anti-phOx antibodies to solid phase-bound phOx bovine serum albumin by 50%.

e: The affinity factor indicates the quotient of the relative affinity of NQ2/16.2 divided by that of a particular antibody, i.e. antibodies with an affinity factor of >l exhibited a higher affinity while those with an affinity factor of <l have a lower affinity than NQ2/16.2.

f: The analysis of the light and heavy chain variable genes to gene families has been performed with computer programs (see section Materials and Methods) and according to *Dildrop* (1986). When possible, the trivial names of genes are indicated in brackets.

g: The J gene segments of the L- and H-chain are indicated by numbers.

h: The asterisk (*) indicates an identical known germline gene indicated by its reference number of the European Bioinformatic Institute (EBI). An EBI number without an asterisk indicates the next related, but *not* identical germline gene.

i: The appraisal as a new germline gene relies on the sequence comparisons, but is not proven at the genomic level.

n.d.: not determined

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can be assumed that a large proportion of serum IgG antibodies is the product of these bone marrow plasma cells and can be transferred to the offspring for a long period of time. This favours the idea that maternal antibodies really represent a large proportion of the entire immunological experience of the mother and have the potential to guide the education of the neonatal immune system.

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