Prevention of Th2-mediated murine allergic airways disease by soluble antigen administration in the neonate

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ABSTRACT It has been demonstrated recently that neonatal antigen administration in the mouse can lead to priming for Th2-mediated immune responses. This observation has important implications for the development of vaccination strategies in humans, particularly for individuals who may be predisposed to atopy or asthma. In this paper it is shown that although i.p. administration of antigen (100 μg) in adjuvant to the neonate does indeed prime for Th2-mediated disease in mice [allergic airways disease (AAD)], when the same relatively low dose of antigen is given in soluble form no priming occurs. Further, administration of a larger dose of soluble antigen (1 mg) actually prevents the ability to prime for a Th2 response subsequently and so prevents the induction of AAD. Protection from disease was associated with evidence of functional inactivation of both Th1 and Th2 ovalbumin-specific T cells. In contrast, administration of a very low dose of antigen (10 μg) primed for a Th2 response in a similar fashion to antigen in adjuvant. We suggest that the adjuvant lowers the "effective" dose of antigen administered in the neonate and thereby primes for Th2-type immune responses. These findings demonstrate that neonatal antigen administration can inhibit Th2-mediated diseases, such as AAD, but the dose of antigen may be critical to avoid predisposition to disease.

In classical experiments, Medawar and coworkers (1) demonstrated in 1953 that neonatal mice become tolerant to foreign cells rather than rejecting them as observed in adult mice. This observation led to the concept that the perinatal period is immunologically privileged so that non-self is recognized as if it were self (2–7). Various hypotheses have been proposed and tested in attempts to delineate the mechanism of this phenomenon. Initially, it was thought that self-reactive cells encountering antigen in the perinatal period were deleted in the central lymphoid organs (8). However, in the majority of these early experiments, Th1-type immune responses regulating allograft rejection or survival were used as indicators of the induction of tolerance.

The absence of data regarding the role of Th2 immune responses in the development of tolerance led investigators to speculate that protective immunity induced by antigen exposure in the neonate may in fact reflect skewing of the immune response away from a Th1 toward a Th2 phenotype. This hypothesis was recently tested by a number of investigators (9, 10). These studies provided evidence that Th2 responses were indeed primed in neonatal mice previously thought to be neonatally tolerized. Sarzotti *et al.* (9) demonstrated that perinatal injection of mice with high dose Cas-Br-M murine leukemia virus led to neurological disease, characterized by the

absence of a virus specific cytotoxic T lymphocyte response and of interferon-γ (IFN-γ) production from adult splenocytes stimulated *in vitro* (9). Furthermore, adult splenocytes from these mice were capable of producing interleukin 4 (IL-4) when stimulated *in vitro*, suggesting that high amounts of virus given in the neonatal period lead to deviation of the anti-viral immune response from Th1 to Th2 rather than inducing T cell tolerance. In other studies neonatal mice given antigen with incomplete Freund's adjuvant (IFA) subsequently failed to mount a Th1 response to that antigen, even when the subsequent dose was given in the strong Th1 adjuvant CFA (complete Freund's adjuvant) (10). Serum from mice tolerized by this protocol contained antigen specific Ig of the IgG1 class, but not of the IgG2a or IgM classes. Furthermore, splenocytes from these neonatally tolerized mice secreted IL-5, but not IFN-γ in response to recall antigen, confirming the induction of a Th2 immune response.

Collectively, these results have profound implications for neonatal vaccination in humans and underscore the potential risks for the induction or exacerbation Th2-mediated diseases. Notably, if antigen administration in the neonate primes for Th2 immune responses, then current neonatal vaccination strategies need to be reassessed for individuals who are predisposed to atopy and/or asthma. In the present investigation we have used a murine model of allergic airways disease (AAD), as a very sensitive indicator of Th2-mediated responses, to specifically determine the impact of neonatal antigen administration on the development of a defined immunopathological process. Administration of 50 μg ovalbumin (OVA)/alum to adult mice has previously been shown to predispose to allergic Th2-type responses in the airways on subsequent aeroallergen challenge with this antigen (11). In response to aeroallergen challenge mice develop pronounced AAD that is characterized by blood and airways eosinophilia, pathological changes to the respiratory epithelium, airways hyperreactivity to β-methacholine, and the production of OVA-specific IgE and IgG1. Some of these features of AAD are also hallmarks of clinical asthma. The airways eosinophilia is IL-5-dependent as mice treated with antibody to IL-5 or deficient in this cytokine fail to develop eosinophilia. Furthermore, adoptive transfer of IL-4/IL-5-producing CD4$^+$ T cells to IL-5-deficient mice restores the eosinophilia and AAD (12).

METHODS

Antigen Treatment of Mice. BALB/c neonates were treated within 24 h of birth with OVA administered by i.p. injection in

50 μl PBS. In some neonates OVA was administered i.p. in an IFA emulsion. Controls received PBS alone. OVA was administered to groups of 4-week-old mice by one of three regimes; first mice were sensitized with OVA (50 μg) in 1 mg alum (alhydrogel) adjuvant (CSL Ltd., Parkville, Australia) by i.p. injection and 12 days later aerosolized with OVA (10 mg/ml) in 0.9% saline for a period of 30 min three times at hourly intervals on every second day for 8 days. Second, neonatally OVA-treated mice received the aerosol regime at 4 weeks of age without prior systemic sensitization. Mice were assessed for evidence of AAD 24 h after the last aerosol. Third, neonatally OVA or PBS-treated mice were immunized at 4 weeks of age with OVA (50 μg) in CFA in the tailbase, and 12 days later cellular and humoral immune responses were assessed. In each case PBS was used as a control.

Aerosolization of OVA treated mice with an irrelevant antigen (BSA) did not induce AAD (data not shown).

Analysis of Lung Histology, Peripheral Blood Leukocytes, and Bronchoalveolar Lavage Fluid (BALF). Specimens of lung tissue representing the central (bronchi-bronchiole) and peripheral (alveoli) airways were fixed in 10% phosphate-buffered formalin, sectioned, and stained with May–Grunwald–Giemsa solution or haematoxylin/eosin. Leukocytes in the blood, BALF, and lung tissue were identified by morphological criteria and quantified as described (11).

Measurement of Airways Hyperreactivity. Airways constriction was measured with a bronchospasm transducer (Ugo Basil 7020) that was coupled to a Lab Mac/8 computer (Ad Instruments, Sydney, Australia) and analyses were performed during cumulative i.v. administration of β-methacholine as described (11). The increase in respiratory overflow volume provoked by β-methacholine was represented as a percentage of the maximal overflow volume (100%) obtained by totally occluding the tracheal cannula.

Measurement of Serum OVA-Specific Antibody Levels by ELISA. Anti-OVA antibody titers were measured by isotype-specific ELISA utilizing mouse Ig isotype specific secondary antibodies (Caltag, South San Francisco, CA) (13, 14).

Cytokine Production by Cultured Splenocytes or Lymph Node Cells. Lymphocyte suspensions were prepared from spleens or lymph nodes. Lymphocytes (2×10^6 cells/well) were incubated in RPMI 1640 medium/10% fetal calf serum (200 μl) in 96-well microtiter plates and stimulated for 72 h with OVA, purified protein derivative of mycobacterium tuberculosis (50 μg/ml) (CSL Ltd.), or anti-CD3 (5 μg/ml). [^{3}H]Thymidine incorporation was determined in a scintillation counter after 6 h.

Cytokine Assays. IFN-γ and IL-5 concentrations in culture supernatants were determined by ELISA as described (13, 15). IL-4 was determined by using a bio-assay with CTS-4 cells (13).

Statistical Analysis. The significance of differences between experimental groups was analyzed by using the unpaired Student's *t* test. Differences in experimental groups were considered significant if $P < 0.05$.

RESULTS

Administration of High Dose Soluble OVA (1 mg) to Neonates Resulted in Decreased Th1 Immune Responses. To test whether high dose OVA treatment at birth would result in a decreased Th1 immune response in the adult, mice were treated at birth with either 1 mg OVA or PBS then immunized at 4 weeks of age with OVA emulsified in the Th1 adjuvant, CFA. OVA-treated neonates had decreased levels of the Th1 dependent antibody subclass IgG2a compared with PBS-treated controls (Fig. 1). Furthermore, a significant reduction in proliferation and IFN-γ production was observed in OVA-stimulated splenocytes taken from CFA/OVA immunized mice as compared with neonatally PBS-treated controls (Fig. 1).
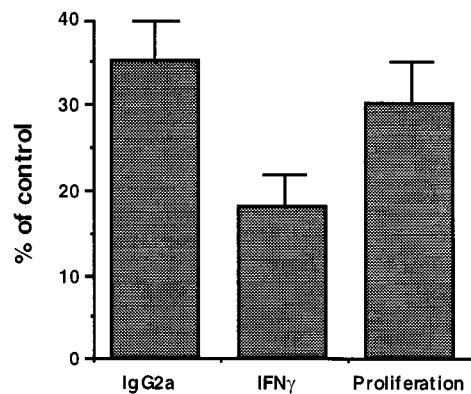

FIG. 1.   Th1 immune responsiveness from mice treated with OVA at birth after immunization with OVA (50 μg)/CFA. Serum OVA-specific IgG2a levels from OVA-treated mice were reduced to 35% of serum levels from PBS treated control mice ($P < 0.05$, $n = 5$). IFN-γ levels produced by spleen cells from mice treated with OVA were reduced to 18% of the level produced by spleen cells from PBS-treated controls ($P < 0.05$, $n = 5$). Proliferation of splenocytes from OVA-treated mice was reduced to 31% in comparison to PBS treated controls ($P < 0.05$, $n = 3$).

Administration of High Dose Soluble OVA (1 mg) to Neonates also Resulted in Decreased Th2 Immune Responses. Based on the results of Sarzotti *et al.* (9) and Forsthuber *et al.* (10) we next determined whether the reduction in Th1 responsiveness resulting from high dose OVA treatment at birth could be explained by deviation to a Th2 immune response. Initially, Th2 immune responses to OVA were measured in the adult after immunization at 4 weeks of age with OVA/CFA. Neonatally OVA-treated mice that had been immunized with OVA/CFA had significantly decreased levels of the Th2 dependent serum antibody IgG1, compared with neonatally PBS-treated controls (data not shown). Furthermore, isolated splenocytes from CFA/OVA immunized mice that had been OVA treated neonatally had reduced proliferative responses and IL-5 production when stimulated with OVA compared with splenocytes from PBS-treated neonates (data not shown). Because our results did not show immune deviation to a Th2 response as recently reported, OVA-treated neonates were immunized at 4 weeks with OVA in the Th2 adjuvant alum and were subsequently OVA aerosolized in an attempt to induce Th2-driven AAD. However, even this stimulus failed to induce antigen-specific Th2 responses. The levels of total IgG and IgG1 isotype antibodies against OVA after OVA/alum and OVA-aerosol challenge were lower in the serum from neonatally OVA-treated mice compared with mice given PBS at birth (Fig. 2). Furthermore, OVA-specific IgE was not detected in OVA-treated neonates in response to subsequent injection with OVA and aeroallergen challenge (data not shown). IL-4 and IL-5 production by cultured bronchial lymph node and spleen cells taken from OVA/alum immunized and aerosol challenged mice treated with OVA at birth was significantly decreased when compared with neonatally PBS-treated control mice [Fig. 3 and IL-4 (data not shown)]. Splenocytes from neonatally OVA-treated mice also had reduced OVA-stimulated proliferation compared with neonatally PBS-treated mice (Fig. 4). Contrary to reported findings (9, 10), our results show that neonatally OVA-treated mice not only had decreased Th1 immune responses, but also decreased Th2 immune responses to recall antigen, even when immunized with a potent Th2 adjuvant.

High Dose Soluble OVA Treatment of Neonatal Mice Resulted in Protection from Th2-Dependent AAD. To further determine the ability of neonatally OVA-treated adult mice to develop Th2 responses, attempts were made to induce AAD. Initially, neonatal mice were given 1 mg or 100 μg of soluble
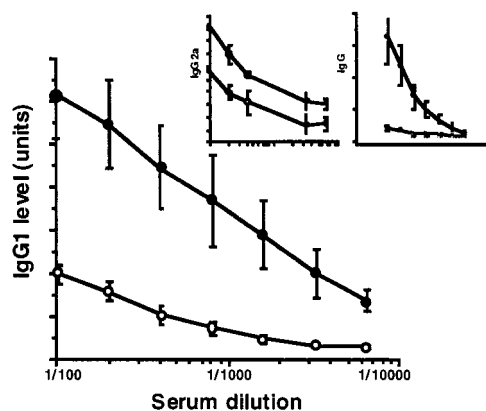
FIG. 2. Titration of serum OVA-specific IgG1. (*Inset*) OVA-specific, IgG2a and total IgG levels from mice treated with PBS or high dose OVA at birth. Serum levels of antibody were determined in PBS (●) or OVA-treated (○) mice following i.p. injections of OVA (50 μg)/alhydrogel at 4 weeks of age and subsequent aeroallergen challenge 12 days later. Titers in PBS-treated mice were at least 32-fold greater than OVA-treated mice. Horizontal axis represent doubling dilutions of serum (log scale) and vertical axis represent units of Ig. ($P < 0.05$, $n = 8$).

OVA and aerosol challenged at 4 weeks. None of these mice developed AAD (Fig. 5a, Table 1). In addition, no antibodies against OVA were present and IL-5 production was not detected in supernatants of antigen-stimulated T cell cultures (results not shown). Mice given these doses were therefore primed with OVA/alum and aerosol to determine if the neonatal antigen had switched off the ability to become primed. Our results show that mice neonatally tolerized with 1 mg of OVA and then exposed to OVA/alum sensitization and aeroallergen challenge, failed to develop AAD (Fig. 5b, Table 1). This failure was accompanied by a reduction in eosinophil numbers in the BALF and blood (Fig. 5b, and blood data not shown). Furthermore, airways reactivity to β-methacholine, a hallmark of AAD, was also significantly attenuated in mice treated with OVA at birth compared with PBS controls (Fig. 5c, Table 1). Consistent with these observations, the morphology of the airways of aeroallergen challenged OVA-treated mice was also normal (Fig. 6). However, mice that were exposed to 100 μg soluble OVA as neonates and then exposed to OVA/alum sensitization and aeroallergen challenge did develop AAD (Fig. 5b, Table 1). Thus delivery of 100 μg soluble OVA to the neonate does not inhibit subsequent
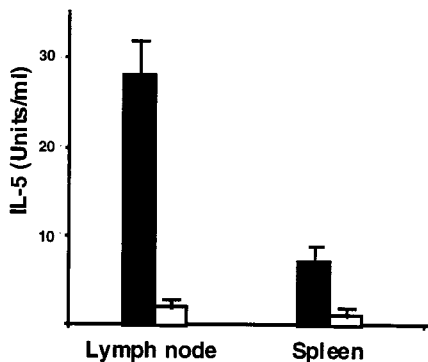

FIG. 3. IL-5 production by cultured bronchial lymph node and spleen cells taken from mice treated with 1 mg OVA at birth and immunized with OVA (50 μg)/alhydrogel at 4 weeks of age then subsequently aerosol challenged 12 days later. Lymph node and spleen cells were stimulated for 72 h with OVA (100 μg/ml), and then IL-5 levels were measured by ELISA. Cells from mice treated at birth with OVA (1 mg) produced significantly less IL-5 than cells from PBS-treated mice (28 vs. 2 units/ml and 7 vs. 1 units/ml, $P < 0.01$, $n = 3$).
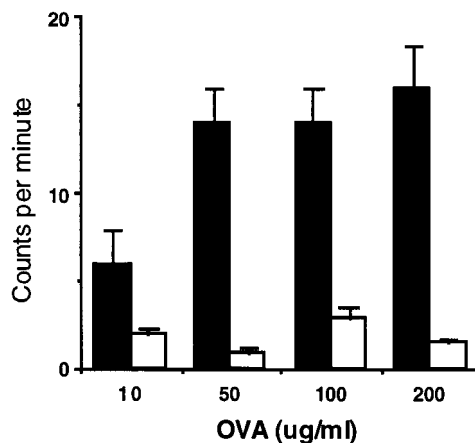

FIG. 4. Proliferation of spleen cells from mice treated with 1 mg OVA at birth and immunized with OVA (50 μg)/alhydrogel at 4 weeks of age then subsequently aerosol challenged 12 days later. Spleen cells were stimulated *in vitro* with OVA for 72 h and then ³H incorporation determined. OVA-specific T cell proliferation was determined as incorporation levels (cpm) above background and normalized to incorporation levels from anti-CD3 stimulated cells. Splenocytes from mice treated neonatally with OVA (open bars) show significantly reduced proliferative ability at all concentrations of OVA compared with splenocytes from PBS-treated control mice ($P < 0.05$, $n = 3$).

systemic priming of the Th2 arm of the immune system as 1 mg OVA does.

OVA/Adjuvant Treatment of Neonatal Mice Resulted in Priming for Th2-Dependent AAD. In contrast to the findings after administration of 1 mg or 100 μg soluble OVA to neonates, administration of 100 μg of OVA in IFA did prime for Th2-dependent AAD. In OVA/IFA treated mice aeroallergen challenge induced a pronounced BALF eosinophilia and high anti-OVA IgG1 titres (>1:12,800, Fig. 5a, Table 1).

DISCUSSION

Neonatal antigen administration has previously been shown to induce antigen-specific unresponsiveness in the adult (2–7, 16–18). Recently, it was demonstrated that in some models of neonatal tolerance, the induction of unresponsiveness was associated with the loss of antigen-specific Th1 cells, but the persistence of primed antigen-specific Th2 cells (9, 19). Thus, immune deviation has been proposed as a mechanism to explain unresponsiveness in the neonatal period. The finding that neonatal antigen administration can prime Th2 immune responses raises important issues for early childhood vaccination strategies, particularly for individuals who are likely to be atopic and/or asthmatic. In contrast to the finding that neonatal antigen/adjuvant administration primes for Th2 immune responses, we provide evidence here that administration of soluble antigen to the neonate can result in unresponsiveness in both the Th1 and Th2 compartments.

Administration of 1 mg OVA to neonatal mice resulted in decreased Th1 and Th2 immune responses following subsequent challenge with OVA in the Th1 adjuvant CFA. Furthermore, even when the immunizing dose was administered in the strong Th2 adjuvant, alum, the neonatally OVA-treated mice were unable to mount a Th2 response to OVA. To further test for low level priming of antigen-specific Th2 responses we attempted to induce AAD in neonatally treated mice. However, in contrast to the findings of Sarzotti *et al.* (9), Forsthuber *et al.* (10), and Sing *et al.* (20), and despite the prediction that the induction of neonatal unresponsiveness may exacerbate Th2-dependent diseases, we found that administration of OVA resulted in a decrease in OVA-specific Th2 immune responses and protection from the development of AAD.
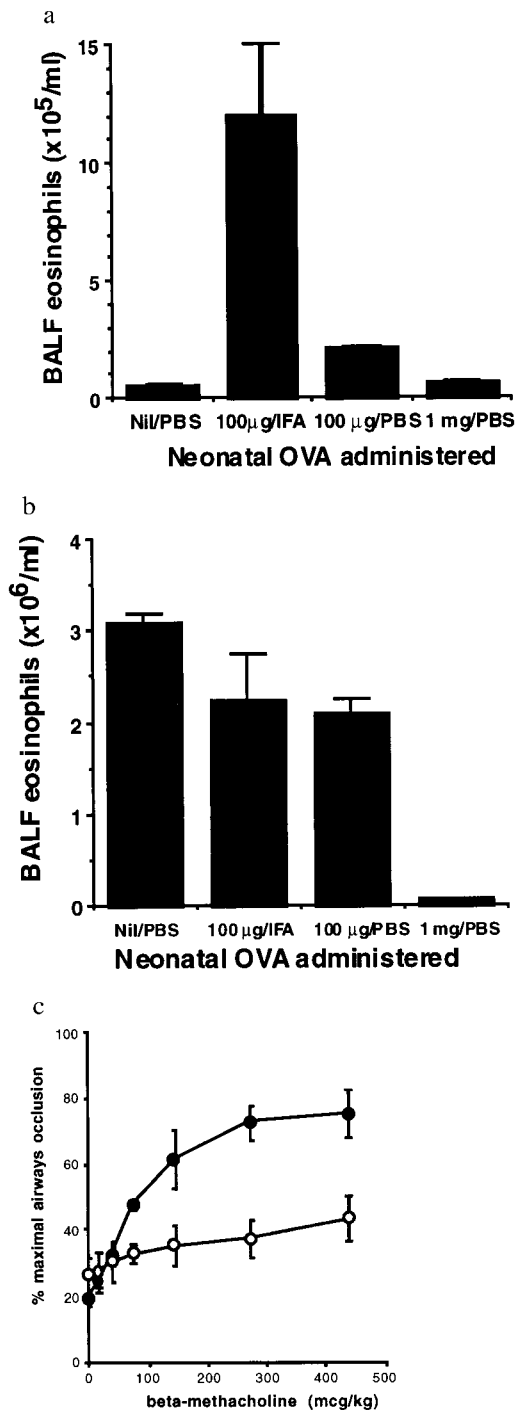
Table 1.   Summary of findings on the effect of neonatal antigen administration on subsequent AAD development

n	Birth	4 weeks	6 weeks	AAD
4	—	—	Aerosol	No
9	—	OVA/alum i.p.	Aerosol	Yes
6	OVA (1 mg)	Aerosol	—	No
9	OVA (1 mg)	OVA/alum i.p.	Aerosol	No
6	OVA (100 μg)	Aerosol	—	No
3	OVA (100 μg)	OVA/alum	Aerosol	Yes
3	OVA (100 μg)/IFA	Aerosol	—	Yes
3	OVA (100 μg)/IFA	OVA/alum	Aerosol	Yes
1	OVA (10 μg)	Aerosol	—	Yes
3	OVA (10 μg)	OVA/alum	Aerosol	Yes

Soluble OVA at doses of 100 μg or 1 mg did not prime for Th2 responses and AAD. Importantly, 1 mg OVA prevented subsequent priming and induction of AAD. In contrast, 100 μg OVA in IFA primed for Th2 responses and subsequent aerosol induced AAD. Interestingly, administration of 10 μg of soluble OVA also primed for a Th2 response and subsequent AAD.

purpose. In 1953 Billingham *et al.* (21) demonstrated that inoculation of newborn mice with tissue clumps, single cells and cell debris from testis, kidney, and spleen, failed to tolerize mice to skin grafts from the same donor strain. However, they subsequently demonstrated that tolerance to allogeneic grafts can be achieved by administration of haemopoetic cells to neonatal mice (1). Recently, Ridge *et al.* (22) have repeated these earlier studies using nonallogeneic haemopoetic cells that differed only in that the donor cells were male and carried the HY antigen (22). They too showed the induction of tolerance to HY antigen as measured by cytotoxicity assays.

FIG. 5.   (*a*) Measurement of eosinophil numbers in the BALF from mice treated with PBS, OVA, or OVA/IFA at birth, and aeroallergen challenged at 4 weeks of age. Eosinophilia was measured after aerosol. (*b*) Measurement of eosinophil numbers in the BALF from mice treated with PBS, OVA, or OVA/IFA at birth. Eosinophilia was measured in mice given PBS, OVA, or OVA/IFA at birth after i.p. injections of OVA/alhydrogel at 4 weeks of age and aeroallergen challenge 12 days later ($P < 0.01$ for 1 mg OVA vs. others, $n = 8$). (*c*) Measurement of airways constriction in response to β-methacholine stimulation after OVA/alum and aeroallergen challenge. Mice were neonatally treated with 1 mg OVA (○) or PBS (●). Results are presented as a percentage of maximal airways occlusion obtained by totally occluding the tracheal cannula ($P < 0.01$, $n = 4$).

To understand the different mechanisms that may be involved in the development of neonatal unresponsiveness, it is important to look at the variation in models used for this
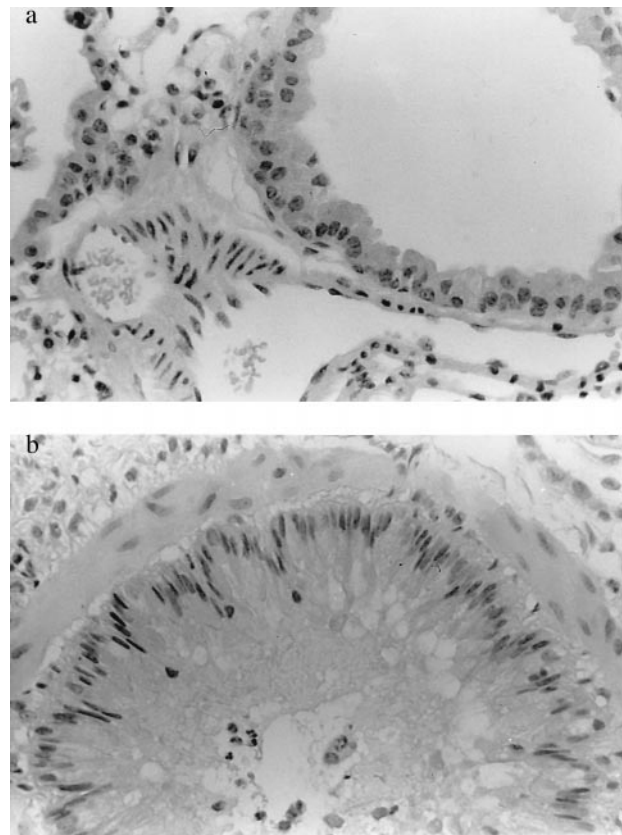

FIG. 6.   Histology. Representative histological appearance of lungs in mice given 1 mg OVA or PBS at birth and sensitized with OVA/alum and OVA aerosol. (*a*) Lungs in mice treated at birth have a normal appearance. (*b*) In contrast, lungs from mice treated with PBS demonstrate AAD characteristics: eosinophilia and parenchymal disruption.

Furthermore, although HY$^+$ splenocytes administered to neonatal females were able to induce tolerance, HY$^+$ dendritic cells induced immunity, arguing strongly that it is the donor cell type and its ability to deliver a second signal that determines the tolerogenic or immunogenic response of the recipient (22).

By using a different model that involved infection of neonatal mice with high doses of Cas-Br-M murine leukemia virus, Sarzotti *et al.* (9) recently showed that induction of unresponsiveness was dependent on the dose of antigen. At low dose a typical Th1 immune response developed in adult mice infected as neonates, and virus infection was resolved, whereas a high dose led to nonresponsiveness and the mice succumbed to infection. Interestingly, in mice that had been treated with high dose virus neonatally, only the Th1, but not the Th2 effector arm of the T cell compartment, was attenuated in response to specific antigen in the adult. Thus, deviation of the immune system to a particular T helper phenotype may be determined by the specific pathways invoked during processing of viral antigen; in other words, whether the mouse ultimately becomes immune responsive or tolerant, it is the Th1 compartment that is regulated.

In another model of neonatal unresponsiveness Forsthuber *et al.* (10) demonstrated an antigen-specific Th2 bias in splenocytes from adult mice that had been neonatally exposed to HEL (hen egg lysosyme). On this occasion, injection of HEL in the adjuvant IFA may have resulted in the persistence of a low level of antigen in the spleen, thereby biasing the naive T cell pool toward a Th2 immune response.

To test the idea that administration of antigen in adjuvant may bias toward a Th2 immune response, we looked for evidence of AAD in mice that had been given OVA/IFA as neonates. Indeed, the presence of IFA resulted in priming for OVA-specific Th2-mediated AAD, as evidenced by airways eosinophilia. It is interesting to note that priming for OVA-specific Th2-mediated AAD also resulted from very low dose soluble antigen administration (Table 1). Taken together, these results suggest that adjuvant may act by sequestering antigen such that an "effective" lower dose is administered to the neonate resulting in priming for a Th2-type immune response.

Our work clearly demonstrates that in response to neonatal administration of soluble antigen both Th1 and Th2 antigen-specific immune responses can be inactivated. Furthermore, inactivation prevents, rather than exacerbates, subsequent antigen sensitization and induction of Th2-mediated AAD. Thus, according to our data, and the work of others, the type of immune response or tolerance that develops after antigen administration to the neonate is dependent on a number of different parameters. These include the following: the number of appropriate antigen presenting cells, the microenvironment of the compartment where sensitization occurs, the type of costimulatory signals involved in antigen processing, the type of adjuvant, and the "effective" dose of antigen used during its delivery. The results presented here highlight the latter point, confirming that the use of adjuvant in delivering antigen to the neonatal mouse can prime for Th2-mediated diseases. These results have important implications for the design of delivery systems used for vaccination strategies in individuals who are atopic and/or asthmatic.

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