Selective Enhancement of Systemic Th1 Immunity in Immunologically Immature Rats with an Orally Administered Bacterial Extract

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Infant rats primed during the first week of life with soluble antigen displayed adult-equivalent levels of T-helper 2 (Th2)-dependent immunological memory development as revealed by production of secondary immunoglobulin G1 (IgG1) antibody responses to subsequent challenge, but in contrast to adults failed to prime for Th1-dependent IgG2b responses. We demonstrate that this Th2 bias in immune function can be redressed by oral administration to neonates of a bacterial extract (Broncho-Vaxom OM-85) comprising lyophilized fractions of several common respiratory tract bacterial pathogens. Animals given OM-85 displayed a selective upregulation in primary and secondary IgG2b responses, accompanied by increased gamma interferon and decreased interleukin-4 production (both antigen specific and polyclonal), and increased capacity for development of Th1-dependent delayed hypersensitivity to the challenge antigen. We hypothesize that the bacterial extract functions via enhancement of the process of postnatal maturation of Th1 function, which is normally driven by stimuli from the gastrointestinal commensal microflora.

During the preweaning period, the immature immune system is faced with antigenic challenges that are qualitatively and quantitatively different from those encountered during fetal life. In particular, it must learn to discriminate between antigens on pathogenic microorganisms and trivial antigens from domestic animals and plant sources (e.g., danders and pollens), and it must also develop the capacity to respond in a fashion that is qualitatively and quantitatively appropriate to these different types of challenges.

Failure to develop such immune competence in a timely fashion after birth confers increased risk of development of a number of diseases. For example, it is well recognized that transient maturational deficiencies in immune and inflammatory functions predispose infant animals and humans to infections (42). Therefore, interest in the concept that similar deficiencies may predispose toward allergic sensitization against environmental allergens and development of some autoimmune diseases (16, 19) is growing. The precise nature of these maturational deficiencies remains to be determined. However, a common feature appears to be an imbalance between the T-helper 1 (Th1) and Th2 arms of the cellular immune response (e.g., see references 1, 17, 27, and 33).

As a result of a series of regulatory mechanisms that selectively dampen aspects of Th1 function, such as gamma interferon (IFN-γ) production (18, 41), the fetal immune system appears constitutively biased toward Th2 function, and this imbalance is not usually redressed until biological weaning. Antigen challenge during this period evokes relatively low-level immune responses, which prime selectively for Th2 immunity (3–5, 35), and the relative deficiency in Th1 memory generation can be partially corrected by the use of potent Th1-selective adjuvants (4).

Accumulating evidence suggests that the normal postnatal maturation of immune competence, and in particular the selective postnatal upregulation of Th1 functions, is driven by contact with microbial stimuli, especially signals provided by the commensal flora of the gastrointestinal tract (16, 38). There is increasing interest in the potential therapeutic use of such immunostimulatory stimuli, especially in relation to immunocompromised subjects, who are at increased risk of mucosal infections. There is a particular need for the development of safe and effective immunostimulants for use in immunocompromised children, but there is currently little clinical or experimental information on the utility and mechanism of action of such agents in early postnatal life. The present study examines an animal model designed to systematically address this issue.

We report below on a rat model to study potential methods of boosting the development of humoral and cellular immunity to antigen challenge during the early postnatal period. We have utilized an oral bacterial extract (Broncho-Vaxom OM-85) derived from a mixture of heat-killed respiratory pathogens, which has previously been used in a number of clinical and experimental settings. These include studies of immunostimulation in normal adult experimental animals (7, 8) and double-blind multicenter clinical trials with humans with chronic obstructive pulmonary disease (12, 30). The principal end points employed for the present study are production of immunoglobulin G1 (IgG1) and IgG2b subclass antibodies, which in the rat are respectively dependent upon Th2 versus Th1 cytokines (14, 36). Our findings confirm earlier reports indicating that immunization in the neonatal period selectively primes for production of Th2-dependent IgG subclass antibodies and further demonstrate that oral administration of the bacterial extract OM-85 circumvents this Th2 bias via selective
upregulation of Th1-dependent IgG subclass production. Furthermore, this switch toward Th1 immunity is accompanied by increases in antigen-specific and polyclonal lymphoproliferation and IFN-γ production in vitro and development of anti-gen-specific delayed-type hypersensitivity (DTH) in vivo.

MATERIALS AND METHODS

Animals. Inbred PVG.RT1° rats were bred free of common rat pathogens in house at the TVW Telethon Institute for Child Health Research and housed under specific-pathogen-free conditions. Neoborn rat pups within 24 h of birth and 8- to 12-week-old adult male rats were used.

Immunization procedures. Rats were anesthetized under ether and administered primary immunization with ovalbumin (OVA; Sigma Chemical Co., St. Louis, Mo.) dissolved in phosphate-buffered saline (PBS) intraperitoneally (i.p.), or combined with incomplete Freund's adjuvant (IFA; Flow Laboratories, Sydney, Australia) subcutaneously (s.c.) on an approximate dose-per-body-weight basis. The i.p. route was avoided for IFAs, because this adjuvant can cause prolonged peritonitis.) Newborns were given 25 μg of OVA, and adults were given 100 μg. An antigen challenge was given 28 days after immunization containing 100 μg of OVA in PBS alone (i.p.) or combined with IFA, complete Freund's adjuvant (CFA; Flow Laboratories) s.c., or aluminum hydroxide (Alhydrogel; Wyeth Amherst) i.p.

Oral delivery of OM-85 and placebo. OM-85 (Broncho-Vaxom; OM Pharma, Geneva, Switzerland) is a lyophilized extract of eight common respiratory pathogens (Haemophilus influenzae, Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus viridans, Klebsiella pneumonieae, Klebsieilla ozuenae, Staphyloccocus aureus, and Moraxella catarrhalis), currently in use in many countries as an oral immunostimulant. OM-85 and placebo (lyophilized extract vehicle) was dissolved in sterile water to 400 mg/ml and delivered by mouth to newborn rats at 1 μl per g of body weight. Feeding was provided for 14 consecutive days and then every second day until day 28.

Media and reagents. Cell isolation procedures were performed in ice-cold PBS supplemented with 0.2% bovine serum albumin (BSA; CSL, Melbourne, Australia) and 0.5 g each of CaCl2 and MgCl2 per ml (DAB/BSA). The tissue culture medium used was RPMI 1640 (Gibco, Life Technologies) supplemented with 2 g of sodium bicarbonate per liter and 2 mM-glutamine, 5 m. Alternatively, the medium used was RPMI 1640 (Gibco, Life Technologies) supplemented with 2 g of sodium bicarbonate per liter and 2 mM-glutamine, 5 m.

The limits of detection of both subclasses are 1 ng/ml. The limits of detection in the assay are 0.01 AU/ml.

Skin testing for DTH. Eight-week-old rats immunized neonatally with OVA in IFA, fed OM-85 or placebo, and challenged with OVA in PBS at 4 weeks of age or immunized, age-matched (8 weeks old) control rats received an intradermal (i.d.) injection of 20 μg of OVA in 10 μl of PBS or PBS alone in the left and right ears, respectively. Ear swelling was measured after 24 h with a micrometer, and the increments (mm/2 mm) were obtained by subtracting values for the thickness of the PBS ear from the test ear. Untouched animals with untouched ears were included to establish background variation.

Statistics. Statistical comparisons of mean values were performed with the nonparametric Mann-Whitney U test for unpaired samples by using the StatView software package (SAS Institute, Cary, N.C.).

RESULTS

Primary and secondary IgG subclass antibody responses in newborn versus adult rats. In the experiments shown in Fig. 1,
newborn animals <1 day old, together with adults, were primed with soluble OVA i.p., and they were bled 2 weeks later (determined in preliminary experiments to be the peak of the primary response). All animals were rechallenged with soluble OVA i.p. 4 weeks postpriming, bled 2 weeks thereafter, and assayed for IgG1 and IgG2b anti-OVA antibody. It can be seen that this prime-challenge protocol elicits very low primary responses, particularly in the newborns. It is additionally evident that strong secondary responses (indicative of successful initial priming) occurred for both IgG subclasses in the adults; however, the newborns demonstrated weak secondary IgG1 responses, but displayed no evidence of priming for the IgG2b subclass.

Figure 2 further contrasts the capacity of newborn and adult animals to express secondary immune responses, in this case when potent adjuvants are employed to unmask earlier priming. In the adults, the highest IgG1 and IgG2b recall responses occurred following respective challenge with OVA in AH ver-
sus OVA in CFA, a finding consistent with the known Th2 versus Th1 selectivity of these two adjuvants.

Effects of the oral bacterial extract OM-85 on IgG subclass responses. In Fig. 3, newborn or 12-week-old rats were primed i.p. with soluble OVA and given daily doses of OM-85 thereafter for 14 consecutive days, prior to serum collection for determination of peak primary response titers of IgG1 and IgG2b. OM-85 administration was continued as detailed in the legend to Fig. 3, prior to elicitation of a secondary response via rechallenge i.p. with soluble OVA. In the adult, i.p. priming was effective, as shown by the log-scale increase in titers following rechallenge, and OM-85 administration significantly enhanced priming for the IgG1 subclass.

In contrast, titers of both the Th1-dependent and Th2-dependent IgG subclasses in primary responses of infant animals were boosted by OM-85 feeding. Consistent with the pattern demonstrated in Fig. 1, i.p. immunization of newborn animals resulted in weak priming for IgG1 subclass responses, but not for IgG2b, and this priming was not enhanced by OM-85.

Unmasking of the enhancing effects of oral bacterial extract OM-85 by the use of adjuvants. In the experiments shown in Fig. 4, newborn rat pups were primed with soluble OVA i.p. and given doses of OM-85 up to day 28 as in Fig. 3. However, unlike the animals in Fig. 3, which were rechallenged on day 28 with soluble OVA, these animals were challenged with OVA together with the adjuvant IFA. It can be seen that the use of IFA unmasks substantial levels of priming for IgG1 antibody (c.f. titers in the placebo group in Fig. 4 versus those in the same group in Fig. 3B) and that the titers attained are equivalent to those for immunocompetent adults. OM-85 did not further boost these responses.

In contrast, and consistent with the data shown in Fig. 1B and 3B, IgG2b responses following secondary challenge were extremely low in the placebo group, indicating poor development of immunological memory in response to priming. However, corresponding responses in the OM-85-treated group were more than 1 log fold higher than those in placebo controls, suggesting that the vaccine had facilitated development of significant levels of immunological memory.

In Fig. 5, an alternative prime-challenge protocol was examined, in which OM-85 treatment of infant rats was carried out prior to initial OVA priming; OM-85 administration was con-
continued thereafter up until secondary challenge, as detailed in
the legend. This resulted in higher levels of OM-85 stimulation
of IgG2b production, particularly in the secondary response.

**Effect of OM-85 on in vitro T-cell responses.** In Fig. 6,
newborn rats were primed with OVA in IFA, with or without
accompanying OM-85 administration, as detailed in the legend
to Fig. 5. The methodology employed is standard in the field
for T-helper-cell activation, and the cytokines produced in
these cultures are primarily from T cells, but small and variable
contributions from other cell types cannot be ruled out. The in
vitro cytokine responses were assessed 21 days later, a time
point identified in earlier experiments as the peak or plateau of
T-helper-cell reactivity. Several key observations are illus-
trated. First, feeding of the OM-85 extract increases levels of
spontaneous IFN-γ production (note medium controls), in
particular in the MLN draining the gut, and a reciprocal pat-
tern of decreased IL-4 production is also evident. Maximal
IFN-γ response capacity, as determined by polyclonal ConA
stimulation, was also increased in MLN, again accompanied by
decreased IL-4 release. A similar pattern of markedly in-
creased antigen-specific IFN-γ production and a parallel de-
crease in the IL-4 response were observed in lymph nodes and,
to a lesser extent, spleens.

The experiments shown in Fig. 7 examined the effects of
OM-85 on lymphoproliferative responses during primary and
secondary responses to OVA, focusing on time points during
active in vivo expansion of specific T cells. It can be seen that
OM-85 significantly enhanced OVA-specific lymphoproliferative responses are significantly
enhanced in OM-85-treated animals during both the primary
and secondary responses in all lymphoid organs tested, in
particular MLN. In Fig. 8, the effects of OM-85 on splenic lym-
phoproliferative responses were examined by employing an
alternative polyclonal stimulant, anti-TCRβ-anti-CD28. In
these experiments, donor animals did not receive any immu-

**Effects of OM-85 on priming in vivo DTH responses.** In the
experiments shown in Fig. 9, animals were OVA primed by a

FIG. 5. Effect of OM-85 preexposure on priming for IgG subclass antibody response of newborn rat pups. Newborn rat pups were given oral
doses of OM-85 or placebo (400 μg per g of body weight) on day 1. Dose administration was continued for 4 consecutive days, and on the 5th day,
the pups were immunized s.c. with 25 μg of OVA in IFA. Administration of OM-85 or placebo was continued each day for a further 14 days
(postimmunization), when serum was collected and primary OVA-specific IgG1 and IgG2b antibody titers were measured by ELISA (A). OM-85
or placebo was then given every second day until the day of challenge (3 weeks postimmunization). An i.p. challenge dose of 100 μg of OVA in
PBS was given to all rats, and serum was collected after a further 2 weeks for measurement of IgG1 and IgG2b recall responses (B). The data are
representative of four separate experiments, and each bar represents the group mean (n = 6 to 7 rats) ± standard error. Mann-Whitney U
test-generated P values indicate significant differences in antibody titers due to administration of OM-85 (*, 0.05 > P > 0.01; **, 0.01 > P > 0.005;
and ***, 0.005 > P > 0.0001).
protocol known to elicit DTH responses and given a dose of OM-85 or placebo, prior to secondary immunization and subsequent intradermal challenge (in the ear) with soluble OVA. DTH responses were assessed as changes in ear thickness 24 h after challenge. It can be seen that DTH responses are significantly elevated in the OM-85-fed group.

DISCUSSION

It is generally acknowledged that while the transition from fetal to adult life involves “maturation” of several aspects of innate and adaptive immune function, the nature and degree of the maturational deficit in newborns may vary significantly.
between species (2, 18, 26). However, the two species studied in the most detail, humans and mice, share as a common feature the generalized Th2 bias, which is characteristic of the fetal compartment.

In murine systems, this bias is manifested as differential expression of Th2-polarized immunological memory in response to priming during the preweaning period, together with diminished capacity to develop Th1-polarized immunity (3–5, 10), which appears attributable principally to deficiencies in the antigen-presenting cell (APC) compartment (34). In humans, it has been demonstrated that early postnatal responses to environmental allergens (31, 32) and microbial antigens (35) also display an intrinsic Th2 bias, and recent findings from our laboratory suggest that this bias is associated with reduced capacity of infants to generate long-lasting Th1-polarized memory in response to vaccines (35a). Underlying this Th2 bias in human infants is reduced capacity to generate T-cell IFN-γ responses in vitro (24, 35a, 43), which appears to be derived from functional deficiencies in both the T-cell and APC compartments (2, 17, 24, 26, 43).

As noted in the introduction, the functional consequences of “inefficient” postnatal maturation of Th1 function in humans are increasingly being considered as potential etiologic factors in a variety of immunoinflammatory diseases, as well as risk factors for infections in infancy and childhood. Accordingly, potential avenues for selective boosting of Th1 activity during early life warrant further investigation.

Our studies reported here were carried out with an infant rat model, which shares the principal characteristics of the established murine models and of infant humans, notably reduced capacity to generate Th1 (as opposed to Th2)-polarized memory responses (Fig. 1 and 2). We have employed this model to investigate the possibility that microbial extract provided orally may be able to enhance the capacity of infant animals to develop a balanced Th1-Th2 memory response against parenterally administered antigen.

Our initial interest in this approach derives from the extensive literature on germfree animals, which has established that the principal signals for maturation of immune function in mammals are provided by the gastrointestinal microflora that are established in early postnatal life. It is evident from studies with germfree rats (13), and in particular from recent experiments with germfree mice (38), that denial of gastrointestinally derived microbial stimulation effectively prevents the infant immune system from developing a balance between the Th1 and Th2 arms of the adaptive immune response, effectively “locking” it into the Th2 bias characteristic of the fetal compartment. Additionally, microbial conventionalization of the gastrointestinal tract redresses this imbalance (38).

Additional impetus for these studies was provided by reports on the immunostimulatory effects of the OM-85 oral bacterial extract in animal models (7, 8) and in human clinical trials (12, 30). This agent, which is an extract of cell walls from eight bacterial species commonly responsible for respiratory infections, has been demonstrated to exert a variety of stimulatory effects upon humoral and cellular immunity and upon the expression of protective immunity at mucosal surfaces.

The salient findings from this study on the effects of OM-85 in infant rats are as follows. First, treatment of animals with the extract during primary immune responses had variable effects, which were related to the intensity of antigen rechallenge and the timing of administration of OM-85 relative to initial antigen priming. Thus, administration of OM-85 clearly boosted priming for Th1-dependent IgG2b responses, providing an adjuvant (IFA in the experiments shown) was employed during secondary challenge (Fig. 4). The magnitude of the OM-85-
boosted IgG2b response in Fig. 5B is approximately threefold that in Fig. 4. This indicates that the effects of the extract are maximal if it is given for several days before priming (Fig. 5), suggesting that time-dependent “maturation” of one or more elements of the immune response was required before the optimal immunostimulatory effects occur.

The results of experiments in Fig. 6 to 8 suggest that one cell population implicated in the effects of the OM-85 extract are Th cells. These findings establish that concomitant with upregulation of the IgG2b component of the memory response, the overall capacity of the Th cell compartment to expand upon polyclonal activation and the capacity for expansion of OVA-specific Th cells are increased. More importantly, accompanying these changes are alterations in the Th1-Th2 balance, as demonstrated by upregulation of the Th1 cytokine IFN-γ and concomitant downregulation of IL-4 production. These effects are most notable in the MLN, which directly drains the site of administration of the OM-85 extract, but are also observed at distal sites in the immune system. Further confirmation of the efficacy of the oral vaccine in preferential upregulation of Th1 immunity is the demonstration in Fig. 9 that treated animals develop enhanced memory DTH responses.

Whether this stimulation is the direct result of effects of the oral extract on Th cells remains to be established. However, the finding that the boosting effects of OM-85 were only observed when adjuvant was employed in the secondary response (cf. Fig. 3 and 4) suggests a possible common cellular target or targets for both agents. A likely candidate for these effects are APC, which have been demonstrated in the mouse to display a maturational deficiency in Th1-stimulatory capacity during the neonatal period and to accordingly prime preferentially for Th2 immunity (34). APC are also acknowledged to play a central role in mediating the effects of immunological adjuvants, and studies with other systems have demonstrated modulatory effects of OM-85 on functions of several cell types that display APC activity (6–8, 23, 25). In particular, it has been shown that the extract stimulates IFN-γ production by CD4+ T cells via induction of IL-12 secretion in APC (9). Given the important role of APC-derived IL-12 in stimulating the preferential development of Th1 immunity (15), this pathway appears to be a likely target for the effects of OM-85 in this model. Accordingly, more detailed studies on the antigen processing and presentation functions and costimulatory activity of APC following OM-85 treatment, appear warranted.

In conclusion, this study has demonstrated that repeated oral administration of the bacterial extract Broncho-Vaxom OM-85 to rats during the preweaning period selectively amplifies Th1 function and in doing so appears to accelerate the normal postnatal maturation of adaptive immune competence. It appears plausible that the vaccine may function via mechanisms analogous to those employed via the normal gastrointestinal microflora, which have been shown in other systems to drive this natural process postnatally. The Th1-stimulatory effects observed here are likely to contribute to the clinical
efficacy of this bacterial extract in enhancing resistance to infections, as demonstrated in human trials (11, 12, 30).

REFERENCES


