NEONATAL ADMINISTRATION OF IDIOTYPE OR ANTIIDIOTYPE PRIMES FOR PROTECTION AGAINST *ESCHERICHIA COLI* K13 INFECTION IN MICE

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Antipolysaccharide antibodies directed at the capsular antigens of pathogenic bacteria correlate with protection against infection by these organisms (1–3). Antibody responses to most polysaccharides, however, develop late in ontogeny and do not reach adult levels until 8–12 wk in mice (4, 5) and 2–5 yr in man (6–8). Although conversion of a thymus-independent (TI)[1] polysaccharide (Ps) antigen to a thymus-dependent (TD) antigen has been shown to stimulate adult-level antibodies in 3–4-wk-old mice, this conjugate antigen failed to stimulate anti-Ps antibody responses in newborns (9). For this reason we have begun studies of alternative approaches to stimulating anti-Ps antibody responses in neonatal mice.

Rubinstein et al. (10, 11) have shown that both idiotype (Id) A48 (10), a bacterial levan (BL)-binding myeloma protein, and anti-Id (11), when given within 24 h after birth, can prime mice for an A48-dominant antibody response upon subsequent immunization with BL. Using this model, we have investigated the ability of Id 150C8, an IgM monoclonal antibody directed against the Ps capsule of *Escherichia coli* K13, and anti-Id 5868C, an IgG1 monoclonal anti-150C8, to prime the immune system for protection against infection with *E. coli* K13. We report here that administration of either Id or anti-Id within 24 h after birth can prime weanling mice for protection against *E. coli* K13 infection.

Materials and Methods

Animals. Mice used in the immunization experiments and the CAF_1 mice used for the production of anti-Id ascites were obtained from The Jackson Laboratory, Bar Harbor, ME. BALB/c mice used in the protection experiments were bred in the Animal House of the Institute of Medical Microbiology, University of Göteborg, from breeding stocks obtained from the Chester Beatty Research Institute, London, England. Sprague-Dawley rats were obtained from Taconic Farms, Inc., Germantown, NY.

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[1] *Abbreviations used in this paper:* BL, bacterial levan; CFA, complete Freund's adjuvant; HAI, hemagglutination inhibition; HIB, *Haemophilus influenzae* type b; Hy, hemocyanin; Id, idiotype; IFA, incomplete Freund's adjuvant; NeuNAc, *N*-acetylneuraminic acid; PBS, phosphate-buffered saline; Ps, polysaccharide; SRBC, sheep erythrocyte; TD, thymus dependent; Th, helper T cell; TI, thymus independent.

Polysaccharides. *E. coli* capsular Ps K1, K13, K20, and K23 (Table I) were purified and characterized as described by Vann et al. (12, 13). *E. coli* K1 Ps, a homopolymer of α-(2 → 8)-linked *N*-acetylneuraminic acid (NeuNAc), and K20 and K23 Ps were provided by Dr. Willie Vann. *Haemophilus influenzae* type b (HIB) capsular Ps and K13 Ps coupled to horsehoe crab hemocyanin (K13-Hy) prepared as described (14) were provided by Dr. Rachel Schneerson.

Bacteria. *E. coli* strain 06:K13:H1, obtained from the Statens Seruminstitut, Copenhagen, Denmark, was grown overnight in liquid medium containing beef extract and peptone. After overnight growth the cells were subcultured in the same medium and grown to ~5×10^8 cells/ml. A vaccine was prepared by inactivating the cells with 0.5% formalin in phosphate-buffered saline (PBS) for 60 min at room temperature. The concentration was adjusted to 1×10^9/ml and the vaccine was stored at 4°C for up to 1 wk.

Cultures used for challenge with live bacteria were adjusted to the desired concentration in PBS and 0.5 ml was injected intraperitoneally per mouse. Challenge doses were verified by direct plate count. In preliminary experiments the LD_{50} for 4–5-wk-old BALB/c mice was determined to be 6×10^6 cells/mouse.

Hybridomas. Anti-K13 Ps hybridomas were prepared in BALB/cANN mice as described by Söderström et al. (15). One of those hybridomas, 150C8, an IgM anti-K13, was used to prepare a monoclonal anti-Id antibody. A/HeJ mice were immunized with a complete Freund's adjuvant (CFA) emulsion of a precipitate formed by incubating 0.4 ml of 150C8-containing ascites with 0.4 ml of a 1 mg/ml solution of K13 Ps for 24–48 h in the cold. The resulting precipitates were pelleted by centrifugation, washed twice in PBS, resuspended in 0.4 ml PBS, and emulsified with 0.4 ml CFA containing H37Ra tubercle bacilli (Difco Laboratories, Inc., Detroit, MI). Mice were injected subcutaneously in the footpads (0.05 ml per footpad) and in the inguinal and axillary folds (0.025 ml in each region). The first injection was given in CFA and weekly injections thereafter were given in incomplete Freund's adjuvant (IFA). 1 d after the seventh injection, the spleen was removed from one mouse and used for fusion with SP 2/0 as described (15). Cells were cloned on pristane-induced peritoneal exudate cell feeder layers. Anti-Id was selected by an enzyme-linked immunosorbent assay (ELISA) in which K13 Ps was coated onto polyvinyl microtiter plates (at 50 μg/ml for 1 h) followed by a 1:50,000 dilution of 150C8-containing ascites. At this dilution, the alkaline-phosphatase–conjugated polyvalent goat antiimmunoglobulin (Cappel Laboratories, Cochranville, PA) reacted very weakly with the 150C8 itself and very well after addition of anti-Id. One anti-Id clone, 5868C, an IgG1, was selected for study. Ascites was produced in CAF_1 mice.

Hemagglutination Inhibition (HAI). HAI was performed using the chromic chloride method of Gold and Fudenberg (16) as described by Lieberman et al. (17) except that anti-Id was coated onto the erythrocytes (RBC). Ammonium sulphate–precipitated 5868C was used for coating RBC at a concentration of 25 μg/100 μl. An S-300 (Pharmacia Fine Chemicals, Piscataway, NJ) column fraction of 150C8-containing ascites was ammonium sulphate precipitated and the purified IgM was used to agglutinate 5868C-coated sheep RBC (SRBC). Titrations consisting of serial twofold dilutions were performed in every experiment. The concentration of 150C8 chosen for HAI was two dilutions before the endpoint of agglutination and usually fell between 10 and 100 ng/ml. 5868C-coated SRBC were specifically agglutinated by the IgM idiotype, 150C8. Another IgM (150B2) and two IgG (156E8, 147D11) hybridoma anti-K13 antibodies as well as MOPC 104E (IgMλ) and TEPC 183 (IgMκ) myeloma proteins (Litton Bionetics, Rockville, MD), at concentrations of 1 mg/ml, failed to agglutinate 5868C-coated SRBC and to precipitate 5868C by immunodiffusion.

Immunizations. Mice were injected intraperitoneally with 0.2 ml of either 5 μg K13 Ps in PBS, 1×10^8 killed K13 bacteria in PBS (K13 vaccine), or 30 μg K13-Hy in CFA, and were bled as indicated in Table II. A second immunization was given 1 mo after the first as stated above except that K13-Hy was given in IFA. Mice primed at birth were subsequently given K13 vaccine or 2.5 μg K13 Ps in PBS, injected intraperitoneally. Rats

were anesthesized with CO_2, immunized intraperitoneally with K13 vaccine once a month for 6 mo, and bled 1 wk after the last injection.

Neonatal Priming. Mice were primed within 24 h after birth by an intraperitoneal injection of 50 μl containing the substances indicated in Tables IV and V, diluted in sterile, pyrogen-free saline.

Challenge with E. coli. Mice were challenged with 0.5 ml live *E. coli* 06:K13:H1 in PBS given intraperitoneally 1 wk after immunization when they were 5 wk old (Table IV) or 13 wk old (Table V). Deaths were recorded 24 h after challenge. The challenge doses are given in Tables IV and V.

Ouchterlony Analysis. Immunodiffusion was performed in 0.9% agarose (Indubiose A37; Accurate Chemical and Scientific Corp., Hicksville, NY) in PBS. Sera and ascites were used undiluted, IgM myeloma and hybridoma proteins at a concentration of 1 mg/ml, and IgG myeloma and hybridoma proteins at a concentration of 0.25 mg/ml.

Results

Production and Characterization of 5868C Anti-Id. The sera of A/HeJ mice hyperimmunized with immune complexes of IgM anti-K13 (150C8)-K13 Ps appeared to contain anti-Id antibodies after the 5th wk of immunization as determined by Ouchterlony analysis (data not shown). One mouse was chosen for the production of monoclonal anti-Id. Selection for anti-Id on Id plus antigen (K13 Ps)-coated plates was successful in this instance, probably because not all of the combining sites of the IgM Id were bound to antigen. The same technique was not successful in the selection of anti-Id antibodies reactive with IgG idiotypes. Several independent clones were derived from this fusion and 5868C, an IgG1, was chosen for further study. By Ouchterlony analysis, 5868C precipitates 150C8 but not two IgM myeloma proteins, MOPC 104E and TEPC 183, nor does it precipitate with normal mouse serum or a variety of IgM and IgG anti-K13 hybridomas.

A reverse HAI assay was used to study the effect of antigen on the Id-anti-Id reaction and to assess the presence of the 150C8 Id in immune sera. This assay was used because Id-coated RBC were not agglutinated by the IgG anti-Id. Table I shows that agglutination of anti-Id (5868C)-coated RBC by Id (150C8) was specifically inhibited by K13 and not by two unrelated Ps, K1 and HIB. In addition, a weak inhibition due to cross-reactivity, not seen by Ouchterlony analysis, was observed with two Ps, K20 and K23, which are very closely related to K13.

Presence of 150C8 Id in Serum. The 150C8 Id, as detected by HAI, was found in both the preimmune and immune sera of three strains of mice that differ at the Igh locus: BALB/c, Igh^a; C57BL/6, Igh^b, and CBA, Igh^j (Table II). Increases in Id related to immunization were seen in BALB/c and CBA strains and only early after a primary immunization. In both strains the level of Id declined to below preimmune levels at 14 and 21 d after a primary immunization and 7 d after a secondary immunization in BALB/c mice. The same trend was seen in C57BL/6 mice, although the differences were not significant. In contrast, two of three Sprague-Dawley rats (Table III) that had been hyperimmunized with K13 vaccine showed high levels of 150C8 Id whereas the pooled normal rat serum showed no HAI activity. When the presence of the 150C8 Id was followed in another group of six Sprague-Dawley rats (data not shown) before and 7, 14, and 21 d after a primary, and 7 d after a secondary immunization with K13, no

TABLE I
Inhibition of Id-Anti-Id by Antigen

	Polysaccharide*	HAI titer‡
K13	3)-β-Ribofuranosyl-(1 → 7)-β-KDO-(2 → 4 ↑ Acetyl	44
K20	3)-β-Ribofuranosyl-(1 → 7)-β-KDO-(2 → 5 ↑ Acetyl	5
K23	3-β-Ribofuranosyl-(1 → 7)-β-KDO-(2 →	6
K1	8)-α-NeuNAc-(2 → 8)-α-NeuNAc-(2 →	<1
HIB	3(-β-Ribofuranosyl-(1 → 1)-Ribitol-5-(PO_4 →	<1

* Ps solutions were used at a starting concentration of 10 mg/ml in saline and serial twofold dilutions were used for inhibition.
‡ Log 2.

TABLE II
150C8 Id in Mouse Serum

Strain	Immunogen	HAI titer*				
		Preimmune	Days after immunization			
			Primary			Secondary
			7	14	21	7
BALB/cJ		4‡				
	K13 Ps		7	2	2	2
	K13 vaccine		7	2	2	2
	K13-Hy		6	2	2	2
CBA/J		3				
	K13 Ps		5	3	1	ND§
	K13 vaccine		6	2	1	ND
C57BL/6J‖		3				
	K13 Ps		4	2	2	ND
	K13 vaccine		4	4	3	ND

* Log 2, starting with a 1:2 dilution of serum; sera were pooled from six mice per group.
‡ A pool of serum from unimmunized BALB/cCu mice was used in place of preimmune BALB/cJ serum.
§ Not done.
‖ All sera were absorbed with an equal volume of packed SRBC before testing.

TABLE III
150C8 Id in Rat Serum

Serum	HAI titer*
Pooled NRS‡	1
Rat 1§	<1
Rat 2	6
Rat 3	6

* Log 2.
‡ NRS, normal rat serum.
§ Rats were hyperimmunized with K13 vaccine over a period of several months. They were bled 1 wk after the last immunization.

TABLE IV
Effect of Neonatal Priming with Id and Anti-Id When BALB/c Mice Are Challenged at 5 Wk of Age

Exp.	Treatment at birth	Immunization at 4 wk of age	No. of mice	Percent survival
1	—*	—	6	0‡
	—	K13 vaccine	9	44
	K13 Ps, 2.5 μg	K13 vaccine	6	33
	150C8, 1 μg	K13 vaccine	15	87
	5868C, 50 ng	K13 vaccine	15	93
	5868C, 10 μg given to mothers after delivery§	K13 vaccine	6	83
2	Saline	K13 Ps, 2.5 μg	6	0‖
	K13 Ps, 2.5 μg	K13 Ps, 2.5 μg	6	33
	150C8, 1 μg	K13 Ps, 2.5 μg	8	75
	150C8, 50 ng	K13 Ps, 2.5 μg	10	40
	5868C, 1 μg	K13 Ps, 2.5 μg	5	80
	5868C, 50 ng	K13 Ps, 2.5 μg	9	78

* No injection.
‡ Mice were challenged with 20 LD_{50} live *E. coli* 06:K13.
§ Anti-Id was injected intraperitoneally into the mother within 24 h after delivery of her pups.
‖ Mice were challenged with 30 LD_{50} live *E. coli* 06:K13.

change was found with immunization. In this group of rats, titers of 4–6 (log 2) were found in individual preimmune sera and they did not change in immune sera at any time. The 150C8 Id was not found in burro and human sera known to contain high titers of anti-K13. It remains to be determined whether the Id in C57BL/6 and some rat sera is present on K13-binding antibodies.

Effects of Id and Anti-Id Priming at Birth. In earlier experiments we had found that 150C8 anti-K13 would confer passive protection against intraperitoneal infection with *E. coli* K13 in mice (18). We therefore designed experiments to determine if either Id (150C8) or anti-Id (5868C), when administered within 24 h after birth, could prime for protection against *E. coli* K13 infection in both young mice (5 wk old) and adults (13 wk old). In the first experiment (Table IV), mice injected at birth with 150C8 or 5868C and immunized at 4 wk of age with K13 vaccine were significantly protected from infection with 20 LD_{50} *E.*

coli K13 as compared with controls either uninjected or immunized with K13 Ps at birth. In addition, this experiment shows that anti-Id can prime for protection even when administered through milk, i.e., by administration of anti-Id to the mother within 24 h after delivery.

To rule out any effects of lipopolysaccharide on the protection, inasmuch as the mice were given a whole cell vaccine at 4 wk of age, the experiment was repeated, except that the mice were immunized with highly purified K13 Ps. Again, both Id and anti-Id were able to prime for protection when the challenge dose of *E. coli* was 30 LD_{50} (Table IV). When the time of immunization was delayed until 12 wk of age (Table V), however, only anti-Id and not Id was able to prime for protection. Only the 50 ng dose of 5868C gave a significant priming effect, but it should be noted that a 50 LD_{50} challenge dose was used in this experiment.

Discussion

We have shown that a monoclonal anti-Id (5868C) as well as the Id anti-K13 Ps (150C8) can prime neonatal mice for protection upon immunization and subsequent challenge with live *E. coli* K13. The data also show that the priming effects of anti-Id can be transferred to pups via the milk as the pups of a mother given anti-Id within 24 h after delivery were also protected from challenge with *E. coli*. The properties of this system that may be important to the ability of Id and anti-Id to prime for protection are that (*a*) 150C8, itself, can confer passive protection (18), (*b*) 150C8 is bactericidal in vitro (15), and (*c*) that the site recognized by 5868C anti-Id is related to the 150C8 combining site because Id-anti-Id is specifically inhibited by K13 Ps. Both Id and anti-Id primed for challenge at 5 wk of age. These studies confirm the results of Rubinstein et al. (10, 11) who demonstrated that treatment at birth with A48 (10), a BL-binding myeloma protein, or anti-A48 (11), primed for an A48-dominant response upon immunization with BL at 4 wk of age. Their experiments showed that the administered A48 alone was not responsible for the effect and that immunization with antigen was required. Inasmuch as IgM has a short half-life, a direct effect of 150C8, 5 wk after its administration, is unlikely.

In our experiments, when immunization was delayed until 12 wk of age, only

TABLE V

Effect of Id and Anti-Id Priming When BALB/c Mice Are Challenged at 13 Wk of Age

Treatment at birth	Immunization at 12 wk of age	No. of mice	Percent survival*
—‡	—	9	0
—	K13 Ps, 2.5 μg	20	25
150C8, 1 μg	—	5	0
150C8, 1 μg	K13 Ps, 2.5 μg	27	22
5868C, 1 μg	K13 Ps, 2.5 μg	19	16
5868C, 50 ng	K13 Ps, 2.5 μg	30	78

* Mice were challenged with 50 LD_{50} live *E. coli* 06:K13.

‡ No injection.

the mice primed with anti-Id were protected. In the experiment shown in Table V, 50 ng but not 1 μg of 5868C primed for protection. The reason for the lack of priming by 1 μg of 5868C is unknown but it may reflect a dose effect of anti-Id. Experiments involving 5868C priming of adult mice indicated that nanogram quantities of 5868C primed for anti-K13 whereas microgram quantities, depending on the amount, either did not prime or primed for Id on non–antigen-binding molecules (T. Söderström and K. E. Stein, unpublished). The differences seen in the effects of neonatal Id and anti-Id priming on the adult response to challenge may reflect differences in the cell populations being stimulated by Id and anti-Id. Rubinstein et al. (10, 11) have shown that the effects of Id priming can be transferred to unprimed animals by helper T cells (Th) (10), but that the effects of anti-Id priming can be transferred by B cells (11). We speculate that the primed B cells (by anti-Id) are long-lived whereas the Id-specific Th cells either are short-lived or fail to be expanded during environmental antigen exposure. This would be the case if the 150C8 Id were not a dominant Id in the response to K13; we have no data to suggest that it is.

Both in mice (4, 5) and in man (6–8), the immune response to TI Ps antigens develops relatively late in ontogeny although responses to TD antigens are present at birth (9). The development of responses to Ps antigens parallels the development of a subset of B cells in mice, Lyb-5^+ cells, regardless of whether the Ps is presented in a TI or TD form (9). One explanation proposed by Stein et al. (9) for the failure of neonates to respond to Ps antigens was that the Lyb-5^- B cells present in the neonate are tolerized by exposure to environmental Ps. The data presented here, as well as the data of Rubinstein et al. (11) showing that anti-Id given within 24 h after birth can prime for a subsequent anti-Ps response, coupled with the additional findings of Rubinstein et al. (11) that the priming effect of anti-Id can be transferred to adoptive recipients by B cells, strongly suggest that B cells are present at birth that have surface Ig specific for Ps antigens. These cells would be activated by anti-Id such that they become refractory to the tolerizing effects of environmental Ps. The implication is that if the B cells present at birth can be activated before exposure to environmental Ps, they will respond, rather than be tolerized, upon subsequent antigen exposure.

To our knowledge, this is the first demonstration of the ability of a monoclonal anti-Id antibody to prime for protection against a microbial infection. These experiments show that a monoclonal anti-Id, directed against a single site on an anti-K13 antibody, can alter the entire biological response to a bacterial infection so that mice primed with anti-Id survive challenge with live *E. coli* K13, whereas unprimed mice die. Polyclonal anti-Id priming has been shown to induce protection in adult mice against trypanosomiasis (19) and to induce neutralizing antibodies against rabies virus (20) and antibodies to the surface antigen of hepatitis B virus (21) in mice. It seems likely that in infections where antibodies are protective and can be induced by anti-Id reagents, anti-Id antibodies can induce or prime for protection. The use of anti-Id antibodies as vaccines in human infants, however, is not yet practical.

Our data suggest that Id, or passive antibody, can also prime for protection when antigen is encountered early in life. Although the priming effect of Id was

not seen in mice challenged as adults, priming is not as important at this age inasmuch as adults respond well to Ps antigens. Passive antibody of protective idiotypes could thus have a dual effect in infants: it would provide immediate passive protection and it could prime for subsequent exposure to antigen. The concept of passive antibody protection of newborns is not a new one. In 1951, Cohen, Schneck, and Dubow (22) immunized pregnant women against diphtheria and pertussis and measured the levels of antibodies in infants born of these mothers. They found that the infants had protective levels of antibodies for a period of 3 mo and they advocated immunization of these infants at 4 mo of age, a time when most of the maternal antibody was gone. The possibility of a priming effect of maternal antibody was suggested by the data of Levi et al. (23) which showed that the response to vaccination with pertussis-diphtheria-tetanus vaccine is greater in babies with antibodies in the cord blood. More recently, Gill et al. (24) immunized pregnant women with tetanus toxoid and found that infants whose mothers were immunized responded better to subsequent immunization than infants of unimmunized mothers. Maternal antibody may not always have a priming effect. When high cord blood antibody levels exist and immunization is performed at a young age (2 mo), reduced responses may be seen, as reported by Burstyn et al. (25) for IgG antibodies to the leukocytosis-promoting toxin of *Bordetella pertussis*. A similar finding was reported by Osborn, Dancis, and Julia (26) on the effects of circulating antibody in the response to diphtheria toxoid. In contrast, McCormick et al. (27) found that children born of mothers immunized during pregnancy with meningococcal groups A and C Ps respond as well to these antigens as children born of unvaccinated mothers. These children, however, were immunized at 6 mo of age, when maternal antibodies had declined to levels indistinguishable from those in children born of unvaccinated mothers. Taken together, the data suggest that immunization of infants with high levels of maternal antibody should be delayed until a time when the antibody level has declined substantially. The role of maternal antibody, as opposed to immune gamma globulin from unrelated donors, may be important for Id-anti-Id regulation if such regulation requires the presence of allotype-linked *Igh-V* genes, as was recently demonstrated for the anti-Id induction of protection against trypanosomiasis by Sacks and Sher (28). The priming effect of maternal antibody on immune responses in infants with regard to Ps antigens as compared with protein antigens remains to be investigated. We suggest that the role of network regulation via Id-anti-Id interactions will see increasing study as protective idiotypes are described in man.

Summary

Antibodies directed against the capsular polysaccharides (Ps) of encapsulated pathogenic bacteria can protect the host against infection with such organisms. The immune response to Ps, however, does not develop until relatively late in ontogeny. We have, therefore, studied alternative ways to stimulate anti-Ps antibody responses in neonates, namely priming with idiotype (Id) and anti-Id. We believe that these studies provide the first demonstration of the use of an anti-Id antibody to prime for protection against a bacterial infection and the first demonstration of the ability of a monoclonal anti-Id to prime for protection

against any microbial infection. We have used a monoclonal IgM Id, anti-K13 capsular antibody, and a monoclonal IgG1 anti-Id in studies of the effects of administration of anti-Id or Id within 24 h after birth on the ability of mice to respond to subsequent immunization and challenge with live bacteria. These studies show that neonatal administration of 1 μg of Id or 50 ng of anti-Id lead to significantly enhanced protection in mice immunized at 4 wk of age and challenged at 5 wk with an intraperitoneal injection of 20–30 LD_{50} of *E. coli* 06:K13:H1, as compared with unprimed or antigen (Ps)-primed controls. Mice primed at birth, immunized at 12 wk of age, a time when they can respond fully to Ps itself, and challenged 1 wk later, were still significantly protected by anti-Id priming but no longer showed the effects of Id. We conclude that administration of protective Id early in life may serve a dual function in providing immediate passive protection as well as priming for protective antibodies upon subsequent antigen exposure.

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Note added in proof: H. Kohler, M. McNamara, and R. E. Ward have shown that immunization of BALB/c mice with 4C11, a monoclonal anti-Id T15, that was coupled to keyhole limpet hemocyanin, led to enhanced survival after infection with *Streptococcus pneumonia. In* The Molecular Basis of Cancer. R. Rein, editor. Alan R. Liss, Inc., New York. In press.

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