Longitudinal Development of Specific and Functional Antibody in Very Low Birth Weight Premature Infants¹

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ABSTRACT. We evaluated the formation of specific and functional antibody in preterm infants born weighing less than 1500 g (mean 1088 g) and less than 32 wk gestational age (mean 28.8 wk). Plasma IgG antibody against tetanus and diphtheria toxoids were measured by an enzyme-linked immunosorbent assay. Opsonic activity of heat-inactivated plasma was measured using radiolabeled bacteria, adult polymorphonuclear leukocytes and exogenous human complement. In the presence of complement, the strain of coagulase negative staphylococcus used was opsonized by IgG antibody, and the strain of *Escherichia coli* by IgM. Geometric mean plasma levels of tetanus and diphtheria IgG antibody fell from birth to 4 months chronological age, but rose significantly by 9 months (approximately 2 months after the third dose of diphtheria, tetanus, pertussis vaccine). However, at 9 months they remained lower than the respective geometric mean levels in 9-month-old term infants (tetanus: $p < 0.001$; diphtheria: $p = 0.02$). The preterm infants' mean plasma IgG staphylococcal opsonic activity fell from birth to 2.5 months, but by 9 months was comparable to that of term infants of the same age. Mean IgM opsonic activity for *E. coli* was very low at birth in both preterm and term infants. It rose with chronological age, correlating with the rise in total IgM ($r = 0.48$, $p < 0.001$) and by 9 months the mean preterm and term infants' levels of IgM opsonic activity for *E. coli* were comparable. In the newborn period, the preterm infants' levels of plasma IgG tetanus and diphtheria antibody, but not IgG staphylococcal opsonic activity, correlated with their mothers' levels (tetanus: $r = 0.84$, $p < 0.001$; diphtheria: $r = 0.86$, $p < 0.001$; staphylococcal: $r = 0.17$, $p = 0.38$). Placentally transferred maternal tetanus, diphtheria, and opsonic staphylococcal IgG antibody did not inhibit the infants' subsequent antibody responses to the respective antigens. We conclude that the ability of preterm infants to form specific and functional antibody may place them at lower risk for infection than might be expected from their very low plasma levels of total IgG and IgM antibodies. In addition, the good correlation between newborn and maternal plasma levels of IgG antibody to tetanus and diphtheria toxoids, but not IgG-dependent staphylococcal opsonic activity, suggests preferential transport of some IgG antibod-

ies across the placenta or, alternatively, that the function of some IgG antibodies may be impaired by placental transport. (*Pediatr Res* 23: 14-22, 1988)

Abbreviations

NICU, neonatal intensive care unit
VLBW, very low birth weight
DTP, diphtheria, tetanus, pertussis
ELISA, enzyme-linked immunosorbent assay
TIG, tetanus immune globulin
PMN, polymorphonuclear leukocytes
cfu, colony forming units
PBS, phosphate-buffered saline
gel HBSS, Hanks' balanced salt solution with 1.0% gelatin
cpm, counts per minute
PHS, pooled human serum

Previously we reported that VLBW premature infants (less than 1500 g birth weight) develop severe prolonged hypogammaglobulinemia in the first few months of life (1, 2). From the time of discharge from the nursery until 10 months chronological age, these infants' overall rate of infection and their rate of lower respiratory tract infection, in particular, were higher than those in term infants. However, although their IgG levels are lowest after discharge from the NICU, only two of the 89 premature infants enrolled in our study to date have developed a serious bacterial infection after discharge. Most patients with congenital immune deficiency who have circulating levels of immunoglobulin as low as those seen in preterm infants have a markedly increased susceptibility to recurrent and severe bacterial infections, and require maintenance immune serum globulin replacement therapy (3, 4). However, a small group of patients with hypogammaglobulinemia, those with transient hypogammaglobulinemia of infancy, do well without replacement therapy (5). The ability of patients with this syndrome to form antibody in response to antigenic challenge distinguishes them from other patients with congenital (4) and acquired hypogammaglobulinemia (3), and may help explain why they do not usually require immune serum globulin therapy. Herein, we set out to determine whether or not, despite their very low plasma levels of immunoglobulin G, M, and A (1), premature infants could respond to antigenic challenge with the formation of specific and functional antibodies. We measured their plasma IgG antibody responses to immunization with tetanus and diphtheria toxoids, their IgG-dependent opsonic activity for coagulase negative staphylococcus, and their IgM-dependent opsonic activity for *Escherichia coli*.

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MATERIALS AND METHODS

Study population. After obtaining informed written parental consent, we enrolled infants with birth weights less than 1500 g who were appropriate for gestational age (6) and were either inborn or transferred within the first 24 h of life to the NICU at the University of Connecticut Health Center. Infants with major congenital or metabolic defects or intrauterine infections were not enrolled in the study. To date, 39 infants have been followed until 12 months, and another 12 until at least 6 months chronological age. Because the infants reported herein are part of an ongoing study to determine the effects of feeding formula *versus* human milk on the development of the immune system, two infants who developed necrotizing enterocolitis, and five who were unable to tolerate enteral feeds by 28 days of life were withdrawn from the study. Two other babies were excluded after double-volume exchange transfusions. When blood transfusions were required, red blood cells were tightly packed (hematocrit greater than 80%) and resuspended in saline in order to reduce the amount of immunoglobulin in the transfusion.

The mean ($\pm$ SD) gestational age was 28.8 ($\pm$ 1.8) wk (median 29; range 24–32) for the 51 infants followed until at least 6 months of age. The mean birth weight was 1088 ($\pm$ 204) g (median 1115; range 659–1439). There was a good correlation between gestational age and birth weight ($r = 0.80$, $p < 0.001$). There were 30 males and 21 females. Forty-six infants were Caucasian and five were black. The pattern of infection in the first 43 infants enrolled has been reported previously (1). Subsequently, of the 89 babies enrolled to date, one baby with severe, oxygen-dependent bronchopulmonary dysplasia died at 5.5 months of age with pneumococcal sepsis and respiratory syncytial virus pneumonia. Another infant developed pneumococcal meningitis at 4.5 months of age and recovered without sequelae.

Beginning in the 1st wk of life (median = day 6), heparinized blood samples were obtained by heel stick every 2 wk until discharge from the NICU. Infants were seen in the Neonatal Follow-up Clinic at the University of Connecticut Health Center at approximately 4, 6, and 10 months chronological age. Heparinized blood was obtained by venipuncture at follow-up clinic visits. Plasma was stored at -70°C in small aliquots. Plasma was used instead of serum to allow the recovery of lymphocytes for other studies of the immune system. We have found that the antibody concentrations and function measured in this study are comparable in plasma and serum (Ballow M, Cates KL, unpublished data).

After informed consent was obtained from the parents, plasma samples also were obtained from 10 healthy term infants in the 1st wk of life, and from 37 others at 9 to 10 months of age. All 9- to 10-month-old term infants had received three doses of DTP vaccine.

DTP immunization. The age at the time the preterm infants were immunized with DTP vaccine varied because of the infants' clinical condition. The first dose was given at a mean ($\pm$ SD) of 69 ($\pm$ 24) days chronological age (median 62; range 41–170), the second at 142 ($\pm$ 38) days (median 140; range 84–324), and the third at 201 ($\pm$ 31) days (median 197; range 140–328). Eight infants did not receive three full doses of vaccine. In six of these infants, one of the three injections (five first dose; one third dose) consisted of only one-half or the recommended dosage. Two other infants received half doses for two of their three injections (first and second; first and third).

Tetanus and diphtheria toxoid IgG antibody determination. IgG antibodies to tetanus and diphtheria toxoids were measured by an ELISA. Tetanus toxoid IgG antibodies were quantified as previously described (7) with minor modifications. Dilutions (0.025 to 0.0018 tetanus antitoxin U/ml) of TIG (human Hyper-Tet; 250 U/ml; Cutter Laboratories, Berkeley, CA) were used for the standard curve. Plasma samples from infants <200 days chronological age initially were diluted 1:5, and those >200 days, 1:10. Five 2-fold serial dilutions were used to construct the

dilution curve. The concentrations of IgG antibody to tetanus toxoid (U/ml) in the infants' plasma samples were calculated by computer from the linear portion of the standard curve by polynomial regression. The lower limit of sensitivity of the assay was 0.01 U/ml. A human serum pool was assayed on each plate as an internal control. The coefficient of variation was 10.1% (7).

IgG antibody to diphtheria toxoid (refined concentrate, preservative free; Wyeth Laboratories, Marietta, PA) was quantified by ELISA as for tetanus antibodies except that, because of the lack of availability of a standardized human antiserum, PHS derived from 10 healthy adults was used for the standard curve. The PHS contained 0.96 diphtheria antitoxin U/ml (kindly measured by Dr. George R. Siber, the Massachusetts Public Health Biologic Laboratories, Boston, MA). Six 2-fold serial dilutions (1:50 to 1:1600) of the standard PHS pool and infant plasma samples were tested. The area under the dilution curve for PHS and each infants' sample was calculated as described by Sedgwick *et al.* (7) and Sparks and Ballow (8). The area under the curve for PHS was normalized to 1000 U/ml (arbitrary units). The amounts of IgG antibody to diphtheria toxoid in the infants' plasma samples were expressed as a percentage of that in the PHS and (1% corresponding to 0.0096, or approximately 0.01 U/ml). The lower limit of sensitivity of this assay was 1% of the PHS. The coefficient of variation was 10.8% for the reproducibility of one sample on different days and different microtiter plates.

Determination of total plasma IgG and IgM. Total plasma IgG and IgM concentrations were measured by ELISA (1).

Determination of opsonic activity. Opsonic activity was measured by a modification of the methods previously described by Peterson *et al.* (9) and Cates *et al.* (10) for measuring uptake of radiolabeled bacteria by PMN. Two organisms were chosen for opsonic studies based on 1) their ability to resist killing by serum or plasma alone, in the absence of PMN (*i.e.* serum resistance), 2) their ability to be opsonized well by plasma from most adults, and 3) their requirement for heat-stable opsonins (most likely immunoglobulin), in addition to complement. The last requirement was necessary since the purpose of our study was to evaluate the development of functional antibody, not complement, in premature infants. Studies of complement have been performed in this population by other investigators (11, 12).

The strain of coagulase negative staphylococcus used was a clinical isolate derived from the blood of a septic premature infant in the NICU at the University of Connecticut Health Center. It was identified as coagulase negative staphylococcus by standard techniques (Ref. 13, identification kindly performed by Dr. Richard C. Tilton, Clinical Microbiology Laboratory, the University of Connecticut Health Center). We selected it from among five clinical isolates of coagulase negative staphylococcus because its uptake by human PMN was more dependent on a heat-stable plasma factor, in addition to complement, than the other strains.

The test organism was grown overnight on Mueller-Hinton agar (Regional Media Laboratories, Lenexa, KS) and was stored in small aliquots in skim milk at -70°C . The night before the assay, a portion of skim milk was subcultured onto Mueller-Hinton agar and incubated aerobically overnight at 37°C . The following morning, a large loopful of organisms was inoculated into 10 ml of Mueller-Hinton broth (Beckton Dickinson, Cockeysville, MD) and 150 μl of methyl [^3H]thymidine (specific activity, 5 Ci/mmol; Amersham Corporation, Arlington Heights, IL) were added. The organism was grown for 2.5 to 3 h in a shaking 37°C water bath (100 cycles/min) to a concentration of approximately 8×10^8 cfu/ml. The radiolabeled bacteria were washed twice with PBS (pH 7.4) and resuspended in PBS to a final concentration of approximately 2×10^9 cfu/ml as determined by spectrophotometry and confirmed by colony counts. The final suspension of organisms had approximately 7×10^5 cpm/ml.

The other organism used was a strain of *Escherichia coli*, serotype 022:H16 (generously provided by Dr. Paul G. Quie, the University of Minnesota Hospitals, Minneapolis, MN). It was stored in skim milk at -70°C . Two nights before the assay, a portion of skim milk was cultured onto Mueller-Hinton agar and incubated aerobically overnight at 37°C . The following evening, 20 colonies were touched with a loop and inoculated into 10 ml of Mueller-Hinton broth and the culture was incubated aerobically overnight (approximately 18 h) at 37°C . On the morning of the assay, 1.5 ml of the overnight culture was inoculated into 13.5 ml of Mueller-Hinton broth, and 35 μl of methyl [^3H] thymidine were added. The organism was grown for 2.5 to 3 h in a shaking 37°C water bath (60 cycles/min) to a concentration of approximately 8×10^8 cfu/ml. The radiolabeled bacteria were washed twice with PBS and resuspended in PBS to a final concentration of approximately 2×10^9 cfu/ml as determined by spectrophotometry and confirmed by colony counts. The final suspension of organisms had approximately 9×10^5 cpm/ml.

Human PMN were isolated daily from heparinized peripheral venous blood of healthy adult volunteers as previously described (10), and resuspended to a final concentration of 10^7 cells per ml in HBSS (Gibco Laboratories, Grand Island, NY) with 1% gelatin HBSS (Becton Dickinson, Cockeysville, MD).

The babies' plasma samples contained a variable amount of complement activity because they could not always be frozen at -70°C immediately after sampling. Thus, all samples were heat-inactivated at 56°C for 30 min before use and then tested for opsonic activity in the presence of exogenous human complement. The source of complement was serum from an adult with common variable hypogammaglobulinemia (serum IgG: 22 mg/dl, IgM: 14 mg/dl, IgA: 8 mg/dl), but normal total hemolytic complement activity [CH50; 277 hemolytic units (normal 193 to 361 U)]. This serum was stored in small portions at -70°C .

Radiolabeled organisms were preopsonized in a reaction mixture containing 50 μl of radiolabeled staphylococci or *E. coli*, 12.5 μl of complement, and 12.5 μl of the plasma sample to be tested for the staphylococcal assay (final complement and plasma concentrations: 2.5% each) or 25 μl of complement and 50 μl of plasma to be tested for the *E. coli* assay (final complement concentration: 5%; final plasma concentration: 10%), and sufficient gel HBSS to bring the final volume to 500 μl . After 5 min incubation in a 37°C shaking water bath, the preopsonized bacteria were centrifuged at $1500 \times g$ for 15 min at 4°C . The supernate was discarded, and the organisms were resuspended in 1 ml of gel HBSS to a density of 10^8 cfu/ml.

The phagocytosis mixture consisted of 100 μl of preopsonized radiolabeled staphylococci or *E. coli* (10^8 cfu/ml), and 100 μl of the PMN suspension (10^7 /ml), giving a final ratio of bacteria to PMN of 10:1. Organisms that had been preopsonized with each plasma sample were tested in triplicate pairs with PMN, and in a control pair without PMN. The tubes were incubated in a 37°C shaking water bath (100 cycles/min for staphylococci and 60 cycles/min for *E. coli*) for 15 min for the staphylococcal assay, and 12 min for the *E. coli* assay. The reaction was stopped by adding 3 ml of iced PBS to each tube. Differential centrifugation was performed by spinning one tube of each pair at $125 \times g$, 4°C for 5 min to pellet PMN and intracellular organisms (slow spin), and the other tube of the pair at $1500 \times g$ for 15 min to pellet all the organisms (fast spin). Uptake of radiolabeled bacteria by PMN was quantitated as previously described (10) by calculating the ratio of PMN-associated counts in the pellet from the slow spin to total bacteria-associated counts in the pellet from the fast spin. To control for day to day variability, opsonic activity of the test plasma was expressed as a percent of the uptake of bacteria induced by opsonization with PHS, in the presence of exogenous complement. Uptake induced by the respective concentrations of PHS plus complement for each organism (as noted above) was assigned an arbitrary value of 100% since it approximated the upper limit of the assays (mean

$\pm$ SD of 58 staphylococcal experiments was $75.1 \pm 7.5\%$ and for 27 *E. coli* experiments was $51.7 \pm 11.6\%$ uptake of total cpm added). When complement alone was used as an opsonin, the mean of 53 staphylococcal experiments was $17.2 \pm 11.8\%$ and for 27 *E. coli* experiments was $4.0 \pm 2.9\%$ of control uptake. The coefficient of variation when one sample was tested repeatedly on different days with PMN from different donors was 10.0% for staphylococcal experiments, and 13.4% for *E. coli* experiments. Opsonic activity of individual healthy adults was $83 \pm 11\%$ control uptake for coagulase negative staphylococcus ($n = 11$), and $109 \pm 13\%$ for *E. coli* ($n = 10$).

Statistics. Statistical analyses were performed on the Apple IIe computer. Simple linear regression and the Student's *t* test were performed using the Stats Plus program. A *t* test for paired samples was used when testing infant: mother pairs, or changes in individual infants' values over time. The *t* test for unpaired samples was used for comparing the mean or geometric mean values of different groups. Log transformation was performed on the total plasma IgG and IgM, and tetanus and diphtheria IgG concentrations, and geometric means were used. Results of the opsonic assays are expressed as arithmetic means.

RESULTS

IgG antibody to tetanus and diphtheria toxoids. As has been reported (15), we found no difference in the geometric mean values of antibody responses to tetanus or diphtheria toxoids among the eight infants who had received half doses of DTP vaccine for one or two of their injections, and those of the 22 infants who had received three full doses of vaccine. Plasma samples were available for four of the five infants who received half doses of vaccine for the first injection. Their geometric mean tetanus and diphtheria toxoid IgG antibody levels after the first dose were comparable to those of the 21 infants who had received full doses (tetanus: 0.024 versus 0.028 U/ml, $p = 0.68$; diphtheria: 2.3 versus 6.0%, $p = 0.27$). Similarly, the antibody levels for the four infants who had received half doses and whose plasma was available after the second dose of vaccine, were comparable to those of the 20 infants who had received two full doses (tetanus: 0.037 versus 0.047 U/ml, $p = 0.61$; diphtheria: 21.1 versus 17.1%, $p = 0.68$). After the third dose of vaccine, plasma was available on all eight infants who had received one ($n = 6$) or two ($n = 2$) half doses, and on 22 infants who had received three full doses. Again, the antibody concentrations were comparable for the two groups (tetanus: 0.108 versus 0.089 U/ml, $p = 0.55$; diphtheria: 31.4 versus 32.9%, $p = 0.55$). Therefore, for purposes of analysis, data from the infants who had received one or two half doses were combined with those of the infants who had received three full doses of DTP vaccine.

Changes with age in geometric mean IgG antibodies to tetanus and diphtheria toxoid are shown in Tables 1 and 2 and Figures 1 and 2. As can be seen in Table 1 and Figure 1, in the first 2 wk of life, the preterm infants' geometric mean tetanus IgG antibody level is lower than that of their mothers, but the difference is not significant ($p = 0.06$). The geometric mean level of tetanus IgG antibody fell from the newborn period to reach its lowest point at 4 to 6 months chronological age. It rose significantly from 6 to 9 months ($p = 0.001$), after the third dose of vaccine. However, at 9 months chronological age, the preterm infants' geometric mean level of IgG antibody to tetanus toxoid was significantly lower than that of their mothers ($p < 0.001$) or of 9-month-old term infants ($p < 0.001$).

As noted above ("Materials and methods"), the ELISA assay used to measure IgG antibody to tetanus toxoid was standardized with TIG of known toxin neutralizing activity. Tetanus antitoxin levels of >0.01 U/ml as measured by tetanus toxin neutralization assays correlate with protection (16). Nine of our infants had one tetanus IgG level of 0.01 U/ml, and another three infants had a level of less than 0.01 U/ml as measured by ELISA on one of their plasma samples. Five of the nine samples with levels of

Table 1. *Tetanus IgG concentrations*

	Age (mo)	n	Doses DTP	Geometric mean* (U/ml)	p
Preterm infants	0.5	27	Pre	0.22 (0.06–0.75)†	0.04
	2	16	Pre	0.09 (0.02–0.42)	
	4	25	1	0.03 (0.01–0.10)	0.01
	6	23	2	0.04 (0.02–0.09)	
	9	22	3	0.10 (0.04–0.21)	0.001
	12	7	3	0.07 (0.04–0.15)	
Term infants	9	25	3	0.34 (0.12–1.00)	<0.001‡
Mothers		25		0.35 (0.04–3.25)	<0.001‡

* Measured 2 or more wk after the indicated dose of vaccine.

† The ranges in parentheses were determined by taking the antilog of (mean logarithm $\pm$ 1 SD of the logarithms).

‡ Compared with 9-month-old preterm infants.

Table 2. *Diphtheria IgG concentrations*

	Age (mo)	n	Doses DTP	Geometric mean* (U/ml)	p
Preterm infants	0.5	28	Pre	17.9† (5.0–64.4)‡	0.009
	2	16	Pre	5.7 (1.2–26.8)	
	4	25	1	5.4 (1.0–29.0)	0.03
	6	23	2	16.6 (3.0–92.3)	
	9	22	3	30.9 (8.7–109.4)	0.17
	12	7	3	30.7 (16.3–57.9)	
Term infants	9	25	3	67.6 (23.8–192.3)	0.02§
Mothers		25		18.7 (6.3–55.8)	0.5§

* Measured 2 or more wk after the indicated dose of vaccine.

† Results are expressed as a percent of a normal human serum pool (see text).

‡ The ranges in parentheses were determined by taking the antilog of (mean logarithm $\pm$ 1 SD of the logarithms).

§ Compared with 9-month-old preterm infants.

0.01 U/ml were obtained just before the second dose of DTP, three after the second, and only one was obtained after the third dose. Plasma samples were available on eight of nine mothers of these infants, and they had a median tetanus IgG level of 1.04 U/ml (range 0.48 to 2.46 U/ml). Two of the three infant samples with levels less than 0.01 U/ml were obtained before the first dose of vaccine, and the mothers of both infants also had levels of less than 0.01 U/ml (the lower limit of the assay). The third infant sample with a level of <0.01 U/ml was obtained on day 78 of life, 22 days after the first dose of vaccine. This infant's mother had a level of 0.74 U/ml.

Fig. 1. Plasma tetanus IgG concentrations in very small premature infants (<1500 g), in their mothers, and in 9-month-old term infants. Bars represent geometric mean $\pm$ SD. Preterm infants 0.5 and 2 months of age had received no DTP immunizations; those 4 months had received one dose, those 6 months, two doses and those 9 and 12 months, three doses, respectively. All 9-month-old term infants had received three doses of DTP vaccine.

In the first 2 wk of life, the preterm infants had a geometric mean diphtheria IgG antibody level comparable to that of their mothers (Table 2). The preterm infants' geometric mean diphtheria IgG level fell and was lowest at 2 to 4 months chronological age, but rose significantly after the second dose of DTP vaccine (6-month sample) to a level comparable to that seen in the first 2 wk of life ($p = 0.38$) and to that of the mothers ($p = 0.5$). However, even at 9 and 12 months, the preterm infants' diphtheria IgG level was lower than that of 9-month-old term infants ($p = 0.02$).

A level of 0.01 diphtheria antitoxin U/ml measured by toxin neutralization assays corresponds with protection (17). The lower limit of the ELISA assay we used for determining diphtheria IgG antibody was 1% of the activity of PHS, which contained 0.96 diphtheria antitoxin U/ml. Therefore, the lower limit of the assay corresponded to approximately 0.01 diphtheria antitoxin U/ml. Nine infants had diphtheria IgG antibody levels that were below the level of detection of our assay, two just before the first dose, three just before the second dose, and four before the third dose. The median diphtheria IgG antibody level in the seven mothers whose plasma was available was 13.8% (range 2.1 to 255%), six of seven had levels of 20.6% or less.

There was no correlation between gestational age and plasma tetanus IgG ($r = 0.214$, $p = 0.283$), or diphtheria IgG values ($r = 0.155$, $p = 0.435$) in the first 2 wk of life. However, there was a very good correlation between paired maternal and preterm infant tetanus or diphtheria IgG antibody levels in the newborn period (tetanus: $n = 18$ pairs, $r = 0.84$, $p < 0.001$; diphtheria: n

Fig. 2. Plasma diphtheria IgG concentrations (see legend to Fig. 1).

= 19 pairs, $r = 0.86$, $p < 0.001$). In contrast, there was no correlation between maternal and preterm infant values at 9 months chronological age, after three doses of DTP (tetanus: $n = 27$ pairs, $r = -0.314$, $p = 0.11$; diphtheria: $n = 25$ pairs, $r = 0.12$, $p = 0.57$). Similarly, as would be expected from the lack of correlation between maternal and 9-month infant samples, there was no correlation between infant geometric mean tetanus or diphtheria IgG antibody concentrations at birth and those at 9 months ($n = 19$ pairs, tetanus: $r = -0.356$, $p = 0.13$; diphtheria: $r = 0.032$, $p = 0.61$), suggesting that passively acquired maternal antibody did not inhibit the infants' antibody response to immunization.

Plasma opsonic activity for coagulase negative staphylococcus. The nature of the opsonic requirements for the test strain of coagulase negative staphylococcus is demonstrated by results of a representative experiment shown in Table 3. There was relatively poor uptake of staphylococci by PMN after opsonization with human complement or heat-inactivated PHS alone, compared with PHS which had been stored in aliquots at -70°C preserve complement activity, or heat-inactivated PHS plus complement. Heat-inactivated PHS was absorbed on an IgM Isolation System (Isolab, Inc., Akron, OH) to remove about 95% of its IgG content, leaving about 90% of the original IgM. In the presence of complement, opsonic activity of the absorbed PHS was comparable to that of complement alone (Table 3). In contrast, in the presence of complement, an IgG pool which contained $>99\%$ IgG (14), generously provided by Dr. John R. Schreiber, Albany Medical College and Dr. George R. Siber) had opsonic activity comparable to that of PHS tested at the same or greater concentrations of total IgG. These data suggested that the heat-stable factor required for optimal plasma opsonic activity was IgG antibody.

Table 3. Opsonic activity for coagulase-negative staphylococcus

Opsonin	% Control uptake*
C†	18
Δ‡ PHS§	34
PHS	110
Δ PHS + C	100
Δ Absorbed PHS + C	19
IgG¶ (3.1 mg/dl) + C	91
IgG (6.25 mg/dl) + C	100
IgG (12.5 mg/dl) + C	129
IgG (25 mg/dl) + C	127
IgG (50 mg/dl) + C	100
IgG (100 mg/dl) + C	100
IgG (200 mg/dl) + C	107

* Uptake of radiolabeled staphylococci by 10% Δ PHS and 5% complement was assigned a value of 100% for this experiment and other results are expressed as a percentage of this control value (see text).

† Complement (final concentration, 5%).

‡ Heat-inactivated.

§ PHS (final concentration, 10%).

|| Absorbed to remove IgG, leaving IgM (see text).

¶ Purified IgG (see text).

In contrast to tetanus and diphtheria IgG levels, in the newborn period, the premature infants' mean plasma opsonic activity for coagulase negative staphylococcus was significantly lower than that of their mothers ($53.5 \pm 20.1\%$ control uptake *versus* $97.0 \pm 12.5\%$, $p < 0.001$, Table 4 and Fig. 3). Opsonic activity fell in the first 3 wk of life, and began to rise again at 4 months chronological age. At 8 and 11 months, it was comparable to that of 9-month-old term infants ($p = 0.27$ and 0.59 , respectively). Mean opsonic activity of both 11-month-old preterm and 9-month-old term infants was lower than that of the preterm infants' mothers ($p = 0.02$ and <0.001 , respectively).

There was no correlation between gestational age and staphylococcal opsonic activity at birth ($r = 0.221$, $p = 0.18$); nor was there a correlation between maternal levels and preterm infant levels in the newborn period ($n = 28$ pairs, $r = 0.17$, $p = 0.38$), or infant levels at 8 to 11 months ($n = 16$ pairs, $r = 0.32$, $p = 0.23$). Also, there was no correlation between the premature infants' opsonic activity for coagulase negative staphylococcus in the newborn period and that at 11 months of age ($n = 23$ pairs, $r = 0.134$, $p = 0.55$), suggesting that high levels of activity at birth did not adversely affect the ability to produce opsonic antibody for this organism later in the 1st yr of life.

Because it appeared that the heat-stable opsonic factor for this particular strain of coagulase negative staphylococcus was IgG, we examined the correlation between infants' plasma opsonic activity for this organism and their total plasma IgG concentrations. In 248 plasma samples we found a weak ($r = 0.201$), but significant ($p = 0.001$), correlation between staphylococcal opsonic activity and total IgG concentrations. Since, in the first few weeks of life, most infant IgG is of maternal origin, we reanalyzed the correlation between total plasma IgG and staphylococcal opsonic activity in 156 samples from 38 infants obtained prior to, and including, each infants' lowest IgG value (at about 2 months of age) (1), and then in 130 samples obtained from the lowest IgG value until 11 to 12 months of age. The correlation coefficient (r) between coagulase negative staphylococcal opsonic activity and the initial fall in IgG concentration was 0.095 ($p = 0.24$), but with the subsequent rise in IgG was 0.392 ($p < 0.001$). These data suggested that there was a better correlation between coagulase negative staphylococcal opsonic activity and the infants' own plasma IgG than with transplacentally acquired maternal IgG. The correlation coefficient between the mothers' total plasma IgG and their opsonic activity for this organism was 0.39 ($n = 32$, $p = 0.03$).

Plasma opsonic activity for E. coli. The opsonic requirements for the *E. coli* strain employed are shown in Table 5. As was seen with the staphylococcus (Table 3), there was very little uptake of *E. coli* by PMN in the presence of complement or heat-inactivated PHS alone. We used purified IgM and IgG, as well as the whole serum from which they were derived (14) to evaluate opsonic activity of IgM and IgG antibody. In the absence of complement, these preparations had no significant opsonic activity for *E. coli* (Cates KL, unpublished data). However, in the presence of complement, 10% heat-inactivated whole serum from which the purified IgM and IgG had been derived (containing 10 mg/dl of IgM and 60 mg/dl of IgG) had opsonic activity (110%) comparable to that of 10% heat-inactivated control PHS and complement (100%). In the presence of complement, purified IgM tested at 2 and 5 mg/dl had 37 and 49% control uptake, respectively. Limited availability of IgM precluded testing of

higher concentrations. In contrast, in the presence of complement, purified IgG tested at concentrations of 10 and 30 mg/dl had only 5 and 7% control uptake, respectively.

The premature and term infants had very low levels of opsonic activity for *E. coli* in the newborn period (Table 6). In preterm infants, the level rose gradually beginning at 4 months chronological age and, at 8 and 11 months, was comparable to that of 9-month-old term infants. However, opsonic activity of both groups of infants at those ages was still lower than that of the preterm infants' mothers ($p < 0.001$, Table 6; Fig. 4). There was a good correlation ($r = 0.48$, $p < 0.001$) between the preterm infants' total plasma IgM concentrations and opsonic activity for *E. coli*.

DISCUSSION

Previously we reported that VLBW (less than 1500 g) preterm infants develop severe prolonged hypogammaglobulinemia in the first few months of life (1, 2). Compared with term infants who have a mean plasma IgG level of 427 mg/dl (range 206–1125) at 4 to 6 months of age (18), at 4 months chronological

Table 4. Opsonic activity for coagulase-negative staphylococcus

	Age (mo)	n	Mean $\pm$ SD (% control)*	p
Preterm infants	0.5	39	53.5 $\pm$ 20.1	<0.001
	0.75	24	46.6 $\pm$ 21.4	
	1.25	31	45.7 $\pm$ 25.1	
	1.75	20	49.4 $\pm$ 33.1	
	2.5	23	45.1 $\pm$ 25.4	
	4	30	60.6 $\pm$ 27.2	<0.02
	6	26	80.4 $\pm$ 25.0	
	8	11	76.2 $\pm$ 19.7	<0.001
	11	28	88.9 $\pm$ 12.4	
				NS
Term infants	9	26	85.2 $\pm$ 10.7	NS†
Mothers		28	97.0 $\pm$ 12.5	0.02† <0.001‡

* Results are expressed as a percentage of the opsonic activity of a normal human serum pool (see text).

† Compared with 11-month-old preterm infants.

‡ Compared with 9-month-old term infants.

Table 5. Opsonic activity for *E. coli*

Opsonin (mg/dl)	% Control uptake*	IgM (mg/dl)	IgG (mg/dl)
C†	2	0	0
Δ ‡ PHS§	16	NT	NT
Δ PHS + C	100	NT	NT
Δ Serum¶ + C	110	10	60
IgM + C	37	2	0
IgM + C	49	5	0
IgG + C	5	0	10
IgG + C	7	0	30

* Uptake of radiolabeled *E. coli* by 10% Δ PHS and 5% complement was assigned a value of 100% and other results are expressed as a percentage of this control value (see text).

† Complement (final concentration, 5%).

‡ Heat-inactivated.

§ PHS (final concentration, 10%).

|| Not tested.

¶ Whole serum from which purified IgM and IgG were derived (final concentration, 10%).

Fig. 3. Plasma opsonic activity for coagulase negative staphylococcus in very small premature infants, in their mothers, and in 9-month-old term infants. Bars represent mean $\pm$ 1 SD.

age, all of our preterm infants had IgG levels of less than 200 mg/dl and more than two-thirds had levels less than 100 mg/dl. By 6 months, more than 60% of the preterm infants still had IgG levels of less than 200 mg/dl (1). The present study was undertaken in order to determine whether these preterm infants could make specific antibodies despite their profound hypogammaglobulinemia. We found that VLBW preterm infants were capable of producing specific IgG antibody in response to immunization with tetanus and diphtheria toxoids. In addition, by 8 to 11 months chronological age, the preterm infants had developed levels of functional opsonic activity for an IgG-dependent strain of coagulase negative staphylococcus and an IgM-dependent strain of *E. coli* which were comparable to those of 9-month-old term infants.

Bernbaum *et al.* (19) previously reported the response of preterm infants to DTP immunization. Our study differs from theirs in that we examined the changes in absolute levels of tetanus and diphtheria IgG antibody in preterm infants over the 1st yr of life, not the percentage of infants who developed "protective" levels of antibody, especially since this relatively small amount of antibody (0.01 U/ml) is found in most preterm

infants even after only the first dose of vaccine (19; and present study). We also quantitated tetanus and diphtheria IgG antibodies in order to compare the ability of preterm infants to make specific antibody with that of term infants.

We found that tetanus and diphtheria IgG antibody levels in newborn preterm infants were comparable to those of their mothers. Einhorn *et al.* (20) reported that IgG subclass 1 antibody is preferentially transferred across the placenta. They found that term infants' IgG1 levels were almost twice those of their mothers, whereas, IgG2 levels were similar in infants and their mothers. Approximately 90% of tetanus IgG antibody is subclass 1 (21, 22). Therefore, it is possible that even though total IgG levels are significantly lower in preterm infants than their mothers (1), tetanus and diphtheria IgG antibody levels are not lower because of preferential transport of IgG subclass 1 antibodies. The decline in transplacentally derived tetanus and diphtheria IgG antibody paralleled the decline in these infants' total plasma IgG concentrations in the first few months of life (1). The preterm infants' rate of antibody production overcame the rate of catabolism of maternal antibody, on average, only after the third dose of vaccine for tetanus IgG, and after the second dose of diphtheria IgG. After the lowest levels were reached, all infants developed tetanus IgG antibody levels ≥ 0.01 U/ml as measured by ELISA. Also, all of the infants had diphtheria IgG antibody levels $>1\%$ of control, which correspond to approximately 0.01 diphtheria antitoxin U/ml. After three doses of DTP vaccine, the preterm infants' tetanus and diphtheria IgG levels remained significantly lower than those of term infants who had received three doses of vaccine, and their tetanus, but not diphtheria, IgG levels were lower than their mothers'. (The low maternal levels of diphtheria antibodies, compared to those of full term infants at 9 months of age, probably reflect that the mothers had received tetanus, but not diphtheria boosters.) However, the preterm infants clearly were capable of forming specific antibody in response to challenge with these two antigens.

There have been conflicting reports as to whether or not passive transport of maternal tetanus or diphtheria antibody interferes with the infants' response to immunization with the respective antigen (23-26). We found no correlation between maternal or newborn preterm infant tetanus and diphtheria IgG antibody levels, and the antibody levels to these antigens at 9 months of age. These data suggest that maternally derived tetanus and diphtheria IgG antibodies do not influence the infants' response to these antigens upon immunization with DPT.

The two organisms used in the opsonic assays were chosen

Table 6. Opsonic activity for *E. coli*

	Age (mo)	n	Mean $\pm$ SD (% Control)*	p
Preterm infants	0.5	28	5.3 $\pm$ 3.2	>0.1†
	0.75	6	4.7 $\pm$ 1.9	
	1.25	22	5.2 $\pm$ 2.7	
	1.75	4	5.2 $\pm$ 2.9	
	2.5	12	6.0 $\pm$ 3.3	
	4	19	13.4 $\pm$ 17.9	
	6	17	23.8 $\pm$ 20.9	
	8	7	37.8 $\pm$ 39.4	
	11	13	62.8 $\pm$ 17.1	
Term infants	Newborn	10	7.5 $\pm$ 2.7	>0.1†
	9	37	50.9 $\pm$ 27.2	
Mothers		18	87.3 $\pm$ 14.9	<0.001‡

* Results are expressed as a percentage of the opsonic activity of the normal human serum pool (see text).

† Compared with 8- or 11-month-old preterm infants.

‡ Compared with 9-month-old term or 11-month-old preterm infants.

Fig. 4. Plasma opsonic activity for *E. coli* in very small premature infants, in their mothers, and in newborn and 9-month-old term infants. Bars represent mean $\pm$ 1 SD.

because of their ubiquitous nature and their ability to be opsonized by most normal individuals. Whereas, many adults and most infants lack good antibody levels for such known pathogens as K1-positive *E. coli* (27) or group B streptococci (28), most normal adults have functional serum opsonic activity for non-K1 *E. coli* (29) and coagulase negative staphylococci (30). In addition, newborn infants rapidly become colonized with both of these organisms.

In vitro opsonic requirements of coagulase negative staphylococci vary (31), but many strains require both heat-stable and heat-labile factors. The test isolate used in this study required a heat-stable plasma factor in addition to complement for most efficient opsonization. We found that this factor was predominantly IgG, as is the case for most gram-positive organisms which require heat-stable opsonins (32).

The pattern of development of the preterm infants' opsonic activity for the coagulase negative staphylococcus in the 1st yr of life was similar to that of total IgG (1), and that for IgG antibody to tetanus and diphtheria toxoids in that there was an initial fall in opsonic activity, followed by a rise with age. However, the pattern of staphylococcal opsonic activity differed from that of tetanus and diphtheria IgG not only in that the preterm infants' mean level of staphylococcal opsonic activity in the newborn period was significantly lower than that of their mothers, but also in that there was no correlation between maternal and newborn levels.

The effect of placental transport on the function of IgG antibodies has not been examined extensively. Hemming *et al.* (33) found that maternal IgG opsonins for three types of group B streptococci crossed the placenta and appeared in paired cord specimens from most infants. However, most other studies comparing antibodies in paired infant:mother samples have been quantitative (34), not qualitative (*i.e.* functional). When function has been studied, the function of newborn infants' antibodies usually has been compared with that of healthy adults, not the infants' own mothers (34). Our finding that newborn opsonic activity for coagulase negative staphylococcus does not correlate with maternal activity corresponds with the results reported by Fleer *et al.* (36), and lends further support to their contention that transfer of IgG across the placenta does not necessarily reflect transfer of functional antibody. It is possible that the good correlation between newborn and maternal levels of tetanus or diphtheria IgG antibody, but not opsonic activity for coagulase negative staphylococcus, may reflect the quantitative but not functional nature of the tetanus and diphtheria IgG ELISA assays. Alternatively, it is possible that there is preferential transfer of some IgG subclasses across the placenta. Although most studies have not demonstrated significant differences in the transfer of different IgG subclasses across the placenta (37-41), as was noted above, Einhorn *et al.* (20), have demonstrated preferential transport of IgG1 over IgG2 across the placenta in term infants. Another possible explanation for the poor correlation between infant and maternal antibody functional activity that has been suggested by Fleer's group (36), is that the IgG molecule is damaged in transport across the placenta. A third possibility is that heat-stable factors other than IgG antibody are responsible for opsonization of this organism. However, this is unlikely because we found that, in the presence of complement, purified IgG antibody had opsonic activity comparable to that of pooled normal human serum containing the same amount of IgG.

The usual screening test for functional IgM antibodies is the measurement of isoagglutinins (42). However, since not all infants develop isoagglutinins (type AB), and anti-B isoagglutinins are present in only a small proportion of infants by 1 yr of age, we used an assay for detecting IgM opsonic activity for *E. coli* to study functional IgM antibody in our population of preterm infants. In previous studies, no correlation was found between maternal opsonic activity and opsonic activity for *Pseudomonas aeruginosa* (43), *E. coli* (43, 44), or *Serratia marcescens* (44). Many gram-negative organisms require IgM antibody as well as

complement, particularly the alternative pathway, for efficient opsonization (44, 45). Thus, the poor opsonic activity of newborn plasma for these organisms can be predicted because of the poor transfer of IgM and alternative pathway complement proteins across the placenta (11, 12, 43, 44). Despite high levels of maternal opsonic activity for *E. coli* and the addition of exogenous complement, opsonic activity for *E. coli* was very poor in both preterm and term infants in the newborn period. Opsonic activity in both groups of infants rose steadily with age, corresponding well with the rise in total plasma IgM antibody levels. As was seen with staphylococcal opsonic activity, preterm infants' plasma opsonic activity for *E. coli* was comparable to that of 9-month-old term infants by 8 to 11 months chronological age. Our finding that neither term nor preterm infants developed as much opsonic activity as the preterm infants' mothers could be anticipated by the fact that by 1 yr of age, even term infants' total plasma IgM is only $55 \pm 23\%$ of adult levels (18).

The results presented herein may be somewhat biased toward representing "healthier" preterm infants because seven infants were withdrawn from the study because of feeding difficulties. It is possible that factors such as poor nutrition may have made those infants more susceptible to later infections or, conversely, that the stress of necrotizing enterocolitis could have stimulated their immune systems. Nevertheless, since the infants who did not tolerate feedings represented only 8% of the 89 infants enrolled (14% of the 51 infants followed to at least 6 months of age), we conclude that the ability of most VLBW preterm infants to form specific and functional antibody may place them at lower risk of infection than might be expected from their severe, prolonged hypogammaglobulinemia in the first several months of life (1). Other patients who have similar levels of hypogammaglobulinemia and who cannot form specific antibody, such as those with Bruton's agammaglobulinemia (4) or common variable hypogammaglobulinemia (3), usually require immune serum globulin therapy. However, patients with transient hypogammaglobulinemia of infancy, who are capable of responding to antigenic challenge with the production of specific antibody, do not ordinarily need such therapeutic intervention (5). Although preterm infants have a high rate of serious infections in the NICU, we found that only two of 89 babies enrolled in this study to date have developed invasive bacterial infections after discharge from the nursery, even though they are discharged when their plasma IgG levels are still falling (*i.e.* about 2 months chronological age) (1). Several infants developed lower respiratory tract infections (1), but it is unclear whether hypogammaglobulinemia or residual lung disease was the more important predisposing factor. It is possible that the ability to produce specific and functional antibodies, may protect premature infants from having as many serious infections as would be expected from the hypogammaglobulinemia alone. Thus, the ability to recognize specific antigens and to form functional antibody may obviate the need for immune serum globulin therapy in most VLBW infants after discharge from the nursery.

Recently, it was reported that there was an inverse correlation between birth weight and age at immunization with DTP vaccine in 103 special care nursery graduates (46). Our finding, as well as that of Bernbaum *et al.* (19), that VLBW preterm infants respond well to tetanus and diphtheria antigens when administered at approximately the ages recommended by the Committee on Infectious Diseases of the American Academy of Pediatrics (47), suggests that special care nurseries should be more aggressive in developing DTP immunization programs.

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