

NATURAL KILLER CELL REGULATION OF AGE-RELATED AND TYPE-SPECIFIC VARIATIONS IN ANTIBODY RESPONSES TO PNEUMOCOCCAL POLYSACCHARIDES

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Early demonstrations that antibodies to type-specific capsular polysaccharides of *Streptococcus pneumoniae*, *Hemophilus influenzae* type b, and *Neisseria meningitidis* protect against lethal infections with these organisms (1) led to the development of vaccines comprising one or more of the respective purified bacterial capsular polysaccharides. Although effective in clinical trials in adults (2), two major problems have lessened the impact and utility of these vaccines: (a) the relatively weak antibody response in all age groups to the type-specific capsular polysaccharide antigens of certain specific organisms, e. g., *S. pneumoniae* type 6; and (b) the fact that children <2 yr of age, a group with particularly high susceptibility to lethal infection with these organisms, fail to respond not only to type 6 pneumococcal polysaccharide (PPS 6),¹ but to most other bacterial capsular polysaccharides currently used in the vaccines (3-7).

The frequent failure of adults as well as infants to mount adequate antibody responses to such antigens as PPS 6, and the age-specific inability of infants and young children to produce protective levels of antibody to pneumococcal and other bacterial polysaccharides remains unexplained. Studies in mice, a species with a type-specific and age-specific pattern of antibody responses to PPS antigens similar to that of humans, suggest that although PPS antigens are T-independent, PPS-specific antibody responses are regulated by amplifier and suppressor T cells (8-13). Thus, deficiency or immaturity of specific B cells, excessive suppressor cell activity, and deficient amplifier activity might all be considered as factors that potentially influence age-related and type-specific variations in PPS antibody responses.

Some of the cells that regulate PPS antibody responses have properties of "natural" regulatory cells (14) since they are detected in nonimmunized animals. This, together with recent evidence that NK cells, in addition to their role in tumor surveillance and control of hemopoietic stem cell populations, act as natural regulators of antibody production (15-19) led us to investigate the possibility that NK cells are natural regulators of antibody responses to PPS antigens. To address this question we measured the effects of depleting or augmenting NK activity in mice of various strains and ages on their antibody

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¹ Abbreviations used in this paper: ALS, antilymphocyte sera; PPS, pneumococcal polysaccharide.

responses to type 3 and type 6 PPS, prototypic "strong" and "weak" PPS immunogens. Our findings indicate that NK cells physiologically downregulate anti-PPS responses, and that this NK regulatory effect is the primary determinant of age-related and type-specific variations in these responses.

Materials and Methods

Mice. BALB/c mice were bred in our own facilities. C57BL/6, C57BL/6-beige, and SJL mice, age 6–8 wk, were obtained from The Jackson Laboratory, Bar Harbor, ME. Athymic nude mice, age 6–8 wk, were obtained from Charles River Breeding Laboratories, Inc., Wilmington, MA. Animals of both sexes were used in these experiments.

Immunization. Type 3 and type 6 purified PPS were obtained from American Type Culture Collection, Rockville, MD. A mixture of 0.5 μ g PPS 3 and 1.0 μ g PPS 6 diluted in normal saline was injected intraperitoneally into each recipient. Previous studies (11, 12) have shown these doses to be optimal.

Anti-Asialo GM1. Rabbit antiserum to anti-asialo GM1 was obtained from Wako Chemicals, Dallas, TX. Mice were given a single intraperitoneal injection of 50 μ l of antibody diluted to 0.25 ml in normal saline. Control animals were injected with a similar dilution of normal rabbit serum, or remained uninjected before immunization with PPS antigens.

Poly(IC). Poly(IC) (Sigma Chemical Co., St. Louis, MO) was diluted in normal saline and given as a single intraperitoneal dose of 100 μ g per recipient.

Antibody Determinations. Serum specimens from individual mice were tested for antibody to PPS 3 and PPS 6 by ELISA. To conduct the assays, 48 wells of a 96-well, flat-bottomed polyvinyl microtiter plate were coated for 48–72 h with PPS 3 or PPS 6 diluted to 10 μ g/ml carbonate-bicarbonate coating buffer. The other 48 wells were incubated with coating buffer alone. After washing three times with PBS and 0.05% Tween (PBS-Tween), plates were incubated overnight at 4°C with 0.5% BSA in coating buffer to block nonspecific protein-binding sites. Serial twofold dilutions of test samples in PBS-Tween were added in triplicate to both PPS-coated and noncoated wells. After overnight incubation at 4°C the wells were washed three times with PBS-Tween, and incubated for 4 h at 37°C with 1:1,000-diluted alkaline phosphatase conjugates of either polyvalent goat anti-mouse Ig (Boehringer Mannheim Biochemicals, Indianapolis, IN), μ chain-specific goat anti-mouse IgM, or γ chain-specific goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD). After three more washes, the plates were incubated with substrate (*p*-nitrophenylphosphate; Sigma Chemical Co.) for 30 min at room temperature. Reaction was stopped by addition of 3 N NaOH, and absorbance of light of each well at 405 nm wavelength was determined with a spectrophotometer. Blanks consisted of uncoated wells containing substrate alone. The amount of antibody-specific binding was calculated using absorbances that were corrected for nonspecific binding by subtraction of the absorbances of the parallel uncoated wells. The titer of test serum was considered to be the highest serum dilution giving a corrected optical density ≥ 0.1 in the case of assays using polyvalent enzyme-conjugated goat antiserum, and ≥ 0.3 in assays using IgM and IgG isotype-specific enzyme-conjugated antisera. In all instances, this corrected optical density reflected a statistically significant difference (as determined by Student's *t* test) between the optical densities obtained in replicates of antigen-coated and uncoated wells.

NK Assay. NK activity of spleen cells was determined in a 4-h chromium-release assay against YAC-1 target cells. Effector cells (spleen cells isolated by passage through wire mesh) were washed and suspended in medium (RPMI 1640 with 10% FCS). Target cells were labeled by incubating 10^6 cells with 200 μ Ci 51 Cr (New England Nuclear, Boston, MA) for 60 min at 37°C and then were washed three times. Target cells were added in 0.1 ml medium to V-bottomed wells (10^4 cells/well) in a microtiter plate. Each well then received 0.1 ml of medium containing various dilutions of effector cells. The plates were centrifuged at 40 *g* for 5 min and then incubated at 37°C in a humidified CO₂ incubator. After 4 h, plates were centrifuged at 200 *g* for 5 min, and 0.1 ml supernatant was collected

and counted in a gamma counter. % specific cytotoxicity = $100 \times [(text\ release) - (spontaneous\ release)/(80\% \text{ of the total label}) - (spontaneous\ release)]$.

Statistics. One-way analysis of variance with a priori contrasts was used to assess the effects of treatment on each of the antibody responses. One-way analysis of variance was also used to assess the effects of age on each of the antibody responses within each treatment group.

Results

Antibody Responses of Weanling and Adult BALB/c Mice to PPS 3 and 6 after Depletion or Augmentation of NK Activity. To examine the possibility that NK cells play a role in type-specific and age-dependent variations in antibody responses to antigens, we determined the effect of in vivo treatment with anti-asialo GM1 and poly(IC) on the anti-PPS responses of infant and adult mice. Mice injected with rabbit antibody to asialo GM1 manifest little or no NK activity 24–72 h after injection (20), whereas mice injected with poly(IC), an IFN inducer, have increased NK activity which peaks 18–24 h after injection (21).

Groups of weanling (2–3-wk-old) and adult (6–10-wk-old) BALB/c mice were injected intraperitoneally with anti-asialo GM1 or poly(IC) 24 h before immunization with optimal doses of PPS 3 and PPS 6. Controls included unimmunized mice and mice given no treatment before immunization. All mice injected with PPS 3 and PPS 6 were bled on day 5 after immunization at the time of peak antibody response. Anti-PPS 3 and anti-PPS 6 titers of these and control mice are shown in Fig. 1. Consistent with previous findings of age-related and type-specific variations in anti-PPS responses (11, 12), adult mice developed a strong antibody response to PPS 3 (geometric mean titer, 750; $p = 0.001$), but little or no response to PPS 6 (geometric mean titer, 31; $p = 0.793$). The responses of weanling mice to both PPS 3 and PPS 6 were minimal (geometric mean titers, 40; $p = 0.084$ and 31; $p = 0.545$, respectively). The weak anti-PPS 6 responses of adult mice, and the weak anti-PPS 3 and anti-PPS 6 responses of weanling mice were all dramatically altered when injected with anti-asialo GM1 1 d before immunization. Anti-asialo GM1-treated adult mice showed a 71-fold increase in anti-PPS 6 response ($p < 0.0005$), achieving an anti-PPS 6 level (geometric mean titer, 2,208) even greater than that of the anti-PPS 3 response of anti-asialo GM1-treated mice (geometric mean titer, 880). The latter was not significantly different than the anti-PPS 3 response of untreated adult mice. Anti-asialo GM1-treated weanling mice showed marked increases in antibody titers to both PPS 3 and PPS 6 (54-fold and 46-fold, respectively; $p < 0.005$). Thus, pretreatment with anti-asialo GM1, which depletes NK activity, eliminated the age-related and type-specific variations in anti-PPS responses. The likelihood that this resulted from elimination or inactivation of suppressor NK cells is supported by the finding that pretreatment with poly(IC), which augments NK activity, resulted in a 14-fold reduction in anti-PPS 3 responses of adult mice ($p = 0.003$).

Kinetics and Ig Isotype of Adult BALB/c Anti-PPS 6 Response after Depletion of NK Activity. To determine whether NK cell depletion alters the Ig isotype of the anti-PPS 6 response, and to investigate the possibility that the marked differences in antibody responses after depletion of NK activity are in time course rather than extent, PPS 6-specific IgM and IgG antibody responses of anti-

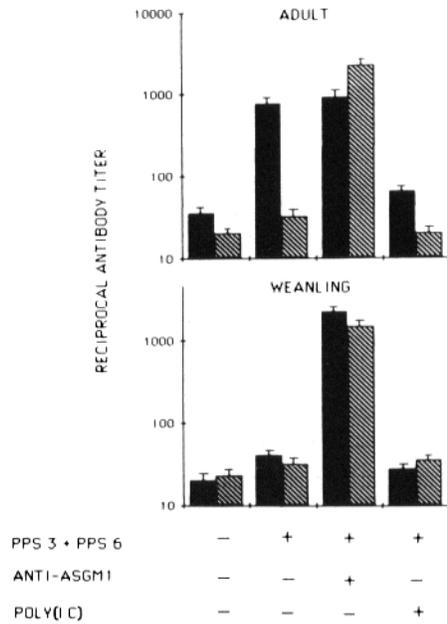


FIGURE 1. Adult (age 6–8 wk) and weanling (age 2–3 wk) BALB/c mice were immunized intraperitoneally with a mixture of 0.5 μ g PPS 3 and 1.0 μ g PPS 6 on day 0. On day -1, groups of five mice were pretreated with 50 μ l rabbit anti-asialo GM1 or 100 μ g poly(IC). Controls included unimmunized mice and mice receiving PPS immunization alone. Day +5 serum antibody titers to PPS 3 and PPS 6 were determined individually for each mouse by ELISA. The data represent the geometric mean titers (+1 SD) of five identically treated mice. Solid bars, anti-PPS 3; striped bars, anti-PPS 6.

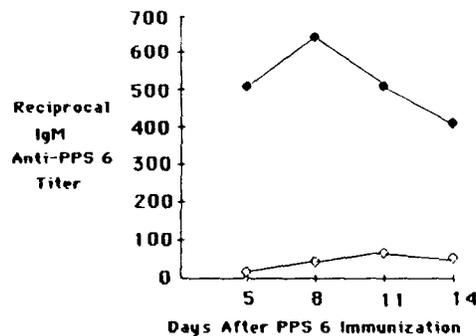


FIGURE 2. Adult (age 6–8 wk) BALB/c mice were immunized intraperitoneally with 1.0 μ g PPS 6 on day 0. On day -1, mice were pretreated with 50 μ l rabbit anti-asialo GM1 or normal rabbit serum and were bled 5, 8, 11, or 14 d after immunization. PPS 6-specific IgM and IgG antibody titers were determined individually for each mouse by ELISA. Each point represents the geometric mean of the IgM anti-PPS 6 titers of three identically treated mice. IgG anti-PPS 6 antibody was not detected in any of the sera tested. Filled squares, anti-asialo GM1; open squares, normal rabbit serum.

asialo GM1-treated or control normal rabbit serum-treated adult BALB/c mice were tested on days 5, 8, 11, and 14 after immunization (Fig. 2). IgM but not IgG anti-PPS 6 antibody was detected at each of the time points tested. As in the previous experiments, mice treated with anti-asialo GM1 before immuniza-

TABLE I
Effect of Pretreatment with Anti-Asialo GM1 on Antibody Responses to PPS Antigens in Athymic Nude Mice

Treatment	Reciprocal antibody titer on day 5*		NK activity on day 0 [†]
	PPS 3	PPS 6	
None	10, 10	10, 10	35, 51
PPS 3 + PPS 6 (day 0)	40, 10	10, 10	ND
Anti-Asialo GM1 (day -1), PPS 3 + PPS 6 (day 0)	640, 640	2,560; 2,560	ND
Anti-Asialo GM1 (day -1)	10, 10, 10	10, 10, 10	1, 4

* Athymic nude mice were injected intraperitoneally with 0.5 μ g PPS 3 and 1.0 μ g PPS 6 on day 0. Some immunized mice received an intraperitoneal injection of 50 μ l rabbit anti-asialo GM1 on day -1. Control unimmunized mice were injected on day -1 with anti-asialo GM1. All mice were bled on day +5. The data represent determinations on individual mice.

[†] Specific lysis of YAC in a 4-h chromium-release assay at E/T ratio of 100. NK activity of spleen cells from untreated mice and mice treated with anti-asialo GM1 1 d earlier was tested against YAC target cells in a 4-h chromium-release assay.

tion with PPS 6 developed considerably higher antibody levels than control mice. The anti-PPS 6 titers in anti-asialo GM1-treated mice were high at day 5, peaked at day 8, and declined only slightly over the next 6 d. By contrast, the anti-PPS 6 titers of control normal rabbit serum-treated mice were low at day 5, and showed little if any tendency to increase throughout the time period examined. These findings indicate that the marked increase in anti-PPS 6 responses seen after depletion of NK activity is due to a true increase in an otherwise weak primary IgM anti-PPS 6 response, and is not simply the result of an alteration in the kinetics of that response.

Depletion of NK Activity in Athymic Nude Mice Increases Their Antibody Responses to PPS 3 and PPS 6. Although in vivo treatment with anti-asialo GM1 has been shown (20) to have no effect on T cell functions, anti-asialo GM1 has been found (22, 23) to react in vitro by complement lysis and by immunofluorescence with some T cells. This, together with the previous evidence for the existence of T cell suppressors of anti-PPS responses (8-10), led us to examine the possibility that the augmenting effect of anti-asialo GM1 treatment on PPS-antibody responses might be due to elimination or inhibition of a T cell rather than NK cell suppressor population. This was accomplished by determining the effects of pretreatment with anti-asialo GM1 on the anti-PPS 3 and anti-PPS 6 responses of adult athymic nude mice, mice that possess NK cells but lack T suppressor cells. As shown in Table I, untreated nude mice responded poorly to both PPS 3 and PPS 6. Pretreatment with anti-asialo GM1 abrogated NK activity against YAC-1 targets measured 1 d after treatment, as expected, and resulted in marked increases in the response of the nude mice to both PPS 3 and PPS 6. The increased anti-PPS responses were not due to nonspecific activation of anti-PPS responses by anti-asialo GM1 since no measurable anti-PPS 3 or anti-PPS 6 antibody was detected in mice treated with anti-asialo GM1 alone. These findings indicate that increased anti-PPS responses resulting from pretreatment with anti-asialo GM1 are due to elimination or inactivation of NK cells rather than of T suppressor cells.

TABLE II
Antibody Responses to PPS Antigens in Strains of Mice with Normal or Low NK Activity

Strain	Endogenous NK activity	Reciprocal antibody titer on day 5*	
		PPS 3	PPS 6
BALB/c	Normal	320; 80; 5,120 1,280; 1,280	160; 10; 10 10; 10
Nude	High-normal	40; 10	10; 10
C57BL/6	Normal	10; 10; 80	10; 10; 80
C57BL/6-Beige	Low	640; 640; 2,560	1,280; 2,560; 2,560
SJL	Low	2,560; 2,560; 5,120	2,560; 2,560; 2,560

* Each value represents the serum antibody titer of an individual adult mouse 5 d after intraperitoneal injection of a mixture of 0.5 μ g PPS 3 and 1.0 μ g PPS 6.

Comparison of Antibody Responses to PPS in Inbred Strains of Mice with Normal or Low Endogenous NK activity. To further examine the relationship between NK cells and anti-PPS responses, we compared the responses to PPS 3 and PPS 6 of adult BALB/c, athymic nude, and C57BL/6 mice (strains with normal or high normal endogenous NK activity) with those of age-matched SJL and C57BL/6-beige mice (strains with low to absent endogenous NK activity [24, 25]). As shown in Table II, the anti-PPS 6 responses of SJL and C57BL/6-beige mice were greater than those of BALB/c, athymic nude, and C57BL/6 mice, and their responses to PPS 3 were greater than athymic nude and C57BL/6 mice, but were similar to those of BALB/c mice.

Discussion

Two problems have hampered the clinical utility of polyvalent PPS vaccines: the relatively low immunogenicity in all age groups of certain purified PPS, exemplified by PPS 6, and the failure of infants <2 yr of age to respond adequately to this and several other PPS antigens contained in the vaccine. The immunological factors responsible for these two phenomena have not been previously established. The findings obtained here in mice, a species with a pattern of anti-PPS responses similar to that of humans, indicate that physiological down regulation of anti-PPS responses by NK cells plays a major and possibly a decisive role. Pretreatment of mice with anti-asialo GM1, which has been shown to deplete NK cytolytic activity without affecting cytotoxic T cells or macrophages (20), increased the weak anti-PPS 6 responses of adult mice to levels similar to that of their anti-PPS 3 responses, and increased the weak-to-absent anti-PPS 3 and anti-PPS 6 responses of weanling mice to the same levels achieved by adult mice. By contrast, pretreatment with poly(IC), which augments NK activity, resulted in a 14-fold reduction in the anti-PPS 3 responses of adult mice. In addition, strains of mice with low or absent endogenous NK activity were found to have considerably stronger anti-PPS responses than strains of mice with normal NK activity.

Are the markedly increased anti-PPS levels seen 5 d after immunization in mice pretreated with anti-asialo GM1 due to a change in the time course rather than extent of antibody production? After immunization with PPS 6, sera obtained 5–14 d after immunization from control normal rabbit serum-pre-

treated adult mice contained low levels of anti-PPS 6 antibody, which changed little during the period of measurement, whereas sera from anti-asialo GM1-treated mice showed relatively high levels of anti-PPS 6 antibody, which peaked on day 8 and declined slightly over the next 9 d. This, together with previous studies (12) showing that the peak primary anti-PPS 6 response normally occurs 5–8 d after immunization, indicates that the amount of anti-PPS 6 antibody seen on day 5 in anti-asialo GM1-treated animals is representative of a truly augmented response and does not simply represent the acceleration of an otherwise normal antibody response.

Previous studies (8–10, 12) have shown that euthymic but not athymic nude mice have increased anti-PPS 3 and anti-PPS 6 responses after *in vivo* treatment with heterologous anti-lymphocyte sera (ALS), and that the ability of ALS to enhance anti-PPS responses is abrogated by *in vitro* thymocyte absorption. These findings indicate that T cells exist that suppress anti-PPS responses. Nevertheless, the enhancement of anti-PPS responses in mice treated with anti-asialo GM1 seen here was almost certainly due to inactivation or depletion of NK cells and not T suppressor cells since this enhancement occurred in athymic nude mice as well as in euthymic mice. Thus, both the T cell and NK cell populations contain natural suppressors of anti-PPS responses. However, age-dependent and type-specific variations in anti-PPS responses appear to be primarily due to NK cell rather than T cell regulatory effects since such variations are negated after *in vivo* depletion of NK activity, whereas they are, if anything, exaggerated after ALS treatment (11, 12).

There are several possible mechanisms by which NK cells could physiologically downregulate anti-PPS responses. They could directly affect antigen-activated B cells, or they could indirectly inhibit the differentiation and/or proliferation of such cells by affecting amplifier T cells or macrophage accessory cells. The observation that anti-PPS responses of nude mice were enhanced by pretreatment with anti-asialo GM1 to roughly the same degree as the anti-PPS responses of euthymic mice rules out the possibility that inhibition of amplifier T cells, which nude mice presumably lack, is the sole mechanism by which NK cells suppress these responses. This conclusion is further supported by our finding that only the IgM and not IgG anti-PPS 6 response increased in NK-depleted mice. As seen here, only IgM antibody is produced in the normal murine anti-PPS response (10, 12). By contrast, it has been shown that IgG antibody is produced after immunization with PPS 3 attached to a T-dependent carrier antigen (e. g., sheep erythrocytes), or after *in vivo* removal of suppressor T cells by ALS treatment. This implies that suppressor T cells normally prevent T amplifier cells from inducing the IgM-to-IgG switch. The failure of mice to produce IgG anti-PPS 6 antibody after NK cell depletion therefore suggests that unlike suppressor T cells, NK cells are not major contributors to the physiological inhibition of amplifier T cell function. Whether NK cells directly suppress PPS-activated B cells or inhibit the function of PPS antigen-exposed accessory cells is not clear from the findings presented here, and is the subject of future investigations.

If, as our results imply, NK suppression rather than a deficiency or immaturity of B cells, T cells, or macrophages is the primary determinant of type-specific

and age-dependent variations in anti-PPS responses, then it would appear that NK cells suppress some anti-PPS responses (e. g., anti-PPS 6 responses) more effectively than others (e. g., anti-PPS 3 responses), and that NK suppression of some anti-PPS responses (e. g., anti-PPS 3 responses) is more efficient in newborns than in adults. Given the current uncertainty as to the target cell(s) for NK suppression and the nature of NK-target cell recognition in general (26), at the moment one can only speculate how this might occur. At least two possible mechanisms might be considered: (a) age-dependent differences in the number or activity of NK cells that suppress anti-PPS responses; and (b) age-dependent differences in the number of functional activity of PPS-responsive cells susceptible to NK-mediated suppression. It is of interest to examine how well each of these mechanisms fits with what is currently known about age-dependent variations in NK activity and NK target susceptibility. Thus, it is well established that, compared to adult mice, infant mice <3 wk of age show little if any NK activity against such standard target cells as YAC (27). At first glance this would appear to render unlikely the possibility that NK-mediated suppression of anti-PPS responses is more efficient in infants than in adults because of increased numbers or activity of NK cells. However, evidence suggests that the low NK activity of spleen cells from infant mice is not due to a quantitative or qualitative deficiency of NK cells, but results instead from the presence in the spleens of these mice of nonadherent non-T inhibitory cells (28). The mechanism by which these cells inhibit anti-YAC NK activity in vitro is not known. One possibility is cold-target inhibition, i. e., competitive inhibition for NK recognition of labeled YAC target cells by unlabeled physiological NK target cells prevalent in the spleens of infant mice. That infant mice may possess increased numbers of physiological NK targets is supported by the observation (29) that neonatal thymocytes are considerably more susceptible as physiological NK targets than are adult thymocytes. By analogy, PPS-reactive B cells of infants may be more susceptible to NK activity in vivo than those of adults. The abundance of these and other physiological NK targets in infant mice cells may then account for the age-dependent inverse relationship between anti-YAC NK activity and NK suppression of anti-PPS responses.

In addition to their relative inability to mount adequate antibody responses to certain PPS antigens, infants and young children fail to respond adequately to immunization with type-specific capsular polysaccharide antigens derived from other clinically important bacteria including *H. influenza* type b, and *N. meningitidis*. If, as the present findings suggest, NK suppression is responsible for the weak responses of infants to PPS antigens, it is possible that NK suppression may also play a role in the weak responses of infants to these other antigens. Further clarification of the role and mechanism of action of NK cells in controlling the antibody response to PPS antigens and other bacterial capsular polysaccharides could facilitate current efforts to devise more effective methods of immunizing infants against these bacterial pathogens.

Summary

Pretreatment of mice with anti-asialo GM1, which has been shown to deplete NK cytolytic activity without affecting cytotoxic T cells or macrophages, in-

creased the weak anti-PPS 6 responses of adult mice to levels similar to that of their anti-PPS 3 responses, and increased the weak-to-absent anti-PPS 3 and anti-PPS 6 responses of weanling mice to the same levels as achieved by adult mice. By contrast, pretreatment with poly(IC), which augments NK activity, resulted in a 14-fold reduction in the anti-PPS 3 responses of adult mice. The enhancement of anti-PPS responses in mice treated with anti-asialo GM1 was due to inactivation or depletion of NK cells, and not T suppressor cells, since this enhancement occurred in athymic nude mice as well as in euthymic mice. In addition, strains of mice with low or absent endogenous NK activity were found to have considerably stronger anti-PPS responses than strains of mice with normal NK activity. NK depletion enhanced anti-PPS 6 IgM but not IgG response, and resulted in a true increase in antibody production rather than an alteration in the time course of the response. These findings indicate that NK cells physiologically downregulate anti-PPS responses, and that age-dependent and type-specific variations in these responses are primarily determined by NK regulatory effects.

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