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Effects of Theophylline on the Neonatal Immune Response

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Summary

Theophylline has recently been shown to affect lymphocyte reactivity. In view of its widespread use in newborn intensive care units, the effects on both lymphocyte proliferation and immunoglobulin production at varied throphylline concentrations were measured. In 12 adults and 10 term infants lymphocyte proliferation, as assessed by a whole blood micromethod, was significantly decreased *in vitro* at 7.5 μg/ml. Immunoglobulin production in adults was decreased, by both a plaque forming cell assay and a radioimmunoassay *in vitro* at 12.5 μg/ml. Ten premature infants on theophylline, mean serum level 7.8 ± 0.4 μg/ml, followed for 3-5 wk, showed a slight increase in lymphocyte proliferative responses to pokeweed mitogen. These data demonstrate no *in vivo* suppression of lymphocyte proliferation in theophylline-treated neonates at low theophylline levels.

Speculation

Low dose theophylline therapy in premature infants does not interfere with mitogen-induced lymphocyte proliferation. Further studies of the effects of theophylline on immunoregulatory function in neonates may demonstrate adverse effects.

After reports of a significant reduction in apnea among theophylline-treated premature infants (21), this drug has become one of the more widely used medications in neonatal intensive care units. Theophylline has several well known toxic side effects, including tachycardia, gastritis, hypertension, hyperthermia, diuresis, and central nervous system irritability, with occasional reports of severe toxicity leading to seizures and death (19). More recently, and less well known, theophylline has clearly been shown to affect immune reactivity, both *in vitro* (8, 9, 18) and *in vivo* (6, 13). Should this occur in the premature infant, it could be associated with a decreased resistance to infection. This is of particular concern because the premature infant's immune system is undergoing marked maturational changes and drug-induced alterations could become permanent.

To determine if theophylline affects immune responses, we measured *in vitro* immunoglobulin production in adult lymphocyte cultures, and *in vitro* adult and neonatal mitogen-induced lymphocyte proliferation at varying theophylline concentrations. On the basis of these data, we then prospectively monitored the lymphocyte proliferative responses among 10 theophylline-treated premature infants.

MATERIALS AND METHODS

Assay for lymphocyte proliferation. Lymphocyte proliferation was assessed using a whole blood micromethod (16). Blood samples from young adults were collected by antecubital venipuncture. Blood was also collected from 3-day-old, healthy, term infants, as well as premature infants on theophylline therapy, via heel stick skin puncture or umbilical artery catheter. Sterile, heparinized, 250 μl Natelson glass pipettes were used for the skin puncture blood samples. Blood was then diluted 1:11 with RPMI-1640 (GIBCO, Grand Island, NY), containing penicillin 250 units/ml, streptomycin 100 μg/ml, and Hepes Buffer (Microbiological Associates, Bethesda, MD) 2.5 ml/100 ml. After dilution, one hundred and ninety μl of diluted blood was pipetted into microtiter plate wells (No. 3040, Falcon Plastics), already containing 10 μl of pokeweed mitogen (PWM, Grand Island Biological Co.) and varying theophylline concentrations. This stock PWM, diluted 1:5 with sterile normal saline, induced optimal proliferation in preliminary studies with adult and neonatal lymphocytes. All cultures were made in triplicate. The plates were incubated at 37°C in humidified 5% CO_2 for 72 h, after which 1 μCi of [^{3}H]-methylthymidine (specific activity 24 Ci/mmole) was pipetted into each well. After an additional 24 h incubation, the cultures were harvested with a multiple microharvester, using washes of both water and saline. The radioactivity in the filter disc was measured in a Searle Isocap 300 liquid scintillation counter and responses were expressed as mean cpm of the triplicates.

Assays for immunoglobulin production. Heparinized adult venous blood mononuclear cells were separated by Ficoll-Hypaque (Pharmacia Fine Chemicals) gradients, according to the method of Boyum (2). The lymphocytes were cultured and the indirect protein A plaque forming cell assay performed, as described by Fauci *et al.* (7). Five × 10^5 mononuclear cells in 1 ml of RPMI-1640 containing penicillin, streptomycin, and 10% heat-inactivated fetal calf serum (GIBCO) were incubated in 12 × 75 mm round bottom plastic tubes. Cultures were activated with PWM (10 μl/ml of a 1:5 dilution with sterile normal saline) and then incubated at 37°C 5% CO_2 in air at 100% humidity for 6 days. After this, the cells were harvested, washed, and resuspended in cold RPMI-1640. One tenth ml cell suspension mixed with 0.85 ml agarose (Accurate Chemicals, Hicksville, NY) and 0.60 ml 15% solution protein A-coated sheep red blood cells were poured onto an agarose precoated petri dish (Falcon No. 1007) and incubated at 37°C for 2 h. One ml of the IgG fraction (1:100 in RPMI-1640) of rabbit antihuman polyvalent immunoglobulin (Cappel Labs, Cochranville, PA) was then layered on the plate and incubated again for 2 h, after which the antisera was removed and 1 ml of guinea pig complement (1:40 in veronal buffered saline) added. After a further 1 h incubation, the complement was removed and PFC read at ×4 magnification, and expressed as PFC per 10^6 cultured cells.

Patient population. The first 12 premature infants placed on theophylline, from whose parents informed consent was obtained, were studied. All infants were begun on theophylline for apneic and bradycardic episodes. No infant with positive blood and/or cerebral spinal fluid cultures before onset of therapy was accepted into the study. Mean birth weights and gestational ages were 1164 g (range 740–1440 g) and 30 wk (range 26–35 wk), respectively. Seven infants were clearly appropriate weight for gestational age (AGA) and one small weight for gestational age (SGA). Two

infants had 10th percentile weights for their gestational age. Immune responses on each infant were followed for 3–5 wk. Two infants died of multiple complications of prematurity.

Loading doses of 5 mg/kg theophylline were used, followed by 1–2 mg/kg/day. Initial therapy was intravenous. Many infants were later given oral therapy. Serum theophylline levels were measured in each blood specimen studied using high pressure liquid chromatography.

Written informed consent was obtained for all individuals studied.

Statistical analysis. All data was analyzed with the paired *t* test. Significance was considered achieved at $P < 0.05$.

RESULTS

Effects of in vitro theophylline on immune responses in adults and neonates. Adult and neonatal lymphocyte proliferative responses to pokeweed mitogen (PWM) were measured, utilizing the whole blood micromethod. In both adults and healthy term neonates, lymphocyte proliferation was significantly decreased at theophylline levels of 7.5 μg/ml ($P < 0.05$), with further decreases in proliferation at higher theophylline levels (Table 1). In the adult, responses fell from 13,985 ± 2067 cpm (mean ± S.E.) in cultures with no theophylline to 8112 ± 1971 cpm in cultures with theophylline levels of 15.0 μg/ml. Similarly, in the neonate, control cultures generated a mean of 18,310 ± 2219 cpm, whereas cultures with 15.0 μg/ml theophylline had 13,030 ± 2009 cpm.

Immunoglobulin production was assessed (in adult lymphocyte cultures) with two different methods. The mean release of immunoglobulin as determined by double antibody radioimmunoassay (22), in PWM-activated adult lymphocyte cultures with theophylline was inversely proportional to the drug concentration. IgG and IgM production was significantly reduced at 12.5 μg/ml theophylline ($P < 0.02$) and IgA, at 25.0 μg/ml ($P < 0.01$). IgM fell from 1643 ng at zero levels to 1093 ng at levels of 12.5 μg/ml, whereas IgG fell from 1054 ng to 789 ng with the same drug dosage. IgA production at zero levels was 782 ng and fell to 476 ng at 25.0 μg/ml (Fig. 1). The IgG production at 25.0 μg/ml of theophylline was not significantly different, with a *P* value of 0.052, but again reached significance at 50.0 μg/ml.

Table 1. *Adult and neonatal lymphocyte proliferative responses to pokeweed mitogen*

Theophylline (μg/ml)	Adult cpm ± S.E.	Neonatal cpm ± S.E.
n	12	10
0	13985 ± 2067	18310 ± 2219
2.5	12810 ± 2251[1]	18645 ± 2691
7.5	10695 ± 2018[3]	16825 ± 2221[1]
12.5	9982 ± 2112[2]	16791 ± 2201[2]
15.0	8112 ± 1971[2]	13030 ± 2009[3]

[1] $P < 0.05$
[2] $P < 0.01$
[3] $P < 0.001$

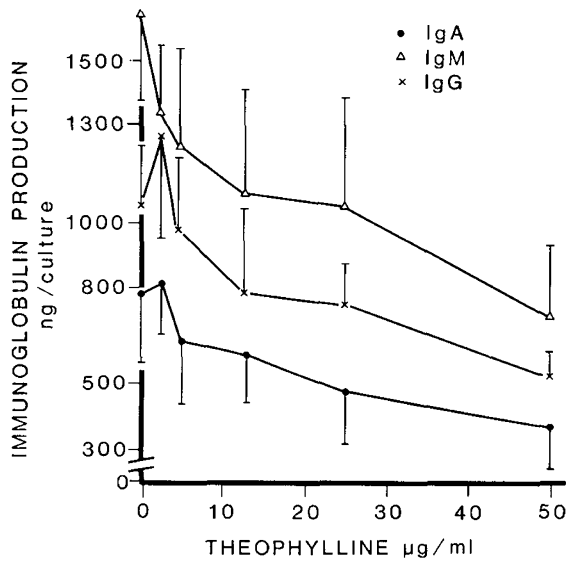

Fig. 1. Theophylline effect on adult immunoglobulin production.

Table 2. *Adult plaque forming cell response to pokeweed mitogen*

Theophylline (μg/ml)	Plaque forming cells/10^6 mononuclear cells ± S.E.
n	12
0	14681 ± 3379
2.5	12490 ± 3079[1]
7.5	10905 ± 3078[1]
12.5	6763 ± 1611[2]

[1] $P < 0.05$
[2] $P < 0.01$

Table 3. *Lymphocyte proliferative responses in 10 theophylline-treated prematures*

Time	cpm ± S.E.
0 h	20370 ± 7598
24 h	22237 ± 6186
72 h	20355 ± 4067
1 wk	31599 ± 6266
3 wk	30120 ± 5803
5 wk	24628 ± 2357[1]

[1] $P < 0.02$

The adult lymphocyte plaque forming cells response was significantly reduced ($P < 0.05$) with theophylline at 2.5 μg/ml. The mean number of plaques formed was decreased from the baseline level by 54% at a theophylline level of 12.5 μg/ml (Table 2), at which drug level the radioimmunoassay (RIA) first demonstrated a significant reduction.

Effects of in vivo theophylline on lymphocyte proliferation in premature infants. The lymphocyte proliferative response to mitogen stimulation in 10 theophylline-treated premature infants was followed. These infants were studied at 0, 24, and 72 h after the initial dose and thereafter weekly for 3–5 wk. Serum levels of theophylline were measured with each specimen and the mean level was 7.8 ± 0.4 μg/ml. The mean of the highest theophylline level in each infant was 11.8 ± 0.9 μg/ml. Two children initially had no response to mitogen but subsequently developed responses within the following 72 h. One of these infants developed staphylococcus aureus sepsis during the course of therapy, which was associated with no change in his mitogen responses.

No decrease in lymphocyte proliferative responses to PWM stimulation was detected in any of these infants (Table 3). In contrast, there was a significant ($P < 0.02$) increase in proliferation after 3–5 wk.

Because all of the infants in the study received blood transfusions at some point in their course, the effect on lymphocyte proliferation of an initial blood transfusion was studied in three premature infants, who received antibiotics but were not on theophylline. They had a mean birth weight of 1270 g and a gestational age of 30 wk. Two of the infants received packed red blood cell transfusions and the third received a whole blood transfusion. The response to PWM in the transfused blood was also measured and was less than the response found in the infant both before and after his transfusion. No marked changes in lymphocyte proliferation were discovered in any of the three infants 24 hr after receiving the transfusion (Table 4).

The variation in responses to PWM expressed as mean cpm was examined in nine cord blood samples obtained from normal term newborns. Six different dilutions with RPMI 1640 were made in

Table 4. *Effects of a blood transfusion on lymphocyte proliferation*

Infant	Donor blood	Donor blood mean cpm	Infant pretransfusion mean cpm	Infant posttransfusion mean cpm
1	PRBC[1]	6550	24910	38149
2	WB[2]	2919	16913	18043
3	PRBC[1]	14457	15884	15297

[1] PRBC, packed red blood cells
[2] WB, whole blood

Table 5. *Mononuclear cells dilutional effects on lymphocyte proliferation*

Whole blood dilution	cpm mean ± S.E.	*P* value[1]	Mononuclear cells × 10^5/ml mean ± S.E.
1:9	21414 ± 2623	< 0.50	5.7 ± 0.6
1:11	21755 ± 3319		4.7 ± 0.5
1:13	22124 ± 3011	< 0.50	3.9 ± 0.4
1:15	22194 ± 3614	< 0.50	3.4 ± 0.3
1:20	20518 ± 3552	< 0.30	2.6 ± 0.3

[1] Compared to the 1:11 dilution used in each experiment.

each cord sample. There were no significant differences in responses to PWM in those dilutions ranging from 1:9 to 1:20 (Table 5). These dilutions represented a range of 2.6–5.7 × 10^5 mononuclear cells/ml. There were technical difficulties at the 1:5 dilution resulting from quenching due to excess hemoglobin remaining in the harvested sample.

In spite of substantial changes in mononuclear cell numbers per well, there are nonsignificant variations in responses to PWM. This is probably indicative of the degree of recruitment of nonspecific cells to proliferate after a few mitogen-specific T cells are stimulated, as indicated by Tse *et al.* (20).

DISCUSSION

The mechanism by which theophylline affects various biological systems has been postulated to be its inhibition of phosphodiesterase, and secondary elevation of intracellular c-AMP (14). Recently, there have been several reports regarding the effects of theophylline, as well as other agents which increase intracellular c-AMP levels, on *in vitro* and/or *in vivo* immune function.

In animal studies, theophylline has been shown to affect macrophage function (5), and T and B cell-mediated immunity (15). It inhibits phagocytosis (5), macrophage cytotoxic activity (17) and allograft rejection (13). Low doses of theophylline stimulate immunoglobulin formation, whereas high doses are inhibitory (3). Interestingly, early exposure of lymphocytes has been shown to be excitatory, whereas late exposure inhibits antibody production (4).

In humans, Gelfand *et al.* (8) have shown theophylline to augment T suppressor cell activity, and Gupta *et al.* (9) have demonstrated a decrease in T cells *in vivo* with receptors for IgM (Tμ), generally considered to be helper cells. In contrast, Katz and Fauci (11) found an enhancement of B cell activation by a selective effect on the T cells, and postulated an inhibition of suppressor T cells. Furthermore, it has been shown to reduce T lymphocyte E rosette formation (12) and lymphocyte transformation (10). Human IgE-mediated skin tests are decreased by theophylline therapy (6). Also, Sherman *et al.* (18) have found increased immunoglobulin synthesis by normal human peripheral lymphocytes, however, the cells were only briefly exposed to theophylline. These results are consistent with animal studies showing induced stimulation of immune response by early, low doses of theophylline (3, 4). Finally, Bourne *et al.* (1) have shown that theophylline inhibits the candidal killing ability of human granulocytes. Thus, previous work has shown a wide range of effects by theophylline on both animals and humans.

We found significant decreases in lymphocyte proliferation in adults and term infants. Interestingly, we found slight but significant increases in the premature infants' responses to mitogen after a period of 3–5 wk, rather than a decrease. We consider this to be due most probably to maturational changes in the infants, possibly masking any depression caused by theophylline. It could also be due to the generally low theophylline levels generated in the infants. Another explanation might be that *in vitro* studies are not necessarily comparable to data obtained *in vivo*.

Interestingly, we also found a slight but insignificant increase in adult IgA and IgG production in human lymphocyte cultures with theophylline levels of 2.5 μg/ml by radioimmunoassay. This is consistent with the animal studies demonstrating stimulation by low dose theophylline exposure (3); however, at all other doses in the radioimmunoassay and in the plaque forming cells assay, theophylline inhibited immunoglobulin production.

Although we found no suppression of lymphocyte proliferation in premature infants during theophylline treatment, this does not exclude the possibility of changes in immuno-regulation, because changes in the suppressor/helper cell system may not be detected in assays measuring only lymphocyte proliferation. Further studies of the effects of theophylline on immunoregulatory function in neonates are needed.

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