Retinol Inhibition of *in Vitro* Human Neutrophil Superoxide Anion Release

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ABSTRACT. Retinol deficiency has been clinically associated with visual impairment as well as altered growth and differentiation of various epithelial cell populations. An additional factor that may be operative in the setting of retinol deficiency is impaired immunomodulation of the neutrophil inflammatory response. Our study demonstrated a concentration-dependent inhibition by retinol of activated neutrophil superoxide anion (O_2^-) release. Concentration of drug (retinol) causing 50% inhibition of initial velocity O_2^- production (IC_{50}) was $42.6 \pm 10.9 \mu M$. Latter phases of the O_2^- release reaction displayed significantly lower IC_{50} values. Similarly, as time of retinol-neutrophil incubation was increased, IC_{50} decreased. Retinol inhibition of neutrophil O_2^- did not appear to involve activation/desensitization, cytotoxicity, or free-radical scavenging mechanisms. Retinol was shown to inhibit intact PMN O_2^- release even after addition of the respiratory burst stimulus. Moreover, retinol inhibited O_2^- release in membranes isolated from activated neutrophils. In addition to promoting proper organization and differentiation of epithelial cells, appropriate plasma/tissue retinol levels may also modulate the neutrophil tissue inflammatory response. (*Pediatr Res* 27: 574-579, 1990)

Abbreviations

BPD, bronchopulmonary dysplasia
CB, cytochalasin B
FMLP, *N*-formyl-methionyl-leucyl-phenylalanine
HBSS, Hanks' balanced salt solution ($^{++}$, complete; $^-$, incomplete, without Ca^{2+} and Mg^{2+})
HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
 IC_{50} , concentration of drug causing 50% inhibition of activity
LDH, lactate dehydrogenase
 O_2^- , superoxide anion
PK-C, protein kinase C
PMA, phorbol myristate acetate
PMN, polymorphonuclear leukocytes
PMSF, phenylmethylsulfonyl fluoride
SOD, superoxide dismutase
 V_i , initial velocity
 V_{2-3} , velocity between 2 and 3 min
 V_{4-5} , velocity between 4 and 5 min

Advances in public health and infant nutrition have essentially eliminated vitamin A deficiency in healthy term neonates living in developed countries. However, in the premature infant with respiratory distress syndrome, retinol deficiency has been postulated to inhibit airway epithelial growth and differentiation and predispose to the development of BPD (1-3). In addition to its other characterized actions (constituent of rhodopsin in the retina, cofactor for growth and differentiation of epithelial, nervous, and bone tissue), vitamin A has been shown to have phagocytic immunomodulatory effects. Because development of BPD is also associated with a pulmonary influx of apparently activated PMN (4-6), it is conceivable that an inadequate PMN immunomodulatory effect might exist in the lungs of malnourished (vitamin A-deficient) infants developing BPD. An unmodulated PMN inflammatory response could result in pulmonary host autoinjury (7). Our study examined the potential of a specific vitamin A metabolite, retinol, to suppress PMN activation as assessed by its *in vitro* inhibition of PMN O_2^- release.

MATERIALS AND METHODS

Reagents. Cytochrome c (type III), FMLP, CB, SOD, retinol (70% all *trans*), DMSO, xanthine, xanthine oxidase, NADPH tetrasodium salt Type I (NADPH), sodium phosphate dibasic, sodium deoxycholate, PMSF, ethylene glycol, sodium azide (N_3), phorbol 12-myristate, 13-acetate (PMA), Ficoll 400, and HEPES were purchased from Sigma Chemical Co. (St. Louis, MO); 90% Hypaque-M from Winthrop Laboratories (Des Plaines, IL); and HBSS $^{++}$ and HBSS $^-$ from GIBCO Laboratories (Grand Island, NY). All other chemicals were of reagent grade and used without purification.

PMN isolation. PMN were isolated from healthy adult venous blood (anticoagulated by 10 IU of heparin/mL) using Ficoll-Hypaque cushion centrifugation (8). Contaminating erythrocytes were eliminated by hypotonic lysis. Cell numbers were quantitated with a cell counter (Coulter Electronics, Hialeah, FL). PMN were initially suspended in HBSS $^{++}$ at a concentration of 10 to 20×10^6 PMN/mL and then aliquots made at a concentration of 5×10^6 PMN/mL. Individual experiments used PMN isolated the same day from a single donor.

Preparation of activated membrane. Purified PMN were resuspended in HBSS $^{++}$ /1 mM N_3^- . Equal volumes PMN (10×10^6 PMN/mL) and PMA ($2 \mu g/mL$) were incubated separately for 5 min at $37^\circ C$, then combined and incubated for 30 min at $37^\circ C$, after which an equal volume of ice cold HBSS $^{++}$ /1 mM N_3^- was added to quench PMN activation. Thereafter, the suspended PMN were centrifuged at $400 \times g$ for 5 min; $4^\circ C$; taken up in 0.25 M sucrose, 1 mM PMSF; sonicated at 20 W three times for 10 s each with 1 min intervals; and recentrifuged at $400 \times g$, 5 min, $4^\circ C$. The resulting supernatant was ultracentrifuged in a SW60 rotor, $30,000 \times g$, $4^\circ C$ and the pellet taken up in 25% ethylene glycol, 1 mM PMSF by Potter Elvehjem homogenization followed by sonication, as noted above. Activated PMN membrane could be stored at $-70^\circ C$ for many months without loss of oxidoreductase activity.

Enzyme assays. NADPH oxidoreductase (PMN superoxide

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anion synthetase) initial velocity, continuous enzyme kinetics were followed spectrophotometrically (DU 50 Series Spectrophotometer, Beckman Instruments Inc., Irvine, CA) according to the previously described method (9). Assays were conducted at 550 nm within microcuvettes, with a total reaction volume of 1 mL using cytochrome c as O_2^- trap. Final concentrations of reactants included cytochrome c (100 μ M), CB (0.01 mM), PMN (0.5×10^6 /mL), retinol (4.38–70 μ M), and FMLP (1 μ M) in HBSS⁺⁺, pH 7.4. DMSO (solvent for stock solutions of PMA and retinol) never exceeded 0.1% and did not interfere with reaction kinetics. Cytochrome c, CB, PMN, buffer (HBSS⁺⁺) with or without retinol (total volume 0.9 mL) were warmed for 5 min at 37°C. FMLP (0.1 mL) was used to initiate the reaction. SOD (100 IU/mL) was used to detect nonsuperoxide sources of cytochrome c reduction. Enzyme-specific activity was expressed as nmol of O_2^- released (nmol of cytochrome c reduced) per min per 10^6 PMN or per mg of membrane protein. Reaction rates for V_i , V_{2-3} , and V_{4-5} were determined for each assay. To ascertain if retinol might have had any inhibitory action on O_2^- production after PMN had already been activated, oxidoreductase assays were conducted in the usual manner except that another constituent (0.10 mL of HBSS⁺⁺ or retinol in HBSS⁺⁺) was added 1.75 min after reaction initiation. V_{2-3} was compared to V_i for each series of reactions after correction for reaction dilution (1.0 to 1.1 mL) was made.

Assay of NADPH oxidoreductase in membrane recovered from activated PMN was similarly performed. The reaction cocktail (1 mL total) contained 100 μ M each of cytochrome c and NADPH in 0.05 M HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.05% DOC, pH 7.4 buffer. Reactions were initiated by addition of 0.10 mL of the activated membrane in 25% ethylene glycol, 1 mM PMSF, and followed again as reduction of cytochrome c.

To quantitate possible retinol free-radical scavenging activity, a cell-free O_2^- -generating system was used, using xanthine (100 μ M) and buttermilk xanthine oxidase (0.005 units) again with cytochrome c (100 μ M) as the O_2^- trap and colorimetric reagent in 0.05 M HEPES/HBSS⁺⁺, pH 7.5, total volume 1 mL (10).

To evaluate possible activation action of retinol on PMN O_2^- production, a PMN O_2^- -generating system was assembled as above but used retinol (70 μ M, 35 μ M, and 3.5 μ M) instead of FMLP as the reaction initiator.

Cytotoxicity experiments. Possible cytotoxicity due to retinol was assessed with the following protocol. PMN (5×10^6 /mL) were incubated in HBSS⁺⁺ with and without retinol (8.75 μ M) for 5 min at 37°C, stimulated with FMLP for 5 min at 37°C, and cooled on ice. Reaction tubes were centrifuged at $200 \times g$, 20 min, 4°C. Supernatants were assayed for LDH, using 50 mM sodium lactate, 5 mM NAD in 0.05 M Na_2HPO_4 , pH 7.4 buffer (11). Supernatant (0.1 mL), the LDH source, was used to initiate the reaction. Change in absorbance was followed spectrophotometrically at 340 nm, for 5 min at 37°C. LDH activity was compared with supernatant obtained after sonication of PMN (20 W, twice, 10 s each, with 1 min intervals) followed by ultracentrifugation (SW60, rotor, $100\,000 \times g$, 60 min, 4°C).

PMN incubated as noted above were recovered after centrifugation, resuspended in HBSS⁺⁺, quantitated and assayed for NADPH oxidoreductase activity. Cell counts and enzyme activities were compared with those obtained from PMN incubated in buffer at 4°C for the entire duration of the experiment.

To demonstrate the possible effect of prolonged incubation of cells with retinol, PMN NADPH oxidoreductase was assayed under the following conditions: 1) PMN incubated with buffer for 60 min at 4°C, and for 5 min at 37°C; 2) PMN incubated with buffer for 60 min at 4°C and with retinol (8.75 μ M) and buffer for 5 min at 37°C; and 3) PMN incubated with retinol (8.75 μ M) and buffer for 60 min at 4°C and for 5 min at 37°C.

Experimental design/data analysis. As noted above, PMN were aliquoted (5×10^6 PMN/mL) after isolation. This was performed to minimize decay of PMN oxidoreductase activity (repeated resuspension causing cell lysis). In concentration-re-

Table 1. IC_{50} values for retinol relative to neutrophil NADPH oxidoreductase V_i , V_{2-3} , and V_{4-5} reaction velocities from five separate experiments

Experiment	Retinol IC_{50} values (μ M)		
	V_i	V_{2-3}	V_{4-5}
1	40.3	18.4	14.1
2	53.5	38.5	36.6
3	25.8	13.9	14.8
4	50.9	19.4	20.6
5	42.5	17.8	22.2
Mean $\pm$ SD	42.6 ± 10.9	21.6 ± 9.7	21.7 ± 9.1

Fig. 1. Concentration-response effect of retinol on intact neutrophil O_2^- generation induced by FMLP. Differential effects on V_i , V_{2-3} , and V_{4-5} are demonstrated. Note that reaction velocities (ordinate) have been normalized to reactions containing no retinol.

sponse experiments, seven identical PMN aliquots were assembled, one for each of the five retinol concentrations examined and one each as an early and late control. Real times of all assays were recorded. In all experiments, control reactions were assayed at the start and end of assays. Although the multiple aliquot technique was used to minimize repeated PMN trauma, a decline in V_i was noted (mean $\pm$ SD = $15.2 \pm 6.3\%$, range 6.4–22%, $n = 5$) for the long concentration-response experiments. This well-recognized spontaneous enzyme decay was corrected for assuming natural logarithmic decay kinetics (9).

All assays represent the mean $\pm$ SD of 3–4 determinations. For calculation of IC_{50} , five separate concentration-response experiments were conducted (five retinol concentrations, early and late controls, four assays plus one SOD background for each condition). An equation describing the effect of oxidoreductase activity (ordinate) relative to retinol concentration (abscissa) was fitted by regression analysis for each experiment. IC_{50} was calculated by solving this equation for retinol concentration when enzyme activity = 50% of maximum, (i.e. without drug). Traditional approaches using reciprocal plots of $1/v$ versus $1/S$ at various inhibitor concentrations to derive a true K_i are not appropriate in the intact cell system used here, because the concentration of reactants, (e.g. cytosolic NADPH) cannot be precisely controlled.

Statistical analysis of results was made using the t test with the Bonferroni correction for multiple comparisons (n). Significance was assumed when $p \leq 0.05/n$.

Table 2. Effect of prolonged incubation of neutrophils with 8.8 μ M retinol on subsequent FMLP-induced NADPH oxidoreductase activity*

	Incubation/assay conditions		
	A	B	C
	Incubated 60 min, 4°C w/o retinol; No retinol in assay	Incubated 60 min, 4°C w/o retinol; Retinol in assay	Incubated 60 min, 4°C with retinol; Retinol in assay
V_i	13.15 $\pm$ 0.86	9.88 $\pm$ 1.35 $p < 0.05:AV_i$	7.58 $\pm$ 0.30 $p < 0.001:AV_i$ $p < 0.05:BV_i$
V_{2-3}	6.92 $\pm$ 2.03	5.04 $\pm$ 0.98 $p \text{ NS}:AV_{2-3}$	2.69 $\pm$ 0.19 $p < 0.05:AV_{2-3}$ $p < 0.02:BV_{2-3}$
V_{4-5}	4.88 $\pm$ 0.89	4.09 $\pm$ 0.88 $p \text{ NS}:AV_{4-5}$	3.04 $\pm$ 0.17 $p < 0.05:AV_{4-5}$ $p \text{ NS}:BV_{4-5}$

* All values represent the mean $\pm$ SD of three determinations, expressed as nmol O_2^- /min/ 10^6 PMN. When included, retinol was present at a concentration of 8.8 μ M. p values reflect differences of that variable with respect to the variable after the colon.

Table 3. Inhibition of NADPH oxidoreductase by retinol added after FMLP activation of neutrophils*

Condition	V_i nmol O_2^- /min/ 10^6 PMN	V_{2-3} nmol O_2^- /min/ 10^6 PMN	$V_{2-3}/V_i \times 100$ (%)
0.10 mL HBSS** at 1.75 min	11.19 $\pm$ 0.56	5.48 $\pm$ 0.55	49.0 $\pm$ 4.92
0.10 mL 200 μ M retinol at 1.75 min	13.09 $\pm$ 0.28	3.78 $\pm$ 0.33	28.9 $\pm$ 2.50

* Each assay contained 5×10^5 PMN and was initiated by addition of 1 μ M FMLP final. At 1.75 min into the reaction, 0.10 mL of HBSS** or retinol was added. V_i was determined between 0.5–1.5 min into the reaction. Raw V_{2-3} data were corrected for dilution ($1.1/1.0 = 1.1$). Each reaction condition reflects the mean $\pm$ SD of three assays.

Table 4. Effect of retinol on O_2^- production by crude membrane recovered from PMA-stimulated PMN (nmol O_2^- /min/mg protein)*

	Control	Retinol (35 μ M)	Retinol (70 μ M)
V_i	255.5 $\pm$ 23.0† 159.0 $\pm$ 23.1‡	251.8 $\pm$ 4.3 156.9 $\pm$ 27.1	293.6 $\pm$ 19.6 217.4 $\pm$ 32.1
V_{2-3}	126.8 $\pm$ 19.5 114.1 $\pm$ 11.4	82.9 $\pm$ 7.4 ($p < 0.05$) 43.4 $\pm$ 13.5 ($p < 0.01$)	69.7 $\pm$ 6.5 ($p < 0.01$) 52.7 $\pm$ 14.8 ($p < 0.01$)
V_{4-5}	70.6 $\pm$ 26.9 72.5 $\pm$ 2.5	49.7 $\pm$ 6.9 12.5 $\pm$ 8.3 ($p < 0.001$)	38.4 $\pm$ 0.4 19.4 $\pm$ 14.6 ($p < 0.001$)

* Assays containing 100 μ M each of cytochrome c and NADPH in a total volume of 1.0 of buffer were initiated by addition of membrane. Data reflect two experiments using different membrane preparations. Each value is the mean $\pm$ SD of three determinations. The p values in parentheses indicate significance of difference between that value and corresponding control values.

† Experiment 1.

‡ Experiment 2.

RESULTS

Polymorphonuclear isolation and activity. PMN yield from healthy adult donors was $2.1 \pm 0.05 \times 10^6$ ($n = 17$) PMN per mL of whole blood and the NADPH oxidoreductase activity (measured as V_i in the absence of retinol) was 12.14 ± 2.34 (range 6.68–14.10, $n = 16$) nmol of O_2^- /min/ 10^6 PMN.

Concentration-response data from five separate experiments are displayed collectively in Table 1, where individual and mean IC_{50} values reflecting the effect of retinol on intact neutrophil NADPH oxidoreductase V_i , V_{2-3} , and V_{4-5} are tabulated. Over

Fig. 2. Lack of effect of retinol on O_2^- synthesis by xanthine oxidase.

the actual range of concentrations of retinol examined in these studies (4.4–7.0 μ M), inhibition of PMN O_2^- synthesis varied from minimal to pronounced. When the data for each experiment are normalized relative to corresponding baseline reaction velocities (no retinol, $V = 100\%$), all the data may be combined and displayed for illustrative purposes as in Figure 1. Both Table 1 and Figure 1 indicate a steeper concentration inhibition slope for later reaction velocities (V_{2-3} , V_{4-5}) as compared with V_i . This is reflected in the lower IC_{50} values for V_{2-3} and V_{4-5} again compared to V_i ($p < 0.01$).

IC_{50} values noted above were established under conditions of retinol contact with PMN for only 5–7 min (the preincubation period at 37°C before initiation of the assay with FMLP). As detailed in Table 2, if lower concentrations of retinol (*i.e.* 8.8

Table 5. Effect of brief incubation (37°C, 10 min) and then washout of retinol ($\pm$ FMLP) on intact neutrophil NADPH oxidoreductase activity*

	Conditions				
	No incubation	-Retinol -FMLP	-Retinol +FMLP	+Retinol -FMLP	+Retinol +FMLP
V _i	13.38 $\pm$ 0.75	13.05 $\pm$ 1.26 (NS)	13.87 $\pm$ 1.19 (NS)	10.66 $\pm$ 1.24 (<i>p</i> < 0.05)	12.20 $\pm$ 1.09 (NS)
V ₂₋₃	5.02 $\pm$ 0.82	5.99 $\pm$ 0.46 (NS)	6.34 $\pm$ 0.93 (NS)	3.36 $\pm$ 0.35 (<i>p</i> < 0.05)	3.05 $\pm$ 0.17 (<i>p</i> < 0.02)
V ₄₋₅	3.05 $\pm$ 0.06	5.38 $\pm$ 0.44 (<i>p</i> < 0.001)	4.79 $\pm$ 0.86 (<i>p</i> < 0.05)	2.88 $\pm$ 0.09 (<i>p</i> < 0.05)	2.39 $\pm$ 0.15 (<i>p</i> < 0.01)

* PMN (5×10^6 in 1 mL) were left gently agitated at 4°C (No incubation) or incubated at 37°C for 10 min with or without retinol (8.8 μ M, 10 min) and FMLP (1 μ M, 5 min). Thereafter, the cells were gently sedimented (200 $\times$ g, 10 min 4°C) and then resuspended in HBSS for assay of FMLP-induced O₂⁻ production. The *p* values indicate differences with the indicated variable compared to cells with "No incubation."

μ M), ordinarily associated with minimal oxidoreductase inhibition, are incubated with PMN for longer periods of time (*i.e.* 60 min), significant inhibition of O₂⁻ synthesis is demonstrable. More pronounced inhibition of late phases of the reaction under brief retinol contact conditions may simply reflect the longer exposure of PMN to retinol during the later part of the assay.

Table 3 indicates that even when added after FMLP activation of PMN, retinol causes significant inhibition of later phases of the reaction (here, V₂₋₃).

When FMLP was omitted from the reaction cocktail and instead retinol (final concentration 3.5–70 μ M) was added last in an attempt to initiate the PMN respiratory burst, no O₂⁻ production was appreciated. Hence, retinol in this concentration range does not appear to stimulate O₂⁻ synthesis.

Retinol similarly was assessed in two separate experiments for its ability to inhibit O₂⁻ production by previously PMA-activated neutrophil membrane. Table 4 indicates that V_i was not affected, even at the highest concentrations of retinol examined. However, both V₂₋₃ and V₄₋₅ were inhibited significantly at both 35 and 70 μ M retinol.

Inhibition of neutrophil O₂⁻ production by retinol does not appear to involve free-radical scavenging action, as illustrated in Figure 2. When evaluated in conjunction with the cell-free xanthine oxidase O₂⁻-generation system, no inhibition of any reaction velocities was noted with 70 μ M retinol.

Possible toxic effects of retinol on intact PMN were evaluated by incubating PMN with 8.8 μ M retinol in the presence or absence of FMLP for 10 min, 37°C; removing the bulk of drug from cells by centrifugation; and then reassaying the cells for FMLP-inducible oxidoreductase activity, and the supernatants for LDH activity. PMN supernatant recovered from sonicated cells demonstrated an LDH activity of 17.3 ± 3.3 nmol lactate oxidized/min/0.10 mL of supernatant. No LDH activity could be ascertained in any of the incubated cell supernatants. Inhibitory effect of retinol on PMN could not be eliminated by washout of the drug by centrifugation and resuspension of PMN in retinol-free media. As indicated in Table 5, once again these effects were most pronounced on later reaction velocities.

DISCUSSION

The hypothesis of our study, that vitamin A may represent an *in vivo* modulator of the PMN inflammatory response, supposes that in the clinical setting of BPD, vitamin A deficiency leads not only to adverse alterations in epithelial membrane structure and function, but conceivably also to impaired regulation of neutrophil activation.

Other investigators using discontinuous assay techniques have demonstrated inhibition of PMA-induced PMN chemiluminescence by retinoic acid (12); PMA-induced O₂⁻ production by retinoic acid (13); and FMLP-induced O₂⁻ production by retinol (14).

Our investigation differs significantly from these studies with

respect to the assay system used. Because the respiratory burst is a relatively short-lived event, particularly with FMLP stimulation, a continuous assay allows examination of all phases of the reaction including activation, catalysis, and termination (9, 15, 16). For example, later phases of the O₂⁻-generating reaction appear to be inhibited by retinol to a greater extent than initial velocity. No effect on extent of lag time after addition of the respiratory burst stimulus but before onset of O₂⁻ production was appreciated in our study. Important subtleties of PMN O₂⁻ synthesis, like these examples, are completely ignored by discontinuous assay schemes. A continuous assay system has previously been used to examine inhibition of PMA-induced O₂⁻ synthesis by retinol, retinyl acetate, and retinoic acid (17).

IC₅₀ values (μ M) for retinol inhibition of FMLP-induced O₂⁻ generation derived from our study (V_i, 42.6; V₂₋₃, 21.6; V₄₋₅, 21.7) are comparable to those generated using a discontinuous assay system with FMLP-induction (80 μ M) (14). Other studies have demonstrated inhibition of PMA-induced O₂⁻ synthesis by various vitamin A metabolites including: retinoic acid (discontinuous assay), IC₅₀ = 45 μ M (12) and 100 μ M (13); and retinol, retinyl acetate, and retinoic acid (continuous assay), IC₅₀ = 200, 200, and 250 μ M, respectively (17).

Using O₂ consumption as an index of the PMN respiratory burst, one study claimed that low concentrations of retinoic acid (≤ 19 μ M) actually stimulated the respiratory burst (18). Auto-oxidation was noted at retinol concentrations > 80 μ M. Although 33 μ M retinol and 17 μ M retinoic acid were reported to initiate O₂⁻ production, as assessed by cytochrome c reduction, the data were not shown (18). When FMLP was used as a PMN activator, this same study found inhibition of O₂ consumption by retinoic acid (18). Attempts in our study at stimulating the PMN respiratory burst with 3.5–7.0 μ M retinol were uniformly unsuccessful. Thus, mechanisms involving PMN activation (by retinol) with subsequent PMN desensitization towards FMLP are probably not operative. Inhibition of PMN O₂⁻ production by retinol was not explained by possible free-radical scavenging effects (Fig. 2), inasmuch as retinol did not impair cytochrome c reduction by O₂⁻ in a cell-free system using xanthine and xanthine oxidase. This finding is in accord with data of other investigators (14, 17). Similarly, our investigation confirmed (19) that retinol in the concentrations examined did not appear to mediate PMN cytotoxicity as assessed by PMN LDH release.

Normal plasma values for retinol are in the 1–4 μ M range, with definite deficiency ≤ 0.3 μ M. Toxicity can be seen at concentrations > 5 μ M, particularly if retinol-binding protein is low. The IC₅₀ value for retinol reported here is an order of magnitude greater than the upper limit of this normal range. But note that this IC₅₀ value reflects only a 5–7 min interaction between retinol and the PMN. If longer periods of incubation are used, the IC₅₀ concentration is reduced considerably (Table 2). Although our investigation established concentration-response data for exogenous retinol and its effect on the PMN respiratory burst, it did not examine the effect of exogenous retinol on the PMN retinol

compartment. Presumably, there is a retinol equilibrium between retinol micelles or plasma transthyretin (the retinol-binding protein complex with prealbumin) and PMN retinol (membranes and intracellular retinol binding protein). Ideal experimental design would quantitate actual PMN retinol content as a function of alterations in PMN oxidative metabolism. It is conceivable that normal physiologic concentrations of retinol, in continuous equilibrium with PMN at body temperature, may exert some baseline modulatory effect on the extent of PMN activation. It is known that PMN isolated from various individuals differ significantly with respect to activity of the respiratory burst. Perhaps some of this variability relates to the *in vivo* concentration of vitamin A metabolites in the PMN in equilibrium with plasma.

Although the mechanism of activation of PMN O_2^- production remains incompletely understood, three phases of this process have been formulated (20, 21): 1) stimulus recognition by the PMN at cell membrane or intracellular receptors; 2) signal transduction, which involves a number of diverse events including changes in plasmalemma electrical potential, activation of phospholipases with release of fatty acids and synthesis of a variety of lipid metabolites, increased phosphoinositide turnover, enhanced calcium influx and intracellular storage release, translocation and phosphorylation of proteins, and alterations in guanosine 5'-triphosphate-binding proteins; 3) activation of the oxidase complex, which may be mediated by assembly or alteration (acylation, methylation, phosphorylation) of the components (NADPH-binding flavoprotein, cytochrome b245, ubiquinone species). Many of these events might be postulated to be sensitive to alterations in PMN retinol concentrations.

Four observations gleaned from our investigation provide clues regarding the possible mechanism of action of retinol in suppressing PMN O_2^- release. One, attempts at retinol removal (by PMN washing) after exposure to PMN does not reverse inhibition of O_2^- production (Table 5). This likely relates to the hydrophobicity of the retinol molecule and its strong interaction with the PMN membranes or intracellular binding protein. Two, later phases of the respiratory burst are inhibited by retinol to a greater extent than V_i . This may simply relate to length of exposure of the PMN to retinol (Table 2) relative to the rate constant for achieving equilibrium between exogenous and PMN retinol. Three, even when added after the respiratory burst stimulus (FMLP), retinol can still restrain PMN O_2^- production (Table 3). Four, retinol inhibits O_2^- generation even in previously activated PMN membrane. In this situation, initial velocity is unaffected, but later reaction phases are significantly inhibited (Table 4).

Retinol (35–70 μ M) has been shown to induce erythrocyte membrane perturbations characterized by plasmalemma indentations and vacuolizations (22). It is known that activation of the PMN respiratory burst is associated with alterations in membrane fluidity (19) and phospholipid remodeling (23), as well as possible translocation of the oxidoreductase complex components. It is possible that retinol affects the dynamic properties of the plasma membrane of activated PMN such as the association of the various respiratory burst oxidoreductase components (20, 21, 24, 25). Retinol suspended in an aqueous solvent, perhaps in the form of micelles, likely requires time (minutes) to effectively interact with the PMN, thus accounting for the more pronounced effects on later respiratory burst reaction velocities. In the case of an activated respiratory burst complex (activated PMN membrane), retinol may act by promoting complex dissociation, hence the effect on V_{2-3} and V_{4-5} , but not V_i . In addition to inhibiting O_2^- release, previous investigators have also demonstrated that retinoids inhibit the exocytosis of granule enzymes such as lysosome and β -glucuronidase. Hence, the effect of these compounds is not specific to oxyradical metabolism (19).

Retinol may affect the expression of genes or gene products (26). Activation of the respiratory burst does not involve transcription or translation. Moreover, effects on these processes (by retinol) would be expected to require hours, not minutes. It has

been stated that retinol may directly inhibit PMN PK-C (17, 27). Moreover phosphorylation of oxidoreductase components may be required for activation (20, 21). PMN activation by PMA follows PK-C activation (21). In the presence of retinol, PK-C activation and O_2^- production are concomitantly inhibited (28). PMN activation by the receptor ligand FMLP, however, involves other signal transduction in addition to PK-C activation, (e.g. calmodulin-dependent kinase) (20). Hence, inhibition of PK-C by retinol is not sufficient to explain retinol's effect on FMLP-induced PMN.

Retinoids have been termed "labilizers of lysosomes" (14) because of their tendency to increase lysosomal fragility. NADPH oxidoreductase (O_2^- synthetase) is known to be exquisitely sensitive to endogenous PMN proteases (29). Release of proteases from PMN granules by retinol could perhaps explain its inhibitory effect on O_2^- production.

Alterations in PMN membrane fluidity and permeability by retinol might result in other aberrations such as altered FMLP receptor conformation or increased calcium influx leading to permanent depolarization. Higher concentrations of receptor agonist (FMLP) might reverse some of the retinol inhibition and thus implicate this receptor as a specific target of retinol.

If retinol works through a depolarizing mechanism, transient activation of O_2^- synthesis might be expected, followed by inhibition. Activation of O_2^- synthesis by retinol was not observed in our investigation, but has been reported by others (18).

Use of adult rather than neonatal PMN in our study was rationalized by the observation that neonatal and adult PMN are essentially identical with respect to qualitative and quantitative aspects of the respiratory burst (16, 30). Assuming a yield of 2×10^6 PMN per mL of blood, 15–30 mL of blood was required for each of the individual experiments in our investigation. Obtaining this amount of blood from neonates (5–10% of total blood volume of a 4-kg infant) could not be justified. However, it would be of great interest to assay the respiratory burst in vitamin A-deficient PMN obtained from vitamin A-deficient infants (presumably at risk to develop BPD) compared with vitamin A-sufficient infants.

Vitamin A metabolites play an essential role in regulating various aspects of epithelial structure and function (1–3, 26). Deficiency of vitamin A in the setting of respiratory distress syndrome and iatrogenic-induced primary pulmonary injury may impair airway healing and contribute to the pathogenesis of BPD. Based on the results of our *in vitro* study, it is intriguing to speculate that the major vitamin A derivative, retinol, at physiologic levels, may orchestrate modulation of the PMN inflammatory response. Insuring adequate vitamin A levels in BPD-susceptible infants may be important for a number of positive effects. Further elucidation of the precise action of retinoids on PMN metabolism will help define their role in the clinical setting.

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