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Inhibition by Retinoic Acid of Multiplication of Virulent Tubercle Bacilli in Cultured Human Macrophages

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The immunologically active vitamin retinoic acid (RA) was tested for the ability to increase the resistance of cultured human macrophages (MP) to experimental infection with virulent *Mycobacterium tuberculosis* Erdman (tubercle bacilli [TB]). It was added to MP in various concentrations and addition regimens. Protection against TB was measured by counting live TB (CFU) in lysates of samples of MP taken at 0, 4, and 7 days after MP infection. RA was protective when added after infection at the pharmacologic concentration of 10^{-5} M and when added before infection at the physiologic concentration of 10^{-7} M. The protection lengthened intracellular generation times for TB, occasionally caused bacteriostasis, and regularly kept CFU counts at 7 days (end of the period of infection) 1 to 2 $\log_{10}$ CFU below control values. Significant protection was seen in a series of 16 experiments with MP from seven different donors, but the degree of protection varied considerably. The protection depended partly on and was inversely proportional to concentrations of a serum substitute or autologous serum used as a supplement in the RPMI 1640 MP culture medium. It was strongest at concentrations of serum below 1%. RA at concentrations used in the MP cultures did not inhibit TB in the absence of MP. These results suggest that RA (vitamin A), like vitamin D, may have some immunoprotective role against human tuberculosis, as historically intimated by the regular use of vitamin A- and D-rich cod liver oil for the treatment of tuberculosis before the introduction of modern chemotherapy.

Vitamin A is best recognized for its roles in maintaining vision, the reproductive system, and the integument (27). However, it also affects immunologic responses, notably increasing the induction of those which are cell mediated (12, 27, 34). A deficiency of vitamin A lowers resistance to various infections (3, 12), and supplementation with it raises resistance (12, 27).

The immunologic activities of vitamin A have begun to be studied in vitro (34). One activity especially relevant to cellular immunity is the stimulation of monocytic differentiation by the active metabolite of vitamin A (13, 18), retinoic acid (RA) (14, 20, 25). The active form of vitamin D, 1,25$(OH)_2$-vitamin D_3, does this also (24). Since 1,25$(OH)_2$-vitamin D_3 recently has been found to increase the resistance of cultured human monocytes (30) and macrophages (MP) (8) to tubercle bacilli (TB), it seemed worthwhile to test whether RA also might have some antituberculosis capability. It is worth recalling that vitamins A and D, in the form of cod liver oil, were regularly prescribed for the prevention and treatment of tuberculosis before the introduction of specific antituberculosis chemotherapy (22).

The results reported here show that RA is able to protect cultured human MP against virulent TB as well as 1,25$(OH)_2$-vitamin D_3 and suggest that vitamin A may have a natural role in human tuberculoimmunity.

MATERIALS AND METHODS

Media and ingredients. Human peripheral blood monocytes obtained by centrifugation on Ficoll-Hypaque were washed and plated as described fully elsewhere (7, 9, 15) and, briefly, as follows. Arm vein blood (50 ml) was centrifuged on Ficoll-Hypaque (Ficoll 400 [Pharmacia Fine Chemicals, Piscataway, N.J.] and Hypaque [Winthrop-Breon, New York, N.Y.]). The leukocyte layer was collected, and the cells were washed four times in Hanks solution. They were suspended at 10^7 nucleated cells per ml in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) for plating. Plating medium was supplemented with 2 mmol of L-glutamine, 0.5 mM sodium thioglycolate, and 1% unheated autologous serum. Culture medium (RPMI 1640 with 1% unheated autologous serum) was the same except that the sodium thioglycolate was omitted. In some experiments 1% unheated type AB serum or 10% serum substitute (SS) (8) was used in place of 1% unheated autologous serum.

RA (catalog no. 2625; Sigma Chemical Co., St. Louis, Mo.) was dissolved at 1.5 mg/ml in 95% ethanol to make a stock solution, which was stored at −20°C. This stock solution was diluted into the postinfection culture medium to make a final concentration of 10^{-5} M. The small amount of ethanol also added to the cultures as a solvent for the RA had no effect on the results.

Bacteriologic culture media (7H9 and 7H10) were purchased from Difco Laboratories, Detroit, Mich.

Methods of culturing and infection of MP. Full descriptions are published elsewhere (7, 15). The following is a brief summary. Three 50-μl droplets of the freshly isolated monocytes were deposited separately in each of the several 35-mm petri dishes (Falcon no. 1008, bacteriologic grade; Becton Dickinson Labware, Oxnard, Calif.) used in an experiment. After incubation for 30 min (37°C, 7.5% CO_2 in air), the nonadherent cells were washed off and discarded. The adherent cells were incubated for 7 days in 1.5 ml of culture medium per petri dish. The resulting MP cultures were infected for 30 min at 37°C. The infecting suspension was discarded, the MP cultures were washed twice with unsupplemented RPMI 1640, and the washed cultures of infected MP were incubated for 7 days in RPMI 1640 with 1% unheated autologous serum, 1% unheated AB serum, or 10% SS, as indicated below, for infection to develop. Pairs of culture plates were taken for each experimental group in an

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experiment to determine CFU counts (see below) immediately after infection and at 4 and 7 days. The numbers and health of the cultured MP were checked by inverted-phase microscopy periodically throughout the experiment.

Detailed characteristics of the MP in these cultures are published elsewhere (7). Each monolayer spot averaged 5×10^4 MP, 98% of which were viable by dye exclusion, 99% of which were positive for nonspecific esterase, and 100% of which took up neutral red.

Bacteria and bacterial counts. The MP cultures were infected with virulent *Mycobacterium tuberculosis* Erdman prepared as described in detail previously (7, 9, 15). The bacillus/MP ratio for the 30-min period of infection in RPMI 1640 with 1% unheated autologous serum was 1:4, and at the end of infection about 10% of the MP had taken up one or two culturable bacilli each (7).

The procedures for bacterial CFU counting and evaluation of the data produced were described and documented in detail previously (7, 10, 15) and, briefly, were as follows. As each culture plate was taken for counting, its supernatant was removed, and the plate and its supernatant were stored separately at −90°C. At the end of an experiment, the collected plates and respective supernatants were processed together. Each plate received 1.5 ml of sodium dodecyl sulfate solution at room temperature for both rapid thawing and lysis of the frozen MP; the supernatants were thawed in a 37°C water bath. After 10 min of sodium dodecyl sulfate lysis, the lysing solution from each plate was transferred to a 6-ml plastic culture tube, neutralized with 0.5 ml of 20% bovine serum albumin, and recombined with the thawed supernatant originally taken from that plate. The mixture was sonicated briefly to ensure dispersion of bacilli, diluted in 10-fold steps from 10^{-1} to 10^{-4}, and plated in quintuplet in 20-μl droplets on 7H10 agar. After 2 weeks of incubation at 36°C, the numbers of colonies of TB per droplet on these 7H10 plates were counted. These counts were converted to CFU per milliliter of MP lysate, where 1 ml of lysate was the product of an average of 10^5 MP. Each count was the mean of five values, with standard errors of the means usually being less than 10% of the means. The counts were plotted semilogarithmically, so as to allow observation of the kinetics of intracellular bacillary growth and calculation of mean generation times for the bacilli (7).

RA was tested for the direct ability to inhibit Erdman bacilli by adding it in various concentrations to 7H9 medium inoculated with the bacilli and observing whether bacillary growth was inhibited over a 7-day period of incubation (10).

MP donors. MP were obtained from several normal subjects, male and female, ranging in age from late 20s to middle 50s (informed consent was obtained).

RESULTS

Nature and kinetics of protection by RA against TB. Various regimens of addition and concentrations of RA were tested in preliminary experiments to learn how the protection observed in early experiments could be regularly reproduced for study. Two modes of use illustrated from a single experiment (Fig. 1) seemed best. In one, RA was added to the medium at 10^{-7} M immediately after plating of the monocytes and kept in the medium throughout the experiments. In the other, it was added at 10^{-5} M (3 μg/ml) to the MP culture medium immediately after infection. Both regimens were significantly protective. However, the postinfection regimen was chosen for further study because it was more regularly protective and because RA was unpredictably toxic for the young monocytes when used at concentrations higher than 10^{-7} M immediately after plating. RA did not harm mature 7-day macrophages.

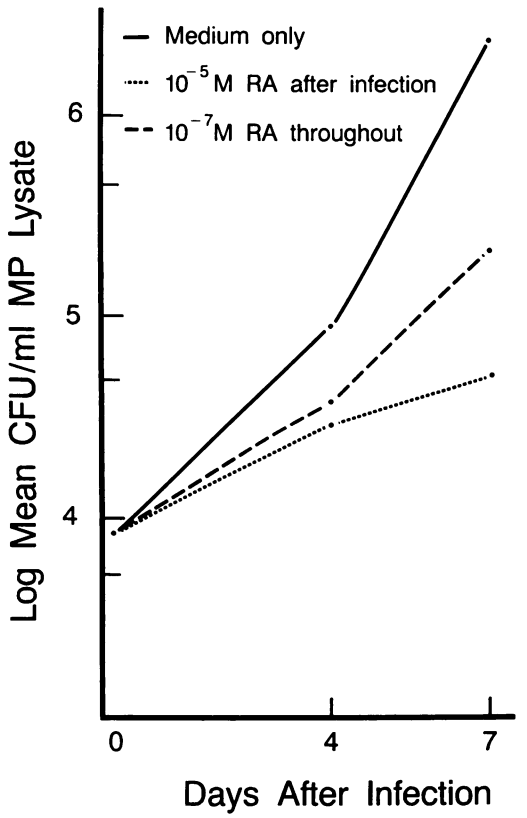

FIG. 1. Inhibition of intracellular TB by two different regimens of RA addition to cultured human MP, one of 10^{-7} M RA in the medium throughout the experiment (14 days) and the other of 10^{-5} M RA in the medium only following infection of the MP (7 days). Each datum point is the mean of five values for CFU per milliliter of MP lysate (average of 10^5 MP per ml of lysate), with duplicate samples taken immediately (zero time) and at 4 and 7 days after infection. Standard errors of the means for 4- and 7-day values were less than 10% of the means.

Reproducibility of protection. Table 1 summarizes results from 16 experiments for the postinfection regimen of RA addition to the MP cultures. RA regularly provided statistically significant protection, but the degree of protection varied considerably.

Minimal protective concentration. The minimal concentration of RA needed for protection when added to the medium immediately after infection was determined by titration (Table 2). Seven-day CFU counts identified this concentration as 0.5×10^{-6} M. The 7-day counts were more sensitive for detecting inhibition than were mean generation times, because the inhibition became greatest in the final days of the infection. Concentrations of RA higher than 10^{-5} M were not practical to test. The minimal effective concentration of RA shown in Table 2 has been verified in several other similar experiments.

Timing of RA addition to MP relative to infection. Adding RA at 10^{-5} M at zero time (immediately after infection) was more protective than adding it at 24 h or 3 days before (Table 3).

Effects of serum on protection by RA. The RPMI 1640 medium normally used for MP culturing in these experi-

TABLE 1. Protection of human MP against TB by RA[a]

Expt	Group	Donor	Counts[b] at:		Intra-MP bacterial mean generation time (h)
			Zero time	Day 7	
1	Medium	A	0.72 (0.09)	87 (4.4)	23.8
	RA		0.71 (0.06)	16 (0.32)	36.3
2	Medium	B	ND[c]	99 (3.0)	22.5
	RA		0.72 (0.05)	4.4 (0.18)	51.6
3	Medium	C	0.61 (0.08)	98 (2.0)	22.0
	RA		0.55 (0.08)	23 (1.8)	27.6
4	Medium	D	1.7 (0.05)	150 (15)	26.9
	RA		1.2 (0.17)	2.1 (0.13)	168.0
5	Medium	E	0.74 (0.09)	200 (19)	22.0
	RA		0.75 (0.08)	2.2 (0.40)	233.1
6	Medium	C	1.5 (0.12)	220 (15)	24.8
	RA		0.72 (0.09)	6.7 (0.20)	56.0
7	Medium	E	0.13 (0.03)	26 (1.8)	23.7
	RA		ND	4.1 (0.21)	27.6
8	Medium	D	0.55 (0.08)	39 (3.9)	29.9
	RA		ND	4.9 (0.42)	41.1
9	Medium	D	0.97 (0.03)	63 (3.8)	27.1
	RA		ND	19 (0.95)	39.9
10	Medium	F	0.53 (0.17)	53 (6.9)	26.4
	RA		ND	11 (2.3)	33.8
11	Medium	F	0.57 (0.07)	48 (3.4)	24.3
	RA		ND	7.6 (0.46)	34.2
12	Medium	G	1.0 (0.16)	230 (21)	22.8
	RA		ND	13 (1.4)	40.4
13	Medium	F	0.76 (0.08)	73 (5.1)	27.4
	RA		ND	31 (1.2)	32.3
14	Medium	D	0.40 (0.04)	43 (7.3)	22.2
	RA		ND	4.0 (0.35)	43.5
15	Medium	A	2.7 (0.25)	290 (12)	25.7
	RA		ND	32 (2.4)	41.5
16	Medium	G	1.5 (0.12)	140 (11)	26.2
	RA		ND	23 (0.94)	44.6

[a] RA was added at 10^{-5} M to MP cultures immediately after infection.
[b] Mean CFU (10^4) per milliliter of MP lysate. Values in parentheses are standard errors.
[c] ND, Not done; zero-time CFU values for medium and RA groups were equivalent before the addition of RA, so one sample was frequently omitted to conserve culture samples.

ments was supplemented with 1% unheated normal human serum. RA protectiveness was affected by serum in the medium (Table 4) and was inversely proportional to the concentration of the serum. Concentrations of serum below 0.063% could not be tested, because they were inadequate for normal MP vitality.

RA protectiveness in medium supplemented with SS. The inhibition of RA protectiveness by serum appears to be a nonspecific concentration-dependent effect of either lipoidal or protein components of the serum, since it could also be seen when medium supplemented with defined SS was used (Table 5). However, some components of whole serum may be more inhibitory than any of the components of SS, since RA was less protective in a mixture of serum and SS equivalent in protein content to 1% SS than in 1% SS alone.

TABLE 2. Titration of RA for protection of MP against TB

RA concn (M)	Mean CFU (10^5)/ml at day 7	Mean generation time (h)	MP numbers[a]	MP health[a]
None	3.9	29.9	4+	4+
10^{-5}	0.49	41.1	4+	3+
0.5×10^{-5}	0.87	35.9	4+	3+
10^{-6}	1.8	30.6	4+	3+
0.5×10^{-6}	1.8	30.7	4+	3+
10^{-7}	2.7	27.3	4+	3+

[a] Checked periodically throughout the experiment with an inverted-phase microscope (see the text). Rated 0 to 4+ relative to control group (no RA).

Direct effect of RA against Erdman bacilli. RA was tested at 10^{-4} M, 10^{-5} M, and successive 10-fold dilutions in 7H9 medium for the direct ability to inhibit the growth of Erdman bacilli. Since the stock solution of RA was in 96% ethanol, concentrations of ethanol equivalent to those used for the respective concentrations of RA also were tested against the bacilli. The ethanol had no effect. RA at 10^{-4} M significantly diminished bacillary growth, but at 10^{-5} M and lower concentrations it had no effect.

DISCUSSION

Clinical observations and experiments with animals suggest that vitamin A is immunologically active (16, 34). It may be protective against some infectious diseases (12, 27), including tuberculosis (see anecdotal evidence in reference 22). Following a trend toward studying it in vitro (29, 31, 34), we tested its active metabolite, RA, for the ability to inhibit TB in cultured human MP (7) and obtained what appears to be the first direct, controlled evidence for vitamin A protection against an infectious disease, specifically tuberculosis. Added to the culture medium following infection of the MP with highly virulent TB, 10^{-5} M RA enabled the MP to slow or stop intracellular bacillary replication. This concentration is about 50 to 100 times higher than normal circulating concentrations (19, 29, 31). Lower, more physiologic concentrations (e.g., 10^{-7} M) also may protect in alternative, perhaps better, regimens of use (Fig. 1). These findings are historically as well as immunologically interesting, because they reveal cellular and molecular explanations for why vitamin A, in the form of cod liver oil, was useful for preventing and treating tuberculosis before modern chemotherapy (22).

How RA protects human MP against TB remains to be determined. Retinoids are immunologically active and can enhance the induction of cell-mediated immunologic re-

TABLE 3. Protection of MP against TB by RA added at different times relative to infection

Time of addition of 10^{-5} M RA relative to infection	Mean generation time (h)	Mean CFU (10^6)/ml at day 7
Immediately following	51.6	0.04
24 h before	20.6	1.2
3 days before	23.2	0.87
None added	22.5	0.99

TABLE 4. Effects of supplemental serum concentrations on RA protection of MP against TB

Concn of:		Mean generation time (h)	Mean CFU (10^5)/ml at day 7
Serum (%)[a]	RA (M)		
4.0	0	30.1	3.9
4.0	10^{-5}	29.3	3.4
2.0	0	29.6	2.1
2.0	10^{-5}	30.6	2.1
1.0	0	26.4	5.3
1.0	10^{-5}	33.8	1.05
0.25	0	24.6	5.1
0.25	10^{-5}	52.4	0.17
0.063	0	26.2	3.5
0.063	10^{-5}	516.1	0.05

[a]Macrophage numbers and health were equivalent for all groups (4+ and 4+, respectively) throughout the 7-day period of infection.

sponses (12, 34). They affect T lymphocytes (12). They induce the synthesis of various proteins and enzymes in responding cells (17), including human monocytes (27, 28). They promote monocytoid cell differentiation (20, 28). Specific nuclear receptors for RA in reactive cells have been identified recently (2, 21). It is interesting that they are closely related to receptors for a family of regulatory molecules which include thyroid hormone, corticosteroids, and vitamin D metabolites (17). The receptors bind these molecules with high avidity and thereby are sterically changed so that they become able in turn to bind to various genes in a superfamily of genes (17). Each of these regulatory molecules thus selects several genes for transcription and translation and the production of new enzymes or regulatory molecules in affected cells (1, 4, 23, 29). RA thus might protect human MP by stimulating them to make TB-inhibiting enzymes.

Alternatively, protection of the MP may have a more trivial explanation. Since 10^{-4} M RA is able to inhibit TB directly, a simple 10-fold concentration by the MP of the 10^{-5} M concentration regularly used in these experiments might account for the observed intracellular inhibition. However, this seems unlikely because of the considerable variability seen between experiments in protection for that one concentration of RA, for such variability is not seen in this model with other drugs acting directly against the bacilli (6, 11). Another explanation for the inhibition relates to the surfactant properties of the retinoids, which can damage cell membranes and partly are responsible for the well-known cytotoxicity of vitamin A (20, 26, 33). Thus, although not morphologically evident, RA may have sufficiently altered MP functions to have suppressed their normal propensity to support TB replication (7). These various interpretations are being investigated.

TABLE 5. Protectiveness of RA at 10^{-5} M in medium supplemented with SS

Medium composition[a]	Mean generation time (h)	Mean CFU (10^5)/ml at day 7
1% NS	22.2	4.3
1% NS + RA	43.5	0.4
10% SS	20.6	29.0
10% SS + RA	28.0	17.7
1% SS	23.8	7.9
1% SS + RA	73.7	0.2
0.5% NS + 0.5% SS	24.8	4.1
0.5% NS + 0.5% SS + RA	59.7	0.2

[a] NS, Autologous serum. The protein concentration of 1% SS was equivalent to that of 1% serum. Thus, 0.5% NS + 0.5% SS had the same final concentration as 1% NS or 1% SS. Macrophage numbers and health were equivalent throughout the infection for all groups. (4+ and 3 to 4+, respectively; see Table 2).

RA protection, although consistently evident, varied widely between experiments (Table 1). Some reasons for this may include the native chemical instability of retinoids (19), variations in the interactions of serum with RA (23, 29), and differences in responses of cultured MP to RA because of autoregulatory molecules, such as colony-stimulating factor and prostaglandin E_2, which the MP can make and which are known to synergize with RA in certain biological functions (29).

The inverse relationship between RA protectiveness and concentrations of serum or serum proteins in the medium seems at odds with any potential protectiveness of RA in vivo, where serum protein concentrations would be much higher than those used in the MP cultures. This problem is commonly noted without much concern in the vitamin A research literature (31). Serum proteins and lipids bind retinoids and thus are supposed to reduce their availability to cells being affected by the retinoids (23, 31). Paradoxically, there is some evidence that retinoids need to be bound to certain proteins to be effective (5, 23, 28).

Interest in the clinical use of retinoids is lively (29, 32). Over 1,500 analogs have been synthesized in searches for those with desirable therapeutic effects but no toxicity (28). Perhaps some of these will be better than native retinoids for possible use in the immunomodulatory prevention and treatment of tuberculosis. However, whether or not these become available, the data presented here have some immediate practical value for the general control of tuberculosis by presenting some direct evidence that vitamin A deficiency may lower antituberculosis resistance.

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