Effect of Triacontanol on Numbers and Functions of Cells Involved in Inflammatory Responses (43441)

REED P. WARREN,[1] ROGER A. BURGER, ROBERT W. SIDWELL, AND LELAND L. CLARK
Developmental Center for Handicapped Persons and Departments of Biology and Animal Dairy and Veterinary Sciences, Utah State University, Logan, Utah 84322, and Royal Pharmaceutical Corp, Bountiful, Utah 84010

Abstract. **A preparation of a triacontanol-containing compound was studied for its effect on cells involved in the inflammatory response. C57BL/6 mice were injected intraperitoneally with various concentrations of this compound and investigated for total body weight, wet weight of thymus tissue, number of thymus cells and splenocytes, interleukin 1 production of spleen monocytes, and response of splenocytes to the T cell mitogen, phytohemagglutinin. Mice treated with the triacontanol preparation exhibited decreased total body weight, 24% reduction in thymus weights, 39% decrease in the number of thymus cells, and 21% depression in total splenocytes. Splenic monocytes of these animals produced a significantly reduced amount of interleukin 1 and splenocytes had a significantly depressed response to phytohemagglutinin. It is concluded that triacontanol has an inhibitory effect on at least some of the cells responsible for inflammation.** [P.S.E.B.M. 1992, Vol 200]

A considerable need exists for anti-inflammatory topical preparations that are not steroid based. Compounds containing halogenated steroids in use today cannot be applied safely to certain areas of the body, such as the face, and can be toxic if applied to areas where they can become systemically active. Triacontanol, a naturally occurring preparation found in beeswax and plant products, is a plant growth regulator (1–3). Chemically, this compound is a carbon-30 alcohol with multiple contaminating fatty acids esters and other free acids. An earlier study (4) suggested that a triacontanol-containing preparation may have anti-inflammatory properties on chemically induced irritation, dinitrochlorobenzene-stimulated allergic dermatitis, and thymidine uptake of lymphocytes in guinea pigs. Moreover, L. L. C. (personal observations) has preliminary findings that this compound has anti-inflammatory properties when applied topically.

This study was undertaken to investigate the systemic effects of *in vivo* exposure to triacontanol on inflammation by examining the numbers and functions of cells involved in the inflammatory response, including monocytes, lymphocytes and thymus cells. While this study may not relate completely to the topical or nonsystemic administration of triacontanol, it was designed in this manner to more extensively investigate the immunologic effects of this compound.

Materials and Methods

Mice. Young adult female C57BL/6 mice (Simonson Laboratories, Gilroy, CA) weighing approximately 20 g were used. The animals were caged in shoebox style polycarbonate cages containing ground corncob bedding (Mt. Pulaski Products, Mt. Pulaski, IL) and fed standard mouse chow and water *ad libitum.* The mice were used after a 24-hr quarantine.

Compound. Triacontanol, also known as melissyl alcohol, myricyl alcohol, and 1-hydroxytriacontane, having the chemical formula $CH_3(CH_2)_{28}CH_2OH$, was provided by Royal Pharmaceutical Corp., Bountiful, UT. This compound contained 16.7% carbon-28 alcohol, 66.6% carbon-30 alcohol, and 16.6% carbon-32 alcohol components. Mixture purity of greater than 99.9% was verified by gas chromatography. Hydrocortisone acetate was obtained from Sigma Chemical Co. (St. Louis, MO). Both compounds were suspended in

[1] To whom requests for reprints should be addressed at UMC 6895, Utah State University, Logan, UT 84322.

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0.7% methylcellulose containing 0.7% Tween 80 for these studies.

Interleukin 1 Production Assay. The ability of the monocytes to produce interleukin (IL) 1 was examined in a 24-hr culture of these cells with *Escherichia coli* lipopolysaccharide (Sigma) at a concentration of 20 μg/ml (5). Supernatants from these cultures were tested for IL-1 content by their ability to stimulate the blastogenic activity of mouse thymocytes in a 48-hr assay. The cells were then harvested on glass fiber filter paper disks using a Skatron cell harvester (Flow Laboratories, Irvine, CA) and the uptake of radioactivity was determined using a Packard Tri-Carb 1500 liquid scintillation counter (Packard, Downers Grove, IL).

T Cell Blastogenesis Assay. As described previously (6), splenocytes (2×10^5) were pipetted in triplicate into wells of flat-bottomed 96-well microplates (Corning, Corning, NY) in a volume of 0.1 ml. Phytohemagglutinin (Gibco, Grand Island, NY) of various concentrations in medium containing 20% fetal bovine serum was added to each well in 0.1-ml aliquots. The plates were incubated 48 hr at 37°C. During the last 24 hr of incubation, the cells were pulsed with 0.4 μCi of [^{3}H]thymidine. The cells were then harvested on glass fiber filter paper disks, as described above with the IL-1 assay.

Experimental Design. This investigation was carried out in two parts: a preliminary investigation of the toxicity of the triacontanol-containing compound and a subsequent study to determine immunologic effects of this compound in the murine host. In the preliminary study, triacontanol in concentrations of 200 mg/kg, 64 mg/kg, 20 mg/kg, 6.4 mg/kg, and 2 mg/kg was administered intraperitoneally in a single treatment to five mice per concentration. All mice were weighed immediately prior to treatment and again 24 hr later. They were observed for death daily for 14 days.

For the immunologic studies, mice were injected intraperitoneally with 80 mg/kg, 40 mg/kg, 20 mg/kg, and 10 mg/kg of the triacontanol-containing compound using five animals per dosage level. These concentrations were based upon results of the preliminary toxicity studies and those used in a previous study in guinea pigs in which triacontanol was shown to depress thymidine uptake of lymphocytes (4). Hydrocortisone acetate in a concentration of 200 mg/kg as a positive control and methylcellulose-Tween 80 as a negative control were similarly administered to five mice each. Five untreated control animals, randomized with the treated animals, were also set aside as an additional negative control. Forty-eight hours later, the mice were sacrificed, their thymuses removed and weighed, and thymus cells in each animal were counted by standard techniques. The number of nucleated cells in the spleen of the animals was determined by the use of crystal violet. Spleens were removed from the animals and were processed into a single-cell suspension using a steel mesh. Following separation over a Ficoll gradient, the cells were used in the IL-1 and T cell blastogenesis assays.

Statistical Analysis. Means and standard errors of each group of values were determined. Differences be-

Table I. Toxicity of Triacontanol Administered Intraperitoneally in a Single Injection

Dosage (mg/kg)	Survivors total[a]	Host weight of change[b] (g)
Normal untreated	5/5	+0.30
Diluent treated[c]	5/5	0.00
200	5/5	−1.98
64	5/5	−1.78
20	5/5	−1.82
6.4	5/5	−1.04
2.0	5/5	+0.14

[a] Fourteen-day observation period.
[b] Day 1 weight minus Day 0 weight.
[c] Methylcellulose, 0.2 ml.

Table II. Effect of Triacontanol on the Weight of Thymus Tissue

Treatment[a]	Dosage (mg/kg)	Wet weight of thymus tissue (mg)
Normal untreated		72 ± 4[b]
Diluent treated[c]		65 ± 4
Hydrocortisone	200	32 ± 4[d]
Triacontanol	80	55 ± 4[d]
	40	59 ± 9[d]
	20	61 ± 4[d]
	10	60 ± 4[d]

[a] $n = 5$.
[b] Mean ± SE.
[c] Methylcellulose, 0.2 ml.
[d] $P < 0.05$.

Table III. Effect of Triacontanol on the Number of Thymocytes and Splenocytes

Treatment[a]	Dosage (mg/kg)	Number of cells[b] Thymocytes	Splenocytes
Normal untreated		246.7 ± 20.7	90.2 ± 7.9
Diluent treated[c]		206.2 ± 20.4	99.8 ± 21.6
Hydrocortisone	200	9.2 ± 3.5[d]	3.2 ± 0.8[d]
Triacontanol	80	150.8 ± 24.2[d]	83.0 ± 18.0
	40	165.2 ± 30.6	81.2 ± 14.6
	20	171.8 ± 18.2	78.1 ± 11.4
	10	186.0 ± 13.7	70.9 ± 7.2[d]

[a] $n = 5$.
[b] Total number of cells $\times 10^6$ ± SE.
[c] Methylcellulose, 0.2 ml.
[d] $P < 0.05$.

Table IV. Effect of Triacontanol on the Production of IL-1

Treatment[a]	Dosage (mg/kg)	Mean incorporation at dilution of supernatant[b]	
		1/2[c]	1/4
Normal untreated		24,200 ± 991	21,840 ± 1,027
Diluent treated[d]		23,200 ± 4,397	20,800 ± 2,223
Hydrocortisone	200	17,420 ± 1,321[e]	16,000 ± 2,027
Triacontanol	80	15,840 ± 1,442[e]	14,150 ± 446[e]
	40	16,960 ± 638[e]	17,340 ± 1,022
	20	18,020 ± 1,995	15,720 ± 1,897
	10	19,050 ± 2,054	16,910 ± 1,531

[a] $n = 5$.
[b] Mean total incorporation in counts/min of tritiated thymidine ± SE.
[c] Dilution of supernatant (obtained from incubating mouse spleen cells with lipopolysaccharide) with RPMI 1640 (Gibco).
[d] Methylcellulose, 0.2 ml.
[e] $P < 0.05$.

Table V. Effect of Triacontanol on the Blastogenic Response of Spleen Cells (T Cells) to Phytohemagglutinin

Treatment[a]	Dosage (mg/kg)	Incorporation of 3-H ($\times 10^4$)[b] at PHA in concentrations of		
		2%	1%	0.5%
Normal untreated		5.8 ± 1.1	5.1 ± 0.7	4.3 ± 0.5
Diluent treated[c]		5.3 ± 0.9	4.9 ± 0.6	4.3 ± 0.4
Hydrocortisone	200	4.1 ± 1.2[d]	3.1 ± 0.8[d]	2.9 ± 0.3[d]
Triacontanol	80	4.3 ± 1.1[d]	2.8 ± 0.7[d]	2.0 ± 0.2[d]
	40	5.6 ± 2.4	4.2 ± 0.5	3.3 ± 0.6
	20	5.1 ± 1.9	4.1 ± 0.6	3.1 ± 0.5
	10	6.5 ± 3.4	4.9 ± 0.9	3.7 ± 0.4

[a] $n = 5$.
[b] The amount of tritiated-thymidine incorporated by spleen cells incubated in phytohemagglutinin (PHA). Data are expressed as mean ± SE.
[c] Methylcellulose, 0.2 ml.
[d] $P < 0.05$.

tween the means of each treatment group and the methylcellulose-treated group were compared using Student's *t* test.

Results

The results of the toxicity study are presented in Table I. The triacontanol-containing compound in dosages of at least 200 mg/kg was not lethal to the animals, although host weight loss of 1–2 g was seen at dosages as low as 6 mg/kg (Table I).

The triacontanol-containing preparation at all concentrations studied induced reduction in the wet weight of the thymus (Table II). The 80-mg/kg dosage induced the greatest reduction (approximately 24%), whereas the positive control, hydrocortisone, induced a weight loss of about 55%. The vehicle, methylcellulose, induced a slight (about 10%) reduction in thymus weight that was not significant. The reduction in thymocyte counts was consistent with the thymus weight data (Table III). Mice receiving the triacontanol preparation at a dosage of 80 mg/kg had only depressed numbers of thymus cells. Triacontanol, at all doses studied, appeared to reduce somewhat the number of splenic cells in the mice. However, only at the 10-mg/kg dosage was the reduction significant. Triacontanol did not have significant effect on spleen weight (data not presented).

The effects of the triacontanol preparation on the functions of monocytes and lymphocytes are given in Tables IV and V. Monocyte function was assessed with the production of IL-1 (Table IV). The dosages of 80 mg/kg and 40 mg/kg significantly reduced IL-1 production. The response of spleen cells to the T cell mitogen, phytohemagglutinin, is shown in Table V. The 80-mg/kg dosage was significantly inhibitory to this function; however, lower concentrations were not inhibitory.

Discussion

The inflammatory response originates from an injury that has a mechanical, chemical, or autoimmune basis. This response, a complex series of events, is essentially a protective and restorative reaction that attempts to maintain homeostasis. It is well known that most forms of acute and chronic inflammation are propagated and amplified by humoral and cellular components associated with lymphocytes and phagocytic cells, including monocytes and granulocytes (7). The development of inflammation usually indicates that cells of the immune system have encountered: (i) antigen in an unusual location, (ii) antigen that is difficult to digest, or (iii) an unusually large amount of antigen. In some diseases, such as rheumatoid arthritis, the antigen is unknown or may be a normal host tissue component (8). In other diseases, inherent or acquired abnormalities of immune regulation may help sustain inflammatory processes (8).

The current project attempted to assess the therapeutic potential of triacontanol on inflammation by examining the toxicity of this substance, as well as the systemic effect of this compound on the number and function of some of the cells involved in the inflammatory response. Triacontanol, up to a dosage of 200 mg/kg, was not lethal to the mice, but host weight loss of 1–2 g was seen at dosages as low as 6 mg/kg. The relevance of this weight loss to the immunologic changes seen in the animals is not known; however, the

toxicity data provide evidence that this compound is biologically active.

The finding that triacontanol treatment of C57BL/6 mice clearly had an effect on thymus tissue (both the wet weight of the thymus and the number of cells in the thymus) is important because thymus cells are precursor cells to the various subpopulations of T cells known to regulate and mediate immune responses. In the thymus, it is well known that thymocytes or immature T cells proliferate and differentiate into mature T cells that eventually seed peripheral lymphoid tissue such as the nodes and the spleen. The mechanism by which triacontanol reduces thymus weight and cell number is not known, but it is possible that this compound is toxic to the thymocytes or interferes with their proliferation. Although triacontanol had a clear effect on the thymus, its effect was not nearly as great as that of hydrocortisone, the positive control for this investigation. However, the concentration of hydrocortisone preparation used in the study was greater than that of the triacontanol preparation.

The inhibitory effect of triacontanol treatment on the number of splenocytes provides direct evidence that this compound has an effect on inflammation, because all nucleated cells in the spleen are believed to be involved in inflammatory responses. However, the mechanism by which triacontanol reduces the number of these cells is not clear from the current results. However, it is likely that triacontanol inhibits immune function without being toxic to the cells, because after the spleens were removed from the animals and processed into single cell suspensions, only viable cells were used in the *in vitro* assays. This compound may be acting on one or more of the splenocyte precursor cells, i.e., thymus cells, rather than on the splenocytes themselves. It is interesting that the concentration of 10 mg/kg of triacontanol had the greatest effect on splenocyte counts, whereas 80 mg/kg had the greatest effect on thymus cell counts. The reason for this difference is not clear.

The finding that triacontanol had a suppressive effect on IL-1 production provides additional evidence that this compound has anti-inflammatory effects. IL-1 is an endogenous pyrogen, inducing the febrile responses associated with inflammation and activating endothelial cells, phagocytes, fibroblasts, synovial cells, T cells and B cells (9). Moreover, the observation that triacontanol-treatment reduced T cell response to phytohemagglutinin is evidence that this compound can suppress inflammatory responses. Two major products released by activated T lymphocytes are γ-interferon and colony-stimulating factors. Both of these products have an important modulatory role in inflammation. Among its many activities, γ-interferon enhances phagocytic function in an immunologically nonspecific manner; it activates macrophages that secrete numerous products, such as reactive oxygen products, IL-1, and tumor necrosis factor (10). Colony-stimulating factors are the specific hemopoietins responsible for regulating the circulating concentrations of granulocytes and mononuclear phagocytes (11).

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