Effect of Excess Vitamin A on Acid Phosphatase Content of Guinea Pig Peritoneal Leucocytes.* (27587)

AARON JANOFF,† AND ROBERT T. MCCLUSKEY
(Introduced by Benjamin W. Zweifach)
Department of Pathology, New York University School of Medicine, New York

It has been found that large doses of vit A cause profound depletion of cartilage matrix in the rabbit(1), in cultures of limb-bone rudiments of chick embryos(2), and in larvae of *Xenopus laevis*(3). Interestingly, this action of vit A closely resembles the action of papain-protease on mucoprotein of cartilage ground substance(1,4). It has been postulated, therefore, that the vit A effect is due to release of an endogenous protease in the affected tissues of hypervitaminotic A animals and A-treated organ cultures. This suggestion is supported by the observation that normal embryonic cartilage *ex vivo* contains a protease capable of producing the vit A changes(5). Moreover, this protease was found to possess an acid pH optimum and to resemble the cathepsins that have been isolated from the lysosome fraction of liver cells by de Duve(6). These observations suggested that high levels of vit A result in release of proteolytic enzymes from cells by increasing the permeability of their lysosomes. This view received further support, in turn, from the demonstration by Dingle(7) that vit A added to the lysosome fraction of liver homogenate *in vitro* released a proteolytic enzyme with an acid pH optimum.

The experiments reported here were undertaken to investigate the action of vit A upon lysosomes *in vivo*. Acid phosphatase, an enzyme associated with the lysosomes of macrophages(8) and polymorphonuclear leucocytes (9), was measured in peritoneal exudate cells harvested from guinea pigs treated with excess vit A. Since hydrocortisone has been demonstrated to retard the action of hypervitaminosis A upon bone and cartilage *in vitro*(10), the effect of cortisone and hydrocortisone on the peritoneal leucocytes of vit A treated guinea pigs was also studied.

* This work was supported by grant from USPHS.

† Postdoctoral Fellow, USPHS.

It was found that excess vit A caused a marked decrease in the acid phosphatase activity extractable from peritoneal phagocytes and second, that this effect of the vitamin was not inhibited by moderate doses of corticosteroid hormones.

Methods. Male, albino guinea pigs of the Hartley strain, weighing approximately 250 g, were divided into 6 groups and treated as follows:

Group 1 (9 guinea pigs) received daily, intraperitoneal injections of 4 ml of sterile, heavy, mineral oil containing 34,000 IU (10.3 mg) per ml of vit A-alcohol (Nutritional Biochemicals Corp., Cleveland, Ohio). Each animal received a total of 3 doses (408,000 Units) spaced over a 72-hour interval. The crystalline vitamin was first dissolved in a small volume of warm, absolute ethanol, then diluted with mineral oil to obtain the concentration given above. The preparation of oil-vitamin contained 0.05 ml ethanol/ml.

Group 2 (9 guinea pigs) received 3 daily intraperitoneal injections of mineral oil-ethanol mixture only, in the same doses described above. These animals served as controls for Group 1.

Group 3 (4 guinea pigs) was given the same dose of vit A as Group 1; in addition, these animals were simultaneously treated with an aqueous suspension of cortisone acetate (Philadelphia Ampoule Lab., Phila., Pa.). Intramuscular injections of 8 mg of cortisone were given on day 1 of vit A treatment, and 5 mg were given on days 2 and 3.

Group 4 (4 guinea pigs) was treated in the same way as Group 2 (oil-ethanol only), and also received intramuscular cortisone-acetate in the doses described for Group 3.

Group 5 (4 guinea pigs) was given vit A according to the same schedule described for Group 1, except that half the daily dose of vitamin was administered (17,000 International Units/ml).

TABLE I. Effect of Vitamin A-Alcohol (and Glucocorticoids*) on Total Activity of Acid Phosphatase of Guinea Pig Peritoneal Leucocytes.

Exp Group	No. of Animals	Treatment	mM p-nitrophenol released/hr/10^{10} phagocytic cells† (specific activity of acid phosphatase)	WBC/mm³‡	% PMN + monocytes‡	% lymphocytes‡
1	9	136,000 I.U. vit A in oil, i.p. x 3 doses	2.5 ± 1.4§	3900	63	37
2	9	oil only, i.p. x 3 doses	12.1 ± 3.7	6600	75	25
3	4	136,000 I.U. vit A, i.p. and cortisone acetate, i.m. x 3 doses	3.8 ± .9§	3600	80	20
4	4	oil only, i.p. and cortisone acetate, i.m. x 3 doses	18.8 ± .9	7600	65	35
5	4	68,000 I.U. vit A, i.p. x 3 doses	5.6 ± .9§	5100	59	41
6	4	68,000 I.U. vit A, i.p. and cortisone acetate, i.m. and hydrocortisone, i.p. x 3 doses	2.8 ± .7§	4500	85	15

* Doses of steroid hormones given in text under Methods.
† Mean ± standard error.
‡ Average value.
§ Difference statistically significant from control (Group 2) at 0.1% level (Fisher's "t" test).

Group 6 (4 guinea pigs) was treated as Group 5 except that, in addition to vit A, these animals received cortisone and hydrocortisone during the same interval. Each guinea pig received 5 mg of cortisone acetate, intramuscularly, 24 hours before first injection of vit A. Thereafter, the animals were given hydrocortisone-free alcohol (No. 62921, Merck Inst. for Therapeutic Research, Rahway, N. J.), dissolved directly in the mineral oil-vitamin solution, in doses of 5 mg, 3 mg, and 1 mg on days 1, 2, and 3 of vitamin treatment respectively.

Peritoneal leucocyte enzyme assays. Peritoneal leucocytes were harvested from animals in all groups 24 hours after the last intraperitoneal injection of oil. Each animal was sacrificed by a blow to the head and peritoneal exudates were obtained by a method described earlier(11). The exudate fluids thus collected were sampled for differential and total cell counts, after which exudate cells were concentrated and then lysed by ultrasound(11). These lysates contained few intact cells when examined with phase-contrast microscopy. Enzyme activity released by this procedure was apparently maximal and could not be increased by additional lytic procedures such as repeated freezing and thawing. Aliquots (0.1 ml) of the lysates were assayed for acid phosphatase according to the method of Bessey, Lowry, and Brock(12). Acid phosphatase activity was expressed as millimoles of p-nitrophenol released per hour at 37°C per 1×10^{10} phagocytic cells (polymorphonuclear cells and monocytes).

Results. Leucocyte acid phosphatase. The data presented in Table I show a highly significant reduction in acid phosphatase content of peritoneal leucocytes harvested from guinea pigs treated with 2 different doses of vit A (compare Groups 1 and 5, with Group 2). This action of the vitamin could not be prevented by pretreating the animals with corticosteroid hormones. Thus, large doses of vit A produced an equivalent reduction in leucocyte acid phosphatase in guinea pigs given intramuscular cortisone acetate (Group 3); nor did pretreatment with intramuscular cortisone followed by daily intraperitoneal in-

jections of hydrocortisone prevent the depletion brought about by smaller doses of the vitamin (Group 6). To substantiate the corticosteroid-hormone data, it was necessary to demonstrate that these agents by themselves did not cause reduction in leucocyte acid phosphatase (Group 4).

Leucocyte numbers. Average total white cell counts and differential counts in all experimental groups are also presented in Table I. The average total number of leucocytes/mm^3 of exudate in animals not treated with vit A was 7000 cells (Groups 2 and 4). Modest doses of vitamin (68,000 units daily) reduced the cell count to 4500-5000/mm^3 (Groups 5 and 6). Larger doses of vit A (136,000 units daily) reduced the count still further, to below 4000 cells/mm^3 (Groups 1 and 3). Enzyme activity was expressed per 10^{10} phagocytes (specific activity) to correct for the reduction in leucocyte number in vitamin treated animals.

Phagocytic cells (polymorphonuclear cells plus monocytes) ranged between 60 and 80% of the total; of these, generally three-fourths were monocytes and one-fourth polymorphonuclear leucocytes. In calculating specific activity of enzyme, lymphocytes, which comprised the rest of the cells, were excluded to avoid errors resulting from the variation in their numbers. Failure to make this correction could have resulted in significant error, since the acid phosphatase content of lymphocytes is low in relation to that of the 2 phagocytic cell types.

Differential staining of leucocytes revealed that cytoplasmic granules were frequently in close proximity to or in actual contact with ingested droplets of mineral oil which contained vit A (Fig. 1). It is reasonable to assume, therefore, that the observed effect of vit A upon peritoneal leucocyte enzyme could be attributed to a direct action of the vitamin. The local effect may have been reinforced by the systemic action of vitamin absorbed from the peritoneal fluid. In addition, differential stains showed that peritoneal leucocytes obtained from A-treated guinea pigs were normal with regard to nuclear conformation and cytoplasmic granularity (Fig. 1). Although numbers of cells were reduced by vitamin

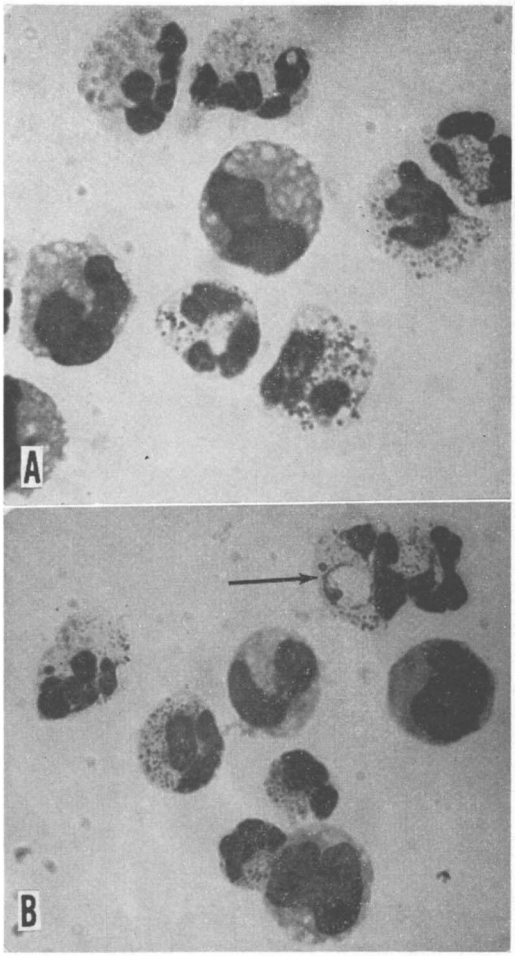

FIG. 1. Appearance of peritoneal leucocytes after Wright's staining. Fields were selected to show many polymorphonuclear cells in addition to the usual numbers of monocytes. Note cytoplasmic granules in close proximity to ingested oil droplets (arrow). A. Leucocytes of control guinea pig. B. Leucocytes of vit A-treated guinea pig. Nuclear conformation and cytoplasmic granularity of surviving cells are unaffected by vit A treatment. Magnification equals 720×.

treatment, apparently viable cells served as the basis for calculation of specific activity of enzyme in the experimental groups.

Discussion. These results show that intraperitoneal injection of a large dose of vit A-alcohol in oil caused a marked loss of acid phosphatase from guinea pig peritoneal macrophages and polymorphonuclear cells. Two other observations are relevant to the observed phenomenon: Thorbecke(8) has shown that the acid phosphatase content of mouse peritoneal macrophages is localized in

cytoplasmic granules; also Cohn and Hirsch (9) have reported that the nonspecific acid phosphatase of human and rabbit polymorphonuclear leucocytes is contained within cytoplasmic granules which closely resemble, both morphologically and biochemically, the lysosomes described by de Duve(6). It can therefore be justifiably assumed that the depletion by vit A of acid phosphatase of peritoneal macrophages and polymorphonuclear leucocytes demonstrates the *in vivo* action of this vitamin upon lysosomes.

In view of the effect of vitamin A upon lysosomes *in vitro*(7), the most likely interpretation of its mechanism of action *in vivo* is that it increases the permeability of lysosomal membranes and promotes loss of enzyme normally contained within intact particles. Following the intracellular disruption of lysosomes, the release of solubilized lysosomal enzymes from the cells of treated animals might be facilitated by an increase in the permeability of the cell membrane caused by the presence of vit A(13).

De Duve has suggested that disruption of lysosomes may play an early and fundamental role in initiation of cell death during a variety of pathological processes(6). Although the number of peritoneal leucocytes in the exudate was reduced in vit A treated guinea pigs, the remaining cells appeared to be morphologically normal despite an apparent intracellular solubilization of their lysosomal enzymes. This observation suggests that, in phagocytic cells, irreversible injury may not be an obligatory consequence of increased lysosomal permeability. The fact that phagocytes of peritoneal fluid and of other tissues initiate intracellular digestive processes following phagocytosis of foreign materials may render them more resistant to the effects of digestive enzymes. It is therefore probable that disruption of lysosomes within such cells would not necessarily lead to irreversible autolysis.

Summary. Guinea pigs were given daily intraperitoneal injections of 136,000 I.U. of vit A-alcohol dissolved in sterile mineral oil. Peritoneal leucocytes, harvested from these animals 24-48 hours after the third dose of vitamin, showed a highly significant depression of total acid phosphatase activity when compared with leucocytes obtained from control animals treated with oil alone. Smaller doses of the vitamin (68,000 I.U.) had a similar action. This effect of vit A was not prevented by simultaneous treatment of the animals with modest doses of cortisone or hydrocortisone. The observation that leucocytes of vit A treated animals show a marked loss of acid phosphatase supports the contention that this agent has a disruptive action on lysosomal membranes *in vivo*.

Hydrocortisone, as the free alcohol, was kindly provided by Dr. H. J. Robinson, Merck Institute of Therapeutic Research. The technical assistance of Miss Nili Kratka is gratefully acknowledged.

1. Thomas, L., McCluskey, R. T., Potter, J. L., Weissmann, G., *J. Exp. Med.*, 1960, v111, 705.

2. Fell, H. B., Mellanby, E., *J. Physiol.*, 1952, v116, 320.

3. Weissmann G., *J. Exp. Med.*, 1961, v114, 581.

4. Fell, H. B., Thomas, L., *ibid.*, 1960, v111, 719.

5. Lucy, J. A., Dingle, J. T., Fell, H. B., *Biochem. J.*, 1961, v79, 500.

6. De Duve, C., in *Subcellular Particles,* New York, Ronald Press, 1959, 128.

7. Dingle, J. T., *Biochem. J.*, 1961, v79, 509.

8. Thorbecke, G. J., Old, L. J., Benacerraf, B., Clarke, D. A., *Histochem. and Cytochem.*, 1961, v9, 392.

9. Cohn, Z. A., Hirsch, J. G., *J. Exp. Med.*, 1960, v112, 983.

10. Fell, H. B., Thomas, L., *ibid.*, 1961, v114, 343.

11. Janoff, A., PROC. SOC. EXP. BIOL. AND MED., 1962, v110, 372.

12. Bessey, O. A., Lowry, O. H., Brock, M. J., *J. Biol. Chem.*, 1946, v164, 321.

13. Lucy, J. A., Dingle, J., *Biochem. J.*, 1962, v82, 31p.

Received April 26, 1962. P.S.E.B.M., 1962, v110.