Stimulatory effect of vitamin A on tumoricidal activity of rat alveolar macrophages

K. Tachibana1, S. Sone2, E. Tsubura2 & Y. Kishino1

1Department of Nutrition and 2Third Department of Internal Medicine, School of Medicine, The University of Tokushima, Tokushima 770, Japan.

Summary  F344 rats were given saline, vitamin A placebo or vitamin A analogues orally for 4 consecutive days. The following day they were killed and their alveolar macrophages (AMφ) were harvested by lavage. The functional integrity of the AMφ was determined by their capacity to phagocytose opsonized SRBC and to kill syngeneic adenocarcinoma cell lines nonspecifically. Results showed that 4 days treatment with >100 IU of vitamin A as retinyl palmitate per gram body weight rendered the AMφ tumoricidal against syngeneic mammary adenocarcinoma cell lines (MADB-100 and MADB-200) and that AMφ activated with retinyl palmitate showed increased ability to phagocytize opsonized SRBC. Other retinoids, such as retinoic acid and retinol, had the same effect of inducing tumoricidal activity in rat AMφ. AMφ harvested from normal rats were also rendered tumoricidal by direct interaction with >103 IU ml−1 of retinyl palmitate for 24 h in vitro. Thus, vitamin A at high doses can increase the phagocytic and tumoricidal activities of rat AMφ.

The important role of cells of the macrophage-histiocyte series in host defence against infection and cancer is now recognized. Murine alveolar macrophages (AMφ) obtained by lavage from normal healthy donors are usually not cytotoxic to tumour cells in vitro (Sone et al., 1980). These noncytotoxic AMφ, however, can be rendered tumoricidal by interaction in vitro with bacterial products, or lymphokines (Sone & Fidler, 1980, 1981). AMφ can also be activated to kill tumour cells by in vivo treatment with preparations of bacteria such as Bacillus Calmette-Guérin, Corynebacterium parvum, or cell wall skeletons of Nocardia rubra (Olivotto & Bomford, 1974; Sone & Fidler, 1982; Zwilling & Campolito, 1977). The resultant cytotoxic AMφ should function as primary effector cells against tumours growing in the lung. In fact, there is encouraging evidence of a close association of the tumoricidal activities of AMφ and eradication of pulmonary metastasis in mice (Fidler et al., 1981; Sone & Fidler, 1982).

Vitamin A and its analogues are known to have antitumour activity against chemical carcinogen-induced or transplanted tumours (Bollag, 1971; Kurata & Micksche, 1977; Moon et al., 1976; Nettlesheim & Williams, 1976; Saffioti et al., 1967; Seifert et al., 1983). There is some evidence that certain retinoids stimulate immune responses: Vitamin A stimulates the induction of cell-mediated cytotoxicity against tumours (Dennert & Lotan, 1978; Dennert et al., 1979; Lotan & Dennert, 1979), enhances natural killer cell activity (Goldfarb & Herberman, 1981), accelerates graft rejection (Floersheim & Bollag, 1972), augments lymphocyte blastogenesis (Abb & Deinhardt, 1980; Lapin et al., 1974; Micksche et al., 1977), and potentiates the antitumour effect of BCG vaccine (Kurata & Micksche, 1977). Retinoids also seem to stimulate the function of the mononuclear phagocyte system, since they have been found to augment defence activity against infection with Listeria monocytogenes (Hof & Emmerling, 1980). These findings suggest that the antitumour effect of vitamin A and its analogues might be mediated indirectly via enhancement of host defence activities. Little is known about activation and/or potentiation of tumoricidal macrophages by vitamin A or its analogues. AMφ may be important in host defence against neoplastic cells developing and/or growing in the lung. Since the lung is often used to evaluate the inhibitory effect of vitamin A on chemical carcinogenesis (Nettlesheim & Williams, 1976; Saffioti et al., 1967), it seemed of interest to determine whether vitamin A could activate AMφ to destroy syngeneic tumour cells.

In this paper, we report that AMφ from F344 rats can be rendered cytotoxic to syngeneic tumour cells by incubation in vitro with retinyl palmitate, and also by oral administration of vitamin A to 1:344 rats for 4 days.

Materials and methods

Animals

Specific pathogen-free inbred F344 male rats of 5–7 weeks old were obtained from the Shizuoka Animal Facility Center (Shizuoka, Japan).
Cell lines

The syngeneic tumours MADB-100 and MADB-200 are mammary adenocarcinomas induced in F344 rats given a single oral dose (20 mg) of 9, 10-dimethyl-1,2-benzanthracene (Sigma Chemical Co., St. Louis, Mo). Assays were always done with cells from cultures in the exponential phase of growth.

Treatment of animals

Retinyl palmitate (Wako Pure Chemicals Co., Tokyo, Japan), retinol and retinoic acid (Sigma Chemical Co., St. Louis, Mo) suspended in soybean oil were given to rats at doses of 100–500 IU g⁻¹ body wt through a plastic stomach tube.

Preparation and purification of AMφ

AMφ were obtained by the tracheobronchial lavage method described fully elsewhere (Sone et al., 1980; Sone & Fidler, 1981). Briefly, the lungs were washed with 5 ml of sterilized saline at 37°C. This process was repeated several times to obtain a total of 50 ml of lavage fluid per rat. The total number of cells collected was determined by cell counts in a hemocytometer (using 2% acetic acid as diluent). The viability of nucleated cells suspended in PBS, measured by trypan blue dye exclusion, was >95%. More than 95% of the lavage cells from normal rats were AMφ, judging by their positive staining for non-specific esterase. The remaining cells were small mononuclear cells or neutrophils, which were eliminated during washing of plated cells (see below). The lavage suspension was washed and resuspended in RPMI 1640 medium supplemented with 5% heat-inactivated foetal bovine serum (FBS), penicillin G, and streptomycin (named CRPMI 1640 medium), and 10⁵ AMφ were plated into wells of a Microtest II plate (Falcon Plastics, Oxnard, Calif.). Nonadherent cells (<10%) were removed by washing the plate 60 min after plating. At that time, >99% of the adherent cells were mononuclear and could phagocytize carbon particles.

In vitro activation of AMφ

Inocula of 10⁴ AMφ were plated and the resulting monolayers were washed 60 min later. Then they were incubated for 24 h with or without retinyl palmitate, muramyl dipeptide (MDP) (Calbiochem, La Jolla, CA) or lipopolysaccharide (LPS) (E. coli 055: B5; Difco Laboratories, Detroit, MI). The AMφ monolayers were then washed and assayed for AMφ-mediated cytotoxicity.

Assay of AMφ-mediated cytotoxicity in vitro

AMφ-mediated cytotoxicity was assayed by measuring release of radioactivity as described in detail previously (Sone et al., 1980; Sone & Fidler, 1981). Target cells in the exponential growth phase were incubated for 24 h in medium containing 0.4 μCi of [¹²⁵I]iododeoxyuridine [¹²⁵I]UdR ml⁻¹ (Sp. act., 5 Ci mg⁻¹; Amersham International Ltd., Bucks, England). The target cells were then washed to remove unbound radiolabel, harvested by brief trypsinization, and resuspended in medium. Then 10⁶–2 × 10⁶ target cells per 10⁵ AMφ were plated in each well. No significant differences were detected in the plating efficiencies of labelled target cells to plastic and to monolayers of AMφ (normal activated). Radiolabelled target cells were re-fed with fresh medium 14 h after the plating of tumour cells. The AMφ-target cell cultures were then incubated for another 58 h at 37°C. Finally the cultures we washed twice with PBS and adherent (viable) cells were lysed by adding 0.1 ml of 0.5 N NaOH. The radioactivity of the lysate was measured in a gamma counter. The cytotoxic activity of the macrophages was calculated as follows:

\[
\% \text{ cytotoxicity} = \frac{\text{cpm in target cells cultured with normal AMφ} - \text{cpm in target cells cultured with test AMφ}}{\text{cpm in target cells cultured with normal AMφ}} \times 100
\]

Quantitative assay of phagocytosis

Opsonized sheep red blood cells (SRBC) labelled with ⁵¹Cr (0.2 ml of 0.4% suspension) were added to AMφ monolayers in wells of 16 mm diameter in tissue culture dishes (Costar, Cambridge, Mass.) (Moriguchi et al., 1983; Sone & Fidler, 1981). After incubation for 2 h at 37°C, the cultures were rinsed once for 10 sec with distilled water to lyse non-phagocytized SRBC and washed twice with PBS. The remaining adherent cells were lysed with 0.5 N NaOH, and the lysate was monitored for radioactivity in a gamma counter. Values were obtained from data in triplicate cultures.

Statistical analysis

The statistical significance of differences between test groups was analyzed by Student's two-tailed t-test.
Results

In vitro activation of rat AMφ by vitamin A

Rat AMφ obtained from the lungs of normal F344 rats were plated for 1 h in CRPMI 1640, and then thoroughly washed and incubated for 24 h in medium with or without 5 μg ml⁻¹ LPS, 25 μg ml⁻¹ MDP, or various amounts of vitamin A. Then the AMφ monolayers were washed and incubated with 2 × 10⁴ MADB-100 cells for 72 h. AMφ treated in vitro with 10⁻³–5 × 10¹ IU retinyl palmitate showed much less tumoricidal activity (14–21%) than that of cells treated with LPS (72%) or with MDP (42%) (Table I).

Table I In vitro activation of tumoricidal activities of rat AMφ by retinyl palmitate

| AMφ treatment          | AMφ-mediated cytotoxicity against MADB-100 |
|------------------------|------------------------------------------|
| Tumour cells alone     | 2359±95*                                  |
| Untreated AMφ          | 2269±130                                  |
| LPS 5 μg ml⁻¹          | 627±42 (72%)                              |
| MDP 25 μg ml⁻¹         | 1306±37 (42%)                             |
| Retinyl palmitate      |                                         |
| 1 IU ml⁻¹              | 2283±239                                  |
| 10                      | 2236±196                                  |
| 100                     | 2142±162                                  |
| 1000                    | 1955±76 (14%)                             |
| 10000                   | 1786±137 (21%)                            |
| 50000                   | 1863±66 (18%)                             |
| 100000                  | 1905±152 (16%)                            |

*Cpm ± s.d. for triplicate cultures. Results were obtained in 3 independent experiments.

*Percent cytotoxicity calculated from results with tumour cells and untreated AMφ (P < 0.05).

Phagocytic ability of vitamin A-treated rat AMφ

Male F344 rats of 5–7 weeks old were given saline, vitamin A placebo (soybean oil) or vitamin A (250 IU g⁻¹ body wt) orally for 4 consecutive days, and 24 h later, their AMφ were harvested by lavage of the lungs. There was no difference in the numbers of AMφ obtained from the lungs of rats given saline, vitamin A placebo and vitamin A. The lavaged cells were plated for 60 min in CRPMI 1640 and then thoroughly washed to obtain a monolayer consisting of > 99% AMφ. The AMφ monolayers in Costar 24 wells of 16 mm diameter were tested for ability to phagocytize opsonized SRBC labelled with ¹¹⁵Cr. The combined data from 3 independent experiments are summarized in Figure 1. AMφ from rats that received > 250 IU retinyl palmitate g⁻¹ body wt showed significantly greater ability to phagocytize opsonized SRBC than those from rats given saline or vitamin A placebo alone.

In vivo activation of AMφ by vitamin A

Next we examined whether oral administration of vitamin A could render AMφ tumoricidal. Rats were given saline, vitamin A placebo or 250 IU vitamin A orally for one or 4 days and 24 h after the last administration, their AMφ were lavaged and assayed for AMφ-mediated cytotoxic activity. As shown in Figure 2, administration of retinyl palmitate for 4 days resulted in significant and reproducible increase in AMφ-mediated cytotoxicity against MADB-100.

Dose response of AMφ to vitamin A in vivo

F344 rats were given saline, vitamin A placebo or different amounts of vitamin A orally for 4 days. Twenty-four hours later, their AMφ were lavaged and plated. The indicated numbers of MADB-100 cells were added to the AMφ monolayers and incubations were terminated 72 h later. AMφ from rats given vitamin A at 100–500 IU g⁻¹ body wt acquired the ability to lyse syngeneic tumour cells in vitro. Under the same conditions, vitamin A placebo, a preparation consisting of soybean oil without vitamin A, did not render AMφ significantly cytotoxic (Table II). In a parallel set of experiments, AMφ activated in vivo with vitamin A were incubated in CRPMI 1640, and at the indicated times, 1.5 × 10⁴ labelled MADB-100 cells were added to the AMφ monolayers. The tumoricidal activity of vitamin A-treated AMφ was...
Table II  Induction of tumoricidal activities of AMφ by oral administration of retinyl palmitate

| Treatment of rats      | Ratio of AMφ/tumour target cells* |
|------------------------|-----------------------------------|
|                        | 5:1  | 10:1 | 20:1 |
| No AMφ,                | 3678±226b | 2039±158 | 936±111 |
| Saline                 | 3457±197 | 2074±64  | 933±13  |
| Vitamin A placebo      | 3501±180 | 1970±122 | 924±14  |
| Retinyl palmitate      |       |       |       |
| 100 IU                 | 2511±135 (27%)c | 1455±78 (30%)c | 816±74 (30%)c |
| 250 IU                 | 2414±137 (30%)c | 1460±86 (30%)c | 805±80 (30%)c |
| 500 IU                 | 2277±180 (34%)c | 1375±51 (34%)c | 718±59 (23%)c |

*aDifferent numbers of labelled MADB-100 cells to give the indicated ratio of effector/target cells were added to the 10^5 AMφ monolayers.

*bCpm±s.d. for triplicate cultures.

*cPercent cytotoxicity calculated from results with tumour cells and AMφ from rats given saline (P<0.05).

Figure 2  Effect of duration of retinyl palmitate administration on induction of AMφ-mediated cytotoxicity. Rats were given vitamin A placebo or retinyl palmitate (250 IU g⁻¹ body wt) orally once or once a day for 4 days. AMφ were plated for 60 min and then incubated with 10^5 labelled MADB-100 cells. Percent cytotoxicity was calculated by comparison with the value for AMφ from rats given saline. Points are means ± s.d. for triplicate cultures.

Figure 3  In vitro maintenance of the tumoricidal state of rat AMφ after oral administration of vitamin A. Percent cytotoxicity was calculated by comparison with the value for AMφ from rats given saline. Points are means ± s.d. for triplicate cultures.

In vivo effects of vitamin A analogues

Retinyl palmitate, retinol or retinoic acid in soybean oil was given to F344 rats orally for 4 days retained for 24-48 h, but was gradually lost by 96 h after the start of culture (Figure 3). At a dose of 250 IU g⁻¹ body wt, and 24 h after the last dose, the AMφ were lavaged and assayed for cytotoxic activity. Figure 4 shows that AMφ from rats given retinyl palmitate, retinol or retinoic acid showed cytotoxic activity against syngeneic mammary adenocarcinoma cell lines (MADB-100 and MADB-200).
receptor functions but in enhancement of their enzyme production (Rhodes & Oliver, 1980). Some vitamin A analogues, as well as agents such as LPS, pertussis or Mycobacterium butyricum, have been shown to labilize lysosomal membranes (Spitznagel & Allison, 1970). These agents also had marked adjuvant effects, and there was a parallel between their lysosome-labilizing capacity and their adjuvant activity. However, it is unknown whether the induction of tumoricidal activity in rat AMφ by vitamin A is related to lysosomal labilization, and other possible mechanisms for activation of AMφ by vitamin A, cannot be excluded. For instance, since vitamin A is known to stimulate lymphocyte blastogenesis in vitro (Abb & Deinhard, 1980; Lapin et al., 1974; Micksche et al., 1977), it is conceivable that when vitamin A is given orally the tumoricidal activity of AMφ could be potentiated via vitamin A-stimulated lymphocytes. In any event, an increase by retinoids in the functions of both the monocyte-macrophage series and lymphocyte systems could account for the antitumour effects observed in vivo in transplanted tumour and carcinogenesis systems (Bollag, 1971; Kurata & Micksche, 1977; Moon et al., 1976; Nettesheim & Williams, 1976; Saffioti et al., 1967).

We found that of three vitamin A analogues tested, all three retinoids (retinyl palmitate, retinol and retinoic acid) induced tumoricidal activity of rat AMφ in vivo. The tumoricidal activity of AMφ was significant when rats were given vitamin A orally for 4 days but not one day, suggesting that administration of vitamin A for 4 days was necessary for full activation of the tumoricidal activities of AMφ in vivo.

Large doses of vitamin A were necessary for in vivo activation of the tumoricidal activity of AMφ in the present work. However, the side effects of vitamin A seem to be less when it is given orally than when it is given i.p. or i.v. (Lapin et al., 1974; Seifter et al., 1983). Since the lung is a frequent target organ for carcinogenesis and cancer metastasis in animals and humans, and there is increasing evidence in animals that activated AMφ are important in host defence against neoplasms in the lung (Fidler et al., 1981; Sone & Fidler, 1982), the present work suggests that use of vitamin A to activate AMφ in situ, in conjunction with other procedures, such as immunotherapy, chemotherapy and radiation therapy, might be of benefit in treatment of primary and/or metastatic cancer in the lung.

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