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Effects of Retinoic Acid (Vitamin A) on Tumor Necrosis Factor Cytolytic Action

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Tumor necrosis factor (TNF) is a monokine produced primarily by macrophages. TNF has a number of activities including direct lysis of certain transformed cells and induction of antiviral activity. One of the protoypical transformed cell lines used for studying TNF cytolysis is murine L-929 cells. Because of the lysis, TNF has not been shown to have antiviral activity in these cells. Since retinoic acid (RA) induces a normal phenotype in the L-929 cells, we sought to determine if their conversion to a normal phenotype would 1) render them insensitive to the cytolytic effect and 2) allow for the development of an antiviral state. We present evidence that both the cis- and trans- forms of RA and to a lesser extent, the RA precursor beta-carotene, can inhibit recombinant human TNF cytolytic activity in mouse L-929 cells. However, blockage of the cytolytic activity does not allow development of an antiviral state.

Vitamin A is a fat-soluble vitamin that has been shown to have diverse biological effects. It is necessary for promotion of general growth, differentiation of epithelial tissues, visual function, and reproduction (1). In addition, natural and synthetic analogues of vitamin A (retinoids) have been shown to be capable of inhibiting the development of chemically induced carcinomas in a variety of animals at many anatomical sites (2, 3). Retinoids have also been shown to decrease carcinogen-induced transformation of cells in tissue culture. These decreases are manifested by restoration of contact inhibition of growth and decreased ability to grow in soft agar (4). In addition, we have shown that RA treatment will decrease the ability of transformed xeno-(human WISH) and allogeneic (murine L-929) cells to induce the production of interferon-alpha/beta (IFN-α/β) in nonsensitized splenocytes (5). The effect was presumed to be due to a decrease in the levels of transformed cell-surface glycoprotein(s) which had been previously been shown to be responsible for the induction of interferon in this system (6).

Tumor necrosis factor (TNF) is a monokine with a multitude of biological activities (7). It was originally described by its ability to preferentially lyse transformed cells. In addition, TNF has been shown to induce antiviral activity through its induction of interferon (8, 9). Mouse L-929 cells are the prototypic cell line that are used for assaying TNF cytolytic

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activity (10) while human HEp-2 cells were one of the original cell lines used to demonstrate its antiviral activity (9). Both of these activities are mediated through specific cell surface receptors for TNF (11). Since RA has been shown to shift transformed cells towards normal phenotypes (4), we determined its effects on TNF cytolytic and antiviral activities in L-929 and HEp-2 cells. We present evidence that both the cis- and trans- forms of RA can inhibit TNF-α cytolytic activity in L-929 cells, that the RA precursor beta-carotene inhibits the lytic activity to a lesser degree, and that the effect is not due to a direct effect on TNF-α. We further demonstrate the kinetics of this response. In addition, while RA has little effect on the induction of an antiviral state by TNF-α in human HEp-2 cells, in the absence of cytolysis in RA treated mouse L-929 cells, the antiviral state is not induced by TNF-α.

MATERIALS AND METHODS

Cells and media.

Mouse L-929 and human HEp-2 cells were obtained from the American Type Culture Collection. Cells were maintained in Eagle's minimal essential media supplemented with Earle's salts, 10% donor bovine serum and penicillin/streptomycin (100μg/ml and 100 units/ml, respectively) (EMEM 10%). Cultures were maintained in plastic tissue culture vessels (Costar, Cambridge, MA) in a 4% CO_2, 37°C atmosphere.

Reagents.

Retinoic acid (all cis and all trans) and beta carotene were obtained from Sigma Chemicals (St. Louis, MO). Recombinant human tumor necrosis alpha (specific activity 10^7 u/mg) was obtained from Suntory Chemicals (Tokyo, Japan). Initial dilutions of RA and beta-carotene were made in 100% ETOH. Further dilutions of RA, beta carotene and TNF for use in assays were made in EMEM 2%. In any instance, the residual ETOH levels following dilution were never higher than 0.01% which had no effect on cellular viability or assay results.

Assays.

Confluent L-929 or HEp-2 cells were treated with RA or beta-carotene for varying periods of time. Following treatment, the cells were washed 3 times with EMEM 2%. Cultures were then treated with TNF at the desired concentrations in EMEM 2% for 24h at 37°C. In the case of L-cells, cells were stained with a 2% crystal violet solution and then washed with tap water to remove excess stain. After drying cells were destained with Sorenson's buffer (0.1M sodium citrate, 0.1N HCl and 95% ETOH). Optical density of the wells was then determined on an automated reader at a wavelength of 590 nm. Destained, non-treated control well values were assumed to be 100% viable and calculations of percent control viability were determined from this value. One unit of TNF cytolytic activity is defined as the dilution at which corresponds to 50% of the non-treated control value.

For determination of antiviral activity, L-cells or HEp-2 cells, following treatment, were incubated with TNF at the desired concentrations. Twenty-four hours later, they were infected with vesicular stomatitis virus at an innput multiplicity of 0.3. After another 24 hours, virus was harvested and yields were determined in a modified plaque reduction assay in L-929 cells as described elsewhere (12).

RESULTS

RA has been shown to induce a transient normal phenotype in transformed L-929 cells (4). Since L-929 cells are the prototypic assay target for TNF and TNF preferentially lyses

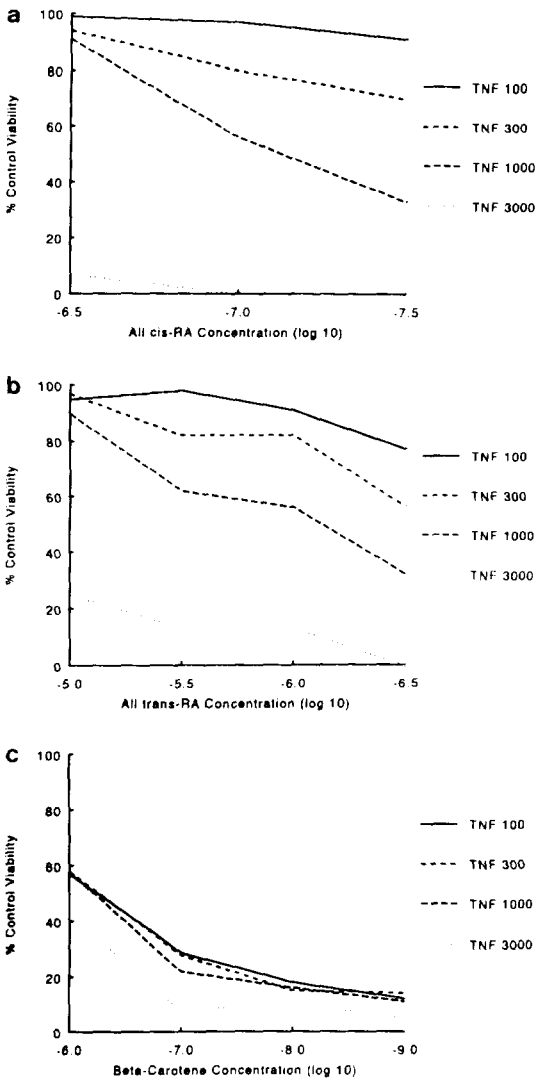

FIGURE 1. Effects of all cis- or all trans- RA and beta carotene on TNF-α cytolytic activity in mouse L-929 cells. L-929 cells in microtiter plates were treated with RA at the indicated concentrations for 18-24 hours. Cells were then washed, following which recombinant human TNF-α was added. Cytolytic activity was determined after 24 h as described in materials and methods.

transformed cells, we determined the effects of RA on lysis in this system. L-929 cells were treated with RA at concentrations ranging from 10^{-5} M to $10^{-6.5}$ M, and $10^{-6.5}$ M and $10^{-7.5}$ M, for trans RA and cis RA, respectively. Higher concentrations of RA were not used due to toxicity. As shown in figures 1a and 1b, there is a dose dependent inhibition of TNF cytolytic activity induced by RA in L-cells. The effect appears to be due to some RA induced alteration in the cell since its effect is stable in cells following extensive washing (data not shown). We similarly tested the effect of the RA precursor beta-carotene . As shown in figure 1c, beta carotene was much less effective than the RA derivatives. Further support for the concept that

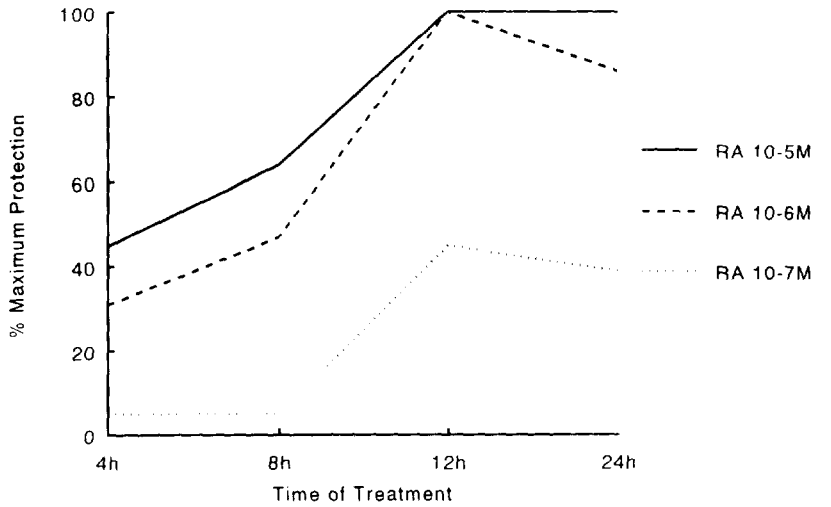

FIGURE 2. Kinetics of induction of RA inhibitory effect. L-929 cells were treated with RA for the indicated time periods following which they were washed, then treated with TNF. Twenty-four hours later, TNF cytolytic activity was determined.

RA induces some cellular alteration come from determining the kinetics of induction of the protective effect since RA requires between 8 and 12 hours to induce its full inhibitory effect (figure 2).

We next determined if RA interacted directly with TNF and in this fashion blocked its activity. To perform this experiment, cis- and trans- RA ($10^{-6.0}$ M) were incubated with 100 units of TNF for 4h at 37°C. Following incubation, TNF was dialyzed free of RA. The samples were then tested for TNF activity in a standard assay on L-cells. As shown in figure 3, the residual TNF activity was identical in all samples regardless of the treatment. Thus,

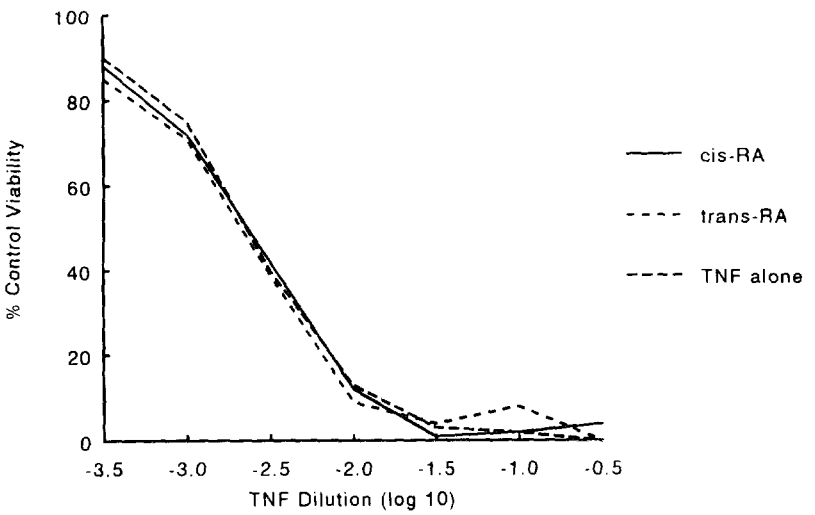

FIGURE 3. Lack of a direct effect of RA on TNF-α. Following co-incubation for 24 h, TNF preparations were dialyzed free of RA as described and assayed for cytotoxic activity.

TABLE 1. Retinoic acid treatment does not affect TNF-α antiviral activity in HEp-2 cells

Treatment	Virus yield (pfu/ml)	Fold Reduction from Control
None (control)	1.4×10^5 (4.8)[b]	--
TNF (3000 u/ml)	5.2×10^3 (1)	26
RA ($10^{-6.5}$M)	4.1×10^5 (4.5)	0
TNF[a] + RA ($10^{-6.5}$M)	6.5×10^3 (4)	22
RA ($10^{-7.0}$M)	4.2×10^5 (4.5)	0
TNF + RA ($10^{-7.0}$M)	3.2×10^3 (2)	44
RA ($10^{-7.5}$M)	3.9×10^5 (3)	0
TNF + RA ($10^{-7.5}$M)	3.7×10^3 (1.5)	38

[a]All combination treatments recieved 3000 u/ml of TNF-α.
[b]Parentheses indicate standard deviation of plaque number at particular virus dilution. Representative of two separate experiments.

neither the cis- nor the trans- forms of RA have any direct effect on TNF cytolytic activity. This finding further supports the concept that RA is altering some cellular property.

TNF has been shown to possess antiviral activity in certain cell lines (8). Thus, we tested its effects on the induction of an antiviral state in HEp-2 cells. RA was incubated for 24 hours on HEp-2 cells at the concentrations indicated in table 1. Following incubation, the cells were washed 3 times and treated with 3,000 and 10,000 units/ml of TNF. From previous experience, these concentrations have been shown to induce a significant antiviral state (9). Twenty four hours later the cells were challenged with virus and yields were determined. As shown in table 1, RA has no significant effect on the antiviral activity of TNF in human HEp-2 cells. In contrast, as shown in table 2, prior treatment of L-929 cells with RA does not result in the induction of an antiviral state by TNF. Similar results were seen in a parallel study using murine TNF-α (data not shown).

DISCUSSION

These studies were designed to investigate the effects of RA on TNF cytolytic and antiviral activities. We have shown that 1) both the cis- and trans- forms of RA can inhibit TNF cytolytic activity in L-929 cells, 2) the RA precursor beta-carotene will not inhibit the lytic activity as effectively, 3) at least 8-12 h is required for RA to induce its effect, 4) the effect is not directly on TNF but appears to be due to an alteration of some cellular process(es), 4) RA has little effect on TNF's induction of antiviral activity in human HEp-2

TABLE 2. RA pretreatment of L-929 cells does not result in development of antiviral activity by human TNF-α

Treatment[a]	Virus Yield (pfu/0.1 ml)[b]
None	2.0×10^6
RA $10^{-5.5}$ M	3.4×10^6
RA + TNF (300 u/ml)	5.5×10^5
RA + TNF (100 u/ml)	5.8×10^5
RA + TNF (30 u/ml)	1.4×10^6
RA + TNF (10 u/ml)	9.4×10^5
TNF (300 u/ml)	8.2×10^5
TNF (100 u/ml)	6.0×10^5
TNF (30 u/ml)	4.0×10^5
TNF (10 u/ml)	6.4×10^5

[a] L-929 cells in 96-well microtiter plates were treated with the indicated concentrations of RA for 8h. Following this period, recombinant human TNF-a was added directly to the cultures at the specified concentrations. Eighteen hours later, supernatant fluids were decanted and the cells were challenged with VSV.

[b] Virus yields were assayed 24h post-challenge as before.

Representative of 2 separate experiments. Similar results obtained with murine TNF-α (data not shown).

cells, and 5) treatment with RA prior to TNF does not allow for the development of an antiviral state in L-929 cells.

We have previously shown that the cytolytic and antiviral activities of TNF can be modulated in a similar fashion by the compound hydrazine sulfate (13). In those studies, hydrazine sulfate, like RA, was capable of inhibiting TNF cytolysis. In contrast, however, hydrazine sulfate greatly potentiated the antiviral activity of human TNF in HEp-2 cells. TNF has been shown to induce its antiviral activity through the production of interferon-beta (9). In light of this, Blalock and Gifford studied the potential of vitamin A to effect the antiviral activity of IFN (14). They demonstrated that vitamin A would inhibit IFN induction of antiviral activity by two mechanisms 1) by directly binding the IFN thereby preventing its action, and 2) through the induction of a protein that could block transcription of a gene controlling IFN synthesis. Since in our studies, RA was extensively washed from the cell cultures following treatment, the possibility of it binding IFN is minimized. There is the potential that some residual RA attched to cellular receptors would directly bind IFN during its synthesis and release, but the period of time required for IFN induction by TNF would also minimize this possibility.

At present, we are uncertain as to the mechanism how RA is inhibiting the cytolytic activity. Our studies would tend to indicate that some cellular alteration is occurring due to the time period required for the RA to have an effect. Since TNF preferentially lyses transformed cells through binding to a specific TNF receptor, one possibility may be a down regulation of TNF receptor. Another possibility may be that RA is not down regulating TNF receptors but altering the lytic processes initiated by TNF. Whatever mechanism being effected, a paradox emerges when one considers the use of RA as an antineoplastic agent. If TNF as a natural mediator is important in controlling the growth of neoplastic cells through cytolysis, RA use might be contraindicated since it appears to inhibit this activity.

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