Inhibition of Interferon Action by Vitamin A

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SUMMARY

Simultaneous treatment of mouse cells with interferon and vitamin A (retinoic acid) resulted in an inhibition of interferon action. Increasing concentrations of calf serum decreased the inhibitory effect of retinoic acid on interferon action. Treatment of interferon with retinoic acid prior to the assay for interferon activity also resulted in a loss of interferon activity. Since the residual retinoic acid present after dilution of the interferon for assay was not sufficient to interfere with the assay, it is presumed that interferon and retinoic acid must interact in some fashion to inhibit interferon activity. Calf serum prevented the apparent interaction of retinoic acid and interferon.

The loss of interferon activity which resulted from treatment of interferon with retinoic acid was dependent on temperature and time of incubation. Retinyl acetate (acetate ester of vitamin A) and retinal (vitamin A aldehyde) only slightly inhibited interferon activity, while retinoic acid (vitamin A acid) and retinol (vitamin A alcohol) were similarly effective at inhibiting interferon activity. Another fat soluble vitamin, vitamin K₁, did not inhibit interferon activity.

INTRODUCTION

We have previously reported that simultaneous treatment of human cells with interferon and vitamin A resulted in an inhibition of interferon action (Blalock & Gifford, 1974). This study shows that vitamin A inhibits interferon by an interaction with the interferon molecule and extends the original observation to include the inhibitory effect of vitamin A on mouse interferon. Other than steroids (Kilbourne, Smart & Pokorny, 1961), vitamin A is the only known metabolite having an adverse effect on interferon action. It is the first instance of an apparent interaction of a metabolite with interferon leading to inactivation of interferon.

METHODS

Cells. Mouse L-929 cells were maintained by weekly passage in Eagle's Minimal Essential Medium (MEM) supplemented with 10% calf serum (CS). This medium contained 125 μg streptomycin and 250 units of penicillin/ml.

Virus. A large plaque variant of the Indiana strain of vesicular stomatitis virus (VSV) was originally obtained from Dr Jan Vilcek (New York University) and was grown in the allantoic cavity of developing chick embryos. This pool of virus contained about $1 \times 10^9$ plaque forming units (p.f.u.)/ml when assayed on L-929 mouse cells. A lentogenic strain of New-
castle disease virus (NDV) was obtained from Dr R. P. Hanson (University of Wisconsin). This virus was grown in the allantoic cavity of developing chick embryos.

Production and assay of interferon. Mouse interferon was produced by infection of L-929 cells with a lentogenic strain of NDV. Supernatant fluids were harvested 24 h after infection and dialyzed against pH 2 buffer for 5 days at 4 °C and then against Gey's balanced salt solution (BSS) to restore the pH to neutrality.

A plaque reduction assay using VSV and L-929 cells was employed. Confluent cell monolayers in 2 oz glass bottles were treated overnight with twofold serial dilutions of interferon preparations. Supernatant fluids were then aspirated and cells infected with 0·2 ml of a dilution of VSV containing about 300 p.f.u. After 1 h incubation at room temperature to permit adsorption of virus, each monolayer was overlaid with 5 ml of MEM containing 1 % methyl cellulose, 5 % calf serum, 25 mm-Hepes buffer, 125 μg/ml streptomycin and 250 units/ml of penicillin (overlay medium). The cultures were incubated for 48 h at 37 °C and monolayers were then stained with crystal violet. Assays were performed in triplicate or quadruplicate. Plaques were enumerated after ×6·5 magnification of the monolayers by use of a photographic enlarger. The 50 % plaque depressing dose (PDD₅₀) was defined as the amount of an interferon preparation, in μl, that inhibited 50 % of the plaques from developing as compared to the controls. The PDD₅₀ units/ml is used as a measure of the potency of an interferon preparation.

Chemicals. All trans forms of retinoic acid (vitamin A acid), retinol (vitamin A alcohol), retinyl acetate (acetate ester of vitamin A) and retinal (vitamin A aldehyde) were obtained from Sigma Chemical Company, St Louis, Mo., Vitamin K₁ (from Natural Phytol) was obtained from Schwarz/Mann, Orangeburg, New York. Dimethyl sulphoxide (DMSO) was obtained from Fisher Scientific Company, Pittsburgh, Pa.

Stock solutions were made by dissolving each vitamin at 2000 μg/ml in DMSO. For experimental purposes further dilutions of the vitamin were made in medium. Controls always received an equivalent amount of DMSO (usually 1 %). The concentrations of vitamin and DMSO employed had no effect on VSV plaque formation.

RESULTS

Effect of simultaneous addition of retinoic acid and interferon on the assay of interferon

Separate interferon assays were simultaneously performed in the presence or absence of different concentrations of retinoic acid. Dilutions of interferon and retinoic acid in MEM with 3 % calf serum (CS) were mixed and added to L cell monolayers which were then incubated overnight at 37 °C. Residual interferon and retinoic acid were removed and cultures challenged with approx. 300 p.f.u. of VSV. Overlay medium was added and cultures incubated at 37 °C for 48 h. Plaques in experimental and control cultures were counted and the percent inhibition of plaque formation was plotted against the logarithm of the dose of interferon in μl. A series of approximately parallel dose response lines were obtained. These were used to estimate the PDD₅₀ units of interferon measured with each concentration of retinoic acid. The percentage of control PDD₅₀ units obtained in the presence of various concentrations of retinoic acid were calculated for two separate experiments and are shown in Fig. 1. Increasing concentrations of retinoic acid resulted in decreasing activity of interferon. A marked reduction (93 %) in measurable interferon was obtained when 20 μg/ml of retinoic acid was employed (from 6·7 PDD₅₀ units in the control to 0·5 units). This concentration of retinoic acid was not demonstrably toxic for the cells since the same number (and
Fig. 1. Effect of simultaneous addition of retinoic acid and interferon on the assay of interferon. Separate interferon assays were simultaneously performed in the presence or absence of different concentrations of retinoic acid in MEM with 3% CS. The percentage of control PDD₅₀ units (6.7 units) obtained in the presence of various concentrations of retinoic acid were calculated for two separate experiments (× or ○).

Similar size) of VSV plaques were found with all concentrations of retinoic acid (and controls) in the absence of interferon. Greater concentrations of retinoic acid could not be used since they were toxic for the L cells.

Effect of calf serum on the simultaneous addition of retinoic acid and interferon to cells

Preliminary experiments indicated that the effect of retinoic acid on the interferon assay was markedly influenced by the concentration of calf serum employed. Various concentrations of calf serum (%, v/v) were employed with 20 μg/ml of retinoic acid or 1% DMSO and a dose of interferon which would normally result in approx. 80% inhibition of VSV plaques. Controls without interferon received an equivalent amount of retinoic acid or DMSO. These mixtures were then assayed for determination of resultant interferon activity. The results (Fig. 2) indicate that concentrations of calf serum from 2.5 to 20% had only a slight effect on the interferon assay as previously reported by Vilček & Lowy (1967). However, increasing concentrations of calf serum decreased the inhibitory effect of retinoic acid on interferon action.

Effect of treatment of interferon with retinoic acid prior to assay for activity of interferon

A loss of measurable interferon activity, when the interferon is assayed in the presence of retinoic acid, could be due to either a combination of interferon with retinoic acid resulting in an inactive product or to some intracellular event, influenced by retinoic acid, which prevented the expression of interferon activity. The previous data showing that increasing concentrations of calf serum prevented the effect of retinoic acid on interferon activity suggested that the event was extracellular. To test this possibility, interferon and retinoic
acid were mixed, incubated, and then diluted beyond the effective range of retinoic acid but with sufficient interferon remaining to significantly inhibit virus replication. Thus, a 1:10 dilution of stock interferon in MEM was mixed with various concentrations of retinoic acid. Control interferon received an equivalent amount of DMSO. Since the stock interferon was made in 10% calf serum, the resultant concentration of calf serum employed was 1%. Following overnight incubation (22 h) at 37 °C, interferon activity was assayed. Prior to assay, the interferon samples were sufficiently diluted in MEM with 5% CS so that the residual retinoic acid was diluted beyond the concentration needed to interfere with the assay (i.e. 0.4 to 0.003 μg/ml). The control activity represented 400 PDD₉₀ units of interferon. Fig. 3 shows that retinoic acid treatment of interferon resulted in a loss of interferon activity under these conditions. This data indicates that interferon and retinoic acid must interact in some fashion to inhibit interferon activity and that this interaction is not rapidly reversible.
**Effect of calf serum on the treatment of interferon with retinoic acid prior to assay**

Since the concentration of calf serum markedly influenced the effect of retinoic acid on the interferon assay when assayed immediately (see Fig. 2), the effect of calf serum was then determined on the interaction of retinoic acid and interferon when incubated together in the absence of cells.

A 1:10 dilution of the stock interferon was made in MEM with 1, 5, or 10% calf serum and with or without 20 μg/ml of retinoic acid. Following overnight incubation (22 h) at 37 °C, interferon activity was assayed after dilution (residual concentration of retinoic acid following dilution was less than 0.2 μg/ml). Fig. 4 shows that calf serum again prevented an apparent interaction of interferon and retinoic acid under these conditions. In another experiment the calf serum concentration was reduced to 0.125%, and interferon activity was thereby reduced to 2.5% (10 units) of the control (400 PDD₅₀ units/ml) when 40 μg/ml of retinoic acid was employed.

**Effect of temperature on treatment of interferon with retinoic acid**

Since it appears that interferon and retinoic acid interact to result in an inactive product, the effect of temperature on this interaction was determined.

Retinoic acid was added to interferon in MEM (400 units/ml in 1% CaS) at a concentration of 20 μg/ml. One ml portions of the interferon and retinoic acid mixture, as well as control interferon prepared without retinoic acid, were placed at 5, 25 and 37 °C for 24 h. The mixtures were then diluted 1:100 in MEM with 5% CS (reducing the retinoic acid concentration to 0.2 μg/ml) and assayed for residual interferon activity. Fig. 5 shows only a slight loss of interferon activity in the control at 25 and 37 °C, while there was a marked loss of interferon activity at these temperatures in the presence of retinoic acid. No loss of interferon activity resulted when the mixture of interferon and retinoic acid were kept at 5 °C for 24 h. This data would indicate that the interaction of interferon and retinoic acid resulting in the loss of interferon activity was temperature dependent.
Effect of time at 37°C on the loss of interferon activity in the presence of retinoic acid

Retinoic acid (20 μg/ml) was added to 400 units/ml of interferon in MEM (1% CS). The mixture was placed at 37°C and portions were removed at various times and placed at 5°C (a temperature at which retinoic acid did not result in a loss of interferon activity). When all samples were collected (23 h), each sample was diluted 1:100 in MEM with 5% CS (reducing the retinoic acid concentration to 0.2 μg/ml) and assayed for residual interferon activity. As shown in Fig. 6, there is a progressive loss of interferon activity in the presence of retinoic acid with increasing periods of time at 37°C.

Effect of treatment of interferon with different forms of vitamin A and with vitamin K₁

Different forms of vitamin A and another fat soluble vitamin, K₁, were tested to determine if they also effected interferon. Retinoic acid, retinol, retinal, retinyl acetate and vitamin K₁ were made 6.7 x 10⁻⁵ M in an interferon preparation diluted 1:10 in MEM. Following 24 h incubation at 37°C, the mixtures were further diluted 1:100 in MEM with 5% CS and assayed for interferon activity. Treatment of interferon with retinyl acetate (the acetate ester of vitamin A) or with retinal (vitamin A aldehyde) only slightly inhibited interferon activity (Table 1). Retinoic acid (vitamin A acid) and retinol (vitamin A alcohol) were similarly effective at inhibiting interferon activity. Another fat soluble vitamin,
K1, did not inhibit interferon activity. This data suggests that the terminal group on the vitamin A molecule is very important in the interaction of vitamin A with interferon. Furthermore, the interaction is probably not common to all fat soluble vitamins.

**DISCUSSION**

As was previously reported for human interferon (Blalock & Gifford, 1974), retinoic acid was shown to inhibit the action of mouse interferon. The inhibition of antiviral activity which was observed when retinoic acid was mixed with interferon and immediately assayed for interferon activity, apparently resulted from an interaction of retinoic acid with the interferon molecule. This conclusion is supported by the observation that treatment of interferon with retinoic acid and incubation in the absence of cells resulted in a loss of interferon activity, when subsequently measured after sufficient dilution to reduce the concentration of retinoic acid to a level which had no effect in the assay. In addition, the inhibitory effect of retinoic acid on interferon activity was prevented by calf serum.
The loss of interferon activity following addition of retinoic acid is characterized by a dependence on time and temperature. Temperatures above 25 °C were required for a pronounced inhibitory effect.

Recent reports have shown that human interferon has hydrophobic binding sites (Davey et al. 1974, 1975; Huang et al. 1974). These reports have indicated that there is no hydrophobic interaction between mouse interferon with ω-carboxypentyl agarose (Davey et al. 1975) and only slight retention of mouse interferon on albumin immobilized on agarose (Huang et al. 1974). Our data is consistent with the concept of a hydrophobic interaction between retinoic acid and interferon. Davey et al. (1975) reported the binding of human interferon to hydrophobic hydrocarbon arms covalently linked to Sepharose was critically dependent on the hydrophilic head group of the hydrocarbon. We have also observed a critical dependence on the character of the head group of vitamin A for the inhibitory effect on interferon. Forms of vitamin A with carboxy or hydroxy groups were effective at inhibiting interferon while vitamin A forms with carbonyl or acetate ester groups were essentially ineffective. Considering the high affinity hydrophobic binding sites on bovine
Table 1. Effect of treatment of interferon with different forms of vitamin A and with vitamin K₁

<table>
<thead>
<tr>
<th>Vitamin added to interferon</th>
<th>% inhibition of VSV plaques*</th>
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</thead>
<tbody>
<tr>
<td>Retinoic acid (vitamin A acid)</td>
<td>29 (23–33)</td>
</tr>
<tr>
<td>Retinol (vitamin A alcohol)</td>
<td>36 (32–41)</td>
</tr>
<tr>
<td>Retinal (vitamin A aldehyde)</td>
<td>64 (61–65)</td>
</tr>
<tr>
<td>Retinyl acetate (vitamin A acetate ester)</td>
<td>72 (71–75)</td>
</tr>
<tr>
<td>Vitamin K₁</td>
<td>82 (81–84)</td>
</tr>
<tr>
<td>Control</td>
<td>84 (83–84)</td>
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* Numbers in parentheses indicate the standard deviation of triplicate determinations.

serum albumin (Jonas & Weber, 1971) the calf serum dependent prevention of the inhibitory effect of retinoic acid on interferon probably results from competitive binding of the retinoic acid to albumin and other serum proteins. However, a doubling in retinoic acid concentration (from 20 to 40 μg/ml) required a fourfold increase in calf serum to results in a similar degree of protection of interferon (our unpublished observation). Bovine serum albumin can be substituted for calf serum in preventing the effect of retinoic acid on interferon (our unpublished observation).

We have assumed that serum interacts with vitamin A to prevent its binding to interferon. It is possible, of course, that serum interacts with interferon to prevent its binding to vitamin A. However, considering the high affinity that albumin has for fatty acids (Jonas & Weber, 1971) and the low affinity for mouse interferon (Huang et al. 1974), it is more likely that serum interacts with vitamin A to prevent vitamin A from binding to interferon.

If retinoic acid exerts its inhibitory effect by binding to hydrophobic regions on interferon molecules, it would seem this binding must be of relatively high affinity as compared with calf serum, since the molar concentration of protein in serum should be considerably higher than the molar concentration of interferon. The interaction of retinoic acid with interferon may prove valuable in studying the interaction of interferon with its cellular receptor. Also, covalent coupling of retinoic acid or retinol to a solid matrix may provide a means for a significant step in the purification of human and mouse interferon by hydrophobic chromatography. This would only be feasible if the interaction of vitamin A and interferon is reversible. Our limited attempts at reversing the reaction have not been successful to date.

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REFERENCES


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