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EFFECTS OF INHIBITORS OF LIPID SYNTHESIS ON THE REPLICATION OF ROUS SARCOMA VIRUS

A SPECIFIC EFFECT OF CERULENIN ON THE PROCESSING OF MAJOR NON-GLYCOSYLATED VIRAL STRUCTURAL PROTEINS

HOWARD GOLDFINE *, JOHN B. HARLEY ** and JOHN A. WYKE

Department of Tumour Virology, The Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London WC2 (U.K.)

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Summary

The effects of two inhibitors of lipid biosynthesis on the replication of Rous sarcoma virus Prague C strain in chick embryo fibroblasts have been examined in media containing delipidated serum. 25-Hydroxycholesterol, which markedly inhibits the incorporation of [1-^{14}C]acetate into sterols, had no effect on the formation of infectious virions or on the synthesis and processing of intracellular virion proteins. Cerulenin stongly inhibited [1-^{14}C]acetate incorporation into fatty acids and partially inhibited its incorporation into sterols in chick embryo cells. Rous sarcoma virus production as measured by focus formation and by the production of [^{35}S]methionine-labeled virions was strongly inhibited within 5 h after cerulenin addition to infected cultures. Examination of extracts of these cells revealed the accumulation of the 76 000 dalton precursor (Pr76) of the major non-glycosylated virion structural proteins, p27, p19, p15 and p12. The failure to process the 76 000 dalton precursor was coincident in time with the decrease in viron production. Neither whole serum nor mixtures of fatty acids plus cholesterol were able to reverse the effects of cerulenin.

* Present address: Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, Pa. 19104, U.S.A.

** Present address: Department of Internal Medicine, Yale University, New Haven, Conn. 06510, U.S.A.

Abbreviations; RSV, Rous sarcoma virus; cerulenin, (2*S*),(3*R*)-2,3-epoxy-4-oxo-7,10-dodecadienoyl amide; SDS, sodium dodecyl sulfate.

Introduction

Methods for controlling the membrane lipid composition of animal cells in vitro have recently become available [1]. They provide new means to study the maturation of enveloped viruses such as the avian retroviruses, which bud from the plasma membrane of infected cells without destroying the host cell. These methods require the growth of animal cells in media containing delipidated sera or, in the case of certain cell lines, in media without serum. In addition, in order to control endogenous lipid synthesis, it is necessary to block the synthesis of sterols and fatty acids. The former has been accomplished by the use of oxygenated sterols with a keto or hydroxyl function in the 6, 7, 15, 20, 22, 24 or 25 positions [2]. These compounds inhibit sterol synthesis in cultured cells by depressing the activity of the regulatory enzyme hydroxymethylglutaryl-coenzyme A reductase [3,4]. Fatty acid synthesis has been decreased by removing biotin from the medium [1]. Cerulenin, which inhibits both fatty acid synthesis and sterol synthesis [5] may also prove useful.

The lipids of Rous sarcoma virus (RSV) and its host cell have been studied in some detail. Although the phospholipid composition of RSV envelopes broadly resemble that of the plasma membrane of its host cell, as is the case for other enveloped viruses, there are some differences in the proportions of phosphatidylcholine and sphingomyelin [6,7].

We have studied the effects of two inhibitors of lipid synthesis on the maturation of RSV in chick embryo cells. 25-Hydroxycholesterol, at concentrations which markedly inhibited sterol synthesis had no detectable effects. Cerulenin, a potent inhibitor of fatty acid synthesis in bacteria, fungi [5] and the slime mold, *Dictyostelium discoideum* [8], inhibits fatty acid synthesis in chick embryo cells. As in other eurcaryotic cells, it partially inhibits sterol synthesis in these cells. We have shown that it inhibits the maturation and production of RSV. This was potentially related to a defect in the cleavage of the 76 000 dalton precursor (Pr76) of the major non-glycosylated viral structural proteins (p27, p19, p15 and p12).

Experimental section

Virus and cell culture. The Prague C strain of Rous sarcoma virus subgroup C was used in these studies. Chick embryo fibroblast cultures of C/E phenotype were prepared from Brown Leghorn Embryos (Wickham Laboratories, Hants., U.K.) by standard techniques [9]. Secondary cultures were infected with virus at a multiplicity of 0.5—1.0 focus forming units per cell and incubated at 41°C for 4 days. The cells were subcultured and grown for 3 days. Fourth passage cells were usually seeded at $2 \cdot 10^6$—$3 \cdot 10^6$ cells per 10 ml in 9.0-cm dishes, 10^6 cells per 5 ml in 5-cm dishes, or $4 \cdot 10^5$ cells in 2 ml in 3.5-cm dishes. The culture medium was Dulbecco's modified Eagle's medium supplemented with 10% tryptose phosphate broth, 5% calf serum and 1% chick serum. This was supplemented with 0.2 mg of polybrene per 100 ml of medium at the time of infection and with 1% dimethylsulfoxide during subsequent passages [10]. 1 day after the fourth passage the medium was removed and the cells washed twice with Dulbecco's modified Eagle's medium. Then medium supplemented

with 5% delipidated fetal calf serum plus biotin, 1.5 μg/ml, was added, unless otherwise noted. At this time uninfected second passage chick embryo cells, equal to 0.5—1.0 of the original inoculum, were added to each dish. The added cells helped to stabilize the resulting confluent cultures, decreasing peeling and loss of cells during later media changes.

RSV was assayed by focus formation in chick embryo fibroblasts [9,11]. Cell growth was measured either by counting in a haemocytometer or in an electronic particle counter.

Acetate incorporation into lipids. Acetate incorporation was measured essentially as described by Kandutsch and Saucier [12]. To each 3.5 cm dish, 10 μCi of [1-^{14}C]acetate, 8 μmol, was added and the cells were incubated for 1 h at 41°C. They were washed three times with phosphate-buffered saline and 0.5 ml of the same buffer was added plus 0.5 ml 95% KOH. 1 mg cholesterol and 1 mg lecithin were added as carriers, and saponification was at room temperature overnight. The hydrolysate was transferred to a 10 ml conical centrifuge tube with three washes of ethanol, total volume 0.5 ml. Extraction of sterols and fatty acids, and precipitation of sterol digitonides were performed as described [12].

Radioisotopic labeling of virus particles. Labeling of virus was carried out as described by Hayman et al. [13] except that Dulbecco's modified Eagle's medium was substituted for F10 and the chase was carried out in medium containing 5% delipidated fetal calf serum and inhibitor where indicated.

Virus purification. The culture fluids were centrifuged at 300 × *g* for 10 min in order to remove cells and other debris. Virus was pelleted from the supernatant fluid by centrifugation at 117 500 × *g* for 1 h. The virus pellet was resuspendend in standard buffer: 0.1 M NaCl/0.01 M Tris · HCl/0.001 M EDTA (pH 7.4), 0 5 ml [14] and layered on a continuous 20—50% (w/v) gradient in the same buffer, 4.5 ml, and centrifuged to equilibrium in an MSE 6 × 5 swinging bucket rotor for 2.5 h at 50 000 rev./min (=240 000 × *g*).

15-drop fractions were collected and 100 μl of each was counted in Aquasol II (New England Nuclear Corp.) in a liquid scintillation counter. Fractions containing virus were pooled, diluted to 9 ml in standard buffer and centrifuged at 117 500 × *g* for 1 h.

Immune precipitation of intracellular virus. Antibody against disrupted avian myeloblastosis virus was the generous gift of Dr. H. Diggelmann, Swiss Institute for Experimental Cancer Research, Lausanne. Antibodies against p27 and gp85/gp37 were kindly provided by Dr. M.J. Hayman, of this laboratory. After labeling with [^{35}S]methionine the cells were washed twice with cold phosphate-buffered saline and extracted with rocking for 10 min in ice-cold disruption buffer [15] containing 1 mM phenylmethylsulfonyl fluoride, 1 ml per 5.0 cm dish. In some experiments the cells were frozen at −25°C for up to 3 days prior to disruption. The cell extracts were centrifuged at 550 × *g* for 10 min in order to remove the nuclei and other debris and 0.5 ml of extract was used for immune precipitation. Usually 10 μl of antibody against whole virus was used per 0.5 ml extract. After a 1 h incubation at 37°C, 50 μl of goat anti-rabbit serum was added, incubation was continued for 1 h at 37°C and the precipitates were allowed to form overnight at 2°C. They were centrifuged for 10 min at 550 × *g* and washed three times in 20 mM Tris · HCl/50 mM NaCl/0.5%

Nonidet P-40, pH 7.25, and once in 0.05 M Tris · HCl, pH 6.8. The pellets were solubilized in 0.2 ml of electrophoresis buffer [16].

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate buffers was carried out on slab gels either on 12% cross-linked gels as described by Laemmli [16] or on a gradient gel as described by Studier [17].

Isotope incorporation into trichloroacetic acid-precipitable material. Aliquots of the cell extracts were put on glass fiber filter disks and dried. The disks were extracted twice for 15 min in 5% trichloroacetic acid at 2°C. They were then washed twice in methanol, dried, and counted in 5 ml of aquasol II.

Materials. Cerulenin was the generous gift of Dr. Omura, Kitasato University, Minato-ku, Tokyo. Identical results were obtained with a sample from Makor Chemical Ltd., Jerusalem 91060, Israel. 25-Hydroxycholesterol was purchased from Steraloids, Inc., Wilton, N.H., U.S.A.

Labeled compounds were purchased from the Radiochemical Centre, Amersham, Bucks., U.K. Phenylmethylsulfonyl fluoride was obtained from Sigma, London. Acrylamide and bisacrylamide were obtained from Bio Rad Laboratory, Bromley, Kent, U.K. and sodium dodecyl sulfate from Serva.

Delipidated fetal calf serum was prepared by ethanol-ether extraction as described by Horwitz et al. [18].

Results

Effects of cerulenin on cells

Chick embryo fibroblast cultures grow well in Dulbecco's modified Eagle's medium supplment with 10% delipidated fetal calf serum and biotin. The addition of increasing concentrations of cerulenin 1 day after plating resulted in progressive inhibition of growth (Fig. 1).

The addition of 12.5 or 25 μg/ml cerulenin resulted in rapid inhibitions of [1-^{14}C]acetate incorporation into cellular fatty acids and sterols (Fig. 2). 2 h after addition, incorporation of labeled acetate into fatty acids was inhibited

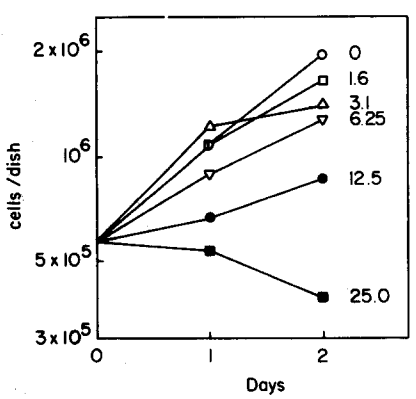

Fig. 1. Inhibition of growth of uninfected chick embryo cells by cerulenin. Cerulenin, in the final concentrations (μg/ml) shown to the right of each of curve, was added at time 0. The cells were plated in Dulbecco's modified Eagle's medium containing 10% delipidated fetal calf serum and biotin, 1.5 μg/ml, 1 day before cerulenin was added.

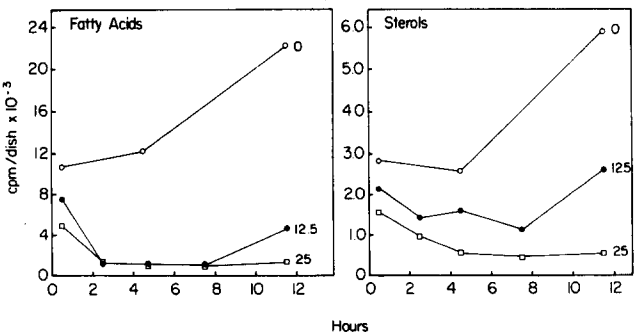

Fig. 2. Effect of cerulenin on the incorporation of [1-^{14}C]acetate into fatty acids and sterols of uninfected chick embryo cells. Second passage cells ($6 \cdot 10^5$) were plated in 3.5-cm dishes in the medium described in the legend to Fig. 1, 1 day before cerulenin was added at time zero. Acetate incorporation was measured as described in Experimental section. ○———○, no cerulenin; ●———●, 12.5 μg/ml; □———□, 25 μg/ml.

by 89—90% and into sterols by 47—63.5% by 12.5 or 25 μg/ml cerulenin, respectively.

In a similar experiment with chick embryo fibroblasts infected with a transformation defective mutant of RSV (LA-24), the incorporation of labeled acetate into sterols and fatty acids was inhibited to a similar extent by 12.5 μg/ml cerulenin. There was less than 5% inhibition of incorporation into sterols and 51% inhibition of incorporation into fatty acids at 4 h after addition of 3 μg/ml cerulenin (data not shown). It can be seen in Fig. 2 that some recovery of incorporation into sterols and fatty acids occurred after 8 h with 12.5 μg/ml cerulenin. Therefore concentrations of 20 μg/ml cerulenin were used in subsequent experiments.

Protein synthesis, as measured by the incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material, was only slightly affected by 20 μg/

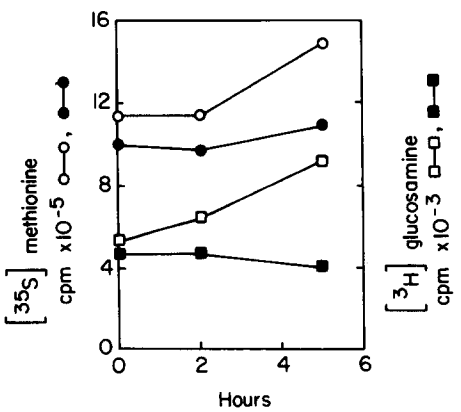

Fig. 3. Incorporation of [^{35}S]methionine and [^{3}H]glucosamine into trichloroacetic acid-precipitable material in uninfected chick embryo cells. Cells were growing in the medium described in the legend to Fig. 1, but with 5% delipidated fetal calf serum, in 5-cm dishes, and were nearly confluent. The cells were preincubated at 41°C with Dulbecco's modified Eagle's medium without methionine for 10 min. This was replaced by the same medium plus [^{35}S]methionine, 5 μCi, 600 Ci/mol and the cells were incubated for 30 min at 41°C. D-[6-^{3}H]Glucosamine, 50 μCi, 20 Ci/mol, was added to dishes without preincubation and the cells were incubated at 41°C for 30 min. Controls are shown by open symbols and cerulenin-treated cultures (20 μg/ml) by closed symbols.

ml cerulenin 2 h after addition. At 5 h, inhibited cells had about the same level of incorporation as at 2 h while the control cells showed a 30% increase in methionine incorporation, presumably resulting from growth (Fig. 3). Similar results were obtained when the incorporation of D-[6-^{3}H]glucosamine into trichloroacetic acid-precipitable material was examined (Fig. 3). Thus, neither protein synthesis nor incorporation of glucosamine into macromolecules was greatly impaired during a 6 h incubation.

Effect of cerulenin on virus production. In order to determine the effect of cerulenin on virus replication, fully infected cultures of chick embryo fibroblasts were prepared by infecting secondary cultures. These were passaged twice more and, usually on the last passage, uninfected secondary cells were added to stabilize the transformed cell monolayers and, at the same time, the chicken and calf serum-containing growth medium was replaced by medium containing 5% delipidated fetal calf serum. The effect of cerulenin, 20 μg/ml, on the production of RSV is shown in Fig. 4. There was a rapid fall in the rate of RSV production and within 5 h there was a 100-fold difference between the control and inhibited cultures, as measured by infectivity. The formation of virus was also measured in a similar experiment by labeling cells with a 15 min pulse of [^{35}S]methionine followed by a 2 h chase in medium with unlabeled methionine. The virus was purified and [^{35}S]methionine labeling determined by counting aliquots of the sucrose gradient fractions in a liquid scintillation spectrometer. Table I presents the time-course of inhibition of virus production. At 1 h after addition of cerulenin there was nearly a 2-fold difference between inhibited and control cultures, and after 4 h there was a 12-fold difference. In the same experiment the effect of cerulenin on the formation of infectious virus was somewhat greater, with a 4-fold difference at 1 h and a 100-fold difference at 4 h. Since it was shown that cerulenin had little effect on incorporation of [^{35}S]methionine into cellular proteins, we next examined its effects on virus protein synthesis.

Effect of cerulenin on intracellular virus proteins

In order to examine intracellular virus protein synthesis, antiserum to disrupted avian myeloblastosis virus was used to precipitate RSV-specific proteins from [^{35}S]methionine-labeled cell extracts. In all experiments it appeared that the amount of antibody-precipitable, labeled protein was appreciably greater in cerulenin-inhibited cells than in control cells (Table I) despite the fact that cerulenin produced lower levels of [^{35}S]methionine incorporation into total trichloroacetic acid-precipitable material (Fig. 3).

The immune-precipitable proteins were examined by SDS-polyacrylamide gel electrophoresis (Fig. 5). The extracts of control cells pulse-labeled from 15 min with [^{35}S]methionine (Fig. 5c and g) produced strongly labeled bands corresponding to the 76 000 dalton precursor (Pr76) previously reported to be present in avian myeloblastosis virus-infected cells [19,20] and RSV-infected cells [13]. In addition to Pr76 other proteins are seen at positions corresponding to 64 000 *, 57 000 *, 19 000 and 15 000 daltons, that are not seen in immune precipitates of uninfected cells (Fig. 5i and j). In extracts of control

* Molecular weights were estimated from a 7% gel.

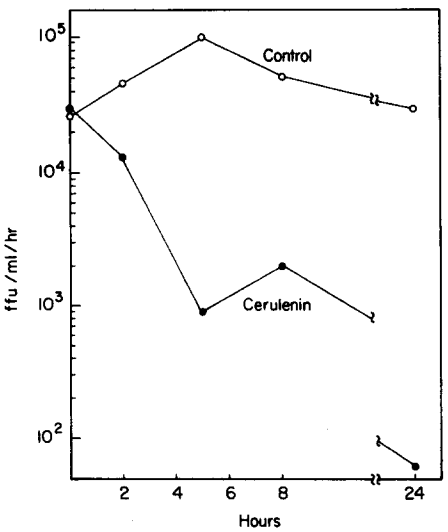

Fig. 4. Effect of cerulenin on replication of Rous sarcoma virus. Infected cells were growing in 3.5-cm dishes in the medium described in the legend to Fig. 3. At time 0 cerulenin, 20 μg/ml, was added to experimental dishes and 40 μl 20% ethanol was added to controls. At the times indicated on the graphs the medium was removed, 1 ml fresh medium was added, and the cells were incubated for 1 h at 41°C. Culture fluids were harvested and assayed for focus forming activity. ffu, focus forming units.

TABLE I

EFFECT OF CERULENIN ON [^{35}S]METHIONINE INCORPORATION INTO VIRUS AND INTO IMMUNE-PRECIPITABLE MATERIAL IN CELL EXTRACTS

Time of pulse (h)	Cerulenin (20 μg/ml)	Radioactive virus released in 2 h chase (cpm) *	Percent of control	Immune-precipitable (cpm) **	Focus forming units per ml	Percent of control
In delipidated serum						
1	+	7 180	56	251 800	$1.4 \cdot 10^5$	25
1	—	12 820	—	222 600	$5.5 \cdot 10^5$	—
2	+	4 240	—	519 800	$3.2 \cdot 10^4$	9.4
2	—	n.d.	n.d.	279 200	$3.3 \cdot 10^5$	—
4	+	3 310	8.5	1 194 600	$8 \cdot 10^3$	1.0
4	—	38 920	—	504 800	$8 \cdot 10^5$	—
In whole serum						
2	+	1 170	8.0	434 200	$8 \cdot 10^4$	5.7
2	—	14 570	—	368 900	$1.4 \cdot 10^6$	—

* Cells were labeled for 15 min with 50 μCi [^{35}S]methionine at 41°C after a 20 min preincubation in Dulbecco's modified Eagle's medium without methionine. The chase was done in 4.0 ml of medium containing 5% delipidated fetal calf serum for 2 h. Cerulenin was present during the chase where indicated. Counts of [^{35}S]methionine represent the sum of the 100-μl samples counted in the peak fraction.

** The washed immune precipitates were solubilized in 200 μl of electrophoresis sample buffer and 20 μl was counted in Aquasol II. The cpm represent the aliquot count multiplied by 10.
n.d., not done.

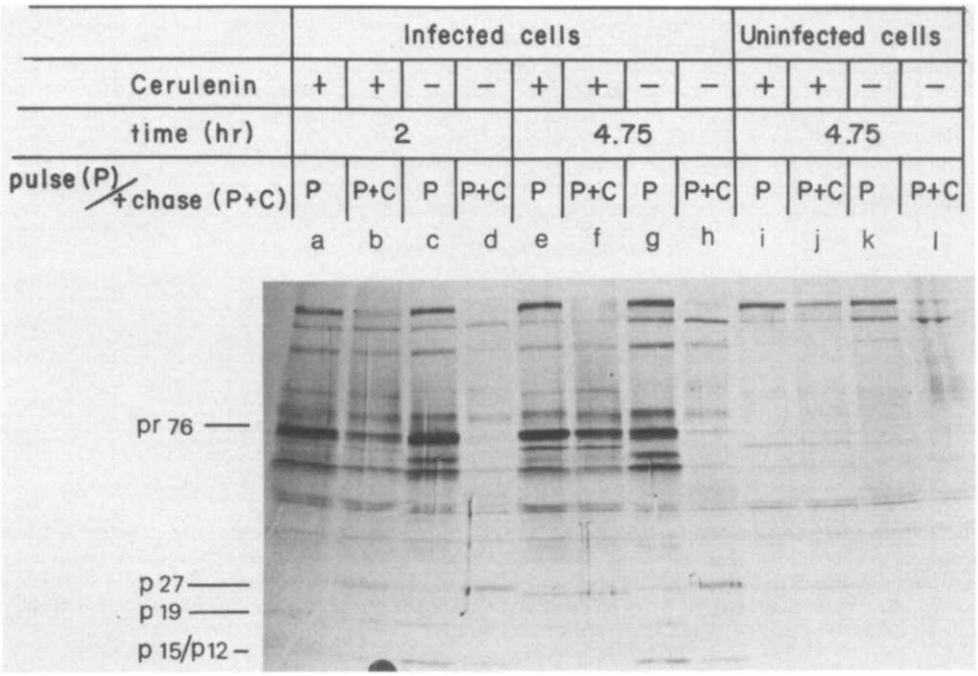

Fig. 5. Effect of cerulenin on the synthesis of intracellular virion proteins. Prague C-infected cells were pulse-labeled with 50 μCi per 5 cm plate of [^{35}S]methionine and either lysed or chased with unlabeled methionine and then lysed. The lysates were precipitated with rabbit anti-avian myeloblastosis virus followed by goat anti-rabbit serum, as described in Experimental section. Lanes a and b were treated with 20 μg/ml cerulenin for 2 h prior to the pulse and lanes c and d were controls. Lanes b and d were chased with unlabeled methionine. Lanes e and f were treated with cerulenin for 4.75 h prior to the pulse and lanes g and h were controls. Lanes f and h were chased with unlabeled methionine. Lanes i, j, k and l were uninfected cells treated the same as the cells in lanes e, f, g and h. Samples were run on a SDS-polyacylamide gradient gel.

cells chased for 2 h with unlabeled methionine (Fig. 5d and h), it can be seen that the bands corresponding to 76 000, 64 000, and 57 000 daltons are much reduced in radioactivity with an increase in the labeling of the major non-glycosylated viral protein p27. In cerulenin-treated cells, on the other hand, the cleavage of Pr76 is much reduced between 2 and 4 h after addition of cerulenin compared to the control (Fig. 5a and b vs. c and d) and appears to be completely inhibited between 4.75 and 6.75 h after addition of cerulenin (Fig. 5e and f vs. g and h). In addition, we observed that the proteins corresponding to molecular weights of 64 000 and 57 000 seen in the control pulse-labeled samples (Fig. 5c and g) were less prominent in the pulse-labeled inhibited cultures (Fig. 5a and e). These may be the intermediates seen by von der Helm [21] in a study of the in vitro cleavage of Pr76 of RSV at 66 000 and 55 000 molecular weight.

Several proteins that accumulate in cells treated with cerulenin are host proteins found in uninfected cells which are either precipitated or coprecipitated during the immune precipitation (Fig. 5i and j). One of these migrates slightly ahead of p27. In SDS-polyacrylamide electrophoresis of the whole cell extracts of infected cells, a very prominent band is seen at this position in

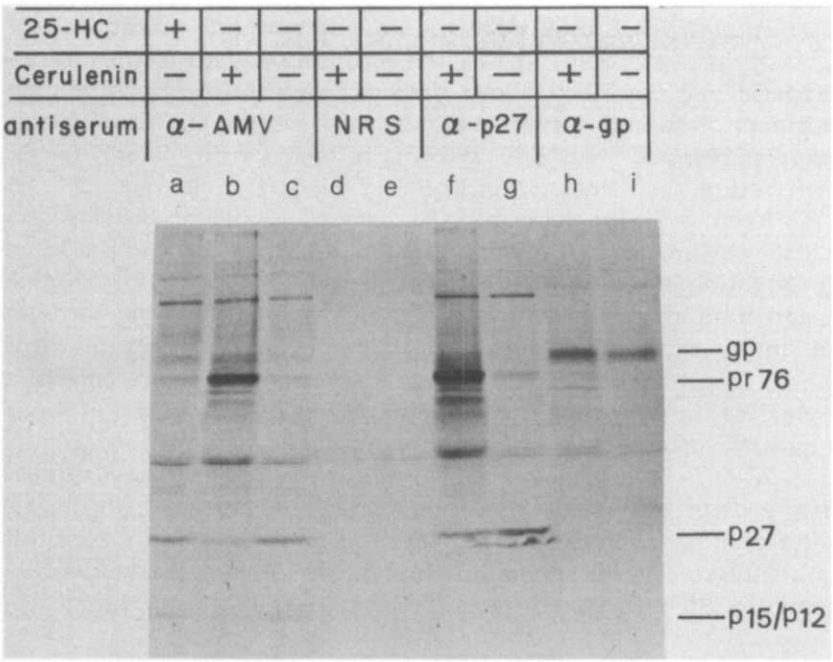

Fig. 6. Precipitation of proteins in inhibited cells with specific antisera. Prague C-infected cells were pulse-labeled with 50 μCi per 5 cm plate of [^{35}S]methionine for 15 min and chased for 2 h. Lane a was treated with 25-hydroxycholesterol, 2.5 μg/ml, for 4 h, prior to the pulse, lanes b, d, f and h with cerulenin, 20 μg/ml, for 4 h and lanes c, e, g and i were control cultures. During the chase inhibitors were also present. Cells were lysed as described in Experimental section and were precipitated with the following antisera: Lanes a, b, and c, rabbit anti-avian myeloblastosis virus (AMV); lanes d and e, normal rabbit serum; lanes f and g, rabbit anti p27; lanes h and i, rabbit anti-RSV glycoproteins. Samples were run on an SDS-polyacrylamide gradient gel.

cerulenin-treated cells but not in control cells (data not shown). Other presumed host proteins are seen migrating between Pr76 and the 64 000 dalton protein, and between the 54 000 protein and p27. The identities of the proteins accumulating in RSV-infected cells inhibited with cerulenin were further studied by treating cell extracts from pulse plus chase-labeled cells similar to those described above. The cells were treated with cerulenin, 20 μg/ml for 4 h, pulsed with [^{35}S]methionine and chased with unlabeled methionine for 2 h. Precipitation of the extracts with antiserum directed against p27 [22] resulted in precipitation of the bands identified as Pr76, the two above-mentioned proteins migrating slightly ahead of Pr76 and p27 (Fig. 6f). Much less of Pr76 and the two faster migrating proteins detected in cerulenin-treated cells are seen in extracts of control cells, as expected (Fig. 6g). (The p27 bands appear split owing to a crack in the gel extending to lanes h and j.) When these extracts were treated with antiserum directed against the major viral glycoproteins [22], it can be seen that the major labeled proteins migrate identically in extracts from cerulenin-treated and control cells (Fig. 6h and i). The amount of radioactivity is somewhat greater in the glycoprotein from cerulenin-treated cells, suggesting that its synthesis is not dependent upon the correct processing of Pr76. Fig. 6 (d and e) also locates proteins which are coprecipitated with

normal rabbit serum on formation of a precipitate with goat anti-rabbit serum.

Are the effects of cerulenin prevented by the addition of lipids to the culture medium? In experiments described above the cells were in media containing delipidated serum prior to and during treatment with cerulenin. When similar experiments were performed with cells growing in media with 5% calf serum plus 1% chicken serum the same results were obtained. The cleavage of Pr76 was strongly inhibited and very little p27 formation was detectable during a 2—4 h period after treatment with cerulenin (Fig. 7a and b). During the same period virus production was inhibited 92% as measured by [^{35}S]methionine incorporation and 94% as measured by the formation of infectious particles (Table I). In a similar experiment the effect of cerulenin on infectious virus production and on the formation of p27 were not reversed by the presence of a lipid mixture yielding the following final concentrations of cholesterol (7.5 μg/ml); oleate (5 μg/ml); stearate (2.5 μg/ml and palmitate (2.5 μg/ml).

The effect of 25-OH-cholesterol on RSV replication. We have also studied the inhibition of growth and acetate incorporation in chick embryo fibroblasts by 25-OH-cholesterol. A concentration of 2.5 μg/ml was found to inhibit growth of chick embryo fibroblasts completely and to inhibit acetate incorporation into sterols by 85% during the period 3—9 h after addition (Harley J.B.

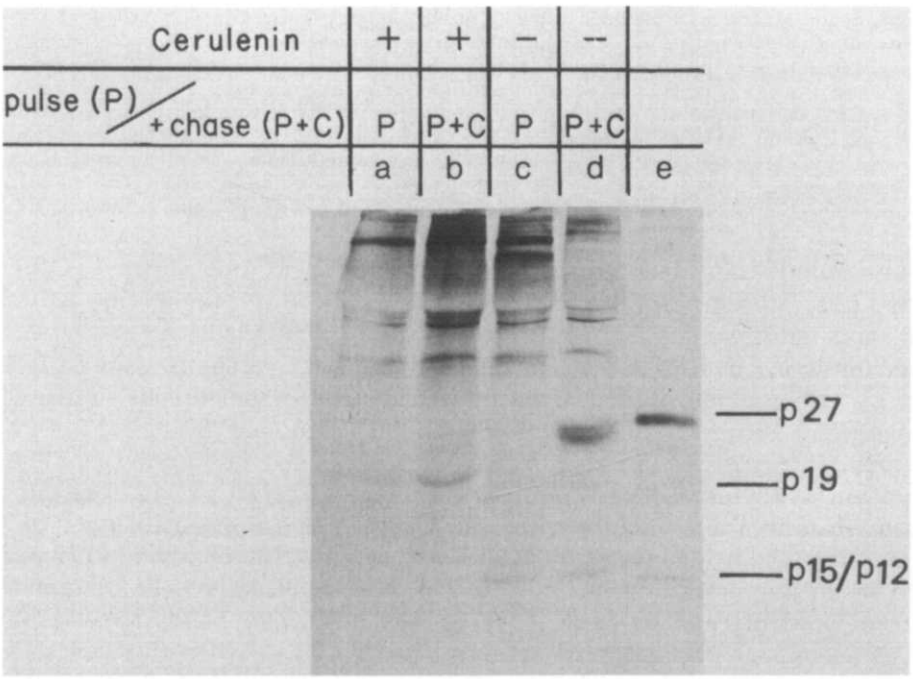

Fig. 7. Effect of cerulenin on Prague C-infected cells grown in complete serum. Cells were grown in Dulbecco's modified Eagle's medium containing 5% calf serum, 1% chick serum and 10% tryptose phosphate broth. Cells were pulse-labeled with 50 μCi of [^{35}S]methionine and either lysed or chased as described in Experimental section. Lanes a (pulse) and b (pulse-chase) were treated with cerulenin, 20 μg/ml, for 2 h prior to the pulse and lanes c (pulse) and d (pulse-chase) were controls. Lysates were immune precipitated as described in the legend for Fig. 5. Samples were run on a 15% SDS-polyacrylamide gel. Lane e, methionine-labeled virus from a control harvest.

and Goldfine, H., unpublished). These results are similar to those reported by Kandutsch and Chen [4] for mouse L cells and for liver cells.

When added to RSV-infected chick embryo fibroblasts growing in delipidated serum, 25-OH-cholesterol had no effect on virus production during short-term incubations. Fig. 6 (lane a) shows the formation of normal amounts of p27 and p15/p12 in cells treated with 25-OH-cholesterol for 6 h. The amount of virus produced during the final 2 h of incubation was 10^6 focus forming units/ml in the treated culture and $7 \cdot 10^5$ focus forming units/ml in the control culture. The cells treated with cerulenin produced $1.4 \cdot 10^4$ focus forming units/ml in this experiment. Similar experiments were done with 25-OH-cholesterol at varying times after addition up to 25 h and no effect was seen on either infections virus production or on the incorporation of [^{3}H]glucosamine into gp85 (data not shown).

Discussion

Our experiments show that the replication of RSV is not affected by 25-OH-cholesterol at concentrations which inhibit acetate incorporation into sterols by 85%. If acetate incorporation into sterol is a measure of de novo synthesis, these results indicate that the virus can replicate normally in cells with strongly reduced sterol synthesis. Whether RSV is formed in regions of the membrane with normal amounts of cholesterol or the new particles produced in the presence of the inhibitor have reduced amounts of sterol in their envelopes, is not known. After growing in the presence of 25-OH-cholesterol, L-cells were found to have plasma membranes with one-half the normal ratio of sterol to phospholipid and to protein [2].

Cerulenin, an inhibitor of fatty acid synthesis and less-effectively an inhibitor of sterol synthesis. profoundly inhibited the replication of RSV. Within 5 h after its addition to RSV-infected chick embryo fibroblasts the production of infectious particles was inhibited on average of 70-fold in five experiments. [^{35}S]Methionine-labeled virion particle production was reduced an average of 9-fold in two experiments at this time. Although the cleavage of Pr76 was not quantitated, the autoradiographs of immune-precipitated cell extracts (Figs. 5 and 6) clearly show that this process was strongly inhibited. Since neither whole serum nor lipids added to the medium were capable of reversing the inhibition of RSV replication by cerulenin, our results cannot be simply interpreted as demonstrating a need for lipids or membrane assembly for RSV replication. Two additional possibilities have to be considered. Either there is a need for de novo lipid synthesis which is not fulfilled by external lipids, or cerulenin has a second effect on the host cell. The inhibition of Pr76 cleavage, the earliest detectable effect, may be the result of a direct effect on a specific protease [21], on an activator of the protease, or on some event needed prior to Pr76 formation which is inhibited in cerulenin-treated cells.

One possibility can be eliminated, however. Virus production continues unabated in the presence of sterol inhibition by 25-OH-cholesterol; therefore, the cleavage block in Pr76 is not a consequence of the inhibition of de novo sterol synthesis by cerulenin.

Glucosamine has also been reported to effect the cleavage of Pr76 in RSV-

infected chick embryo cells [13]. Unlike cerulenin, it also prevents the formation of gp85 and gp37. A carbohydrate-deficient form of gp85 has been found in glucosamine-treated cells by Lewandowski et al. [23]. The connection between the failure to cleave Pr76 and to glucosylate the viral glycoproteins fully is not understood.

The effects of cerulenin and glucosamine on Pr76 cleavage can be studied in the in vitro cleavage system described by von der Helm [21]. Such in vitro experiments should determine whether cerulenin or glucosamine has a second specific action. Meanwhile, the results of studies using cerulenin to investigate membrane biosynthesis and assembly must be interpreted with considerable caution.

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