A toxin from mistletoe, viscumin, inhibited the incorporation of leucine in cells more rapidly than the incorporation of uridine and thymidine, indicating that the toxins act by inhibiting cellular protein synthesis. The presence of galactose, lactose, and melibiose in the medium protected cells against viscumin. The sensitivity to viscumin of different cell lines differed considerably. The cytotoxic properties of viscumin were compared to those of the related toxins abrin, ricin, and modeccin. The profile of the sensitivity of a panel of cell lines to viscumin was dissimilar from those obtained with abrin and modeccin. Cell lines selected for resistance to modeccin and ricin were fully sensitive to viscumin. Ca^{2+} was required for viscumin to express its toxic effect. In contrast, the Ca^{2+} ionophore A23187 protected the cells against viscumin. Whereas the sensitivity of cells to viscumin did not vary much between pH 1 and 9, the cells were much less sensitive at pH 6. Also, cells treated with the two metabolic inhibitors, 2-deoxyglucose and NaF, were insensitive to the toxins. Viscumin taken up by cells in the absence of Ca^{2+} or at pH 6 was able to intoxicate the cells when Ca^{2+} was added or when the pH was adjusted to neutrality. In contrast, cells exposed to viscumin in the presence of 2-deoxyglucose and NaF, which inhibit endocytosis, were not intoxicated when the cells were treated with antiviscumin and then transferred to normal medium. The results indicate that endocytosis is involved in the entry of viscumin.

In the preceding paper (1) we described the isolation and characterization of a toxin from mistletoe, denoted viscumin, and showed that it probably is identical with lectin I, previously isolated from mistletoe by Luther et al. (2) and Franz et al. (3). It was demonstrated that viscumin is remarkably similar in structure and mechanism of action to the plant toxins abrin, ricin, and modeccin and that one of the constituent peptide chains of viscumin, the A chain, inhibits cell-free protein synthesis by inactivating catalytically the large ribosomal subunit. In the present paper we have studied the cytotoxic effect of viscumin on various cell lines, and compared its properties with those of the related toxins abrin, ricin, and modeccin. In particular, we have studied the factors influencing the cellular uptake of the toxic moiety of viscumin.

EXPERIMENTAL PROCEDURES

Toxins—Viscumin was extracted from the green parts of _Viscum album_ L. (mistletoe) and purified as described in the preceding paper (1). For comparison some experiments were also carried out with a sample of viscumin (lectin I) obtained from Dr. P. Ziska, East Berlin. It had been isolated by affinity chromatography on acid-treated Sepharose 4B as described by Ziska et al. (4). Abrin, ricin, and modeccin were isolated as previously described (5-7).

Antitoxins—Antisera to abrin, ricin, and modeccin were raised in rabbits by injection of formaldehyde toxoids (5-7). Antiviscumin was obtained in a similar way in rabbits previously injected with formaldehyde toxoid of viscumin (1). Cell Culture—HeLa S cells, FM cells (human melanoma), Vero cells (from African green monkey kidney), mouse 3T3 cells, 501.1 cells (derived from mouse A9 cells, see Ref. 8), and NRK cells (rat kidney) were propagated in monolayer cultures in Dulbecco-modified Eagle's minimum essential medium with 10% fetal calf serum. Mouse peritoneal macrophages were collected after intraperitoneal injection of 2 ml of 1% hydrolyzed starch solution as described (9).

Toxin-resistant Cells—Toxins were added to cells and surviving clones were selected. The cells here used have been described previously (10, 11).

RESULTS

Inhibition of Macromolecular Synthesis in Cells—In the preceding paper viscumin was shown to inhibit protein synthesis by inactivating the 60 S ribosomal subunits (1). If inhibition of protein synthesis is the reason for the cytotoxic effect of viscumin, it should inhibit protein synthesis before the synthesis of nucleic acids is affected. To study this, viscumin was added to HeLa cells and then, after different periods of time, the ability of the cells to incorporate [^{3}H]leucine, [^{3}H]thymidine, and [^{3}H]uridine into acid-precipitable material was measured. It was found (Fig. 1A) that the incorporation of leucine decreased more rapidly than that of the nucleotides. The inhibition of protein synthesis occurred after a lag time which decreased with increasing toxin concentrations (Fig. 1B). The data are similar to those earlier obtained with other toxins inhibiting protein synthesis in cells (12).

Effect of Sugars on the Sensitivity of Cells to Viscumin—Cell agglutination by the viscumin album lectin is reported to be inhibited by lactose and galactose (2, 13) which protect cells against the related toxins abrin, ricin, and modeccin (5-7). The data in Fig. 2A show that lactose, galactose, and melibiose afforded some protection of cells against viscumin. Thus in the presence of 100 mM of each of these sugars approximately 10 times more toxin must be added to obtain the same toxic effect as in the control. D-Fucose had a slight protective effect, whereas glucose, mannose, and L-fucose (data not shown) did not protect against viscumin. The results indicate that viscumin binds to cell surface receptors containing terminal galactose residues. Differential Sensitivity of Cell Lines to Viscumin and Related Toxins—Cell lines may differ strongly in their sensitivity to toxins, as previously shown with diphtheria toxin (for review, see Ref. 18) and with _Shigella_ toxin (19). The results

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Fig. 1. Rate of viscumin inhibition of macromolecular synthesis in HeLa cells. To HeLa S3 cells growing in 24-well disposable trays, 1.7 x 10^{-4} M of viscumin (A) or the indicated concentrations of viscumin (B) were added, and the cells were incubated at 37°C for the indicated period of time. The medium was then removed, 0.5 ml of leucine-free medium containing the indicated radioactive compounds was added, and the incubation was continued for 10 min more. After incubation with the labeled compound the medium was removed, and the cells were extracted twice with trichloroacetic acid.

The extraction was carried out at 4°C in the case of uridine incorporation and at room temperature in the other cases. Finally, the cells were dissolved in 0.1 M KOH and the radioactivity was measured. The results are expressed as percentage of the control values (no toxin added) which were: 1500 cpm of [14C]leucine; 5000 cpm of [3H]thymidine and 1000 cpm of [3H]uridine. A, additions: • 50 nCi of [14C]leucine; ▲, 5 μCi of methyl-[3H]thymidine; ■, 2.5 μCi of [5-3H]uridine. B, the concentration of viscumin was: □, 10^{-10} M; △, 10^{-9} M; ×, 10^{-8} M; ○, 10^{-7} M.

Fig. 2. Effect of different sugars on the sensitivity of cells to viscumin. To HeLa S3 cells growing in 24-well disposable trays, leucine-free medium without serum and containing 100 mM of the sugars given below was added. Then increasing amounts of viscumin were added and the cells were incubated at 37°C for 2 h and then their ability to incorporate [14C]leucine during 30 min was measured. The results are expressed as percentage of the control values (no toxin added) which were ~30,000 cpm. The sugars added were: □, D-fucose (D-Fuc); ■, galactose (Gal); ▲, lactose (Lac); △, melibiose (Mel); ○, no addition.

in Fig. 3A show that a panel of cell lines derived from different human and animal tissues differ widely in their sensitivity to viscumin. Thus the most sensitive cell lines tested, mouse macrophages and the mouse cell line 501.1, were intoxicated by concentrations that were only 1/100 of those required for baby hamster kidney and HeLa cells.

The results in Fig. 3, B and C, show that the panel of cells tested in Fig. 3A did not show the same relative sensitivities to modeccin and abrin which also bind to galactose-containing sites at the cell surface. Thus, mouse macrophages which were the most sensitive cells to viscumin, were among the least sensitive to modeccin, and mouse 3T3 cells, which were the most sensitive to modeccin and abrin, exhibited intermediate sensitivity to viscumin. The data are consistent with the possibility that different galactose-containing receptors are involved in the uptake of the three toxins here studied.

We also attempted to measure the ability of different cell lines to bind [125I]-viscumin. Such measurements are complicated by the fact that saturation of the binding was not achieved even at viscumin concentrations of 100 μg/ml. The total number of binding sites could therefore not be assessed but it appears to be >10^9/cell. Possibly, viscumin binds to a wide variety of glycoproteins and glycolipids with terminal galactose residues. The insensitive HeLa S3 cells appeared to bind approximately as much viscumin as the sensitive mouse 3T3 cells (data not shown). This indicates that the difference in sensitivity of the different cells is not due to differences in the total binding capacity for viscumin.

Sensitivity to Viscumin of Cells Resistant to Abrin, Ricin, and Modeccin—We have previously shown that cells selected for resistance to ricin are also resistant to abrin and vice versa, whereas there is no cross-resistance between abrin and ricin on the one hand and modeccin on the other (11). In attempts to elucidate the relationship between the receptors for the different toxins, we tested the sensitivity to viscumin of a modeccin-resistant HeLa cell line (Mod^II) which has a normal number of binding sites for modeccin (11), and of a ricin-resistant HeLa line (R^III), which has a reduced number of ricin-binding sites (17). Although the concentrations of modeccin required to inhibit protein synthesis in the resistant variant Mod^II was 10^4 times higher than in the parent HeLa S3 cells (Fig. 4A), Mod^II was as sensitive to viscumin as the parent HeLa S3 cells (Fig. 4B).

The ricin-resistant R^III cell line was highly resistant to ricin (Fig. 4C). When tested with viscumin (Fig. 4D) it was found to be as sensitive as the parent HeLa cells. This indicates that the viscumin receptors are different from those for ricin. Since the two toxin-resistant lines are deficient also in some step in the entry mechanism of modeccin and ricin (10, 11), the results indicate that viscumin does not enter the cells by exactly the same route as these toxins.
Action of Viscumin on Cells

Toxin concentration (M)

Fig. 3. Effect of viscin (A), modeccin (B) and abrin (C) on protein synthesis by various cells. To different cell lines growing in 24-well disposable trays, increasing amounts of toxins were added, and the cells were incubated at 37 °C overnight. Then the medium was removed, and 0.5 ml of leucine-free medium and 25 nCi of [3H]leucine were added to each well and the incubation was continued for 1 h more. Finally, the trichloroacetic acid-precipitable radioactivity associated with the cells was measured. The results are expressed as percentage of the control values (no toxin added) which varied between 2,000 and 10,000 for the different cell lines. The cell lines were: O, mouse macrophages (Macroph.); □, mouse 501.1 cells; O, mouse 3T3 cells; ▲, rat NRK cells; △, Vero cells (monkey kidney); △, FM cells (human melanoma); ▲, HeLa cells; □, BHK cells (fetal hamster kidney).

Requirement of Ca2+ Ions for Sensitivity of Cells to Viscumin—Recent experiments in this laboratory have shown that the entry of abrin and modeccin into cells requires the presence of Ca2+ ions, whereas ricin uptake is less Ca2+-dependent (20). To test if Ca2+ ions are required for sensitivity of cells to viscin, we incubated cells with increasing concentrations of viscin in the absence and presence of Ca2+ and measured the inhibition of protein synthesis. In the experiment without Ca2+, Co2+ was added to prevent the cells from being detached from the plastic surface during the incubation in Ca2+-free buffer. As will be discussed below (Fig. 8), such treatment did not impair the binding of viscin to cells. The results in Fig. 5A show that in the absence of Ca2+, the cells tolerated approximately 100 times more viscin, as measured by inhibition of protein synthesis, than when calcium ions were present, showing that the uptake of viscin, like that of abrin and modeccin, is facilitated by the presence of Ca2+ ions. Calmodulin does not seem to be involved in the uptake of viscin. Thus, cells were equally sensitive in the absence and presence of trifluoperazine, a potent inhibitor of calmodulin (data not shown).

The results in Fig. 5A also show that the trivalent cations La3+ and Fe3+ protected strongly against viscin, as has previously been found for abrin, modeccin, ricin, and diphtheria toxin (20). The reason for this inhibition is not clear since only La3+ and not Fe3+ inhibited influx of Ca2+. The data in Fig. 5B show that the Ca2+ ionophore A23187 afforded some protection against viscin. Clearly, influx of Ca2+ by this artificial method does not facilitate viscin uptake (see “Discussion”). Previously we have made similar findings with abrin and modeccin, but not with ricin (20).

Effect of pH on the Sensitivity of Cells to Viscumin—The sensitivity of cells to abrin, modeccin, ricin, and diphtheria toxin is strongly dependent on the pH of the incubation medium (21). To study how pH affects the sensitivity of cells to viscin, cells were incubated with increasing concentrations of viscin for 2 h at different pH values, then transferred to medium with pH 7.5 and their ability to incorporate...
Toxin concentration (M)

**Fig. 5.** Effect of cations (A) and the calcium ionophore A23187 (B) on the sensitivity of Vero cells to viscumin. To Vero cells growing in disposable trays were added 1 ml of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2, 0.14 M NaCl and, when not otherwise indicated, 2 mM CaCl₂. The cations or the ionophore indicated below were added and the cells were incubated at 37 °C for 15 min. Then increasing amounts of viscumin were added and the incubation was continued for 90 min more. Finally, leucine-free medium and [¹⁴C]leucine were added, and the ability of the cells to incorporate radioactivity during 15 min was measured. The results are expressed as percentage of the control values which were ~2000 cpm. The additions were: ○, 0.5 mM FeCl₃; △, 1 mM LaCl₃; ■, 2 mM CoCl₂, in this case the medium did not contain CaCl₂; □, 2 μM A23187; X, control, no additions.

**Fig. 6.** Effect of pH on the sensitivity of cells to viscumin. To Vero cells in 24-well disposable trays were added 0.5 ml of 0.14 M NaCl, 2 mM CaCl₂, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, adjusted with NaOH to the indicated pH, and then increasing amounts of viscumin. The cells were incubated for 2 h at 37 °C. Then the medium was removed, leucine-free medium (pH 7.5) and [¹⁴C]leucine were added and the ability of the cells to incorporate radioactivity during 15 min was measured. The results are expressed as percentage of the control values which were ~2000 cpm.

[¹⁴C]leucine was measured. It was found (Fig. 6) that with increasing pH values the protein synthesis was increasingly inhibited up to pH 8 where the concentration of viscumin required to inhibit protein synthesis was about 100-fold less than at pH 6. At pH 9, the cells appeared to be less sensitive than at pH 8. Preincubation of control cells for 2 h at the pH values here used did not as such substantially alter their ability to incorporate [¹⁴C]leucine under standardized conditions at pH 7.5.

The presence of NH₄Cl in the medium strongly protects cells against diphtheria toxin due to its ability to increase the pH in intracellular vesicles (22-24). It also protects against modeccin, but rather sensitizes the cells somewhat to abrin and ricin (25). The presence of 10 mM NH₄Cl in the medium also sensitized the cells approximately 10 times to viscumin (data not shown). Clearly, the internalization mechanism for viscumin is more related to that for abrin and ricin than to that for diphtheria toxin.

**Ability of Metabolic Inhibitors to Protect Cells against Viscumin**—The combined treatment of cells with inhibitors...
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of glycolysis and of oxidative phosphorylation was recently found to protect cells against abrin, modecin, ricin, diphtheria toxin, and Shigella toxin (21). The data in Fig. 7 show that such treatment also protected strongly against viscumin. The inhibitor of oxidative phosphorylation, NaN₃, did not protect when given alone, whereas 2-deoxyglucose, an inhibitor of glycolysis, offered partial protection alone. Apparently, the uptake of viscumin is dependent on metabolic energy.

**Effect of Ca²⁺ Deprivation, pH, and Metabolic Inhibitors on Binding and Uptake of Viscumin by Vero Cells—**To test the possibility that the protective effect of Ca²⁺ deprivation, pH 6.0, and metabolic inhibitors could be due to reduced binding of viscumin to cells, we measured the binding of 125I-labeled viscumin to Vero cells under different experimental conditions. As shown in Fig. 8, the binding reached a maxi-

![Graph](image)

**Fig. 8.** Effect of Ca²⁺, Co²⁺, pH 6.0, and metabolic inhibitors on total and lactose-resistant binding of viscumin to cells. From Vero cells growing in 24-well disposable trays (10⁵ cells/well), the medium was removed and 0.3 ml of buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2, except when otherwise indicated), 0.14 M NaCl, and 2 mM CaCl₂ were added. When indicated, the buffer was adjusted to contain 10 mM NaNa and 50 mM 2-deoxyglucose or to contain 2 mM CoSO₄ and no CaCl₂. The cells were incubated at 37 °C for 15 min, then 125I-labeled viscumin (73,000 cpm, 4.6 ng) was added to each well and the cells were incubated at 37 °C for the indicated periods of time. Then the buffer was removed, one well was rinsed three times with the same buffer, whereas a parallel well 1 ml of the same buffer containing 0.1 M lactose was added and the incubation was continued for 15 min at 37 °C. The buffer with lactose was removed and the well was rinsed twice with buffer containing lactose. At the end of the experiment, 0.2 ml of 0.1 M KOH was added to each well to dissolve the cells. The dissolved cells were transferred to plastic tubes and the radioactivity was measured in an LKB autogamma counter. The buffer contained: O and ●, 2 mM CaCl₂; ▼ and ▲, 2 mM CoSO₄; □ and ■, 10 mM NaN₃, and 50 mM 2-deoxyglucose. In one case (△, A) the pH was adjusted to 6.0. Open symbols: cells washed with lactose; closed symbols: cells washed with buffer alone.

![Graph](image)

**Fig. 9.** Ability of viscumin, endocytosed under protective conditions, to intoxicate cells when the protection is released. From Vero cells growing in 24-well disposable trays (5 × 10⁴ cells/well) the medium was removed and 0.5 ml of a buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2, unless otherwise indicated), 0.14 M NaCl), adjusted as detailed below, was added. After 15 min at 37 °C, increasing amounts of viscumin were added and the incubation was continued. A, after 90 min the buffer was removed and 0.5 ml of leucine-free medium containing 500 nCi of [3H]leucine was added. The incubation was continued for 15 min more and the incorporation was measured as in Fig. 3. B, after 60 min the

buffer was removed and 1 ml of medium containing 10% fetal calf serum and 10 µl/ml of rabbit antiviscumin serum (both preincubated at 56 °C for 30 min to inactivate complement) was added and the incubation was continued overnight. The next day the medium was removed, 0.5 ml of leucine-free medium containing 1 µCi of [3H]leucine were added, and the incorporation during 1 h was measured as in Fig. 3. The results are expressed as percentage of the control values which were ~10,000 cpm in A and ~50,000 cpm in B. The buffer was adjusted to contain: ●, 2 mM CaCl₂; ×, 2 mM CoSO₄; ○, pH 6.0; △, 10 mM NaN₃, and 50 mM 2-deoxyglucose.
The results in Fig. 9A show that Co²⁺ and pH 6.0 strongly inhibited before the synthesis of DNA and RNA provides strong evidence that the effect of viscumin on protein synthesis can account for its toxicity to cells. The data here presented show that viscumin, like abrin, ricin, and modeccin appears to bind to galactose-containing cell surface receptors. In spite of this, a panel of cells showed a quite different pattern of sensitivity to the three toxins. Furthermore, variants of HeLa cells that were highly resistant to modeccin and ricin were fully sensitive to viscumin. The most likely interpretation of these results is that the three toxins are bound to different receptors, even though the receptors for all these toxins appear to possess terminal galactose residues.

The uptake mechanism of the toxic moiety of protein toxins is not understood. In attempts to elucidate the mechanisms involved, we have studied the effects of different manipulations of the medium and of the metabolic state of the cells on their sensitivity to the different toxins. These experiments have revealed interesting differences in the uptake mechanism of the toxins (21). Here we have found that when Ca²⁺ was omitted from the medium and Co²⁺ was added to prevent cell detachment from the plastic surface, the cells were much less sensitive to viscumin than when Ca²⁺ was present. In this respect viscumin resembles abrin and modeccin, whereas ricin is much less dependent upon Ca²⁺ in the medium. Also we found that a calcium ionophore A23187 which strongly increases the uptake of Ca²⁺ in cells protected against viscumin. This ionophore, which protects against abrin and modeccin (20), may have an indirect effect on the natural Ca²⁺ gates which may become closed when the cytoplasmic concentration of Ca²⁺ is increased. The results suggest that Ca²⁺ flux into the cells through normal Ca²⁺ gates may be a requirement for viscumin entry.

The toxicity of viscumin was influenced by pH in a similar way as abrin, modeccin, and ricin (21). In striking contrast to these plant toxins, diphtheria toxin is most active at low pH and cells are almost resistant at pH 9 (22–24). Furthermore, similarly to abrin and ricin, cells were not protected against viscumin by the presence of trifluoperazine and NH₄Cl, whereas both compounds strongly protected against modeccin and diphtheria toxin (20, 25).

Toxin accumulated in cells under protective conditions (Co²⁺, pH 6.0) was able to intoxicate cells when antiviscumin was added and the cells were transferred to normal medium. This indicates that endocytosed toxin is able to enter the cytosol when the protection is released. Similar findings were previously made with abrin, ricin, and modeccin (26). Like these toxins, as well as diphtheria toxin, viscumin did not intoxicate cells if both NaN₃, a potent inhibitor of oxidative phosphorylation, and 2-deoxyglucose, an inhibitor of glycolysis, were present. 2-Deoxyglucose alone afforded some protection, whereas NaN₃ alone failed to do so. This may be due to the fact that in malignant cells a large fraction of the ATP made is generated by glycolysis.

When cells were exposed to toxin for 1 h and then incubated overnight, approximately 10-fold lower toxin concentrations were required to inhibit protein synthesis than when the cells were exposed to toxin for 90 min and the rate of protein synthesis was measured during the following 15 min. The reason for this difference may partly be that endocytosed toxin is slowly transferred to the cytosol and partly that viscumin A chain acts catalytically. Similar findings were previously made with the related plant toxins (20, 21).

After the submission of this manuscript, Sargiacomo and Hughes (27) reported that a ricin-resistant mutant of baby hamster kidney cells, RicO¹⁴, is also resistant to a toxic lectin from mistletoe which is probably identical with viscumin. The sensitivity of this cell line increases strongly after neuraminidase treatment, supporting our conclusion that viscumin binding occurs to carbohydrates containing terminal galactose residues.

Altogether, viscumin strongly resembles the toxic lectins abrin, modeccin, and ricin in its structure and mode of action. As judged from the effects of inhibitors, the entry of viscumin into the cytosol is more similar to abrin and ricin that to...
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