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Cytotoxicity of Gelonin Conjugated to Targeting Molecules: Effects of Weak Amines, Monensin, Adenovirus, and Adenoviral Capsid Proteins Penton, Hexon, and Fiber

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SUMMARY

It has been reported previously that ammonium chloride, chloroquine, monensin, and adenovirus-2 potentiate the cytotoxicity of several protein toxins conjugated with various targeting molecules. We have tested whether these agents, and protein components of adenovirus-2, would enhance the cytotoxicity of conjugates of gelonin with J5, an antibody directed against common acute lymphoblastic leukemia-associated antigen, with 5E9, an antibody directed against human transferrin receptor, or with ricin B-chain. We found that none of these agents affected the cytotoxicity of gelonin conjugates to any significant extent. For example, monensin moderately (3-fold) enhanced the cytotoxicity of 5E9-gelonin for Namalwa cells but showed no effect when 5E9-gelonin was tested on HeLa cells. The potentiating effects of these agents for the cytotoxicity of free gelonin varied from marked to nonexistent, depending on the type of cells. In particular, adenovirus-2 potentiated the cytotoxicity of gelonin for HeLa cells but not for Namalwa cells. The three major adenoviral capsid proteins, penton, hexon, and fiber, were isolated. It was shown that penton potentiated the cytotoxicity of gelonin for HeLa cells and that hexon and fiber had no measurable effect on the cytotoxicity of gelonin. However, like the whole virus, penton was not able to affect the cytotoxicity of gelonin conjugates.

An important advantage of immunotoxins made with single-chain RIP or isolated A-chains of double-chain RIP over those made with whole double-chain toxins is that their binding to cells is directed only by their antibody component, making these immunotoxins selective in killing antigen-positive cells. Unfortunately, these conjugates are generally less efficient at killing target cells than are immunotoxins containing double-chain toxins (1, 2).

Numerous efforts have been undertaken to improve the efficacy of toxin conjugates. Ammonium chloride, chloroquine and other weak bases (3–17), monovalent ionophores monensin and nigericin (5, 17–21), and adenovirus-2 (20, 22, 23) were found to potentiate the specific cytotoxicity of some conjugates for cultured cells.

These findings are potentially important because they might lead to the development of efficient anticancer therapies based on such immunotoxins. Clinical trials using potentiators together with ricin A-chain conjugates for *ex vivo* treatment of bone marrow are planned or are in progress (24, 25).

The main reason for the low cytotoxicity of single-chain RIP conjugates for target cells appears to be that these conjugates have a poor capacity to enter the cell cytoplasm (1). We hoped that agents such as ammonium chloride, chloroquine, monensin, and adenovirus-2, which had been reported by others to increase the cytotoxicity of various toxin conjugates, would improve the delivery of conjugates of the single-chain RIP gelonin into the cytoplasm and, thereby, increase the cytotoxicity of such conjugates. We tested the capacity of these "potentiators" to enhance the cytotoxicity of conjugates of gelonin with the monoclonal antibody J5, which is directed against CALLA, and with 5E9, which is directed against the human transferrin receptor. The effect of the potentiators on the cytotoxicity of a ricin B-chain-gelonin conjugate was also tested. It appears from our experiments that these agents are not capable of potentiating the cytotoxicity of gelonin conjugates for cultured cells.

In the course of this study, we tested major capsid proteins of adenovirus-2 for their ability to enhance the cytotoxic action of RIP, and we found that one of these proteins, penton, can enhance killing of cells by RIP.

Materials and Methods

Chemicals and biochemicals. Monensin and chloroquine were purchased from Sigma Chemical Co. (St. Louis, MO). Ammonium chloride (certified ACS) was purchased from Fisher Scientific (Pittsburgh, PA). Ricin B-chain (Worthington Diagnostics, Freehold, NJ)

ABBREVIATIONS: RIP, ribosome-inactivating protein(s); CALLA, common acute lymphoblastic leukemia antigen.

showed a single band of protein (M_r ~ 30,000) when analyzed by polyacrylamide/sodium dodecyl sulfate gel electrophoresis under non-reducing conditions. The sources and purification of gelonin and the murine monoclonal antibodies 5E9 (anti-human transferrin receptor) and J5 (anti-CALLA) have been described elsewhere (26, 27).

Preparation and purification of protein conjugates. Disulfide-linked conjugates between the antibody J5 and gelonin, between the antibody 5E9 and gelonin, and between purified ricin B-chain and gelonin were made as described previously (26–28). These conjugates were free of nonconjugated antibody or ricin B-chain, nonconjugated gelonin, and aggregates of high molecular weight (26, 27).

Adenovirus-2 and its major capsid proteins. Adenovirus type 2 was grown in the laboratory of Dr. Phillip A. Sharp (Massachusetts Institute of Technology). It was propagated in a suspension culture of HeLa cells and was then isolated and purified by the procedure of Pettersson and Sambrook (29). The final virus suspension in Tris-buffered saline (containing 10 mM Tris·HCl, pH 7.2, and 145 mM NaCl), containing 0.05% (w/v) sucrose adjusted to pH 7.9, was kept at −70°. The concentration of virions was estimated by measuring the absorbance at 260 nm in 0.5% (w/v) sodium dodecyl sulfate, using an A_{260} value of 1.0 for 1.1×10^{12} virions/ml. Stock solutions contained 3.5×10^{11} virions/ml. The solutions of adenovirus-2 were γ-irradiated (^{137}Cs source, 26 kRad) before cytotoxicity experiments in order to abolish the infectivity of the virus. Adenoviral capsid proteins hexon, penton, and fiber were purified from the virus by the method of Boulanger and Puvion (30) and were kept in phosphate-buffered saline (containing 10 mM potassium phosphate buffer, pH 7.2, and 145 mM NaCl) at 4° under sterile conditions (filtered through 0.22-μm filters; Schleicher and Schuell, Keene, NH).

Cells and cell culture maintenance. The human B cell line Namalwa and the human epitheloid cell line HeLa were purchased from Flow Laboratories (McLean, VA). Namalwa cells were maintained in exponential growth phase in growth medium composed of RPMI 1640 medium (GIBC0), 10% heat-treated (30 min at 56°) fetal calf serum (GIBCO), and 2 mM glutamine.

Cytotoxicity assays. Cytotoxicity was determined by measuring inhibition of colony formation and by back-extrapolation of cell proliferation curves, as described elsewhere (31, 32). Cells were incubated at 37° for 24 hr in growth medium containing a toxin, a potentiating agent, or both. Then the cells were washed and cultured in fresh growth medium. The surviving fractions established by these two methods were in good agreement and were used interchangeably.

Results

Cytotoxicities of gelonin and its conjugates with the antibodies J5 (anti-CALLA) or 5E9 (anti-human transferrin receptor) or with ricin B-chain have been measured in the presence or absence of ammonium chloride, chloroquine, monensin, or adenovirus-2. The results of these experiments are shown in Fig. 1.

Ammonium chloride was not toxic for Namalwa or HeLa cells at concentrations up to 20 mM. Even at these high concentrations, it had no effect on the cytotoxicity of gelonin conjugates for Namalwa or HeLa cell lines. Also, ammonium chloride had no effect on the cytotoxicity of free gelonin for HeLa cells and only marginally (2-fold) reduced the IC_{50} of free gelonin for Namalwa cells.

Ramakrishnan and Houston (11) reported that chloroquine potentiated the cytotoxicity of immunotoxins at relatively high concentrations of the agent (40 to 100 μM). We found chloroquine to be toxic for Namalwa cells at concentrations above 1 μM and for HeLa cells above 10 μM (24-hr exposure). For this reason, the potentiating effects of chloroquine could not be tested on these cells at the concentrations used by these researchers. At 10 μM, chloroquine did not affect the cytotoxicity of free or conjugated gelonin for HeLa cells (Fig. 1, a to c).

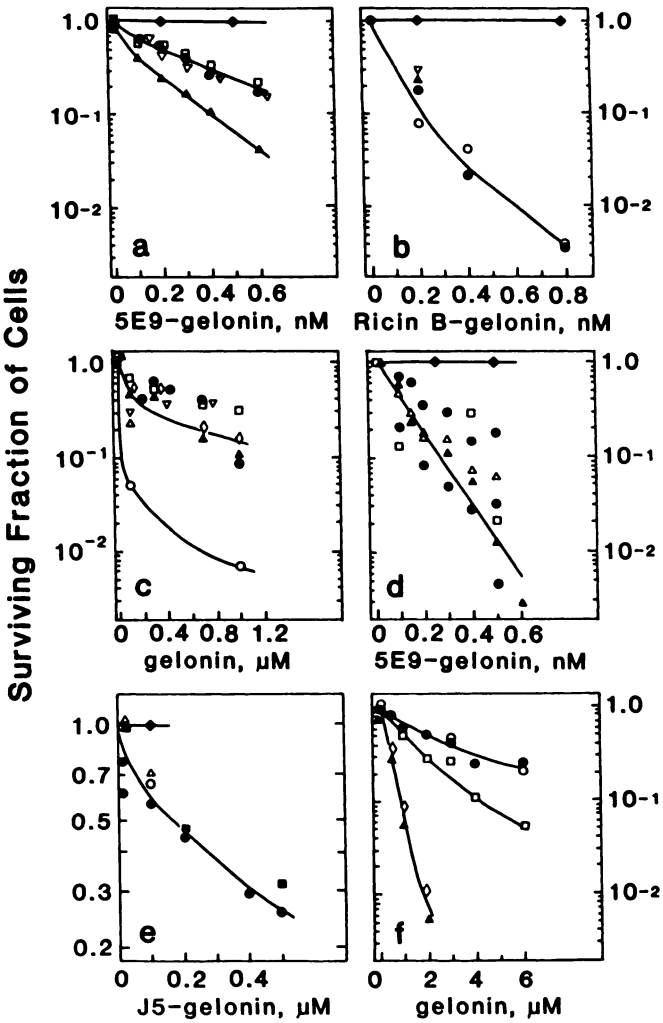

Fig. 1. Effects of potentiators on the cytotoxicity of gelonin and its conjugates. HeLa cells (a to c) or Namalwa cells (d to f) were incubated with gelonin or with a gelonin conjugate, either with or without one of the potentiators, at 37° for 24 hr, and then the surviving fraction of cells was determined by methods described in Materials and Methods. ●, No potentiators; ○, adenovirus (10^{11} virions/ml); □ and ■, ammonium chloride, 10 and 20 mM, respectively; △, ◇, and ▲, monensin, 10, 30, and 100 nM, respectively; ▽, chloroquine, 10 μM; ◆, conjugates of gelonin in the presence of a blocking reagent, as follows: 5E9 antibody (1 μM) in a and d, α-lactose (30 mM) in b, and J5 antibody (1 μM) in e.

It has been reported previously that monensin in the range of concentrations 10 to 100 nM vastly increased the cytotoxicity of ricin A conjugates (5, 17–21). Our observation differed from these reports. Monensin moderately (3-fold) reduced the IC_{50} of 5E9-gelonin for HeLa cells (Fig. 1a) but did not affect the cytotoxicity of this immunotoxin for Namalwa cells (Fig. 1d). Monensin had no effect on either the cytotoxicity of ricin B-gelonin for HeLa cells or that of J5-gelonin for Namalwa cells (Fig. 1, b and e). This agent did not affect the cytotoxicity of free gelonin for HeLa cells but caused a 7-fold increase in cytotoxicity of free gelonin in similar experiments with Namalwa cells (Fig. 1, c and f).

Adenovirus-2 did not change the cytotoxicity of ricin B-gelonin or J5-gelonin (Fig. 1, b and e). The virus markedly enhanced the cytotoxicity of free gelonin for HeLa cells but did not affect its cytotoxicity for Namalwa cells (Fig. 1, c and f).

We set out then to test whether any of the three major surface proteins of adenovirus-2, in purified form, was able to increase the cytotoxicity of gelonin for HeLa cells. We hoped that this component, in pure form, would also be able to increase the cytotoxicity of gelonin conjugates. The three major capsid proteins of adenovirus-2, penton, hexon, and fiber (33), were isolated according to a published procedure and tested for their ability to enhance the cytotoxicity of gelonin for HeLa cells (Fig. 2). Only penton and not hexon or fiber was capable of enhancing the cytotoxicity of gelonin in the same manner as whole inactivated virus. This result supports previous observations linking early cytopathic changes in cells infected by adenovirus to penton and is in agreement with the data of Seth *et al.* (34), which showed that an anti-penton base antibody was capable of blocking the enhancement of cytotoxicity of a conjugate between the epidermal growth factor and *Pseudomonas* exotoxin.

Neither penton nor the two other capsid proteins affected the cytotoxicity of gelonin, J5-gelonin, or ricin B-gelonin for Namalwa cells (data not shown), consistent with the results with the intact inactivated virus.

Discussion

Researchers had studied three types of potentiators or agents that seem to increase the specific cytotoxicity of various toxin conjugates (3–5, 7, 8, 10–15, 17–21, 23, 35, 36). These included acidotropic bases such as ammonium chloride and chloroquine, monovalent carboxylic ionophores such as monensin and nigericine, and adenovirus-2.

Contradictory results concerning the potentiating effects of acidotropic bases have been reported. In contrast to the reports mentioned above, ammonium chloride or chloroquine only marginally enhanced, at best, the cytotoxicity of several conjugates of ricin A-chain and a conjugate of gelonin (6, 9, 20, 35, 37–39). More consistent results were reported for carboxylic ionophores or adenovirus-2, which, in all reports to date, appeared to enhance the cytotoxicities of toxin conjugates.

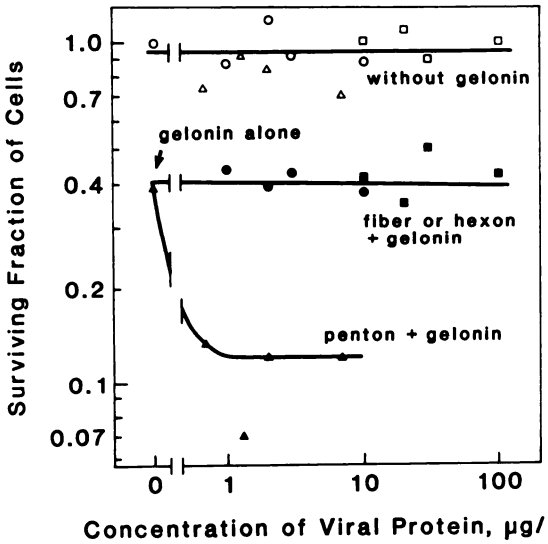

Fig. 2. Effects of adenoviral capsid proteins on the cytotoxicity of gelonin for HeLa cells. HeLa cells were incubated at 37° for 24 hr, with gelonin (0.1 μM) (*closed symbols*) or without it (*open symbols*), with penton (*triangles*), hexon (*squares*), or fiber (*circles*). The surviving fraction of the cells was determined by measuring the inhibition of colony formation, as described in Materials and Methods.

We, however, found that these three types of potentiators either did not affect, or only marginally affected, the cytotoxicities of gelonin conjugates.

Mechanisms of potentiation of toxin conjugates by acidotropic bases or monovalent carboxylic ionophores have not been identified (see Ref. 40 for a review), and it is not clear why the cytotoxicities of only some toxin conjugates are potentiated.

Weak bases accumulate in acidified intracellular compartments such as endosomes and lysosomes and induce increase of the intracompartmental pH, swelling of the compartments, and a variety of other changes in the cellular morphology, cellular metabolism, and intracellular transport (41–43).These changes may, in principle, cause or contribute to the potentiating effect of weak bases. For example, acidotropic base-induced swelling of intracellular vesicles may facilitate the release of the conjugates into the cytoplasm via induced leakage or increased frequency of destruction of the vesicles. Elevation of intralysosomal pH causes the inhibition of lysosomal proteolytic enzymes (42). The cytotoxicity of several toxin conjugates was enhanced by weak bases, perhaps due to the inhibition of their lysosomal degradation (3, 5, 7, 35). However, acidotropic bases failed to potentiate other conjugates of ricin A-chain and gelonin (Fig. 1) (6, 9, 20, 35, 37–39), indicating that lysosomal degradation of these latter conjugates is not a limiting factor in their cytotoxicity. These observations are in accord with previous observations that the extent of degradation of transferrin-ricin A conjugate (19) and a number of antibody-gelonin conjugates (44) is low and does not correlate with their cytotoxicity.

Another possible explanation for the potentiation of certain toxin conjugates by chloroquine (11, 15) is that there is a nonspecific synergistic effect between the potentiator and the conjugate. We found chloroquine to be toxic for Namalwa cells at concentrations above 1 μM and for HeLa cells above 10 μM. Griffin and co-authors (20) found chloroquine to be toxic for the human LoVo cell line at concentrations higher than 8 μM. Synergistic effects of cytotoxic agents with nonrelated mechanisms of action have been reported previously. For example, ricin, a specific inhibitor of ribosomes, showed synergism with Adriamycin in killing murine leukemic cells (45).

The mechanism of potentiation of toxin conjugates by the monovalent carboxylic ionophores monensin and nigericin is also far from clear. Monensin and nigericin affect cells in many ways, apparently as a consequence of primary changes in cytoplasmic Na^+/K^+ balance (42, 46). These effects include changes in the morphology of the Golgi cisternae and mitochondria, inhibition or enhancement of the endocytotic, exocytic, and intracellular transport of certain proteins, swelling of lysosomes, and elevation of the pH inside acidified intracellular compartments (42, 46). As in the case of acidotropic bases, one or several of these cellular changes may be responsible for the potentiation of cytotoxicity of immunotoxins. Raso and collaborators (18, 19) reported that monensin, at a concentration as low as 10 nM, had the capacity to potentiate ricin A conjugates. At this concentration, monensin seems neither to change the pH inside endosomes nor to alter intracellular transport of endocytosed material. In addition, these experiments of Raso and collaborators suggested that the potentiation

of these conjugates was not due to inhibition of their lysosomal degradation. One remaining possibility is that the ionophores cause weakening of endosomes (21), perhaps as a result of their swelling. Remarkably, conjugates of ricin A-chain with the anti-CALLA antibody J5 or with an anti-transferrin receptor antibody were dramatically potentiated by monensin (18, 19), whereas the cytotoxicities of conjugates of gelonin with antibodies directed against the same antigens were barely affected (Fig. 1). This may indicate that potentiation by monensin is specific to conjugates of ricin A-chain and is not a general phenomenon, although, if this were true, it is difficult to understand a common mechanism to explain the enhancement of cytotoxicity of ricin A-chain conjugates and free gelonin, but not gelonin conjugates, on Namalwa cells.

Our data cast doubts on whether monensin would in general improve the therapeutic index of immunotoxins (specific versus nonspecific toxicity). Fig. 1 provides two examples in which monensin does the opposite by enhancing the nonspecific cytotoxicity of a nonconjugated toxin (gelonin) but not of its conjugates with antibodies targeted against Namalwa cells.

The effects of potentiation of ricin A conjugates by weak acidotropic bases and monovalent ionophores might, in part, be explained by contamination of ricin A-chain with ricin B-chain (37), because it is known that the cytotoxicity of whole ricin is increased by weak bases (47–49) and low concentrations of monovalent ionophores (49, 50).

Another agent that seemed promising for potentiation of gelonin conjugates was adenovirus-2. It has been established that infection of cells with certain viruses, adenovirus-2 among them, increases the sensitivity of the cells towards protein toxins such as gelonin, pokeweed antiviral protein and pokeweed antiviral protein from seeds, both purified from *Phytolacca americana*, diphtheria toxin A-chain, abrin A-chain, α-sarcin, mitogillin, restrictocin, and *Pseudomonas* exotoxin (36, 51–53). The proposed mechanism of the potentiation of the cytotoxicity of these protein toxins was that the viruses damage the endosomal membrane, allowing the release of some of the contents of the endosome into the cytoplasm (22, 36, 51).

We found that adenovirus-2 did not potentiate the cytotoxicity of gelonin conjugates. Following antigen-mediated endocytosis, gelonin conjugates are anchored to the antigen on the inner surface of the endosomal membrane via the antibody component of the conjugate. One could speculate that this anchorage may render the conjugates incapable of diffusing out of the endosome via holes that may be produced by adenovirus. This mechanism, however, would not explain the potentiation of the cytotoxicity of a ricin A-chain conjugate with an antibody directed against the carcinoembryonic antigen by adenovirus (20), unless one proposes that the antibody-carcinoembryonic antigen complex is dissociated in an acidified endosome. FitzGerald *et al.* (23, 36) reported that adenovirus-2 increased the cytotoxicity not only of *Pseudomonas* exotoxin but also of its antibody conjugate. It is difficult to explain these latter results, because very little is known about the mechanism of intoxication of cells with *Pseudomonas* exotoxin.

The enhancement of the cytotoxicity of free gelonin by the virus is not a phenomenon universal for all cells; adenovirus-2 enhanced the cytotoxicity of free gelonin for HeLa cells but not for Namalwa cells. The dependence of the potentiating effects on the type of the toxin moiety in the conjugate and on the type of the target cell suggests to us that the mechanism of the potentiating effect of adenovirus is more complex than the simple disruption of cellular membranes.

Conclusive Remarks

The use of acidotropic bases, carboxylic ionophores, or adenovirus as potentiators of immunotoxins has been proposed previously. This proposal was made mostly on the basis of experiments with ricin A-chain immunotoxins and *Pseudomonas* exotoxin immunotoxins.

Our present experiments with immunotoxins made with another protein toxin, gelonin, indicate that neither the acidotropic bases ammonium chloride and chloroquine, the monovalent carboxylic ionophore monensin, nor adenovirus-2 are universal potentiators of cytotoxicity of conjugates of single-chain RIP with molecules that target them to the cell surface. One implication from this observation is that the mechanisms of potentiation by these agents are more complex than those discussed in the literature, such as inhibition of lysosomal degradation, perturbation of intracellular trafficking of endocytosed toxin conjugates, or damaging of endosomal or lysosomal membranes. Also, these experiments cast some doubt on whether these potentiators may be of general use in clinical applications.

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