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Enhancement of Cellular Protein Synthesis Sensitivity to Diphtheria Toxin by Interferon

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In attempts to determine whether, by analogy to cholera and tetanus toxins, diphtheria toxin (DT) can also relieve the antiviral effect of interferon (IF), we found that it rather enhanced the inhibitory effect of IF on the replication of murine leukemia virus in chronically infected NIH/3T3 cells. This enhancement was found to be a consequence of an increased sensitivity to DT of cellular protein synthesis in IF-treated cells. IF stimulated the anti-protein synthesis activity of DT in both mouse cells that are known to be highly resistant to this toxin and in human HeLa cells that are highly sensitive to this toxin. This stimulation was dependent on IF dose. The effect of IF on DT action was strictly species specific, indicating that it was not a consequence of the mere binding of IF to the cell membrane, but rather reflected the cellular changes that followed this initial binding. IF was found to be capable of potentiating intact DT, but could not potentiate its fragments in any combination. IF did not have any effect on the in vitro nicotinamide adenine dinucleotide glycohydrolase activity of DT, suggesting that the effect of IF is not due to molecular modification of the toxin.

The initial step in interferon (IF) action involves its binding (15) to ganglioside-containing receptors (4, 5, 16, 17, 27) located at the outer side of the cell membrane (2, 9, 10, 21, 27, 34). During this interaction with IF, the cell membrane undergoes physical, morphological, and biochemical changes (7, 10, 17, 19), which supposedly trigger several biochemical activities (16, 28), rendering the cells resistant to a wide variety of viruses (16). Besides the antiviral effect, this interaction modifies various other cellular properties (8, 13, 17, 32). Furthermore, IF has been found to interfere with the biological activity of cholera toxin and thyrotropin (20). On the other hand, IF activity has been reported to be inhibited by cholera toxin, tetanus toxin, thyrotropin, and human chorionic gonadotropin (3, 4, 14, 18, 20). Since all of these substances bind to ganglioside-containing receptors in the cell membrane (20, 22, 24, 33), their mutual interference is conceivably due to competition for related binding sites.

IF has also been shown to decrease cellular sensitivity to diphtheria toxin (DT) (6, 23), although the specific receptors of this toxin do not contain gangliosides (25) and apparently are unrelated to IF binding sites. Therefore, it was of interest to determine whether, by analogy to cholera and tetanus toxins, DT can also interfere with IF activity.

During our studies on the effect of IF on murine leukemia virus replication in chronically infected cells, we found that DT enhanced somewhat the inhibition of virus production by IF-treated cells. DT is known to be an inhibitor of cellular protein synthesis due to oxidized nicotinamide adenine dinucleotide (NAD^+)-dependent inactivation of the translation elongation factor EF-2 (11, 25). To understand the significance of the synergistic effect of IF and DT on virus production, we investigated the relationship between DT and IF with regard to cellular protein synthesis and the in vitro nicotinamide adenine dinucleotide-dependent enzymatic activity of the toxin.

MATERIALS AND METHODS

Cells. NIH/3T3 mouse cells chronically infected with Moloney murine leukemia virus [NIH/3T3(MLV) cells] and human HeLa cells were maintained in Dulbecco modified Eagle medium supplemented with 10% newborn calf serum. In experiments with IF the serum content was reduced to 2.5%.

IFs. Partially purified mouse IF containing 10^6 U/mg of protein and human fibroblast IF containing 5×10^5 U/mg of protein were prepared as previously described (1, 30) and were kindly provided by S. Shulman, D. Gurari-Rotman, and M. Revel, Weizmann Institute, Rehovot, Israel. IFs were titrated by the reverse transcriptase assay described elsewhere (1, 30), with reference to National Institutes of Health standard mouse and human IFs. IF quantities are expressed here in standard units.

DT. To prepare DT, a lysogenic strain of *Corynebacterium diphtheriae* (ATCC Park Williams no. 8)

was grown in Elek broth (2% peptone, 0.3% maltose, 0.07% lactic acid, 0.5% NaCl, and 20% horse serum, pH 7.8) with aeration at 35°C for 24 h. The cells were sedimented, and the clear supernatant was fractionated by a stepwise precipitation with $(NH_4)_2SO_4$. DT precipitated at 45 to 60% $(NH_4)_2SO_4$ saturation. This fraction was further purified by diethylaminoethyl cellulose and Sephadex G-100 chromatography, as described previously (12).

Estimation of MLV production. MLV released to the culture medium was quantitated by virus-associated reverse transcriptase activity, as previously described (29).

Estimation of cellular protein synthesis. Cultures in dishes (diameter, 3 cm; Nunc, Copenhagen Denmark) were washed with phosphate-buffered saline and covered with 1 ml of medium containing 30 μCi of [^{3}H]alanine (25 Ci/mmol) per ml. The cells were labeled for 60 min, washed with phosphate-buffered saline, dispersed by trypsinization, and precipitated with 10% trichloroacetic acid. After heating at 80°C for 30 min, the precipitate was collected on Whatman 3MM filters and counted in a toluene-based scintillation liquid.

NAD^+-glycohydrolase activity of DT. Fragment A of DT is capable of NAD^+ glycohydrolysation (11). We recently developed a simple procedure for estimating this activity (manuscript in preparation). Briefly, varying amounts of DT were cleaved by 70 μg of trypsin per ml for 15 min at 37°C and subsequently reduced by 0.1 mM dithiothreitol at 37°C for 15 min. Trypsin inhibitor was added at a final concentration of 70 μg/ml, and the mixture was further incubated for 15 min at 37°C. The fragmented toxin was incubated for 2 min with 0.9 mM NAD^+ at 37°C. NAD^+ was thus glycohydrolysed by fragment A. The remaining NAD^+ was estimated by its reduction in a reaction mixture containing 1 mM lactate and 10 μg of lactic dehydrogenase per ml. Reduced nicotinamide adenine dinucleotide produced in this reaction was measured by its absorbance at 340 nm.

RESULTS

Effects of DT and mouse IF on MLV production. IF is known to suppress the replication of retroviruses (16). In our attempts to determine whether, by analogy to cholera and tetanus toxins (4, 14, 18, 20), DT can also relieve the antiviral activity of IF, we used NIH/3T3(MLV) cells, which chronically release MLV particles. However, DT is a protein synthesis inhibitor which might suppress virus production due to this activity and thus complicate the interpretation of its combined effect with IF. Therefore, we first established the dose curve of DT effect on MLV release. Thus, NIH/3T3(MLV) cells were plated with varying concentrations of DT. After 18 h the cells were washed and covered with fresh medium, and the amount of virus released to the medium was estimated 4 h later by virus-associated reverse transcriptase activity (Fig. 1A).

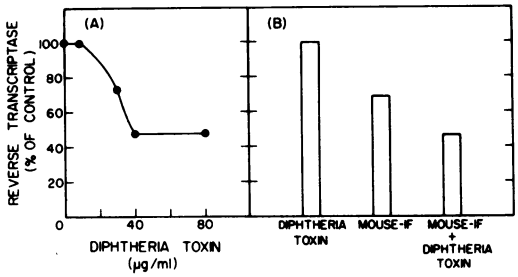

FIG. 1. *Effect of DT and mouse IF on MLV release. (A) NIH/3T3(MLV) cells were plated with various concentrations of DT. (B) NIH/3T3(MLV) cells were plated with or without 15 U of mouse IF per ml, and after 4 h 10 μg of DT per ml was added to the cultures indicated. At 18 h after toxin addition, cells were washed and covered with fresh medium, and virus release during 4 h was estimated by reverse transcriptase activity. Cells grown with no treatment served as a control.*

When the effect of DT on IF action in this system was checked with inhibitory doses of the toxin, we could not detect significant differences in the inhibited virus released in the presence of DT alone or in the presence of DT plus IF (data not shown). It seems that even if DT does relieve the antiviral effect of IF, we would not be able to detect it under these experimental conditions because of its own inhibitory effect on virus production. Therefore, in the next experiment we used the maximal DT dose that still showed no effect on virus release. Thus, cells were plated with or without IF (15 U/ml). After 4 h cultures received 10 μg of DT per ml and were incubated for an additional 18 h, with IF remaining in the IF-treated cultures throughout this incubation. The virus release capacity of the cells was then estimated as described above. This sequence of IF and DT additions was used in order to avoid a possible DT-mediated inhibition of IF binding to the cells. On the other hand, if DT were to be tested for its effect on IF action, it should have been added before the initiation of the development of the antiviral state. As previously reported (29), the antiviral state starts in NIH/3T3(MLV) cells only 4 h after IF addition and reaches its maximal level after 12 to 14 h of IF treatment. Figure 1B shows that IF alone at the concentration used inhibited virus release by 31%. However, when IF was applied together with DT, virus release inhibition increased to 62%, although DT alone was not at all inhibitory. Hence, this experiment demonstrates an enhancement of the inhibition of virus production by combined treatment with IF and DT, rather than a relief.

Enhancement of protein synthesis sensi-

tivity to DT by mouse IF in NIH/3T3(MLV) cells. One possible explanation for the enhanced inhibition of MLV production after combined treatment with IF and DT is that protein synthesis in IF-treated cells probably becomes more sensitive to DT. Under such conditions DT apparently becomes inhibitory for virus production even at the low concentration, therefore increasing the suppression of virus release beyond that rendered by IF. To check this possibility, NIH/3T3(MLV) cells were plated with or without 80 U of IF per ml and 4 h later received varying concentrations of DT. After additional incubation for 18 h, the cells were examined for protein synthesis capacity. Figure 2 confirms that IF treatment indeed potentiated DT, increasing its efficiency in suppressing protein synthesis, although IF alone had no effect in this respect.

Enhancement of protein synthesis sensitivity to DT by human IF in HeLa cells. Mouse cells are known to be relatively resistant to DT (25), presumably because their membrane lacks specific receptors for this toxin (25). It was therefore of interest to determine whether IF potentiation of DT is restricted to such resistant species. Therefore, we examined the effect of human IF on DT in HeLa cells, since these cells are highly sensitive to DT (25). In the experiment shown in Fig. 3A, paired cultures of HeLa cells were plated with increasing concentrations of human IF, and 4 h later one culture of each pair received 1.6 μg of DT per ml. In the experiment shown in Fig. 3B, cells were plated with or without 80 U of human IF per ml and 4 h later received increasing doses of DT. It is evident from these experiments that DT potentiation by IF also occurs in such highly sensitive cells. Furthermore, this potentiation is dependent on IF concentration (Fig. 3A).

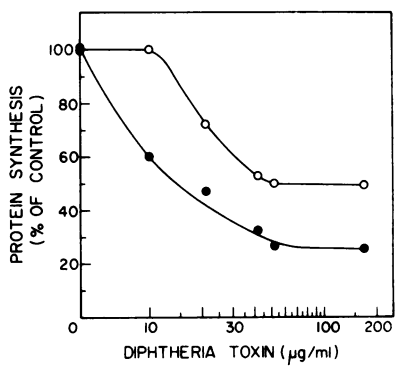

FIG. 2. *Effect of DT and mouse IF on protein synthesis in NIH/3T3 cells. Cells were plated with* (●) *or without* (○) *mouse IF (80 U/ml). After 4 h varying concentrations of DT were added. The cells were incubated for an additional 18 h and then analyzed for protein synthesis by incorporation of [^{3}H]alanine into trichloroacetic acid-insoluble material during 60 min of labeling. Untreated cells served as a control.*

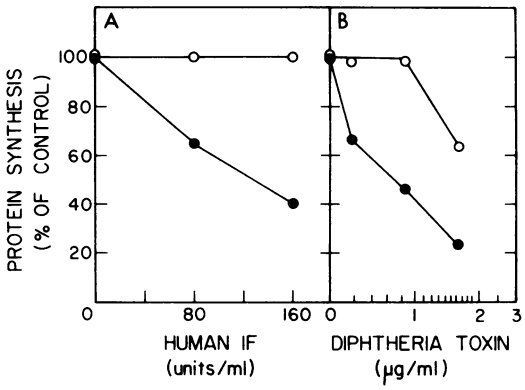

FIG. 3. *Effect of human IF and DT on protein synthesis in HeLa cells. (A) HeLa cells were plated with varying concentrations of human IF. After 4 h one culture of each IF concentration received 1.6 μg of DT per ml* (●)*, and the other received no further additions* (○)*. (B) HeLa cells were plated with* (●) *or without* (○) *human IF (80 U/ml), and after 4 h the cells received varying concentrations of DT. Protein synthesis was determined 18 h later, as described in the legend to Fig. 2.*

Species specificity of DT potentiation by IF. Biological activities of IF are species specific (13, 32), whereas its binding to cell membranes is rather species nonspecific (8, 20, 26). If DT potentiation by IF results from the mere binding of IF to the cell membrane, it should manifest no species specificity. To elucidate this aspect, HeLa cells were plated with or without mouse IF (80 U/ml) and 4 h later received varying doses of DT. As Fig. 4 shows, no potentiation of DT is apparent in this system, indicating that this effect of IF, like its other biological activities, is strictly species specific.

Effect of IF on protein synthesis with fragmented DT. DT can suppress cellular protein synthesis only when it exists as intact molecules. However, this toxin can be split enzymatically into A and B fragments. Fragment A is responsible for the toxic activity of intact DT on animal cells and can exert, even by itself, the enzymatic activity of DT in a cell-free system (25). Fragment B is involved in the binding of intact DT to the cell membrane receptor, but otherwise possesses no other activity in intact cells or in cell-free systems (25). In agreement with other reports (25), Table 1 shows that the two fragments, whether added separately or together, had no effect on protein synthesis in NIH/3T3(MLV) cells. Furthermore, mouse IF could potentiate only intact DT, and it could

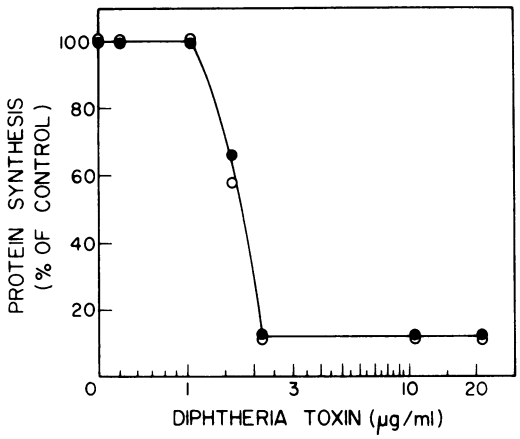

FIG. 4. *Effect of DT and mouse IF on protein synthesis in HeLa cells. Cells were plated with (●) or without (○) mouse IF (80 U/ml) and 4 h later were treated with varying concentrations of DT. After 18 h the cells were examined for protein synthesis, as described in the legend to Fig. 2.*

TABLE 1. *Protein synthesis by NIH/3T3(MLV) cells in the presence of mouse IF and DT fragments*

Addition(s)	Protein synthesis (cpm)[a]
None	9,300
Mouse IF[b]	9,420
DT[c]	9,550
DT + mouse IF	5,230
Fragment A[d]	8,925
Fragment A + mouse IF	9,210
Fragment B[e]	8,950
Fragment B + mouse IF	9,070
Fragment A + fragment B	9,250
Fragment A + fragment B + mouse IF	9,565

[a] Protein synthesis was assayed as described in the legend to Fig. 2.
[b] Mouse IF at 80 U/ml.
[c] DT at 10 μg/ml.
[d] Fragment A at 80 μg/ml.
[e] Fragment B at 80 μg/ml.

not potentiate DT fragments in any combination.

Effect of mouse IF on NAD$^+$-glycohydrolase activity of DT. The cellular protein inhibitory effect of DT is a consequence of the inactivation of translation elongation factor EF-2 by NAD$^+$-dependent adenosine diphosphate ribosylation (11, 25). This enzymatic action is exerted by fragment A, which is produced by cleavage of the penetrating toxin. In the absence of EF-2, fragment A manifests an NAD$^+$-glycohydrolase activity (11). We recently developed a simple procedure to monitor this latter activity of DT in a cell-free system (see above). It could be speculated that during incubation in the presence of IF, DT molecules are probably modified in such a way that the enzymatic activity of fragment A is enhanced. To check this possibility, various amounts of DT were incubated for 18 h with or without 80 U of mouse IF per ml. Then, DT was cleaved by trypsin and tested for NAD$^+$ glycohydrolysation activity. Figure 5 shows no effect of IF on the enzymatic activity of DT. Similar results were obtained when already fragmented DT or its purified fragment A was incubated with IF (data not shown). These observations eliminate the possibility that molecular changes of DT are the reason for its enhanced activity in IF-treated cells.

DISCUSSION

The present study was initiated in an attempt to determine whether, by analogy to cholera and tetanus toxins (4, 14, 18, 20), DT can also relieve the antiviral activity of IF. For this purpose we took advantage of the antiviral effect of IF on the replication of retroviruses (16). When applied to NIH/3T3(MLV) cells, DT suppressed viral release in a dose-dependent pattern. However, when applied to IF-treated cells even at a non-inhibitory concentration, DT increased the inhibition of virus production exerted by IF instead of relieving it. One possible explanation for this enhanced inhibition of virus release is that the toxin somehow increases the sensitivity of the cells to the antiviral effect of IF. An alternative possibility is that IF increases the sensitivity of the cells to the anti-protein synthesis

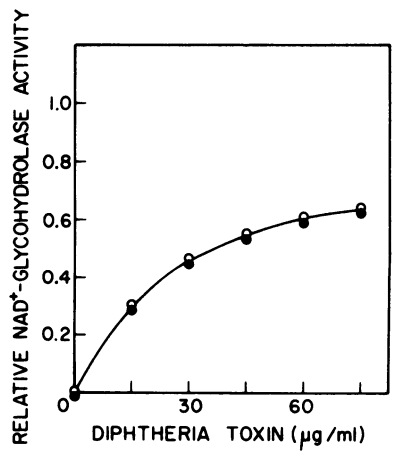

FIG. 5. *Effect of mouse IF on NAD$^+$-glycohydrolase activity of DT. Varying concentrations of DT were incubated at 37°C for 18 h with (●) or without (○) 80 U of mouse IF per ml. DT was then cleaved by trypsin and assayed for NAD$^+$-glycohydrolase activity as described in the text.*

action of DT, so that even at a low concentration it becomes inhibitory for virus production. Therefore, under such conditions, DT further inhibits virus release beyond the inhibition exerted by IF alone. The second alternative proved to be correct. Mouse IF was found to increase markedly the protein synthesis inhibitory effect of DT in NIH/3T3(MLV) cells.

Mouse cells are remarkably resistant to DT (25). It could therefore be argued that IF can potentiate the protein synthesis inhibitory effect of this toxin only in such resistant cells, perhaps by uncovering specific membrane receptors. However, this possibility was ruled out since the potentiation of DT action by IF could also be demonstrated in highly sensitive cells, such as human HeLa cells. The extent of DT potentiation was found to depend on the IF dose applied. It is rather unlikely that this potentiation results from the mere binding of IF to the cell membrane, since this binding exhibits no species specificity (8, 20, 26), whereas IF potentiation of DT is strictly species specific. This specificity indicates that DT potentiation is a consequence of cellular changes that follow the initial binding of IF to the cell membrane. These changes might involve modifications of the cell membrane that enhance DT penetration into the cells. Alternatively, since IF is known to affect the regulatory control of protein synthesis (16, 28), it is possible to speculate that there is a change in the translation control of IF-treated cells that renders their protein synthesis more sensitive to DT.

Restriction of cellular ribonucleic acid and protein syntheses by serum deprivation has been reported to increase considerably the antiviral effect of IF (31, 35). Therefore, it could be argued that DT potentiation, which leads to a partial inhibition of protein synthesis in IF-treated cells, might act similarly to enhance IF antiviral activity in our system. However, this possibility is rather unlikely because specific inhibitors of ribonucleic acid and protein syntheses have been shown to interfere with the development of the IF-induced antiviral state (16, 35). Therefore, it is even possible that the inhibition of MLV production observed after combined treatment with IF and DT represents probably only the DT-related inhibitory effect, whereas no IF-induced antiviral state could develop under such conditions. It is worthwhile to note in this connection that when DT was applied to IF-treated cells at inhibitory doses, no further inhibition of virus release beyond that exerted by DT alone was observed (unpublished data).

The toxic effect of DT on intact cells can be demonstrated only with complete DT molecules and not with any of its split fragments, since DT action must start with specific binding to the cell membrane through its B fragment (25). Nevertheless, it could be speculated that the membrane modification imposed by IF might perhaps allow DT penetration even in a fragmented form, making the initial specific binding to the cell surface unnecessary. However, this possibility was experimentally eliminated by our observation that IF could potentiate only intact DT, not its fragments in any combination.

Another possibility that was considered was that during incubation in the presence of IF, DT might undergo molecular changes, thus rendering it more active when it penetrated into the cells. However, this possibility was also eliminated since incubation of intact or fragmented DT with IF for 18 h did not change its NAD^+-dependent activity in a cell-free system. It is therefore concluded that IF-induced potentiation of DT involves only cellular changes.

Finally, it should be noted that our observation of DT potentiation by IF is in conflict with the reported data of Moehring et al. (23) and Boquet (6), who found inhibition of DT action in IF-treated cells. This variation is probably due to different experimental conditions, cell lines, and quality of IF preparations. For example, Mohering et al. (23) detect the strongest inhibition of DT action when it is added after 20 h of IF treatment, when the antiviral state is apparently fully expressed. It is quite possible that some IF-induced cellular changes are required for the cell resistance against DT. In our experiments DT was added before the establishment of the antiviral state. If the IF-induced cellular changes required for the resistance to DT depend on protein synthesis, it is possible to speculate that under our experimental conditions DT acted as a protein synthesis inhibitor, interfering with the required cellular changes.

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