Antibody-mediated Routing of Diphtheria Toxin in Murine Cells
Results in a Highly Efficacious Immunotoxin*

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The chemical coupling of diphtheria toxin to an antimerine Thy1 antibody resulted in the most efficacious immunotoxin to date. At 1 μg/ml the immunotoxin inhibited protein synthesis of a Thy+ AKR murine cell at a rate of 1.4 logs/h, within the order of magnitude of the efficacy of native toxins. This is unusual since murine cells are highly resistant to diphtheria toxin. The conjugate is highly specific; Thy- AKR cells display no intoxication at 1 μg/ml even after 18 h. The effects of ammonia, acid pulsing of external media, and low temperature reveal some similarities and some differences between intoxication of sensitive cells by toxin and of murine cells by the antibody-toxin conjugate. The differences that result in the high efficacy of the antibody-toxin conjugate appear to result from the antibody-mediated routing. These results imply that murine cells possess an acidic compartment which can mediate toxin cytosolic entry. Unlike the Thy antigen, the toxin receptor on murine cells is unable to route the toxin to this cellular site.

Natural protein toxins, such as the bacterial diphtheria and pseudomonas toxin, and the plant toxins ricin and abrin possess a remarkable capacity to inhibit cellular protein synthesis. These toxins are composed of two subunits, A and B, coupled by a reducible disulfide bond. The high efficacy is due to the presence of multiple domains that function to move the toxin through sequential steps towards cellular intoxification. The A subunit possesses enzymatic activity; the bacterial toxins inhibit protein synthesis through ADP-ribosylation of elongation factor 2 (1); the plant toxins modify the 60S ribosomal subunit (2) through an N-glycosidase activity (3). The initial interaction with a cell is the binding of the toxin via the B subunit to a plasma membrane receptor (4, 5). Endocytosis follows, but the toxin remains within the lumen of the endosome or organelle, inaccessible to the cytosolic substrate. It is the second function ascribed to the B subunit, cytosolic entry, that effectively moves the enzymatic activity past the membrane barrier into the cytosolic compartment resulting in protein synthesis inhibition. For recent reviews on the topic of bacterial and plant toxins, see Refs. 6 and 7.

These toxins are opportunistic in the sense that they utilize cellular receptors and processes that evolved for normal cellular growth. One remarkable exception to toxin potency concerns rodents. Mice, rats, and their isolated cells share a remarkable tolerance to diphtheria toxin. Compared to cell lines derived from sensitive species, such as guinea pigs, monkeys, and man, murine cells require concentrations approximately 10^4 higher for comparable intoxication (8). The insensitivity exists despite the fact that murine cells possess toxin-specific binding sites (9-11) and can internalize levels of radiolabeled diphtheria toxin equivalent to that seen with toxin-sensitive cells (12, 13). Morphological examination of gold-labeled toxin in murine LM cells has demonstrated movement of internalized diphtheria toxin into lysosomes (14). Additionally, murine elongation factor 2 is fully susceptible to enzymatic inactivation by the toxin’s A chain (15). Experiments involving hybrid cells formed between murine and sensitive cells demonstrated that the factor(s) responsible for sensitivity was dominant (16); it was also shown that the gene product for human sensitivity was located on the chromosome 5 (17). It thus appears that murine cells lack an essential element required for cytosolic entry.

Recent work by O’Keefe and Draper (18) and Guillemot et al. (19) has demonstrated that by coupling diphtheria toxin to transferrin or concanavalin A, a 10^4 and 10^10 increase, respectively, in potency was seen. We report here the construction of an anti-murine T cell antibody-diphtheria toxin conjugate that displays the highest inactivation rate of any immunotoxin to date, reaching 1.4 logs/h on Thy+ AKR target cells, at a concentration that displays no toxicity on non-target Thy- AKR cells. Because of the antibody specificity of this conjugate, we can differentiate the novel efficacious route in murine cells from the normally inefficient one.

MATERIALS AND METHODS

Crude diphtheria toxin (Connaught Laboratories) was purified as described previously (9). For cell culture, RPMI medium (Gibco Laboratories) was supplemented with 25 mM HEPES* (Research Organics), 2 mM NaHCO3, and 10% heat-inactivated fetal calf serum. The anti-Thy.1 monoclonal antibody, 0x7 was purified from ascites as described previously (20). 2-Iminothiolane and n-maleimidobenzoyl N-hydroxysuccinimide ester were from Pierce Chemical Co. High performance liquid chromatography was accomplished on a Hewlett Packard 1041B with a 21.5 x 600 mm TSK-G 3000 (Pharmacia LKB Biotechnology Inc.) and a 3 x 25.0-mm Zorbax GP 250 (Du Pont) gel filtration column. L-[U-14C]Leucine (300 mCi/mmol) was from ICN. Centricon microconcentrators were from Amicon. Iron-saturated human transferrin was from Sigma.

Synthesis of Oc7 and Transferrin-Diphtheria Toxin Conjugates—To purified diphtheria toxin (at 20 mg/ml) a 5-fold molar excess of 2-iminothiolane stock was added (at 1 mg/ml) in pH 8.0, 0.16 M borate. Following 90 min at room temperature, the thiolated toxin was desalted on a Zorbax GP-250 column and concentrated back to the original volume in Centricon 30 microconcentrators. To Ox7 or transferrin (at 15 mg/ml in phosphate-buffered saline) was added a 5-fold molar excess of m-maleimidobenzoyl N-hydroxysuccinimide ester (stock at 1 mg/ml in dimethylformamide). After 20 min, the

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‡ The abbreviation used is: HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid.
modified proteins were desalted on a centrifuge column and immediately added to a 10 M excess of thiolated toxin. After 1 h, residual thiol groups were modified with N-ethylmaleimide. Purification of the conjugates was achieved on a 21.5 X 600-mm TSK-G 3000 column.

**Characterization of Conjugates**—Peaks from the TSK-G 3000 column were concentrated with Centricon 30 microconcentrators and then examined on preformed 2-16% acrylamide gradient gels, as described previously (21). The conjugates used in this report contained one toxin per antibody molecule. Protein concentrations were determined by the bicinchoninic acid method (22; Pierce Chemical Co.).

**Protein Synthesis Assay**—The effect of immunotoxin or toxin on protein synthesis on cultured cells was determined as described previously (21). Typically, 10^6 cells in leucine-free RPMI 1640 medium containing 2 mM NaHCO3, 25 mM HEPES, pH 7.4, and 0.1% bovine serum albumin were added to the desired level of immunotoxin and lactose (100 mM), so that the final volume was 0.1 ml. This was done in a 96-well round-bottom plate. After the designated times, a 1 in 10 dilution of stock 1-[U-14C]leucine (0.01 mCi/ml) into leucine-free RPMI was added in a 10-μl volume to the cells. After a 60- or 30-min incubation, the cells were harvested on glass fiber filters with a Titertek cell harvester (Flow Laboratories) and then counted. All protein synthesis assay time points were done in quadruplicate. Unless otherwise specified in the figure legend, data points are the mean value with standard deviations of 10% or less of the mean value. When [14C]leucine incorporation was determined at 15°C the incubation was extended from 1 to 4 h.

**Media Changes for Acid Pulse Experiment**—The mid-incubation media changes in 96-well plates were achieved by aliquoting the 10^6 cells in a volume of 100 μl into V-shaped 96-well plates (Nunc). At the appropriate time, the plates were centrifuged at 400 × 1 g for 5 min in a Beckman TJ-6 centrifuge (TH-4 rotor). The media was then siphoned off, and the cells were resuspended upon addition of new medium with a micro-pipettor.

The above techniques and other general procedures concerning immunotoxin synthesis and characterization have been described previously in greater detail (21).

**RESULTS**

Diphtheria toxin was chemically coupled to either transferrin or an anti-Thy-1.1 antibody (Ox7; Ref. 20) as described under "Materials and Methods." This antibody binds to murine T cell lymphocytes or leukemic cells. Incubation of either conjugate on a murine AKR leukemia-cell line (Thy1.1* AKR SL2) resulted in a rapid loss of protein synthesis (Fig. 1). Under similar conditions, the native toxin at 1000 ng/ml inhibited protein synthesis by 60% of the control (data not shown). Like the intoxication of sensitive cells by diphtheria toxin, there was a lag period during which no loss in protein synthesis occurred, followed by an exponential decline in cellular capacity to incorporate labeled leucine. These effects were concentration-dependent (data not shown) and thus displayed the kinetics generally associated with the efficacious entry process of native toxins.

To demonstrate the specificity of the antibody-toxin conjugate, an 18-h incubation of the Ox7-diphtheria toxin conjugate at varied concentration was carried out on both target (Thy1.1*) AKR SL2 and non-target (Thy1; Ref. 23) AKR K-36 cells. The cells were also exposed to varied levels of the native toxin. The resultant dose-response curve is shown in Fig. 2. Whereas the two cell lines display similarly low sensitivities to the native toxin, there is a 3.6 log difference in the conjugate potencies. With the Thy1* K-36 cell intoxication by the conjugate is through interactions of the toxin moiety with the cell. The potency of the conjugate is less than the native toxin since the toxin moiety has been modified and is now sterically hindered. The specificity for the Thy1* cell demonstrates that it is not the chemical alteration which rendered the toxin efficacious, but rather some feature supplied by the antibody.

Prior to intoxication of sensitive cells, diphtheria toxin requires intravesicular acidification, resulting in a conformational change in the bound toxin molecule that leads to an increased association with, or insertion into, the vesicular membrane (24, 25). The critical nature of this process is demonstrated by the toxin resistance displayed by mutant cells which lack endosomal acidification (26), as well as by the protection of intoxication by the addition of lipophylic amines, such as ammonia (27). As shown in Fig. 3, the antibody-toxin conjugate is also rendered nontoxic by the presence of ammonia. The protection acquired by a sensitive cell through the addition of ammonia can be bypassed, however, through acidification of the medium (28-30); that is, if sensitive cells with prebound toxin are pulsed with a low pH medium, the presence of ammonia offers little protection. Medium acidification increases toxicity by decreasing the lag period for diphtheria toxin intoxication of sensitive cells. Following the procedure established for sensitive cells, AKR cells were preincubated with the diphtheria toxin-Ox7 conjugate at 4°C, washed free from unbound conjugate, and then incubated for 10 min (at 4°C) at either pH 7.4 or 5.0. The cells were then returned to pH 7.4 in the presence or absence of 10 mM ammonium chloride and elevated to 37°C. At the various
times indicated in Fig. 4, the cells were pulsed with [14C] leucine to determine their ability to incorporate label. When the AKR cells were preincubated with the Ox7-toxin conjugate plus ammonia, an acid pulse did not result in the intoxication of the ammonia-protected cells. Additionally, the lag phase was not shortened; in fact, under the conditions specified, 1 h was added to the lag. Acidic pH values were found not to alter the binding of the antibody to the AKR cell. Binding of the iodinated antibody was unaffected in the tested pH range of 4.5–8.0 (data not shown).

To further characterize the acidification process, we examined the timing of the passage of the toxin conjugates through the acid dependent (ammonia protection) step. This method, as originally described for native diphtheria toxin (31), was used to determine the time point at which the first antibody- and transferrin-toxin conjugate molecules pass through the ammonia protection process. Extrapolation of the kinetic data for both conjugates to control values (the protection level afforded by early presence of ammonia) of protein synthesis indicate that both conjugates started passing through this step at 15 min (Fig. 5). This value is longer (by a factor of 3–4) than that seen for native diphtheria toxin intoxication of sensitive cells (31). The inability to achieve the acidification step by a low pH pulse of the medium, as well as the delayed timing of emergence through the ammonia protection step, suggests that the routing which leads to the efficacious intoxication of murine cells by the diphtheria toxin conjugate varies from that of the efficacious intoxication of the sensitive cells by the native toxin.

Another method for examining the routing of proteins through a cell is to lower the temperature of the cells. Various intracellular transport/processing elements are effectively hindered below certain temperature ranges (see "Discussion"). The intoxication of sensitive (non-murine) cells by diphtheria toxin is relatively insensitive to temperature within the range of 15–37 °C (31, 32). The intoxication of murine leukemic cells by the native toxin (see Fig. 6, top) is also relatively insensitive to lowered temperatures. All processes are slowed down at 15 °C, including protein synthesis; thus, the [14C] leucine pulse is extended from 1 to 4 h; in all other respects, the cell plus toxin incubation is identical for all temperatures. When the two toxin conjugates were examined under identical conditions, the intoxication was diminished at lowered temperatures (Fig. 6, middle and bottom). The efficacy seen at 24 °C and above was lost at 15 °C. In a separate, but shorter incubation (6 h), protection was evident at 19 °C (data not shown). The intoxication seen at 15 °C may be through the inefficient temperature-insensitive route seen with the native toxin. While the inefficient intoxication processes of the native toxin is largely unaffected by temperatures below 20 °C, the cellular process that renders the cell sensitive to the toxin moiety does display temperature sensitivity. Thus, the remarkable increase in toxicity coincides with the acquisition of a temperature-sensitive process, presumably routing, sup-

FIG. 2. Dose response of Ox7 conjugate on AKR target and non-target cells. Varied levels of the anti-Thy Ox7-diphtheria toxin conjugate (circles) and the native toxin (squares) were incubated with either the Thy+ AKR SL2 (open symbols) or the Thy- AKR K-36 cell lines (closed symbols) for 18 h. [14C]Leucine incorporation was determined by a 1-h pulse.

FIG. 3. Ammonia protects cells from Ox7-diphtheria toxin intoxication. AKR SL2 cells were incubated with 500 ng/ml conjugate in the presence or absence of 10 mM NH4Cl. The inhibition of protein synthesis was determined as described in the legend to Fig. 1.

FIG. 4. Effect of acid pulse on ammonia protection. Ox7-diphtheria toxin conjugate at 500 ng/ml was bound to AKR SL2 cells (106) at 4 °C in leucine-free RPMI medium, pH 7.4, in 96-well plates. The cells were then centrifuged and resuspended in either pH 7.4 (squares) or pH 5.0 (triangles) medium at 4 °C. After 10 min, the cells were again centrifuged and resuspended in pH 7.4 medium (open symbols) or the same medium plus 10 mM NH4Cl (filled symbols). The cells were then warmed to 37 °C at time 0. Protein synthesis was then determined as outlined in Fig. 1 using 30-min pulses. Data represents the mean of four determinations with a standard deviation of 15% or less of the mean value.
Murine Immunotoxin of High Efficacy and Specificity

Fig. 5. Passage of Ox7- and transferrin-toxin conjugates through ammonia-sensitive cellular compartment. AKR SL2 cells were incubated with either the Ox7- or transferrin (tf)-diphtheria toxin (DT) conjugates at 1000 ng/ml at 4 °C for 2 h, followed by warming to 37 °C at time 0. At the designated times, aliquots were made 10 mM in NH4Cl. The ability of the cells to synthesize protein was then determined at 180 min with a 30-min [14C]leucine pulse. For each time point, there was a control incubation that went through identical manipulations, but lacked the addition of toxin conjugate. The experimental values were normalized to these control values set at 100% protein synthesis.

A similar experiment was performed with the K-36 cell line. This cell is resistant to the native toxin and the anti-Thy antibody-toxin conjugate (Fig. 2); however, it is sensitive to the transferrin-diphtheria toxin conjugate. When exposed to varying levels of this conjugate under the identical conditions described in Fig. 6, 50% inhibition of protein synthesis at 24 °C is reached at concentrations below 10 ng/ml. Reduction of the temperature below 20 °C resulted in greater than a 4 log reduction in potency. Thus, the routing or processing steps that are essential for efficacious diphtheria toxin activity are ligand specificity for the K-36 cells.

The sequence of the ammonia- and temperature-sensitive steps was determined by the inhibitor exchange procedures previously used in the study of toxin entry (29, 33). The results are outlined in Table I. The Ox7-diphtheria toxin conjugate was bound to the murine cells at 4 °C for 1 h. The cells were then washed and the temperature was brought up to 15 °C. After 7 h, the temperature was elevated to 37 °C, and the cells’ capacity to synthesize protein was examined. If ammonia was excluded from all steps in the incubation, protein synthesis was decreased by greater than 80%; however, the addition of ammonia to the medium prior to the elevation to 37 °C resulted in complete protection. This result implies that the temperature-sensitive step precedes the ammonia-sensitive step.

DISCUSSION

The diphtheria toxin-antibody conjugate constructed here has the highest efficacy of any reported immunotoxin. At 4.8 × 10^9 M the Ox7-diphtheria toxin conjugate inhibits target cell (AKR) protein synthesis at a rate of 1.4 logs/h. An AKR cell line lacking the Thy antigen (K-36) shows no toxicity at this concentration following an 18-h exposure. This conjugate can be compared to the highly efficacious anti-T3 (UCHT1)-diphtheria toxin conjugate (0.8 log/h on Jurkat cells; Ref. 34),

Fig. 6. Effect of temperature on intoxication process. AKR SL2 cells were incubated at three different temperatures (X), 24 (triangles), and 37 °C (squares), and at five different concentrations (on x axis) of either diphtheria toxin (DT; uppermost figure), transferrin-diphtheria toxin conjugate (middle figure), and the Ox7-toxin conjugate (bottom) for 20 h. Determination of protein synthesis at the end of the incubation was achieved by a 1-h pulse for the 24 and 37 °C incubations and 4 h for the 15 °C incubation. Each data point (mean of four determinations) is normalized against a non-toxin control.

Table I

| Temperature | Concentration | [14C]Leucine incorporation | % control |
|-------------|---------------|---------------------------|----------|
| 15 °C, 7 h  | +NH3          | 6029 ± 716 | 111.1 ± 13.2 |
| 15 °C, 7 h  | -NH3          | 746 ± 89  | 16.5 ± 2.0 |
| 37 °C, 2 h  | +NH3          | 4984 ± 616 | 112.1 ± 13.9 |

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[Figure 5 and Figure 6 are not included in the text.]
anti-CD5 (T101)-ricin conjugate (0.3 log/h on CEM cells, 0.7 log/h in the presence of 10 mM NH4Cl; Ref. 35), or the anti-CD5 (T101)-ricin A chain conjugate plus monensin (0.4 log/h on CEM cells, Ref. 36), all at or near full receptor (antigen) occupancy. The higher efficacy of the anti-Thy (Ox7)-diphtheria toxin conjugate reported here may in part be due to higher antigen density, 6-2 x 10^5 (21, 37), compared to approximately 10^6 for "T3 on Jurkat (34) and 2 x 10^4 CD5 molecules/CEM cell (36), as well as inherent differences in cell types.

A more informative comparison can be made by examining the inhibition rates of other Ox7-toxin conjugates on the same cell line. Comparisons are accomplished at concentrations of 10^-9 M, the 90% saturation point for Ox7 binding to AKR cells. Ox7-ricin A chain and Ox7-diphtheria toxin A chain (37) conjugates reached a maximal 0.14 and 0.02 log/h rate of protein synthesis inhibition, respectively. The holoricin conjugates in the presence of 100 mM lactose achieved a 0.14 log/h rate for the monoronic conjugate and 0.40 log/h for the biricin conjugate (21). At 5 x 10^-8 M (0.3 receptor occupancy) ricin inhibited AKR protein synthesis at a rate of 5.6 logs/h, whereas a 2-fold higher concentration of diphtheria toxin yielded no detectable toxicity after 6 h (37); a 20-fold increase in toxin concentration resulted in 0.05 log/h (10^-4 M; estimated Kd for diphtheria toxin: 10^-4 M, Ref. 10). Thus, the conjugation of these two natural toxins to the same antibody resulted in a paradoxical shift in efficacy. Coupling ricin to Ox7 decreases efficacy by approximately 1 log; coupling diphtheria toxin to Ox7 results in a greater than 1 log increase. It has been the objective of this report to characterize the cellular mechanisms responsible for this shift.

Because of the antigen specificity of the conjugate, we have been able to demonstrate that the antibody brings about an altered routing of the toxin moiety to a cellular compartment presumably unreached by the conjugate in Thy" cells or the native toxin in murine cells in general. The procedures we used to study routing that follows receptor-mediated endocytosis were acid pulsing of external medium, prevention of vesicular acidification by treatment with amines, and inhibition of specific vesicle fusion reactions by low temperature. Many receptor-bound ligands, such as insulin (38), asialoglycoproteins (39), epidermal growth factors (40), and low density lipoproteins (41) are endocytosed via their receptor and then degraded within lysosomes. Transferrin is unusual in this respect; it is spared from degradation (42, 43). Upon vesicular acidification, the transferrin-bound ferric ions are released, and the receptor-bound spapotransferrin recycles back to the cell surface (44, 45). The routing of transferrin and its receptor through the cell has been extensively studied and may involve more than one non-lysosomal pathway (46). One route involves a juxtanuclear Golgi region. Evidence for this has been both morphological (47-49) and biochemical, as receptor-bound asialo-transferrin and the asialo-receptor are transported, in a limited way, to the sialyltransferase compartment of the Golgi (50, 51), as well as the site of Golgi mannosidase I (52). Thus, a route for bound transferrin to the lysosome is lacking; whereas, movement through the secretory pathway has been established. It is of interest that the addition of anti-transferrin receptor antibody does bring about the delivery of the receptors to the lysosomes (53, 54). This knowledge suggests that the transferrin-diphtheria toxin conjugate's intracellular routing would not involve the lysosome, but could involve other cellular acidifying compartments, such as the Golgi and Golgi-associated vesicles.

The effect of temperature on cellular routings has also been extensively studied. Receptor-bound ligands are not internalized at temperatures below 10 °C, and their delivery to lysosomes is inhibited by temperatures below 20 °C (55, 56). Movement of newly synthesized proteins is also affected by temperature. Transfer of protein from the rough endoplasmic reticulum to the cis face of the Golgi occurs only at temperatures above 10 °C, whereas movement through the trans cisternae and entry into condensing vacuoles is inhibited by temperatures less than 20 °C (57-59). It is of interest that the efficacious route of the diphtheria toxin conjugates is temperature-sensitive, whereas the efficacious route of the native toxin in sensitive cells lacks temperature sensitivity (31, 32), as does the poorly efficacious route of the native toxin in murine cells (Fig. 6). Thus, the conjugation of the toxin to an alternative binding site results in an altered routing of the toxin through the murine cell, possibly through the lysosomal or Golgi pathways. However, toxin bound to murine cells is delivered to the lysosomal compartment, but efficient intoxication of the cell is lacking. The known processes affected so demarcated by moving the temperature below 20 °C are routing functions, presumably involving select types of membrane fusions. This does not preclude a temperature-dependent nonrouting process that is essential for diphtheria toxin intoxication in murine cells.

Whereas the transferrin and antibody-toxin conjugates may be endocytosed by different processes, the two conjugates begin to pass through the ammonia-sensitive compartment at the same time, approximately 15 min. This value differs from that of the native toxin with sensitive cells, which reach that point at 4-5 min. Acidification processes that would be affected by the addition of ammonia are known to occur in both the endosomal/lysosomal pathway as well as the secretory pathway of the trans cisternae of the Golgi (60, 61). Morphological examination of the routing of gold-labeled diphtheria toxin in murine cells has demonstrated rapid internalization and appearance in lysosomes within 2.5-5 min. The toxin is largely excluded from coated invaginations (unlike sensitive cells) as well as from the Golgi region (14, 15). The finding that the Thy antigen, which is also excluded from coated pits (62), effectively routes the toxin to a productive (lethal) intracellular site indicates that the endocytotic mechanism is not a determinant in toxin resistance. Transferrin is internalized through the coated pits (63, 64); thus the two conjugates are internalized by different routes but start completion of their acid-requiring step at the same time. This is consistent with the prior finding that in murine cells labeled a2-macroglobulin (endocytosis through coated invaginations) and diphtheria toxin are found in the same prevlysosomal vesicles (11) and with more recent work directly examining the (common) intracellular pathway of ligands internalized through coated and noncoated invaginations (65).

Our experimental results characterizing the effect of ammonia on the intoxication of a murine cell line by a diphtheria toxin conjugate are similar to and corroborate those of O'Keefe and Draper (18); however, we find differences in the effect of acidification of pre-bound conjugates as it pertains to bypassing ammonia protection. They found that media acidification could bypass the protection by ammonia. These differences may be due to the different murine cell types or different receptors. In the absence of ammonia, their transferrin-diphtheria toxin conjugate (1 µg/ml) resulted in a 1 log decrease in protein synthesis in less than 2 h, and in their 24-h dose response curve, a 1 log decrease was achieved at levels less than 0.01 µg/ml. Media acidification of ammonia-protected cells resulted in a log decrease after 24 h (at a conjugate concentration of 1 µg/ml). This drop in efficacy might be explained by a complicating feature of transferrin; acidifica-
tion of the medium induces release of bound ferric ions, which in turn decreases the affinity of the transferrin (now spotransferrin) conjugate for the cellular receptor (44, 45), which would result in decreased toxicity. Pre-bound labeled Ox7 antibody displays no cellular dissociation upon acidification to below pH 5.0.

Heagy and Neville (66) found that acidifying the media did not bypass the ammonia protection of the native toxin bound to murine cells. In addition to the Ox7-diphtheria toxin conjugate, pseudomonas toxin and modeccin have an amine-sensitive step when examined on murine cells, and like the conjugate, neither of the toxins are potentiated by media acidification (67, 68), as diphtheria toxin is on non-murine-sensitive cell lines (30).

The sequence of the temperature- and amine-sensitive steps is defined by the results in Table I, suggesting that the temperature-sensitive process must occur prior to the amine-sensitive process. This sequence, a temperature-sensitive process followed by vesicular acidification, is known to occur in two intracellular pathways, delivery of endosomal vesicles to the lysosome and movement into (or through) the trans Golgi cisternae. Early endosomal acidification, or for that matter, acidification of plasma membrane-bound conjugate on the murine cell, did not fulfill the acidification requirement as it does in sensitive, non-murine cells (30).

Media acidification of diphtheria toxin bound to sensitive cells results in the bypass of ammonia protection. Why media acidification is inadequate for intoxication of murine cells by the Ox7-diphtheria toxin conjugate is unknown; the toxin moiety may require processing prior to acidification or may require some cellular element for the actual translocation act. This element may be missing on the plasma membrane or the 0x7-diphtheria toxin conjugate is unknown; the toxin has the specificity of the conjugated antibody. Thus, nature has supplied us with a model immunotoxin action of these conjugates in vivo system, this agent has achieved greater than 3 log kills.

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REFERENCES

1. Honjo, T., Nishizuka, Y., Hayaishi, O., and Kato, I. (1968) J. Biol. Chem. 243, 3553–3555
2. Benson, S., Olsnes, S., Pihl, A., Skorve, J., and Abraham, A. K. (1975) Eur. J. Biochem. 59, 573–580
3. Endo, Y., and Tsurugi, K. (1987) J. Biol. Chem. 262, 8128–8130
4. Beenziger, J. U., and Fiete, D. (1979) J. Biol. Chem. 254, 9795–9799
5. Cieplak, W., Gaudin, H. M., and Eidel, L. (1987) J. Biol. Chem. 262, 13246–13253
6. Neville, D. M., Jr., and Hudson, T. H. (1986) Annu. Rev. Biochem. 55, 195–224
7. Jimenez, A., and Vazquez, D. (1985) Annu. Rev. Microbiol. 39, 649–672
8. Moehring, J. M., and Moehring, T. J. (1976) Infect. Immun. 13, 221–228
9. Chang, T., and Neville, D. M., Jr. (1978) J. Biol. Chem. 253, 6866–6871
10. Didsbury, J. R., Moehring, J. M., and Moehring, T. J. (1981) Mol. Cell. Biol. 3, 1283–1294
11. Keen, J. H., Maxfield, F. R., Hardegree, M. C., and Habig, H. W. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 2912–2916
12. Bouvenbre, P. F., Saelinger, C. B., Ivins, B., Woecniki, C., and Amorini, M. (1975) Infect. Immun. 11, 675–684
13. Boquet, P., and Pappenheimer, A. M., Jr. (1976) J. Biol. Chem. 251, 5770–5778
14. Morris, R. E., and Saelinger, C. B. (1983) Infect. Immun. 42, 812–817
15. Moehring, J. M., and Moehring, T. J. (1968) J. Exp. Med. 127, 541–553
16. Dendy, P. R., and Harris, H. (1973) J. Cell Sci. 12, 831–837
17. Creagen, R. P., Chen, S., and Rudde, F. H. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2237–2241
18. O’Keefe, D. O., and Draper, R. K. (1985) J. Biol. Chem. 260, 932–937
19. Guillenot, J. C., Sundan, A., Olanes, S., and Sandvig, K. (1985) J. Cell. Physiol. 122, 193–199
20. Mason, D. W., and Williams, A. F. (1980) Biochem. J. 187, 1–20
21. Marsh, J. W., and Neville, D. M., Jr. (1986) Biochemistry 25, 4461–4467
22. Smith, P. K., Krohn, R. L., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goek, N. M., Olson, B. J., and Klenk, D. C. (1985) Anal. Biochem. 150, 76–85
23. O’Keefe, D. O., and Draper, R. K. (1985) J. Biol. Chem. 260, 932–937
24. Old, L. J., Boyse, E. A., and Stockret, E. (1965) Cancer Res. 25, 813–819
25. Donovan, J. J., Simon, M. I., Draper, R. K., and Montal, M. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 172–176
26. Kagan, B. L., Finkelstein, A., and Colombini, M. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 4900–4904
27. Merion, M., Schlesinger, P., Brooks, R. M., Moehring, J. M., Moehring, T. J., and Sly, W. S. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 5315–5319
28. Kagan, B. L., and Olsnes, S. (1985) J. Cell Biol. 95, 1552–1556
29. Draper, R. K., and Simon, M. I. (1980) J. Cell Biol. 87, 849–854
30. Marnell, M. H., Shih, S.-P., Stookey, M., and Draper, R. K. (1984) Infect. Immun. 44, 145–150
31. Sandvig, K., and Olsnes, S. (1980) J. Cell Biol. 87, 828–832
32. Marnell, M. H., Shih, S.-P., Stookey, M., and Draper, R. K. (1984) Infect. Immun. 44, 145–150
33. Sandvig, K., and Olsnes, S. (1984) J. Cell Biol. 98, 963–970
34. Draper, R. K., O’Keefe, D. O., Stookey, M., and Graves, J. (1984) J. Biol. Chem. 259, 4083–4088
35. Youle, R. J., Uckun, F. M., Vailera, D. A., and Colombetti, M. (1986) J. Immunol. 136, 93–98
36. Leonard, J. E., Wang, Q.-C., Kaplan, N. O., and Boyston, I. (1985) Cancer Res. 45, 5263–5269
37. Casellas, P., Bourrie, B. J. P., Gros, P., and Jansen, F. K. (1984) J. Biol. Chem. 259, 9359–9364
38. Esworthy, R. S., and Neville, D. M., Jr. (1984) J. Biol. Chem. 259, 11496–11504
39. Terris, S., and Steiner, D. F. (1975) J. Biol. Chem. 250, 8389–8396
40. Wall, D. A., Wilson, G., and Hubbard, A. L. (1980) Cell 21, 79–93
41. Carpenter, G., and Cohen, S. (1976) J. Cell Biol. 75, 159–171
42. Anderson, R. G. W., Brown, M. S., and Goldstein, J. L. (1977) Cell 10, 351–364
43. Karin, M., and Mintz, B. (1981) J. Biol. Chem. 256, 3245–3252
44. Octave, J.-N., Schneider, Y.-J., Crichton, R. R., and Trouet, A. (1981) Eur. J. Biochem. 115, 611–618
45. Dautry-Varsat, A., Ciechanover, A., and Lodish, H. F. (1983)
Murine Immunotoxin of High Efficacy and Specificity

Proc. Natl. Acad. Sci. U. S. A. 80, 2258–2262

45. Klausner, R. D., Ashwell, G., van Rensburg, J., Harford, J. B., and Bridges, K. R. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 2263–2266

46. Stein, B. S., and Sussman, H. H. (1986) J. Biol. Chem. 261, 10319–10331

47. Yamashiro, D. J., Tycko, B., Fluss, S. R., and Maxfield, F. R. (1984) Cell 37, 789–800

48. Woods, J. W., Doriaux, M., and Farquhar, M. G. (1986) J. Cell Biol. 103, 277–286

49. Fishman, J. B., and Fine, R. E. (1987) Cell 48, 157–164

50. Regoee, E., Chindemi, P. A., Debane, M. T., and Chartwood, P. A. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 2226–2230

51. Snider, M. D., and Rogers, O. C. (1985) J. Cell Biol. 100, 826–834

52. Snider, M. D., and Rogers, O. C. (1986) J. Cell Biol. 103, 265–275

53. Hopkins, C. R., and Trowbridge, I. S. (1983) J. Cell Biol. 97, 508–512

54. Weissman, A. M., Klausner, R. D., Rao, K., and Harford, J. B. (1986) J. Cell Biol. 102, 951–958

55. Dunn, W. A., Hubbard, A. L., and Aronson, N. N., Jr. (1980) J. Biol. Chem. 255, 5971–5978

56. Weigel, P. H., and Oka, J. A. (1982) J. Biol. Chem. 257, 1261–1207

57. Saraste, J., and Kuismanen, E. (1984) Cell 38, 535–549

58. Saraste, J., Palade, G. E., and Farquhar, M. G. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6425–6429

59. Tartakoff, A. M. (1986) EMBO J. 5, 1477–1482

60. Anderson, R. G. W., and Pathak, R. K. (1985) Cell 40, 635–643

61. Schwartz, A. L., Strous, G. J. A. M., Slot, J. W., and Geuze, H. J. (1985) EMBO J. 4, 899–904

62. Brescher, M. S., Thomson, J. N., and Perase, B. M. F. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 4156–4159

63. Hanover, J. A., Beguinot, L., Willingham, M. C., and Pastan, I. H. (1985) J. Biol. Chem. 260, 15928–15945

64. Harding, C., Heuser, J., and Stahl, P. (1983) J. Cell Biol. 97, 329–339

65. Tran, D., Carpentier, J.-L., Sawano, F., Gorden, P., and Orci, L. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7957–7961

66. Heagy, W. E., and Neville, D. M., Jr. (1981) J. Biol. Chem. 256, 12783–12792

67. Morris, R. E., and Saadler, C. B. (1986) Infect. Immun. 52, 446–453

68. Sandvig, K., and Olanes, S. (1982) J. Biol. Chem. 257, 7504–7513