Kinetics of Protein Synthesis Inactivation by Ricin-Anti-Thy 1.1 Monoclonal Antibody Hybrids

ROLE OF THE RICIN B SUBUNIT DEMONSTRATED BY RECONSTITUTION*

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Richard J. Youle and David M. Neville, Jr.
From the Laboratory of Neurochemistry, Section on Biophysical Chemistry, National Institute of Mental Health, Bethesda, Maryland 20205

Antibody-toxin conjugates of different compositions have been reported to have varying differential toxicities between target and non-target cells in vitro. In this report, we compare the kinetics of protein synthesis inhibition of several different types of anti-Thy 1.1-ricin hybrids. Utilizing two monoclonal antibodies which vary in affinity for the Thy 1.1 antigen by >10^4, thioether-linked hybrids have been made with intact ricin and disulfide-linked hybrids made with ricin A chain. Protein synthesis inactivation kinetics of ricin A chain-S-S-anti-Thy 1.1 IgG is first order and the maximal rate occurs when the Thy 1.1 receptors of AKR cells are saturated. The concentration of the high affinity anti-Thy 1.1 ricin A chain hybrid required to inhibit protein synthesis 90% after 20 h was 10^-2-fold lower than the low affinity hybrid and 10^-3-fold lower than ricin. However, the maximal rate of protein synthesis inhibition by high affinity Thy 1.1-ricin A chain is only one-eighth the rate obtained with ricin. This rate of A chain transport to the cytosol via the Thy 1.1 antigen can be increased 5-fold by adding excess ricin B chain to the cells with no change in the amount of A chain bound to cells. Hybrids between intact ricin and low affinity anti-Thy 1.1 antibodies in the presence of lactose have up to 19-fold faster rates of protein synthesis inhibition than corresponding ricin A chain-antibody hybrids; however, target cell specificity is reduced. We conclude that the ricin B chain facilitates entry of the ricin A chain to the cytosol compartment by a process which is independent of the amount of A chain bound to the surface membrane. These results also show the importance of using kinetic studies to elucidate the variables which affect hybrid-toxin efficacy.

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on the basis of inhibition of protein synthesis was less than 0.1%.

Ricin A chain was purified from ricin D by the method of Cawley et al. (8), taking care to perform the second Sepharose 4B chromatography on the same day.

Anti-Thy 1.1 monoclonal antibody, OX-7, was purchased from Accurate Chemical and Scientific Co., Westbury, NY (16) and anti-Thy 1.1 monoclonal antibody 19E12 (17) was obtained from hybridoma cells (generously provided by R. C. Nowinski, Fred Hutchinson Cancer Research Center, Seattle, WA) grown as ascites in mice and purified over protein A-Sepharose. F(ab')2, prepared as described (18). AKR-SL2 cells were also given to us by R. C. Nowinski.

Protein Synthesis Assay—Protein synthesis was assayed by incubating 10^7 AKR or EL-4 cells in 0.1 ml of RPMI media containing 10% fetal calf serum in 96-well plates. Toxins, lactose, and control solutions were added in 11-μl volumes and incubated with cells for appropriate times and then 20 μl of media containing 0.1 μCi of [3H]leucine were added for 1.5 h. Cells were harvested onto glass fiber filters with a Titertek cell harvester (Flow Laboratories), washed with water, dried, and counted.

Another similar assay was performed with 10^6 cells in 10 ml of media to diminish depletion of high affinity hybrids in the 0.1-ml assay. After the appropriate incubation time, the cells in 10 ml were pelleted and resuspended in 0.1 ml of media, put into 96-well plates, and incubated with [3H]leucine and harvested as in the 0.1-ml assay.

RESULTS AND DISCUSSION

Ricin A Chain Hybrids—Ricin, the ricin A chain, or a variety of hybrids formed between these two toxins and anti-Thy 1.1 monoclonal antibodies was incubated with murine lymphoma cells for 20 h and their effect on protein synthesis was determined (Fig. 1). The least toxic hybrid, the ricin A chain disulfide linked to the F(ab') fragment of 19E12 IgG, (19E12-F(ab')-A) was 7-fold more toxic than ricin A chain on Thy 1.1-expressing AKR cells. Although the affinity of this hybrid was not measured, other studies indicate that it is likely to be about 100-fold lower than the affinity of the bivalent IgG 19E12 (16) which is 10^7 M^-1 at 25 °C. The highest concentration of 19E12-F(ab')-A tested corresponds to 3 × 10^-5 M and saturation of cytotoxicity is not detected. This hybrid shows full A chain enzymatic activity in a cell-free protein synthesis assay (data not shown).

The high affinity A chain hybrid OX-7-A is 10^6 times more toxic than the low affinity hybrid (Fig. 1). The affinity of the OX-7 antibody is >10^10 M^-1 (16). This affinity resulted in

Fig. 1. Dose-response curves of the inhibition of protein synthesis in Thy 1.1 (AKR) or Thy 1.2 (EL-4) cells after 26 h of exposure to ricin or hybrids. All assays were performed in 0.1-ml volume except OX-7-A (AKR), OX-7-ricin (AKR) plus 50 mM lactose, OX-7-ricin (AKR), OX-7-A (AKR), OX-7-ricin (AKR), OX-7-A (AKR), and the more potent curve of ricin, Δ-Δ, which were performed in 10 ml to avoid depletion of the toxins. The less toxic of the two ricin (Δ-Δ) curves was performed in 0.1 ml showing only a 2-fold difference in the two assays. OX-7-ricin in EL-4 cells, □-□; OX-7-ricin plus 50 mM lactose in EL-4 cells, ■-■; ricin plus 50 mM lactose in AKR cells, ▲-▲; ricin plus 50 mM lactose in AKR cells, V-V; ricin plus 50 mM lactose in AKR cells, A-Δ; and A chain in AKR cells V-V.
synthesis, thus 1% surviving cells. Surviving cells after treatment with a saturating dose of ricin extrapolated to 24 h would be $4 \times 10^{-34}$ (where 1% survivors were reached after 1.4 h). Of course there is no assurance that first order kinetics is maintained to this low level of survivors; however, the calculation shows the importance of considering kinetic data to evaluate the efficacy of toxin hybrids. The difference between 1% surviving cells and 10^{-18} could not be observed in standard protein synthesis assays at one time point (3, 20) but would have a vast difference in antitumor efficacy in vivo (21).

**Ricin Hybrids**—We now consider the hybrid formed with ricin and the F(ab)' fragment of the low affinity antibody 19E12, 19E12-F(ab)'. After 20 h in the presence of 50 mM lactose which partially blocks the ricin entry route, this hybrid is 5 times more toxic than ricin plus 50 mM lactose (Fig. 1). The rate of protein synthesis inactivation for 200 ng/ml 19E12-F(ab)'-ricin in the presence of lactose is 14-fold faster than 200 ng/ml ricin in the presence of lactose (data not shown). We can see from Fig. 1 that 240 ng/ml 19E12-F(ab)'-A chain has little toxicity after 20 h and we can calculate that the F(ab)'-ricin conjugate in the presence of lactose is 19 times faster than the F(ab)'-A chain conjugate at inactivating protein synthesis. Apparently the B chain of ricin causes the enhancement of the Thy 1.1 specific entry rate and will be further considered later.

The hybrid formed with the high affinity antibody and intact ricin, OX-7-ricin, is also highly toxic in the 24-h assay performed in 10 ml (Fig. 1). Lactose has a definite effect on reducing the toxicity toward the target cells; however, the hybrid is still 10^6 times more toxic than ricin plus lactose and 10^4-fold more toxic to Thy 1.1 AKR cells than to nontarget Thy 1.2 EL-4 cells (Fig. 1). The inactivation kinetics for OX-7-ricin is shown in Fig. 2D. Comparing the rate of inactivation of OX-7-ricin with that of OX-7-A at saturation (Fig. 2A), it would appear that the presence of the B chain is having a relatively small effect. It is possible that the enhancement of entry rate produced by the B chain in low affinity ricin hybrids or when added to high affinity A chain hybrids (see next section) is not operative for OX-7-ricin. This could be the result of the very tight binding to the Thy 1.1 receptor and the noncleavable character of the chothier linkage between ricin and OX-7 IgG.

**Ricin A Chain Hybrids Plus Ricin B Chain**—The effect of the ricin B chain on enhancing protein synthesis inactivation rates can be clearly demonstrated when B chain is added to mixtures of OX-7-A and target cells. In Fig. 2B, we see the enhancement of the inactivation slopes which is 2-fold for the addition of 170 ng/ml of B and 5-fold for the addition of 1700 ng/ml of B. The maximum effect of ricin B does not seem to have been reached. The target cell specificity in the presence of B is maintained. No toxicity could be detected on EL-4 cells or on AKR cells in the presence of 10 ng/ml of competing OX-7 antibody for 35 ng/ml of OX-7-A plus 1700 ng/ml of B chain (Fig. 2B). The addition of B chain to high affinity ricin A chain hybrids is a way to increase the entry rate of the A chain without unduly sacrificing target cell specificity.

In Fig. 2C, it can be seen that in the presence of 1700 ng/ml of B chain, OX-7-A toxicity still saturates at 176 ng/ml of OX-7-A (compare with Fig. 2A). This indicates that the B chain only facilitates entry of A chain bound specifically to the Thy 1.1 antigen. Another experiment was performed showing that the ricin B chain increased the rate of A chain passage to the cytosol without increasing the number of A chains bound to the cell. OX-7-A chain above saturation was incubated with two series of cells for 1 h. To one series, OX-7-A chain plus 1700 ng/ml of B chain was added and the other series was washed and then treated with 1700 ng/ml of B chain. The total amount of OX-7-A chain in the well equals the amount of OX-7-A chain bound to cells in the unwashed wells. Ricin B chain added to the washed cells accelerated the protein synthesis inactivation beyond that in unwashed wells without ricin B (Table 1). Thus, the accelerated activity of the A chain caused by the ricin B chain was due to some function of the B chain other than binding more A chain to the cell surface. What this activity of the B chain is awaits discovery but most likely requires the galactose binding activity (7). Although OX-7-A exhibits first order inactivation kinetics over 20 h, OX-7-A is not required in the medium past 2 h. Saturating OX-7-A chain was added to cells for 2 h and
and the second incubation was performed with the ricin B chain. These findings have important implications for the time course.

Entry efficiency can be increased by the presence of leucine was added to the cells for 1.5 h and the cells were harvested and their protein synthesis rate determined as described under "Materials and Methods."

Table 1

<table>
<thead>
<tr>
<th>Addition for 1-h incubation</th>
<th>Addition for 3-h incubation</th>
<th>Relative rate of protein synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>880 ng/ml OX-7-A chain</td>
<td>880 ng/ml OX-7-A chain</td>
<td>73</td>
</tr>
<tr>
<td>880 ng/ml OX-7-A chain</td>
<td>1.2 µg/ml ricin B</td>
<td>49</td>
</tr>
<tr>
<td>880 ng/ml OX-7-A chain</td>
<td>12 µg/ml ricin B</td>
<td>29</td>
</tr>
<tr>
<td>None</td>
<td>12 µg/ml ricin B</td>
<td>100</td>
</tr>
</tbody>
</table>

Addition for 2-h incubation: 200 ng/ml OX-7-A chain, 200 ng/ml OX-7-A chain, 200 ng/ml OX-7-A chain, 1.5 µg/ml ricin B, None.

Addition for 4-h incubation: None, 100, 200 ng/ml OX-7-A chain, None, None, None, 1.5 µg/ml ricin B.

replaced with fresh media lacking OX-7-A chain for 18 h. The final rate of protein synthesis was essentially as low as in cells incubated with the same amount of OX-7-A chain over the whole 20 h (Table 1). The OX-7-A chain inhibits protein synthesis only to 80% after 2 h (Fig. 2A); thus, prebound hybrid continues to inactivate protein synthesis over the 20-h time course.

It should be pointed out that there is a major difference in the transport process between ricin, which exhibits a dose-dependent lag period, and OX-7-A or OX-7-A plus B chain which show no detectable lag (Fig. 2). OX-7-ricin at low doses sometimes displays a lag but always less than ricin for similar rates of inactivation of protein synthesis (data not shown) or Fig. 2D. A variety of factors can influence toxin lag periods (22, 23).

In conclusion, the killing of targeted cells by monoclonal antibody-ricin hybrids is a first order process. The high affinity A chain hybrid shows selectivity in dose-response curves at 24 h relative to A chain alone or to the nontarget EL-4 cells of 10^5, yet target cell killing is not greater than 99%, a fact due to the relative low rate of entry of the A chain into the cytosol via the Thy 1.1 receptor. Killing rates are limited by the number of occupied receptors and the efficiency of the entry process. Entry efficiency can be increased by the presence of the ricin B chain. These findings have important implications for the use of hybrid toxins as antitumor reagents and are discussed in detail elsewhere (20).

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REFERENCES
Kinetics of protein synthesis inactivation by ricin-anti-Thy 1.1 monoclonal antibody hybrids. Role of the ricin B subunit demonstrated by reconstitution.
R J Youle and D M Neville, Jr