Hybridoma Cells Containing Intracellular Anti-ricin Antibodies Show Ricin Meets Secretory Antibody before Entering the Cytosol*

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Hybridoma cells which synthesize monoclonal antibodies (mAb) that block ricin toxicity were 50-300-fold resistant to ricin compared with other hybridomas. Two of the mAb blocked two isozymes of ricin, D and E, to different and opposite extents, and the hybridoma cell resistance to the two forms of ricin closely corresponded with the mAb reactivity. The hybridoma cell resistance to ricin was therefore due to the binding activity of the mAb produced by the cells. Neither rabbit polyclonal antibodies, which neutralized extracellular anti-ricin mAb, nor quantitative removal of hybridoma cell surface IgG with papain affected the cellular resistance to ricin. Therefore, neither extracellular or cell surface antibodies contributed to the resistance of the hybridoma cells. In contrast, inhibition of protein synthesis by cycloheximide or puromycin, which selectively decreased levels of intracellular antibody specific for the galactose-binding site of ricin, which selectively decreased levels of intracellular antibody specific for the galactose-binding site of ricin, corresponded with the mAb reactivity. The hybridoma bodies (mAb) that block ricin toxicity were hybridized with monoclonal antibodies in complete Freund's adjuvant. The monoclonal antibodies can also be used to study toxin function within intracellular compartments. An antibody specific for the galactose-binding site of ricin blocks ricin intracellularly, showing that the ricin galactose-binding activity is required in an intracellular compartment for transport of ricin A chain to the cytosol.

Ricin is an extremely toxic protein isolated from castor bean seeds. It has two subunits which have distinct roles in toxicity. The A chain is an enzyme which inactivates eukaryotic ribosomes, whereas the B subunit binds cell surfaces and facilitates entry of the A subunit into the cytoplasm (Olsnes and Pihl, 1982).

How, and from what compartment the toxin crosses the lipid bilayer surrounding the cytosol is unknown. Sandvig and Olsnes (1982) have shown that ricin can enter the cytosol from an intracellular compartment not accessible to extracellular antibodies. However, the results do not rule out additional direct passage of ricin across the plasma membrane, and the intracellular site from which ricin enters the cytosol remains unknown. Morphological studies demonstrate that ricin can enter a variety of intracellular sites including the Golgi apparatus (Nicolson et al., 1975, Gonatas et al., 1980) but, since less than 1% of the internalized ricin enters the cytosol, morphological and cell fractionation studies that localize ricin subcellularly do not distinguish ricin en route to the cytosol from ricin in other compartments not leading to efficient cytosol transport.

We have utilized monoclonal antibodies within hybridoma cells to localize ricin functionally en route to the cytosol. The intracellular antibodies block ricin toxicity indicating that the secretory antibodies meet ricin intracellularly before ricin reaches the cytosol. Our results indicate the other routes to the cytosol, such as direct transport across the plasma membrane, are at most 2% as efficient as the intracellular pathway which meets secretory antibody. Hybridoma cells could be useful to study entry of other proteins, toxins, and viruses into cells.

**EXPERIMENTAL PROCEDURES**

**Cells**—Hybridoma cells were grown in Dulbecco's modified Eagle's medium plus 10% fetal calf serum (FCS), 1 mm 2-mercaptoethanol, and 15 mm Hepes. Jurkat cells, a human leukemic T cell line, were maintained in RPMI and 10% FCS and 10 μg/ml gentamicin.

**Antibodies**—Polyclonal antibodies were raised in rabbits by injection of mouse monoclonal antibodies in complete Freund's adjuvant. Monoclonal antibodies 753B/12, 68 VI/E12, 68 IID3, 68 IA11, 68 IIAS, 68 VD11, and 68 VEG were produced by fusion of SP 2/0 Ag14 myeloma cells with BALB/c spleen cells from mice immunized with purified ricin A or B chains and were generously supplied by Cetus Corp. Monoclonal antibodies 207.E5/E8, 216 BS/C9, 209 H9/G5, 209 G2/F12, 207 A9/E12, and 208 D6/B7 were produced by fusion of NS1 myeloma cells with spleen cells from BALB/c X SJL F1, mice immunized with ricin toxoid made with formaldehyde, according to Pappenheimer et al. (1974), and the mAb, all IgG, will be described in detail elsewhere. Polyclonal and monoclonal antibodies were purified on protein A sepharose (EY et al., 1978).

**Ricin D and E**—Ricin was purified from seeds of Ricinus communis variety Hale, kindly supplied by Dr. Anthony Huang, University of South Carolina, Columbia, SC, by modification of the procedure of Nicolson et al. (1974). Ricin D was freed from agglutinin by passage over a G-75 column of medium at 4°C in PBS plus 0.02% sodium azide, pH 7.1, according to Mise et al. (1977). The ricin peak was applied to a Sepharose 4B column and washed with PBS and sodium azide until nonretained protein and the partially retarded ricin E were washed through as reported previously (Youle and Neville, 1982). Ricin D was then eluted with 10 mM N-acetyl-D-galactosamine in PBS.

**Bioactivity Assays**—Ricin assays were performed in 96 well microtiter plates. Cells at 10^5/well in 100 μl of media (see figure legend for media composition) were added to wells and then toxins, antibodies, or controls were added in 10 μl of PBS ± 0.1% bovine serum albumin. After incubation for appropriate times, [1C]leucine at 0.1 μCi/well was added for 1 h and then cells were harvested with a PHD cell harvester onto glass fiber filters, washed in water, dried, and counted.

**Ricin Uptake**—Uptake of ricin by hybridoma cells was assayed by incubating cells in RPMI plus 5% FCS at 1-1.5 x 10^6 cells/ml with 1211-ricin at 3 x 10^-2 M (specific activity 3.2 x 10^5 cpm/μg). Cells were incubated for 5 min at 37°C and then warmed to 37°C for up to 30

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t On leave of absence from the Istituto di Scienze Immunologiche, Università di Verona, Italy.
**TABLE I**

<table>
<thead>
<tr>
<th>Extracellular and intracellular effect of mAb on ricin</th>
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<tbody>
<tr>
<td>Hybridoma clone</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>207 E5/E8</td>
</tr>
<tr>
<td>75/3B12</td>
</tr>
<tr>
<td>216 B3/C9</td>
</tr>
<tr>
<td>209 H6/C5</td>
</tr>
<tr>
<td>209 G2/F12</td>
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<tr>
<td>207 A9/E12</td>
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<td>68 HI D3</td>
</tr>
<tr>
<td>68 A18</td>
</tr>
<tr>
<td>68 III A5</td>
</tr>
<tr>
<td>68 V D11</td>
</tr>
<tr>
<td>68 V E9</td>
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*Concentration of ricin in ng/ml which inhibits protein synthesis 50% after 3.5 h in RPMI + 10% FCS. Two numbers represent replicate experiments.

**RESULTS**

Hybridoma Cell Resistance to Ricin—Hybridoma cell lines that synthesized anti-ricin monoclonal antibodies were examined for their sensitivity to protein synthesis inhibition by ricin. Cells were washed and exposed to ricin for 3 h and then pulsed with [3H]leucine for 1 h to determine the protein synthesis rate. Three of the 13 clones assayed were 100-fold or more resistant to ricin (Table I). The various monoclonal antibodies synthesized by these 13 cell lines were purified and tested for their effect on ricin toxicity to the human T cell line, Jurkat. The extracellular monoclonal antibodies from the three most resistant clones blocked ricin toxicity more than any of the other antibodies (Table I). At 50 μg/ml the antibody from 75/3B12 (75) and 207E5/E8 clones blocked ricin toxicity 50–100-fold. The hybridoma resistance correlated with the presence of monoclonal antibodies that blocked ricin toxicity.

During the course of this study, the ricin resistance of the 207E5/E8 cell line decreased, as did its antibody production. The hybridoma line was subcloned and one (#8) out of 16 subclones secreted detectable mAb and was also more resistant to ricin toxicity than the other 15 subclones. Fig. 1 shows the ricin dose response of 207E5/E8 clone 8 compared with three other subclones that did not secrete detectable antibody, clones 2, 5, and 11, and compared with the other resistant hybridoma line, 75. The two lines that secreted anti-ricin monoclonal antibodies, 207E5/E8 clone 8 and 75, were more than 100-fold less sensitive to ricin in a 3-h assay than were three hybridoma subclones that did not secrete monoclonal antibody. mAb production closely correlated with hybridoma resistance.

We found that two isozymes of ricin, called D and E (Funatsu et al., 1978), were blocked to different extents by the two mAbs, 75 and 207E5/E8. To determine further that the hybridoma resistance was due to the binding activity of the mAb, we compared the extracellular block of ricin D and E by the mAb with the hybridoma resistance to the two forms of ricin. Jurkat cells had identical sensitivity to ricin D and E and were used to standardize comparisons between antibodies and hybridoma lines. Addition of extracellular 75 mAb blocked ricin D toxicity to Jurkat cells 10-fold more than the block to ricin E. Hybridoma line 75 was 10 times more resistant to ricin D than to ricin E, showing the same isozyme specificity as its mAb (Table II). Conversely, extracellular mAb 207E5/E8 blocked ricin E toxicity to Jurkat 4-fold more than ricin D. The hybridoma line 207E5/E8 was five times more resistant to ricin E than to ricin D (Table II). These results demonstrate that the resistance of the hybridomas qualitatively and quantitatively correlates with the binding specificity of the mAb produced by the hybridoma.

Based on the correlation between the extracellular mAb's effect on ricin and the resistance of 13 hybridoma lines in Table I, along with the correlation of mAb production with hybridoma resistance in 16 subclones and, finally, the isozyme cross-specificity of mAb binding and hybridoma resistance, we conclude that the 75 and 207E5/E8 hybridoma resistance is due to the binding of the mAb produced by the hybridoma.

Localization of Antibody-mediated Resistance to Ricin—The
Extracellular and intracellular mAb specificity for ricin D and E

<table>
<thead>
<tr>
<th>Cells with extracellular or intracellular mAb compared to Jurkat</th>
<th>Fold resistance to ricin D</th>
<th>Fold resistance to ricin E</th>
<th>Ratio of ricin D/racin E resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular 75 (Jurkat) +75/3B12 IgG/Jurkat</td>
<td>21</td>
<td>2.4</td>
<td>0.11</td>
</tr>
<tr>
<td>Intracellular 75 (75/3B12 hybridoma/Jurkat)</td>
<td>19</td>
<td>2.2</td>
<td>0.12</td>
</tr>
<tr>
<td>Extracellular 207 (Jurkat +207E5/8 IgG/Jurkat)</td>
<td>7</td>
<td>27</td>
<td>3.9</td>
</tr>
<tr>
<td>Intracellular 207 (207E5/8 hybridoma/Jurkat)</td>
<td>26</td>
<td>140</td>
<td>5.4</td>
</tr>
</tbody>
</table>

*Extracellular mAb: Jurkat cells were assayed for sensitivity to ricin D or E alone or plus 45 μg/ml purified 75 or 207E5/8 mAb. Incubation lasted for 3 h with a 1-h pulse of [3H]leucine. Fold resistance is calculated as the concentration of ricin D or E plus mAb, which inhibited protein synthesis 50%, divided by the concentration of ricin D or E alone, which inhibited protein synthesis 50%.

*Intracellular mAb: washed hybridoma cells were assayed for sensitivity to ricin D or E for 0 h and then pulsed with [3H]leucine for 1 h. Fold resistance is calculated as the concentration of ricin D or E, which inhibited hybridoma protein synthesis 50%, divided by the concentration of ricin D or E, which inhibited Jurkat cell protein synthesis 50%.

A hybridoma line 75 was resistant to ricin due to the binding of the mAb made by the cell. We examined whether this resistance was due to extracellular IgG, cell surface IgG, or intracellular (secretory) antibody.

The amount of antibody secreted by the freshly washed 75 cells (10^6 cells in 100 μl) within the 3.5-h assay of ricin could have reached 10 ng/ml (Helmreich et al., 1961), or 1000-fold less than the 10 μg/ml level of extracellular 75 required to block ricin toxicity to Jurkat cells 10-fold. To rule out effects of extracellular 75 antibody on ricin, and to block cell surface IgG, we raised polyclonal anti-monoclonal antibodies in rabbits. The effect of the purified rabbit antibodies on the extracellular 75 antibody-block of ricin toxicity to Jurkat cells is shown in Fig. 2A. mAb 75 blocked ricin toxicity approximately 10-fold. Polyclonal anti-75 had no affect on ricin toxicity alone but, when added to 75-treated ricin, completely reversed the block by 75. The polyclonal antibody neutralized at least 45 μg/ml extracellular mAb. We then determined the effect of polyclonal anti-75 on the hybridoma 75 resistance to ricin.

It was shown that extracellular anti-75 did not reduce the resistance of 75 hybridoma to ricin. This result indicates that the mAb imparting resistance to 75 cells is not accessible to extracellular antibodies and that the resistance is not due to secreted or cell surface antibodies.

We directly examined the cell surface IgG levels of 5 ricin-sensitive and resistant cell lines, using flow cytometry. Fig. 3 shows fluorescence profiles of 207E5/8 clone 8, clone 2 and 75 cells stained with goat anti-mouse IgG. The values of cell surface antibody binding obtained in the experiment shown in Fig. 3, along with those obtained in a replicate experiment which included 207E5/8 clone 11 and clone 5, were calculated. The resistant 75 line had a relatively low number of surface antibodies, comparable to the fully sensitive 207E5/8 clone 2, 5, and 11 cell lines and 4-fold lower than the other resistant line, 207E5/8 clone 8. The amount of cell surface IgG does not correlate with resistance.

To rule out further the possibility of cell surface antibodies causing the resistance of 75 cells, we removed the cell surface IgG with dithiothreitol-activated papain (Eady et al., 1974).

Fig. 2. Effect of rabbit antibodies against 75/3B12 monoclonal antibody on extracellular 75/3B12 antibody and on 75/3B12 hybridoma cells. A, Jurkat cells were treated with ricin (□□□□□□), ricin plus 45 μg/ml 75/3B12 (■■■■■■), ricin plus rabbit anti-75/3B12 (△△△△△△), and ricin plus 45 μg/ml 75/3B12 plus rabbit anti-75/3B12 (▴▴▴▴▴▴). Cells were incubated as above in RPMI for 3 h and then pulsed with 0.1 µCi of [3H]leucine for 1 h. B, resistant hybridoma 75/3B12 cells were incubated with ricin alone (◇◇◇◇◇) or ricin plus rabbit anti-75/3B12 antibody (▴▴▴▴▴▴). Sensitive hybridoma 68 V E12 incubated with ricin (ΟΟΟΟΟΩ) is shown for comparison. Assays carried out as in A.

Fig. 3. Flow cytometric analysis of ricin resistant and sensitive hybridoma cell surface IgG. Cells were stained with anti-mouse IgG (——) or negative control anti-human myoglobin (－). A, ricin-sensitive 207E5/8 clone 2; B, ricin-resistant 75/3B12; C, ricin-resistant 207E5/8 clone 8.

Fig. 4 shows the flow cytometric analysis of 75 cells before and after papain removal of cell surface IgG. Fig. 4A shows the negative control staining of 75 cells with a mean free fluorescein equivalent × 10^(-5) of 23, while anti-mouse Fab'
assay of ricin sensitivity, however, lasted 3 h so we checked whether the papain-treated cells re-expressed new or sequestered antibody at the cell surface during the assay period. 75 cells were treated with papain, incubated 3 h at 37 °C as in the ricin assay, and then prepared for flow cytometry. No cell surface antibody was detectable (less than 7% of original levels) on the cells after 3 h (data not shown), which is consistent with a previous report that cell surface antibody slowly reappears after proteolytic removal (Goud and Antoine, 1984). Papain treatment of Jurkat cells or the ricin-sensitive 207E5/E8 clone 5 cells caused no change in ricin sensitivity (Fig. 5 and data not shown). The resistance of 75 cells was therefore not due to secreted or cell surface IgG (see also “Discussion”) and we conclude that the resistance was due to intracellular antibody.

Secretory antibodies are synthesized intracellularly and are cotranslationally segregated into the rough endoplasmic reticulum, transported to the Golgi apparatus, and finally secreted via exocytotic vesicles. These newly synthesized antibodies appear to be causing the intracellular resistance of the hybridoma cells to ricin. If intracellular secretory IgG blocks ricin toxicity, then reducing intracellular levels of secretory IgG should sensitize the cells to ricin. Inhibition of antibody synthesis with cycloheximide or puromycin does not block antibody secretion (Helmreich et al., 1961), and continued incubation of cells with these inhibitors can decrease intracellular levels of mAb by 60% (Helmreich et al., 1962), allowing us to test the model. Protein synthesis in 75 cells was blocked with puromycin or cycloheximide for 1 h at 37 °C to decrease levels of intracellular immunoglobulin, and then cells were washed and assayed for sensitivity to ricin. Fig. 6 shows that inhibition of protein synthesis increased sensitivity of 75 cells 5-fold. Identical treatment of the control cell lines 207E5/E8 clone 5 (Fig. 6) and Jurkat (data not shown) did not affect ricin toxicity. The cycloheximide and puromycin treatments did not affect levels of 75 cell surface IgG (Fig. 4). These results indicate that nascent intracellular mAb accounts for the resistance of 75 cells. Ricin must therefore pass through the compartment containing secretory antibody before reaching the cytosol. Since 75 cells are 50-fold resistant

stained with a mean free fluorescein equivalent of 97. Treatment of 75 cells with DTTC caused no changes in cell surface IgG (data not shown), but treatment with DTTC plus papain decreased the free fluorescein equivalent to 27 (Fig. 4B). This difference reflects more than a 95% decrease in surface IgG levels. Quantitative (20-fold) removal of surface IgG on 75 hybridoma cells in the same experiment as shown in Fig. 4, as well as replicate experiments, caused no significant (always less than a 2-fold) increase in sensitivity to ricin (Fig. 5). The

Fig. 4. Effect of papain, puromycin, and cycloheximide on 75/3B12 cell surface antibody. A, 75/3B12 cells stained with anti-mouse Fab' (-----) or negative control anti-human myoglobin (-----); B, 75/3B12 cells pretreated with papain and then stained with anti-mouse Fab'; C, 75/3B12 cells preincubated with 100 μM puromycin; or D, 100 μM cycloheximide for 1 h at 37 °C and then stained with anti-mouse Fab'.

Fig. 5. Effect of cell surface IgG removal on ricin sensitivity. Resistant hybridoma 75/3B12 cells treated with buffer for 30 min at 37 °C (●●●) or buffer containing 2-3 mg/ml papain (△△△) incubated for 3 h with ricin, and pulsed with [¹³C]leucine. Control, sensitive hybridoma 207E5/E8 clone 5 treated with buffer (○○○) or papain (△△△) and assayed for ricin sensitivity as 75/3B12.

Fig. 6. Inhibition of secretory antibody synthesis and its effect on hybridoma ricin resistance. A, puromycin. 75/3B12 cells were incubated for 1 h at 37 °C in complete Dulbecco’s modified Eagle’s medium (●●●) or media plus 100 μM puromycin (△△△), washed twice, and incubated in leucine-free RPMI + ricin for 1 h and then pulsed with 0.1 μCi of [¹³C]leucine for 1 h. Control ricin-sensitive 207E5/E8 clone 5 cells incubated with medium (○○○) or medium plus puromycin (△△△) and assayed as 75/3B12 cells. B, cycloheximide. 75/3B12 and 207E5/E8 cells were treated exactly as in A except for 50 μM cycloheximide was used in place of puromycin.
to ricin, at least 98% of the native ricin pathway must go through this compartment.

We compared the uptake of ricin and sensitivity to ricin inhibition of protein synthesis of a BALB/c hybridoma, GE-2, and the resistant 75 cells. GE-2 cells secrete monoclonal antibodies against glial cells and serve as a control hybridoma line that does not take up ricin via cell surface antibodies. Table III shows that the GE-2 hybridoma cells were 23 times more sensitive to ricin than 75 cells. Ricin uptake was comparable between the two lines, GE-2 taking up only 1.38 times more than 75 cells. Therefore, intracellular 75 antibody, which blocked ricin toxicity, did not affect ricin binding or uptake by the cells.

**Monensin**—Monensin has been shown to disrupt the Golgi apparatus morphology and to block antibody secretion (Tartakoff, 1983). We examined the effect of monensin on the intracellular ricin resistance of hybridomas making anti-ricin monoclonal antibodies. The 75 cell line sensitivity to ricin was increased 10–100-fold by incubation with 50 nM monensin (Fig. 7). The effect of monensin was not due to decreased extracellular antibody caused by a block in antibody secretion because a block in secretion would cause a larger effect after 4 h rather than 1 h. We found an identical effect of monensin at these two times (Fig. 7). The ricin sensitivity of three control hybridoma cell lines, which displayed no intracellular resistance to ricin, was not affected by monensin (Fig. 7). In monensin-treated cells, where intracellular antibody levels are increased, ricin appears able to circumvent the antibody block en route to the cytosol.

**mAb 207E5/E8**—The cell line 207E5/E8 clone 8 was found to have a much higher level (3–6-fold) of cell surface IgG than 75 cells (Fig. 3). In contrast to their effect of 75 cells, polyclonal antibodies against 207E5/E8 mAb decreased the ricin resistance of 207E5/E8 clone 8 cells several fold, and papain removal of cell surface IgG decreased hybridoma resistance more than 5-fold. These results indicate that the cell surface IgG contributed to resistance of 207E5/E8 clone 8 cells and it serves as a positive control for the 75 cells by showing that the experiments ruling out effects of extracellular and cell surface antibodies in hybridomas is ruling out effects of cell surface IgG. However, 207E5/E8 clone 8 also appears to have an intracellular resistance since blocking or removing cell surface antibody only partially reduced resistance, and inhibition of protein synthesis increased sensitivity to ricin (data not shown).

**DISCUSSION**

Monoclonal antibodies are exquisitely specific probes of cell function. When mixed with intact cells, they selectively bind plasma membrane components facing the medium. However, antibodies can also affect intracellular processes if they can be transferred across the plasma membrane. Antibodies have been introduced into the cytosol compartment by microinjection (Mabuchi and Okuno, 1977), fusion of antibody-containing red blood cell ghosts with cells (Yamaizumi et al., 1979), or by osmotic lysis of endocytotic vesicles containing fluid phase antibody (Okada and Rechsteiner, 1982). One report showed localization of intracellular monoclonal antibodies to the protein secretory compartment by microinjection of mRNA encoding an anti-Golgi monoclonal antibody (Burke and Graham, 1984). The RNA-injected cells synthesized the mAb via the secretory pathway, and the mAb accumulated in the Golgi apparatus, inhibiting vesicular stomatitis virus protein transport to the cell surface.

We report here a nonperturbing, biochemical method of using intracellular antibodies to study toxin entry to the cytosol. We used hybridoma cells which constitutively produce anti-ricin antibodies in the protein secretory pathway. Extracellular immunoglobulin secreted by freshly washed hybridoma cells does not reach significant levels for many hours and is blocked readily by polyclonal antibodies raised against the mAb. Therefore, the only major problem in using intracellular antibodies in hybridomas is ruling out effects of cell surface immunoglobulin. This problem can be tested and overcome in several ways. First, clones can be selected which express low levels of cell surface immunoglobulin. Second, cell surface antibodies can be continually and specifically blocked by anti-mAb polyclonal antibodies (including anti-idiotype). Third, treatment of cells with papain (Eady et al., 1974) or pronase (Goud and Antoine, 1984) can quantitatively

**TABLE III**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Ricin sensitivity</th>
<th>Ricin uptake</th>
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<tbody>
<tr>
<td>GE-2</td>
<td>3 molecules-cell⁻¹-min⁻¹</td>
<td>620</td>
</tr>
<tr>
<td>75B12</td>
<td>70 molecules-cell⁻¹-min⁻¹</td>
<td>450</td>
</tr>
</tbody>
</table>

*Concentration of ricin to inhibit protein synthesis 50% after 3.5 h.
Ricin Entry into the Cytosol

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remove cell surface antibody. Following proteolysis, cell surface antibody slowly reappears, with less than 20% recovery after 3-h incubation at 37 °C (Goud and Antoine, 1984). Finally, selective discharge of secretory IgG allows another distinction between intracellular and cell surface antibody. Incubation of cells with protein synthesis inhibitors at 37 °C for 1 h blocks antibody synthesis, while allowing secretion of accumulated secretory IgG, and decreases intracellular IgG levels by 60% (Helmreich et al., 1962). The treatment does not affect cell surface IgG levels. Unique to this project, a further indication that cell surface antibodies do not inhibit ricin toxicity is available. The 75 mAb binds the ricin B chain specifically at the galactose binding site (Colombatti et al., 1987). Any binding of ricin to cell surface immunoglobulin would mimic binding of ricin to carbohydrate receptors and therefore should increase cell sensitivity, not block it. This effect has already been demonstrated by linking ricin and ricin A chain to several antigens. The hybrid toxins can bind and kill cells via cell surface idiotype binding (Volkman et al., 1982, Morimoto et al., 1983, Killen and Lindstrom, 1984). We have demonstrated, by all of the above criteria, that it is the intracellular, secretory IgG which blocks ricin toxicity. These results are a biochemical confirmation of the morphological demonstration that the endocytotic pathway meets the secretory pathway (Farquhar and Palade, 1981). Our results confirm the conclusion of Sandvig and Olnes (1982) that ricin can enter the cytosol from intracellular vesicles and we extend this conclusion by showing that the intracellular vesicles through which ricin travels to the cytosol contain secretory proteins. Our results also show that at least 98% of the ricin reaching the cytosol does not cross directly across the plasma membrane.

The role of the protein secretory compartment in the activity of other toxins, viruses, and physiologic proteins (or antigenic analogs) which enter cells via receptor-mediated endocytosis will be particularly interesting to examine by this procedure.

Secretory proteins, including immunoglobulins, are compartmentalized during translation into the rough endoplasmic reticulum and are segregated continually from the cytoso by organelle and vesicle membranes as they are transferred between intracellular compartments on their way out of the cell (De Petris et al., 1963, Leduc et al., 1967, Sherr et al., 1971). Following polypeptide synthesis in the ER, the proteins are transported to the Golgi apparatus, undergo posttranslational modifications while migrating through the stacks of the Golgi apparatus, and finally are secreted by fusion of exocytotic vesicles with the plasma membrane. Plasma membrane is internalized continually via endocytic vesicles, retrieving exocytotic vesicle membranes and keeping the cell volume constant. In plasma cells the incoming vesicles fuse with the Golgi apparatus and mix endocytic content with secretory antibody (Ottosen et al., 1980). The endocytotic pathway coupled with the secretory route generates membrane recycling from the Golgi apparatus to the surface and back.

Ricin binds cell surface glycoproteins and glycolipids and is endocytosed to a variety of intracellular compartments including the Golgi apparatus (Gonatas et al., 1975, Olnes and Pihl, 1982, Youle and Neville, 1986). Since plasma cell endocytotic vesicles appear to intersect the protein secretory pathway first at the Golgi apparatus, we propose that the Golgi is the first site of co-compartmentalization of ricin and anti-ricin antibody, and it is the site where the 75 mAb blocks ricin toxicity. This would indicate that ricin does not enter the cytosol via endocytic vesicles or lysosomes. Consistent with this proposal is the observation that neutralizing the low pH in these compartments augments ricin entry to the cytosol (Olness and Pihl, 1982). Perhaps ricin must reach the Golgi apparatus to find a neutral compartment from which it can enter the cytosol. However, a few secretory antibody molecules mislocated in the endosomes could block ricin and explain our results if these few molecules were flushed out of cells by cycloheximide treatment. However, fluid phase uptake of the anti-mAb from the media might be expected to block an effect of mAb in endosomes. The protein secretory compartment would have the capacity to neutralize such incoming anti-mAb with secretory antibodies.

Functions of Ricin B Chain Intracellularly—We have shown that intracellular mAb can be used to localize events subcellularly. Furthermore, antibodies may be used to study toxin mechanism within these locations by using determinant- and function-specific mAb. An important step would be to determine what functions of ricin are required for passage out of the intracellular compartment into the cytosol. The 75 mAb binds the ricin B subunit and blocks ricin toxicity solely by blocking the galactose-binding site on ricin (Colombatti et al., 1987). That 75 mAb blocks ricin toxicity intracellularly means that ricin-galactose interaction within the cell is required for passage into the cytosol.

The ricin B chain specificity of the mAb also rules out a cytoplasmic location of the mAb block, possibly due to low amounts of intracellular mAb mislocated in the cytosol, since the A chain alone in the cytosol inactivates protein synthesis, and its enzymatic activity is not inhibited by B chain specific antibodies.

Previously, it has been shown that the ricin B subunit is required for translocation to the cytosol beyond its role in initial cell surface binding (Youple et al., 1979, Youle and Neville, 1982, McIntosh et al., 1983, Vitetta et al., 1984). We proposed that intracellular galactose receptors mediate this effect of ricin B because Mannose 6-P04- and antibody-ricin conjugates (immunotoxins), which bound cells via new receptors, still required the ricin B chain galactose-binding activity but did not require it at the cell surface (Youle et al., 1981). The results of the present report are consistent with these earlier conclusions in that we have shown that blockage of the ricin galactose-binding site intracellularly (putatively, the Golgi apparatus) blocks toxicity. This study indicates that the previously proposed intracellular receptors required for ricin entry to the cytosol exist in the intracellular compartment containing secretory proteins. Interestingly, the Golgi apparatus contains more mAb-binding sites than any other cellular compartment (Yokoyama et al., 1980), because it is the intracellular site of galactosyl transferase (Roth and Berger, 1982). How galactose binding in the Golgi may help ricin reach the cytosol is unknown. Galactose binding may reduce ricin recycling back to the surface, it may allow ricin to recycle on a proposed pathway from the Golgi to the rough endoplasmic reticulum (Farquhar and Palade, 1981), or it may play a direct role in membrane translocation within the Golgi apparatus.

Thus, by several criteria, we have shown that at least part, if not all, of the ricin B chain translocation activity requires intracellular galactose binding. Therefore, retention of the ricin B chain translocation activity in immunotoxins with concomitant exclusion of the galactose-binding function may not be possible (Youle et al., 1981, Thorpe et al., 1984, Vitetta and Uhr, 1985).

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