points must be considered before this conclusion is established definitively. Among the most important is the fact that although the amount of DNA in the membrane fraction increases with incubation time in supplemented cell suspensions, the amount of DNA in the top fraction, which is initially greater, decreases with incubation time. This inverse correlation could indicate that DNA from the top fraction is released selectively into the membrane fraction with increasing incubation time. For example, deoxyribonuclease activity could increase during incubation of cell suspensions and degrade DNA in the top fraction in quantitative amounts after cell lysis, or DNA could become more sensitive to shear with incubation time. However, no manipulation of cell lysates occurred after addition of the sarcosyl detergent that might cause shearing of the DNA. In addition, if substantial deoxyribonuclease activity were present, little or no DNA would be present in any of the fractions, since acid-washing of the extracts before assay of DNA would presumably remove the products of deoxyribonuclease digestion. Even if some large oligodeoxyribonucleotides were not removed by acid-washing, they would have sedimented in the sucrose gradient in a heterogeneous manner instead of in two main peaks in the top and membrane fractions. Another possibility is that since phospholipids, kinases, and the DNA polymerase are present in the top fraction, DNA is first synthesized in the top fraction and then subsequently bound to the membrane fraction. Although this possibility cannot be eliminated, it must be viewed in relation to the fact that greater amounts of DNA polymerase and deoxyribonucleotide kinase activities are present in the DNA-membrane fraction than in the top fraction. A third possibility, which we favor, is that DNA is replicated in a discontinuous manner, as hypothesized by Okazaki *et al.* (*7*), in the membrane fraction. This would imply (i) that DNA in the top fraction is derived originally from DNA in the membrane fraction but has not yet been covalently bound to DNA in the latter fraction, (ii) that the molecular weight of DNA in the top fraction during early incubation times (less than 10 minutes) is smaller than that of DNA in the membrane fraction, and (iii) that the decrease in percentage of DNA in the top fraction with incubation time reflects the binding of the segments of DNA.

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Selective Destruction of Target Cells by Diphtheria Toxin Conjugated to Antibody Directed against Antigens on the Cells

Abstract. *Monkey-kidney cells bearing new surface antigens induced by infection with mumps virus were lysed selectively by diphtheria toxin conjugated to antibody against mumps antigens.*

Despite evidence that animal and human tumors carry unique antigens (*1*), the conceptual promise of immunotherapy for the control of cancer has resisted practical exploitation. Once autochthonous tumors develop, host immune responses to them are rather weak; furthermore, as they are mediated primarily by delayed hypersensitivity, they are difficult to regulate and to transfer passively to tumor-bearing hosts. Although easier to manipulate, humoral antibodies against tumor-specific antigens have so far proved ineffectual in vivo, despite evidence from sensitive assay methods that they can injure tumor cells in tissue culture; more often than not, circulating antibodies serve to enhance tumor growth (*2*). We now report a method for killing antigen-bearing cells with antibody, using the antibody as a distinctive means for directing a potent cytotoxin to the target cells.

As an optimal model system toward which the antibody-toxin complex could be aimed, we first explored the use of antibodies against viral antigens that appear in abundance on the surface of cells infected with mumps virus, as this was operationally less difficult than working with antibodies against tumor-specific antigens.

Primary cultures of rhesus monkey kidney (*3*) were infected with about 10^4 tissue culture infectious doses of mumps virus, and maintained at 36°C in 1.5 ml of Eagle's minimum essential medium (MEM) which contained 2 percent fetal calf serum that had been previously inactivated at 56°C for 30 minutes. Under these circumstances, mumps virus replicates slowly, so that the cells appeared morphologically intact and excluded a vital dye (erythrosin) for at least 7 to 8 days after infection, although viral antigen in the cell membrane was demonstrable by hemadsorption as early as 4 days after infection.

Diphtheria toxin was purified by the method of Goor and Pappenheimer (*4*), except that we used 0.15*M* phosphate buffer, *p*H 6.5, to elute the toxin from the diethylaminoethyl (DEAE) cellulose column, and phosphate-buffered saline, *p*H 7.4, as our final solvent. The final preparation contained 2000 flocculating (Lf) units per milliliter and 2.5 μg of protein per Lf unit. The immunoglobulin G (γG) fraction was isolated from guinea pig antiserum to mumps virus (5) by chromatography on DEAE cellulose (*6*, pp. 322–25).

Toluene diisocyanate (TDIC) was used to conjugate antibody to toxin (*6*, pp. 150–55). Liquid TDIC (0.1 ml) was added to 20 to 25 mg of γG in 5 ml of phosphate buffer, *p*H 7.5, ionic strength 0.1, and the mixture was stirred at 0°C for 30 minutes. The precipitated TDIC was then removed by centrifugation, and the supernatant was filtered through a Millipore HA filter. After standing at 0°C for another 50 minutes, the clarified solution was then mixed with diphtheria toxin (0.75 mg per milligram of antibody; 1.8 moles

of diphtheria toxin per mole of antibody) in 16 ml of borate buffer, *p*H 9.5, ionic strength 0.1, and the mixture was stirred gently at 36°C for 1 hour to permit conjugation of the two proteins. The mixture was next dialyzed against 1 percent ammonium carbonate at 36°C for 1½ hours and then against phosphate-buffered saline at 4°C for 14 hours. The solution was then concentrated by ultrafiltration in a collodion bag and chromatographed on a Sephadex G-200 column (20 by 350 mm). Antibody-toxin conjugates were eluted in the void volume. Free toxin was retarded; its maximum concentration appeared at 180 percent of the void volume. Complete separation of the two peaks was not achieved, so that small residues of free toxin probably contaminated the preparation. Our stock solution of conjugates contained 0.43 mg of protein per milliliter of phosphate-buffered saline.

Toxin conjugated to antibodies against mumps virus (anti-mumps conjugate) demonstrated a striking cytotoxicity which was selectively directed against cells containing mumps antigen (Table 1 and Fig. 1). Within 18 hours, many cells in the affected cultures had become pyknotic, and by 24 hours, extensive cytolysis was apparent. The selectivity of this phenomenon was not absolute, however. When uninfected cultures were examined 48 hours after treatment with the highest dose of conjugate, slight to moderate cytolysis was visible. This nonspecific toxicity may well reflect contamination of the conjugate with free toxin. In contrast, unconjugated mumps antiserum inflicted no visible injury in mumps-infected cultures.

Confirmatory evidence for the immunologic basis of the selective toxicity exhibited by the conjugate was sought by absorbing it with mumps-infected cells. Conjugate so absorbed lysed cells more slowly and less extensively than did conjugate absorbed with uninfected cells.

Such data did not directly exclude an additional explanation for the selective lysis of mumps-infected cell, namely, that the metabolic burden imposed by viral infection had rendered them hypersusceptible to dipththeria toxin. Consequently, the responses of normal and infected cells to unconjugated toxin were compared. Table 1 shows that both populations were equally susceptible. The data also show that in unin-

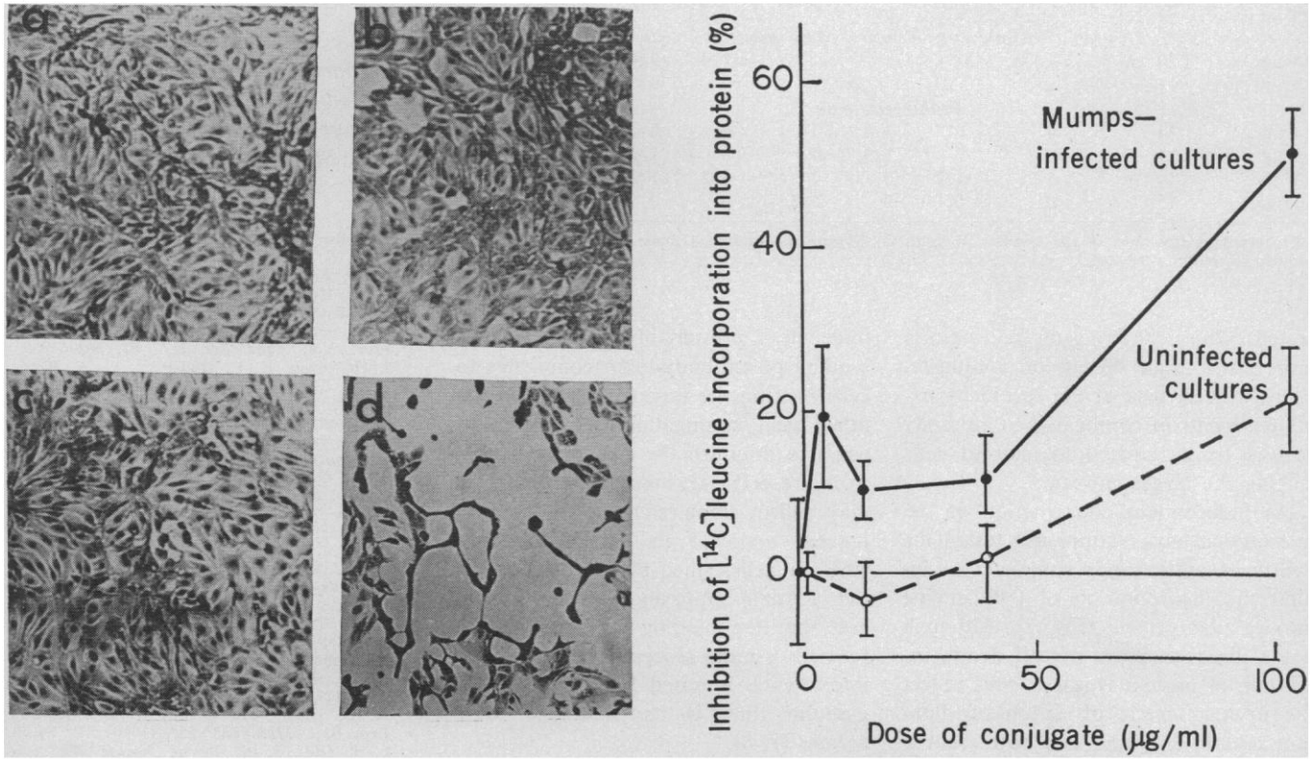

Fig. 1 (left). Effect of mumps antibody-diphtheria toxin conjugate (anti-mumps conjugate) on mumps-infected monkey kidney cells. (a) Normal monkey kidney cells; (b) uninfected monkey kidney cells 24 hours after treatment with anti-mumps conjugate; (c) monkey kidney cells 6 days infection with mumps virus; (d) monkey kidney cells 6 days after infection with mumps virus and 24 hours after treatment with conjugate (× 100). Fig. 2 (right). Inhibition of protein synthesis by anti-mumps conjugate in mumps-infected monkey kidney cells. Cultures were washed with Hanks balanced salt solution, 0.6 ml of serum-free MEM containing the indicated doses of conjugate was added, and the cultures were incubated at 36°C for 5 hours. [^{14}C]Leucine (*11*) was then added (either 0.64 or 0.80 μc, control and conjugate-treated cultures always receiving identical doses), and, after a further hour of incubation, the cultures were processed for assay of the radioactivity in the trichloroacetic acid–precipitable fraction (*12*). The percentage of inhibition was calculated as the ratio of the difference between the radioactivity (count/min) in untreated cultures and that in treated cultures to the radioactivity in the untreated cultures (× 100). Each value is the mean obtained from duplicate cultures of uninfected cells (except for the highest dose, tested in quadruplicate), and from triplicate cultures of mumps-infected cells (except for the highest dose, tested in six replicate cultures). Vertical bars show standard errors of the mean. Cultures that were untreated with conjugate synthesized protein at the same rate, whether mumps-infected or uninfected, incorporating 1.33 plus or minus 0.10 percent and 1.35 plus or minus 0.05 percent, respectively, of the administered leucine into protein during the 1 hour of incubation (mean ± standard error). The responses of infected and uninfected cells to the highest dose of conjugate differed significantly ($P < .01$).

Table 1. Effect of conjugate of mumps antibody with diphtheria toxin on monkey kidney cells infected 5 days previously with mumps virus. After cultures were washed with Hanks balanced salt solution, 1.5 ml of fresh medium and 0.1 ml of the indicated dose of conjugate or free toxin in phosphate-buffered saline, or 0.1 ml of undiluted, heat-inactivated guinea pig antiserum to mumps virus were added. The cultures were incubated at 36°C, and examined microscopically for cytolysis at the specified intervals. Cultures demonstrating less than 5 percent cell loss were scored as 0; higher grades of lysis were scored as follows: Less than one-third of cells lysed, +; one-third to two-thirds of cells lysed, ++; more than two-thirds of cells lysed, +++. Vital staining with erythrosin demonstrated that, in all treatment groups, a grade of 0 was associated with exclusion of the dye by more than 95 percent of the cells. The absorption procedure consisted of mixing 43 μg of conjugate in 0.1 ml of phosphate-buffered saline for 1 hour with either uninfected or mumps-infected cells pooled from five culture tubes. After the suspension was diluted to 0.5 ml with PBS, the cells were removed by centrifugation, and 0.1 ml portions of the supernatant were tested in mumps-infected cultures.

Dose	Time after treatment (hr)	Proportion of cultures displaying different grades of lysis*							
		Uninfected cultures grade				Mumps-infected cultures grade			
		0	+	++	+++	0	+	++	+++
Anti-mumps conjugate									
43 μg	24	9/9	0	0	0	0	1/14	9/14	4/14
8.6 μg	24	1/1	0	0	0	0	2/2	0	0
1.7 μg	24	1/1	0	0	0	1/2	1/2	0	0
Mumps antiserum									
0.1 ml	24					3/3	0	0	0
0.1 ml	45					3/3	0	0	0
Anti-mumps conjugate absorbed with mumps-infected cells									
8.6 μg	30					6/6	0	0	0
8.6 μg	45					0	5/6	1/6	0
Anti-mumps conjugate absorbed with uninfected cells									
8.6 μg	30					1/4	3/4	0	0
8.6 μg	45					0	0	2/4	2/4
Diphtheria toxin									
2.5 μg	24	0	0	3/3	0	0	0	3/3	0
0.8 μg	24	0	0	2/2	0	0	1/2	1/2	0
0.25 μg	24	1/2	1/2	0	0	1/2	1/2	0	0
0.25 μg	45	0	0	0	2/2	0	0	1/2	1/2

* Expressed as the ratio of the number of cultures showing the indicated grade of lysis to the total number of cultures treated.

fected cultures, 0.25 μg of free toxin is more toxic than 43 μg of conjugate. Thus, at least part of the specificity exhibited by toxin conjugated to antibody derives from reduced toxicity to cells lacking the target antigen.

As independent confirmation of the microscopic observations, we tested the ability of anti-mumps conjugate to inhibit the incorporation of [^{14}C]leucine into cellular protein (Fig. 2). Although not a direct measure of cell death, inhibition of protein synthesis does reflect the primary mode of action of diphtheria toxin (*7*), and can be measured more precisely than visual grading of cell lysis. At all doses, anti-mumps conjugate inhibited protein synthesis more strongly in infected than in uninfected cells; with the largest dose this difference was highly significant. Mumps infection itself induced no gross derangements of protein synthesis—infected and uninfected monkey kidney cultures synthesized protein at identical rates.

The selective cytotoxicity of the conjugate probably involves two divergent effects. On the one hand, antibody molecules presumably promote the binding of antibody-toxin conjugates to cells bearing the target antigen. On the other hand, conjugation to antibody appears to diminish the capacity of toxin to injure cells lacking the antigen. This may reflect impaired binding by conjugated toxin to its normal receptor sites on cells, since studies with diphtheria toxin-antitoxin complexes suggest that the binding affinity of toxin for cells is more susceptible to the steric interference imposed by linkage to γ-globulin than is its metabolic toxic actions (*7*, *8*).

Complement was omitted from the experimental system, as its presence caused even unconjugated antibody to be cytotoxic, confirming studies with other viruses (*9*). This complement-mediated toxicity was demonstrable only by vital staining, and was minimal compared to the extensive cytolysis produced by the antibody-toxin conjugate.

Whether conjugates that are highly selective and highly potent in vivo can be made with diphtheria toxin and antibodies directed against tumor-specific antigens remains to be determined. Tumor cells are unlikely to bear tumor-specific antigens on their surface in the abundance characteristic of mumps antigens. Therefore, the major prerequisite demanded of a toxin guided to its target by antibody—extreme potency—assumes particular importance in the case of tumor antigens assumed to be sparsely distributed. Diphtheria toxin nonetheless appears reasonably capable of satisfying such demands, since fewer than 50 molecules of toxin bound to the surface of a cell suffices to produce cytocidal effects (*8*). If some tumor-specific antigens prove too small a target to achieve even this modest concentration of functional toxin molecules, efforts directed toward introducing new antigens onto tumor cells may deserve exploration, possibly using viruses that will tend to become localized selectively in tumors (*10*).

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