Microtubule Function in Immune and Nonimmune Lymphocyte-Mediated Cytotoxicity

Abstract. *Disaggregation of microtubular subunits in effector lymphocytes inhibits their ability to injure target cells. The inhibition is not reversed by deuterium oxide, an agent known to stablize microtubular subunits.*

It has become apparent that there are at least two in vitro models in which lymphocytes may exert an immunologically specific cytotoxic effect on target cells. Lymphocytes explanted from animals sensitized with allogeneic cells or tissue are specifically cytotoxic to cells bearing the sensitizing alloantigens (*1*). The effector cell in this model is a thymus-derived (T) lymphocyte (*2*). In a second in vitro model, nonimmunized, thymus-independent lymphocytes may be cytotoxic to target cells coated with small amounts of antibody to target cells (*3*). The cellular and subcellular basis of the lymphocyte and target cell interaction resulting in target cell destruction is incompletely understood in both models; however, soluble, secreted products of the lymphocyte may be required in the cytotoxic process.

We have demonstrated an in vitro system in which allograft-sensitized rat lymphocytes are selectively cytotoxic to cells bearing the sensitizing alloantigens (*4*). The presence of T lymphocytes (*2*) but not bone marrow-derived (B) lymphocytes is required in the generation of cytotoxicity (*5*) in similar in vitro systems. Macrophages contribute only modestly, if at all, toward producing a cytotoxic effect (*6*). It has also been demonstrated that the in vitro cytotoxic effect of sensitized lymphocytes is modulated by adenosine 3′,5′-monophosphate (cyclic AMP) (*4, 7*) and guanosine 3′,5′-monophosphate (*4, 8*). When minute quantities of antiserum to target cells (Lewis antiserum to Brown Norway rat cells) and unsensitized Lewis splenocytes are added to target cells, injury occurs, while unsensitized cells or antibody alone have no effect. Other investigators have shown that the cytolysis of antibody-coated target cells is effected by thymus-independent (*3*) lymphocytes which are probably B lymphocytes (*9*). Complement is not required in this lytic process (*10*). The antiserums to target cell must contain immunoglobulin G (IgG) (*10*), including its Fc fragment (*11*), in order to facilitate cytotoxicity by nonimmune lymphocytes. Modulation of antibody-facilitated lymphocyte-mediated cytotoxicity by the cyclic nucleotides has also been demonstrated (*12*).

The plant alkaloids, colchicine and vinblastine, specifically bind to microtubule protein and result in disaggregation of microtubular subunits; this process causes destruction of microtubular function (*13*). The effects of colchicine and vinblastine on allograft-sensitized and nonimmune lymphoid populations in assay systems quantitating target cell injury have therefore been studied. There has been one report that the addition of $2.5 \times 10^{-5}M$ colchicine to attacking and target cell mixtures was without effect in a similar system (*14*); however, our study indicates that microtubule function is involved in mechanisms by which both types of lymphocytes injure target cells. These studies were prompted by the known involvement of microtubules in several processes of cellular release (*15–19a*), many of which are modulated by cyclic AMP, as is the cytotoxic effect of lymphocytes.

Male Lewis, Lewis × Brown Norway F_1 (LBN), and Brown Norway (BN) rats were purchased from Microbiological Associates (Bethesda, Maryland). Colchicine was purchased from the Sigma Chemical Company, vinblastine sulfate was purchased from Eli Lilly and Company, RPMI-1640 medium was purchased from GIBCO, and deuterium oxide (D_2O) from Bio-Rad Laboratories. The cytotoxic action of lymphocytes on cells bearing alloantigens to which they are sensitized (immune lymphocyte-mediated cytotoxicity) was quantitated by a modification of Brunner's method (*1*); the methods have been reported (*4*). In brief, splenocytes were explanted from Lewis rats sensitized with a LBN skin allograft. The sensitized Lewis splenocytes were used as attacking cells, and ^{51}Cr-labeled BN thymocytes served as target cells. Cytotoxicity was quantitated by ^{51}Cr release from attacking and target cell mixtures after 4 hours of incubation at 37°C.

The cytotoxic effect of unsensitized Lewis splenocytes on antibody-coated BN thymocytes was similarly quantitated by ^{51}Cr release in mixtures containing 5×10^6 unsensitized Lewis splenocytes, 5×10^4 ^{51}Cr-labeled BN thymocytes and a 1 : 1000 dilution of Lewis hyperimmune antiserum to BN. Lewis antiserum to BN was harvested after Lewis rats received BN skin allografts and subsequently given intraperitoneally 10^7 to 10^8 cells pooled from

Table 1. Effect of colchicine and vinblastine on immune lymphocyte-mediated cytotoxicity. The numbers in parentheses are the ranges of triplicate samples.

	Untreateed (%)	Treated (%)					
		Colchicine			Vinblastine		
		$10^{-4}M$	$10^{-5}M$	$10^{-6}M$	$10^{-4}M$	$10^{-5}M$	$10^{-6}M$
*Agents in mixtures of attacking and target cells**							
Cytotoxicity	36 (34-38)	18 (17–19)	24.5 (23–26)	29.5 (30–28.5)	20 (19–21)	26 (25.5–26.5)	31 (30–32)
Suppression		50 (47–53)	32 (28–36)	18 (17–20.8)	45 (42–47)	28 (26–29)	14 (11–17)
Cytotoxicity	42 (41–43)	23 (22–24)	26 (25–27)	34 (33–35)	25 (24.5–25.5)	29 (27–31)	38 (37–39)
Suppression		45 (43–48)	38 (36–40)	19 (17–21)	40 (39–42)	31 (26–36)	5 (7–12)
Prior incubation of attacking cells†							
Cytotoxicity	43 (42–44)	27 (26–28)	32 (30–34)	39 (39–39)	27 (26–28)	34 (33–35)	41 (40–42)
Suppression		37 (35–40)	25.5 (21–30)	1.7 (1.7–1.7)	37 (35–40)	20 (19–23)	4.6 (2.3–6.9)
Prior incubation of target cells‡							
Cytotoxicity		43 (41–45)			42 (41–43)		
Suppression		0 (− 4.6–4.6)			2.3 (0–4.6)		

* The results of two experiments are shown in which sensitized Lewis splenocytes were used as attacking cells in the presence or absence of the plant alkaloids. † Sensitized Lewis splenocytes were divided into subpopulations. Some populations were untreated, while others were first incubated with colchicine or vinblastine for 30 minutes at 37°C. All cells were washed and compared for cytotoxic capability. ‡ ^{51}Cr-labeled thymocytes were either first incubated with the plant alkaloids for 45 minutes at 37°C or left untreated. All cells were washed and compared for susceptibility to cytolysis.

Table 2. Effect of colchicine and vinblastine on antibody-facilitated nonimmune lymphocyte-mediated cytotoxicity. The numbers in parentheses are the ranges of triplicate samples.

	Untreated (%)	Treated (%)					
		Colchicine			Vinblastine		
		$10^{-4}M$	$10^{-5}M$	$10^{-6}M$	$10^{-4}M$	$10^{-5}M$	$10^{-6}M$
		*Agents in mixtures of attacking and target cells**					
Cytotoxicity	19 (18–20)	8 (6–10)	14 (13–15)	17 (16.5–17.5)	9 (8–10)	15 (13.5–16.5)	18 (18–18)
Suppression		58 (47–68)	26 (21–32)	10 (8–13)	53 (47–58)	21 (13–28)	5 (5–5)
Cytotoxicity	13 (12.5–13.5)	8 (7–9)	10 (10–10)				
Suppression		38 (30–46)	23 (23–23)				
		Prior incubation of attacking cells†					
Cytotoxicity	19 (17–21)	7 (6–8)	9 (8–10)	10 (10–10)	9 (8–10)	12 (11–13)	
Suppression		63 (57–68)	53 (47–58)	47 (47–47)	53 (47–58)	37 (32–42)	
		Prior incubation of target cells‡					
Cytotoxicity		19 (18–20)			19 (17–21)		
Suppression		0 (−5–5)			0 (−10.5–10.5)		

* The results of two experiments are shown in which unsensitized Lewis splenocytes were used as attacking cells in the presence or absence of the plant alkaloids. † Unsensitized Lewis splenocytes were divided into subpopulations. Some populations were untreated, while others were first incubated with colchicine or vinblastine for 30 minutes at 37°C. All cells were washed and compared for cytotoxic capability. ‡ ^{51}Cr-labeled thymocytes were either first incubated with the plant alkaloids for 45 minutes at 37°C or left untreated. All cells were washed and compared for susceptibility to cytolysis.

BN spleen, thymus, and bone marrow at biweekly intervals for a total of seven injections. The antiserum had a lymphocytotoxicity titer of 8500 50-percent lytic units per milliliter. For all experiments RPMI-1640 medium containing 10 percent heat-inactivated fetal calf serum and buffered with HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) was the support medium.

When colchicine or vinblastine was added to sensitized splenocyte and target cell mixtures, a dose-dependent inhibition of cytotoxicity was discerned (Table 1). In order to determine whether inhibition was due to target cell stabilization or a reduction in the cytotoxic capability of sensitized lymphocytes, attacking and target cells separately were first incubated at 37°C for 30 minutes with colchicine or vinblastine. The treated cell populations were washed, and their functional characteristics were compared to those of untreated populations. A dose-dependent inhibition of cytotoxicity was noted in experiments with attacking cell populations that had been treated previously with colchicine and vinblastine (Table 1). The target cells treated with colchicine and vinblastine had an unaltered susceptibility to cytolysis when compared to untreated cells. When attacking cells were first incubated with colchicine or vinblastine at 4°C for 45 minutes, no inhibition of cytotoxicity was discerned.

We have demonstrated that control incubations containing only 5×10^4 target cells and incubations also containing 5×10^6 unsensitized attacking cells released identical low amounts of ^{51}Cr; thus no specific cell lysis was discerned (*4*). Similarly when a 1 : 1000 dilution of Lewis hyperimmune antiserum to BN was incubated under experimental conditions with ^{51}Cr-labeled BN thymocytes no specific cell lysis was noted; however, when unsensitized Lewis lymphocytes, ^{51}Cr-labeled BN thymocytes, and a 1 : 1000 dilution of Lewis antiserum to BN was incubated at 37°C for 4 hours, specific cell lysis was noted. Lysis was not noted if Lewis thymocytes were substituted for BN target cells. A dose-dependent decrease in the cytotoxic effect of unsensitized splenocytes on antibody-coated target cells was noted when colchicine or vinblastine was added to the experimental mixture (Table 2). By first incubating the unsensitized splenocyte and BN target cell populations separately with the plant alkaloids at 37°C, the mechanism of inhibition of cytotoxicity was determined. As in the previous experiments, the cytotoxic activity of splenocytes first incubated with either of the alkaloids was dampened, whereas there was no effect on the susceptibility of target cells to cytolysis (Table 2).

The inhibition of cytotoxicity in both systems (that is, immune and nonimmune lymphocytes) was not altered by a 10-minute delay in addition of the plant alkaloids to the mixture of attacking and target cells. These results are in contrast to their effects on leukocyte histamine release which is dampened by prior exposure to antigen (*18*). Deuterium oxide, a stabilizer of microtubule subunits (*19*), failed to reverse the effects of colchicine or vinblastine when present in the medium at 25, 50, or 75 percent concentration, nor did D_2O alone cause enhanced cytotoxicity. In fact, D_2O when present in the medium at 75 percent concentration caused a modest (20 percent) inhibition in the cytotoxicity produced by immune and nonimmune lymphocytes. Similar experimental data have been obtained while investigating thyroid secretion (*19a*) and interpreted as showing secretion to be dependent on an ability to rearrange microtubules as well as on the presence of intact microtubules. Although the inability of D_2O to enhance cytotoxicity might be due to a stabilizing effect on the target cell, rendering it less susceptible to cell-mediated lysis, D_2O did not have an appreciable effect on antibody-mediated lysis of lymphocytes in the presence of fresh complement.

Microtubules are a universal component of the eukaryotic cell. The role of microtubules in the spindle cell apparatus has been long appreciated. Data have been reported implicating microtubules in many other processes including chemotaxis (*20*), platelet aggregation (*21*), topographical distribution of membrane proteins (*22*), and the release of intracellular stored granules (*15, 16*). Vinblastine has no effect on the release of migration inhibitory factor from sensitized lymphocytes (*23*). It has been noted that many of these functions are also affected by cyclic AMP (*24*). Furthermore, cyclic AMP has a biphasic effect on the binding of colchicine to microtubular subunits (*24*). Our study gives support to the concept that lymphocyte-mediated cytotoxicity is a secretory process, although microtubule function may contribute to the cytotoxic process via other mechanisms, including alterations in membrane surface configuration (*24, 25*).

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25. *Note added in proof*: Since this report was submitted, M. Plaut, L. M. Lichtenstein, and C. S. Henney [*J. Immunol.* **110**, 771 (1973)] have also reported that microtubules (and microfilaments) are involved in the cytotoxic process mediated by immune lymphocytes.

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Observation Is Insufficient for Discovering that the Surface of Still Water Is Invariantly Horizontal

Abstract. *Among women college students who did not know that the surface of still water in a bottle is always horizontal, two types of specific task procedures designed to elicit self-discovery of the principle were ineffective. Failure to acquire the concept was reflected in inaccurate responses on the adjustment task and by inability of students to verbalize a correct strategy.*

Years of research involving hundreds of subjects of preschool through college age indicates that by 12 years of age boys understand the principle that the surface of still water remains horizontal (*1*). Girls, however, lag behind boys at all ages in this respect, and about 50 percent of college women still do not know this principle (*1*). We report here two studies which demonstrate that college women who do not know the principle do not readily learn it in tasks designed to optimize self-discovery of the concept.

Figure 1 shows the two parts of the apparatus. On the left is a disk 27.5 cm in diameter on which is mounted a bottle 9.5 cm in diameter and 14 cm tall. This bottle, called the model, is half filled with red water (the bottle is covered in Fig. 1). On the right is half of an identically shaped bottle similarly mounted and called the pretend bottle. It always appears to be half filled with "red water." What is actually visible through the pretend bottle is part of a rotatable disk 24 cm in diameter, half red and half white.

In use, both bottles are positioned to the same clock-numeral orientations (for example, at 1 o'clock the bottles are tilted 30° to the right as in Fig. 1). The subject's task is always to adjust the pretend waterline to the position she believes the real water has taken.

In both studies women were recruited from introductory psychology sections at Pennsylvania State University. Each subject briefly viewed the water in the model as it was rotated. Then a cover (Fig. 1) was placed on the bottle and the subject was asked to adjust the pretend waterline for eight oblique bottle angles, presented in random order: 1, 2, 4, 5, 7, 8, 10, and 11 o'clock. Before each adjustment the pretend waterline was preset to a randomly selected angle that deviated by at least 45° from the horizontal; then the bottles were moved to their appropriate positions. After the subjects' predictive adjustments, which we call the pretest, their responses to the following two interview questions were recorded: (i) "How did you know where to put the pretend water level?" (ii) "What is the principle or the idea which determines where the water goes in the bottle?"

If seven pretest adjustments were within 4° of the horizontal (*2*), the subject was classified "sophisticated" (So) and dismissed. Remaining subjects were classified "naive" (Na) and retained for training. In study 1, 47 subjects were given the pretest to obtain 30 Na subjects. Of 44 subjects recruited in study 2, 33 were defined Na.

Studies 1 and 2 differ in their training conditions. In study 1, one training trial involved these steps. The subject (i) makes a predictive adjustment with the model covered, as in the pretest; (ii) removes the cover from the model revealing the real waterline; (iii) readjusts the pretend waterline to match the real waterline whenever she perceives her adjustment to be in error; and (iv) replaces the cover on the model. Train-

Table 1. Estimated medians (M) and 95 percent confidence intervals (CI) for absolute errors for naive subjects in studies 1 and 2 and for criterion women and unselected men. Results are given in degrees; *N*, number of subjects.

Bottle orientation	Study 1				Study 2				Criterion women ($N = 38$)		Unselected men ($N = 62$)	
	Pretest ($N = 30$)		Block 4*		Pretest ($N = 33$)		Posttest					
	CI	M	CI	M	CI	M	CI	M	CI	M	CI	M
1 o'clock	8.5-23.0	16.0	4.5-11.5	7.0	8.0-14.0	10.5	5.5-11.5	9.0	2.5-4.5	3.5	1.5-3.5	2.5
2 o'clock	9.0-25.0	13.5	3.5- 9.0	6.0	10.0-18.0	14.0	4.0-11.0	6.5	1.5-2.5	2.0	1.5-3.0	2.0
4 o'clock	15.0-29.0	23.0	8.0-21.5	15.0	11.5-21.0	16.0	3.0- 8.0	5.0	1.0-2.0	1.5	1.0-3.0	2.0
5 o'clock	14.0-28.5	19.5	4.0-11.0	7.0	9.5-16.0	13.0	6.0-12.0	9.0	1.0-3.5	2.0	2.0-3.5	2.5
7 o'clock	10.5-20.0	15.0	5.0- 9.0	6.5	8.5-16.5	12.0	7.0-13.0	10.0	1.5-3.5	2.0	1.0-2.5	1.5
8 o'clock	9.0-24.0	17.5	7.0-18.0	11.0	12.0-27.0	20.0	4.0-14.0	8.5	2.0-3.5	2.5	1.5-3.0	2.5
10 o'clock	10.5-24.5	16.5	4.0-10.0	6.5	8.0-16.0	12.0	5.0- 9.5	7.0	1.5-3.5	2.5	1.5-3.5	2.0
11 o'clock	12.5-28.5	20.5	3.0-12.5	6.0	7.5-13.5	10.0	6.5-11.0	9.0	1.0-2.5	2.0	2.0-3.5	2.5

* Predictive settings only, excludes seven who achieved learning criterion.