logical, since the individual's own lymphocytes would be used to down-regulate the abnormal immune response; and it requires relatively small amounts of antigen. The usefulness of this approach depends on the ability of the cells to suppress the autoimmune response in vivo and on the duration of the putative suppressive effect.

REFERENCES AND NOTES

1. D. M. Fambrough, D. B. Drachman, S. Satyamurti, *Science* **182**, 293 (1973).
2. Y. Ito, R. Miledi, A. Vincent, *Brain* **101**, 345 (1978).
3. A. G. Engel, J. M. Lindstrom, E. H. Lambert, V. A. Lennon, *Neurology* **27**, 307 (1977).
4. J. M. Lindstrom, M. E. Seybold, V. A. Lennon, S. Whittingham, D. D. Duane, *ibid.* **26**, 1054 (1976).
5. K. V. Toyka, D. B. Drachman, A. Pestronk, I. Kao, *Science* **190**, 397 (1975).
6. D. B. Drachman, R. N. Adams, E. F. Stanley, A. Pestronk, *J. Neurol. Neurosurg. Psychiatry* **43**, 601 (1980).
7. R. M. Pascuzzi, H. B. Coslett, T. R. Johns, *Ann. Neurol.* **15**, 291 (1984).
8. A. D. Hess and P. J. Tutschka, *J. Immunol.* **124**, 2601 (1980).
9. G. A. Dos Reis and E. M. Shevach, *ibid.* **129**, 2360 (1982).
10. H. J. Deeg *et al.*, *Exp. Hematol. Suppl.* **9**, 94 (1981).
11. I. F. Hutchinson *et al.*, *Transplant. Proc.* **13**, 412 (1981).
12. J. Elliot *et al.*, *Biochem. J.* **185**, 667 (1980).
13. Responder cells were cultured at 5×10^5 cells per microwell with purified AChR (2.0 pmol/ml) for 7 days in a humidified atmosphere of 95 percent air and 5 percent CO_2. The cultures were performed in triplicate in 96-well flat-bottomed microtiter plates (Corning). Cells were cultured in RPMI 1640 medium with glutamine (Gibco) supplemented with $5 \times 10^{-5} M$ 2-mercaptoethanol, 1:100 dilutions of 100× solutions of sodium pyruvate, nonessential amino acids, and penicillin-streptomycin plus Fungizone (all concentrated stock solutions, Gibco), 1 percent rat serum, and 1 percent horse serum (Gibco).
14. A. Pestronk, D. B. Drachman, R. N. Adams, *Muscle Nerve* **5**, 79 (1982).
15. S. Cammisuli, in *Cyclosporin A*, D. J. G. White, Ed. (Elsevier, Amsterdam, 1982), pp. 243–259.
16. M. B. Rittenberg and K. L. Pratt, *Proc. Soc. Exp. Biol. Med.* **132**, 575 (1969).
17. Lymphocytes were cultured in bulk with CsA (200 ng/ml) plus AChR (2.0 pmol/ml) or with CsA alone (controls). Cultures were performed in a total volume of 5 ml in six-well cluster plates (Costar) at a density of 5×10^6 cells per milliliter.
18. Cells were washed to remove CsA as follows: one wash in RPMI 1640 medium, one spin through horse serum, and two additional washes in RPMI 1640.
19. Various ratios of putative suppressor cells to responder cells or control (CsA-treated) cells to responder cells were used in each suppressor assay. The same ratio that gave the minimal level of "nonspecific" suppression with control cells was used to determine suppression by cells treated with CsA plus AChR. In the spleen cell experiments, nonspecific suppression was 31 percent at a ratio of 1:5 and decreased to 6 percent at a ratio of 1:10 (Fig. 3). In initial experiments, lower ratios (1:20 and 1:40) of control cells to responder cells gave no further decrease of "nonspecific" suppression. In the lymph node experiments, nonspecific suppression was 20 percent at a ratio of control cells to responder cells of 1:1 and 17 percent at a ratio of 1:2. At lower ratios (1:10 and 1:20), no further reduction of "nonspecific" suppression occurred.
20. Responder cells to KLH were prepared as described for AChR except that the rats were immunized with 1 mg of KLH (Sigma) emulsified in Freund's complete adjuvant.
21. D. B. Drachman, R. N. Adams, K. McIntosh, A. Pestronk, *J. Clin. Immunol. Immunopathol.* **34**, 174 (1985).
22. R. J. Brideau *et al.*, *Eur. J. Immunol.* **10**, 609 (1980).
23. P. J. Conlon, J. W. Moorhead, H. N. Claman, *Nature (London)* **278**, 257 (1979).
24. T. Hamaoka, U. Kamashita, T. Takami, M. Kitagawa, *J. Exp. Med.* **141**, 1308 (1975).
25. N. E. Goeken, *J. Immunol.* **128**, 2081 (1982).
26. A. D. Hess, P. J. Tutschka, G. W. Santos, *ibid.* **126**, 961 (1981).

27. Supported by NIH grant 5RO1 HD04817-16 and by a grant from the Muscular Dystrophy Association.

10 June 1985; accepted 6 January 1986

Alloantigen Recognition Is Preceded by Nonspecific Adhesion of Cytotoxic T Cells and Target Cells

HERGEN SPITS, WIM VAN SCHOOTEN, HISKIAS KEIZER, GYS VAN SEVENTER, MATTHIJS VAN DE RIJN, COX TERHORST, JAN E. DE VRIES

T-cell receptors bind antigens only when the antigens are exposed on the cell surface. This can be studied best in the interaction of cytolytic T lymphocytes (CTL) with target cells because the recognition and binding event can be separated from the lytic phase. Studies with CTL clones specific for HLA-A2 and HLA-B7 demonstrated that conjugates of CTL's and target cells can be formed in the absence of specific antigen recognition. Furthermore, T-cell receptor and target antigen cannot interact unless there is conjugate formation. This indicates that nonspecific conjugate formation between CTL's and target cells precedes the recognition of specific antigen by the T-cell receptor.

THYMUS-DERIVED-LYMPHOCYTE (T-cell) recognition and action are mediated by cell-cell interactions. Such cell-cell interactions can be studied in detail with the use of cytotoxic T lymphocytes (CTL's), which play a critical role in the immune response to viral and parasitic infections and in the destruction of tumor cells. The mechanism by which CTL's lyse target cells can be separated into several stages. In the initial phase, cell-cell contact between the CTL and its target cell is established (see Fig. 1). Next, the so called programming for lysis takes place and is followed by the delivery of the "lethal hit." Once the lethal hit has been delivered the presence of the CTL is not required for completion of the lysis of the target cell (*1*).

Until now it has been assumed that the adhesion between the CTL and its target cell is initiated by the recognition of one or more target antigens by the T-cell receptor (*1*). Indeed, monoclonal antibodies directed at the T-cell receptor or the closely associated T3 antigens block cytolysis (*1*). However, these antibodies do not block the formation of adhesions between CTL's and target cells (*2*). This raises the possibility that the interaction between the T-cell receptor and antigen is not required for the formation of conjugates. We reported recently that an HLA-A2–specific CTL clone JR-2-16 was unable to lyse mouse L cells that express the HLA-A2 antigen after gene transfer (*3*). In contrast, human cells transfected with an HLA-A2–specific DNA fragment were all killed (*3*). This finding implies that the presence of specific antigen on the target cell is not sufficient for lysis. Since it has been reported that other T-cell surface structures, the "accessory molecules" T4, T8, T11, and LFA-1, are involved in cytotoxic reactions (*4*), we speculated that target cell structures other than HLA-A2 are required for lysis (*3*). Whether these determinants are the counter-structures of the accessory molecules that may be absent on the surface of L cells remains to be determined.

To study the roles of the T-cell receptor–T3 complex and of the accessory molecules, we used the human HLA-A2–specific CTL clone JR-2-16 (*5*), the HLA-B7–specific CTL clone KOR-132, and various target cells in a single cell assay (*6*). This assay allows the measurement of the number of conjugates formed between killer cells and target cells and an estimate of the number of lysed target cells present in the conjugates. In the single-cell assay, effector cells labeled with carboxyfluorescein diacetate at a concentration of 2×10^6 cells per milliliter were mixed with target cells at the same concentration, incubated for 10 minutes at 30°C, and centrifuged. The fluorescent label

H. Spits, W. van Schooten, H. Keizer, Netherlands Cancer Institute, Amsterdam, The Netherlands.
G. van Seventer, Central Laboratorium of the Blood Transfusion, Service of the Red Cross, Amsterdam, The Netherlands.
M. van de Rijn and C. Terhorst, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115.
J. E. de Vries, Netherlands Cancer Institute, Amsterdam, The Netherlands.

Table 1. The capacity of CTL clones JR-2-16 and KOR-132 to form conjugates and to lyse difference target cells. At least 500 CTL's were counted on each slide. Percentages are given as means ± SEM of *n* experiments. ND, not done.

Target cell	CTL clone	*n*	Conjugates (%)	Conjugates with nonviable target cells (%)
M1A2	JR-2-16	6	46 ± 6	25 ± 3
	KOR-132	3	33 ± 4	3 ± 0.3
M1B7	JR-2-16	6	44 ± 6	2 ± 0.8
	KOR-132	3	37 ± 3	41 ± 5
M1	JR-2-16	7	42 ± 6	2 ± 0.7
	KOR-132		ND	
LA2	JR-2-16	5	7 ± 1	0
	KOR-132	3	2.3 ± 0.3	0
LB7	JR-2-16	3	2.0 ± 0.7	0
	KOR-132	3	6 ± 0.9	0
L	JR-2-16	5	2.4 ± 0.7	0
	KOR-132	3	1.3 ± 0.3	0
Daudi	JR-2-16	8	70 ± 4	5 ± 0.9
	KOR-132	3	57 ± 1	7 ± 0.3
P815	JR-2-16	3	55 ± 5	7 ± 2.3
	KOR-132	3	44 ± 5	3 ± 1.1

does not affect the killing capacity of the CTL's and it is important in distinguishing between CTL's and target cells. After the supernatant fluid was removed, cells were mixed with agarose and were spread on a slide, which was then incubated for 4 hours at 37°C. Viable cells were detected by staining with 0.2 percent trypan blue before fixation in 0.2 percent paraformaldehyde. The total number of fluorescence-labeled CTL's and the number of CTL's conjugated with viable or nonviable target cells were determined with a fluorescence microscope. The percentage of conjugates was calculated as the number of conjugated CTL's relative to the total number of CTL's. The percentage of lysis was calculated as 100 times the ratio of the number of conjugates containing dead cells to the total number of conjugates.

The results of a single cell assay are summarized in Table 1. The CTL clone JR-2-16 was able to form conjugates with the fibroblast cell line M1A2. In contrast, LA2 (or L cells) did not form conjugates with the CTL clone JR-2-16. This was not due to some species barrier as suggested previously (*3*) because the human CTL clone was able to form conjugates with P815 cells, a mouse mastocytoma cell line. The human CTL clone also formed conjugates with the human cell lines M1, M1 cells transfected with HLA-B7 genes (*7*), and Daudi, none of which express HLA-A2 on their surface. However, when the specific target antigen was not present on the cell surface, the cells were not lysed (Table 1).

Similar observations were made with the CTL clone KOR-132, which is specific for HLA-B7 (Table 1). This clone formed conjugates with M1 cells transfected with a DNA fragment containing the gene coding for HLA-B7 (M1B7), M1, and M1A2 cells; but only the M1B7 cells were lysed. Clone KOR-132, which was unable to lyse L cells transfected with HLA-B7 genes (*7*) did not form conjugates with LB7, L, or LA2 cells (Table 1). However, KOR-132 did form conjugates with both Daudi and P815 cells. From the results of the single-cell assays, we concluded that the inability of JR-2-16 and KOR-132 to lyse LA2 cells (*3*) and LB7 cells, respectively, could be explained by the failure to form conjugates between these cells. It appears that a CTL can form conjugates in the absence of the appropriate target antigen. This was confirmed by the observation that CTL clones that are specific for a human major histocompatibility complex (MHC) class I antigen formed conjugates even with Daudi cells, which lack class I antigens. These data provided strong evidence that structures other than the specific antigens and T-cell receptors drive conjugate formation between CTL's and target cells.

We investigated the role of the accessory molecules and the T-cell receptor–T3 complex by carrying out blocking studies with a series of monoclonal antibodies. Conjugate formation between JR-2-16 and the specific target cell M1-A2 was blocked by monoclonal antibodies to LFA-1 (anti-LFA-1) (Table 2). Anti-LFA-1 consistently failed to prevent the lytic event when conjugates were formed. Anti-T8 also blocked the adhesion step, but the lytic event was not affected significantly. In contrast, in the presence of anti-T3 or anti-T-cell-receptor reagents, conjugates were formed but lysis was blocked (Table 2). Monoclonal antibodies directed at HLA-A2 or at a common determinant on all human MHC class I antigens (W6/32) appeared to block both conjugate formation and lysis. Similar results were obtained with clone KOR-132 and M1B7 cells.

Monoclonal antibodies also blocked the nonspecific conjugate formation between JR-2-16 and M1, Daudi, or P815 cells and an HLA-A2–negative Epstein-Barr virus (EBV)–transformed B cell line (QBL) that could not be killed by JR-2-16 (Table 3). The antibody to LFA-1 inhibited the formation of conjugates with each of these target cells. In contrast W6/32 (anti-HLA-A, -B, and -C) and SPV-8 (anti-T8) blocked conjugate formation with M1 cells but not with Daudi or P815 cells. As expected, anti-T3 or anti-T-cell-receptor reagents did not block nonspecific conjugate formation. The effects of monoclonal antibodies to class I HLA antigens on conjugate formation between JR-2-16 and M1A2 and M1 deserves attention. It is possible that the interaction between the T-cell receptor and its antigen contributed to the formation of conjugates. This interaction could have been blocked by antibodies to class I HLA antigens. However, antibodies to class I HLA antigens not only blocked the interaction between the T-cell receptor and HLA-A2 but also inhibited the interaction between T8 and a constant element of class I HLA antigens, as was proposed recently (*2, 8*).

The assumption that the T8–class I HLA interaction would take place during the adhesion phase is supported by the finding that anti-T8 or antibody W6/32, a class I HLA antibody, did not block the conjugate

Table 2. The effect of monoclonal antibodies on different stages of the cytotoxic reaction of CTL clone JR-2-16 against M1A2 cells. At least 500 CTL's were counted on each slide; in the absence of antibody 46 ± 6 percent of the CTL's were conjugated and 25 ± 3 percent were lysed. The final dilution of antibody-containing ascites in the assay was 1:100. Purified immunoglobulin G of WT-31 was added at a concentration of 10 μg/ml. Percentages are given as means ± SEM of *n* experiments.

Monoclonal antibody	Target antigen	*n*	Inhibition of conjugate formation	Inhibition of lysis
SPV-T8	T8	4	36 ± 5	8 ± 4
SPV-L7	LFA-1	6	33 ± 2	1 ± 0.1
SPV-T3b	T3	4	3 ± 0.5	50 ± 8
WT-31*	T-cell receptor	4	2 ± 0.2	60 ± 5
CR-11-351	HLA-A2	4	34 ± 7	63 ± 10
W6/32	HLA-A, -B, -C	6	29 ± 6	49 ± 8

*See (*14*).

formation between JR-2-16 and the Daudi cell (which does not express class I MHC antigen) or the mouse cell line P815. On the other hand, the nonspecific conjugate formation with the EBV-transformed B-cell line QBL was not blocked by anti-T8 or by antibodies to HLA-A, -B, and -C. Whether this failure was caused by the high expression of class I MHC antigens on EBV-transformed B cells as compared to fibroblasts (*3*) or by other unknown factors remains to be determined. It appears that the binding of JR-2-16 to Daudi, P815, and QBL cells is driven by LFA-1 and as yet unknown structures. Our experiments do not permit an evaluation of the contribution of early individual adhesion molecules to the formation of conjugates. It is therefore unclear why in some cases more structures are involved in conjugate formation than in others.

These considerations have led us to propose a new model for the events leading to target cell lysis (Fig. 1). According to our view, the cytotoxic reaction is initiated by nonspecific conjugate formation. LFA-1, T8, and probably also T11 (*9*), are involved in this interaction. On the target cell level, class I HLA antigens play a role in the antigen-nonspecific interaction. A constant portion of class I HLA antigens probably interacts with T8. Since T8 has the ability to form homomultimers (*10*), its role could be to mobilize class I MHC antigens on the interface between CTL's and target cells. Thus, the effector cell–target cell adhesion enables the T-cell receptors to interact with its antigen in a cooperative fashion (*11*). After the interaction between the T-cell receptor and its target antigen takes place, a cascade of reactions leading to target cell lysis is initiated. If the relevant target antigen is not found, the CTL detaches from the target cell.

The "programming for lysis" step is Ca^{2+}-dependent (*12*). Since monoclonal antibodies directed at T3 or the T-cell receptor cause

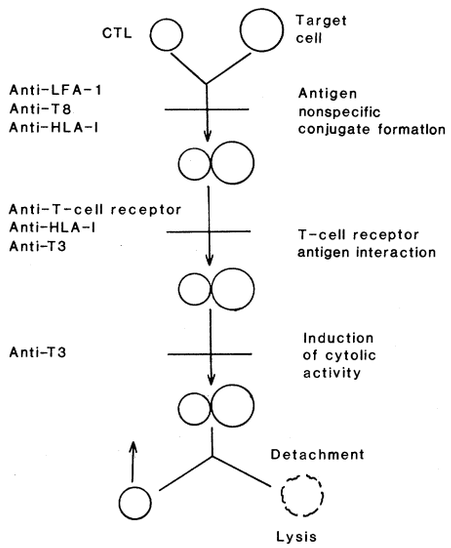

Fig. 1. A model for the involvement of different cell membrane antigens in the different stages of the cytotoxic reaction of a CTL clone and target cells. Anti-HLA-I, antibodies directed at MHC class I antigens.

Ca^{2+} influxes (*12*), it is quite likely that the T3–T-cell receptor complex is involved in triggering of the lytic machinery. Moreover, anti-T3 and anti-T-cell-receptor reagents can cause nonspecific killing (*13*). However, our experiments do not exclude the possibility that antibodies to T3 also block the interaction between the T-cell receptor and its antigen. Perhaps monoclonal antibodies to T3 induce conformational changes in the binding site of the T-cell receptor in a way that prevents binding of the antigen to the T-cell receptor. Thus, it is not possible from our findings to determine precisely which step is blocked by monoclonal antibodies directed at T3; they may block binding of the T-cell receptor to its antigen, or transduction of the activation signal provided by binding of the antigen to the T-cell receptor, or both. Triggering of the transduction signal can be carried by monoclonal antibodies directed at T3 or the T-cell receptor in the absence of T-cell receptor–target antigen interactions (*13*). This is supported by the nonspecific adhesion.

It is clear from these observations that the requirements for a functional interaction between the T-cell receptor and its antigen are just beginning to be elucidated. Cloned T-cell lines and target cells transfected with HLA genes are valuable tools for studying the CTL–target cell interaction. It will be of interest to determine whether the rules governing antigen recognition by CTL's also apply to the recognition of antigen by antigen-specific helper T cells.

Table 3. The effect of monoclonal antibodies on the formation of conjugates between CTL clone JR-2-16 and untransfected M1 cells, Daudi, P815, and QBL cells. In the absence of antibody, 42 ± 6 percent of the total number of CTL's were conjugated with M1, 70 ± 4 percent with Daudi, 55 ± 5 percent with P815, and 75 ± 9 percent with QBL. At least four experiments have been done when no inhibition of conjugate formation was observed. The HLA phenotypes of the QBL line was HLA-A26,26; B818;DR3,3. Percentages are means ± SEM for the number of experiments shown in parentheses.

Monoclonal antibody	Percent inhibition of conjugate formation with			
	M1	Daudi	P815	QBL
SPV-T8	39 ± 4 (8)	0	0	10 ± 2 (3)
SPV-L7	38 ± 4 (5)	48 ± 3 (7)	70 ± 10 (3)	51 ± 10 (3)
SPV-T3b	0	0	1 ± 0.1 (3)	0
WT-31	0	0	0	0
CR-11-351	12 ± 7 (4)	0	0	3 ± 0.9 (3)
W6/32	31 ± 10 (4)	0	0	4 ± 1 (3)

REFERENCES AND NOTES

1. G. Berke, *Prog. Allergy* **27**, 69 (1980).
2. C. D. Tsoukas, D. A. Carson, S. Foray, J. H. Vaughan, *J. Immunol.* **129**, 1421 (1982); H. Landegren *et al.*, *J. Exp. Med.* **155**, 1579 (1982); H. Spits, J. Borst, C. Terhorst, J. E. de Vries, *J. Immunol.* **129**, 1563 (1982).
3. M. van der Rijn *et al.*, *Science* **226**, 1083 (1984).
4. S. J. Burakoff, O. Weinberger, A. M. Krensky, C. S. Reiss, *Adv. Immunol.* **36**, 45 (1984); S. C. Meuer *et al.*, *Science* **218**, 471 (1982); E. L. Reinherz *et al.*, *Nature (London)* **294**, 168 (1981); R. L. Evans *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 544 (1981); A. M. Krensky *et al.*, *J. Immunol.* **131**, 611 (1983).
5. H. Spits, M. H. Breuning, P. Ivanyi, C. Russo, J. E. de Vries, *Immunogenetics* **16**, 503 (1982); H. Yssel, *et al.*, *J. Immunol. Methods* **72**, 219 (1984).
6. E. Grimm and B. Bonavida, *J. Immunol.* **123**, 2861 (1979); B. Bonavida, T. P. Bradley, E. Grimm, *Methods Enzymol.* **93**, 270 (1983).
7. M1B7 cells were obtained by transfection of M1 cells with a genomic HLA-B7 clone, exactly as has been described for the transfection of M1 cells with the HLA-A2 clone JYB3.2 (*3*) KOR-132 lysed with M1B7 cells as determined in a standard chromium 51 release assay (*5*). The percentage lysis was 65 at an effector cell to target cell ratio of 5:1. M1 cells or mouse L cells transfected with HLA-B7 [C. Bernabeau *et al.*, *J. Immunol.* **131**, 2032 (1983)] were not lysed by clone KOR-132.
8. S. L. Swain, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7101 (1981); S. C. Meuer *et al.*, *ibid.* **79**, 4395 (1982).
9. A. M. Krensky *et al.*, *J. Immunol.* **132**, 2180 (1984).
10. P. Snow and C. Terhorst, *J. Biol. Chem.* **258**, 14675 (1983).
11. From such a role of T8, it would be expected that T8 is also associated with events that take place after conjugate formation has occurred. However, anti-T8 did not affect the lytic step mediated by JR-2-16. But inhibition of the lytic event was observed with other $T8^+$ class I MHC-specific CTL clones as effector cells, and EBV-transformed B cells as target cells (H. Spits unpublished data), suggesting that in some cases T8 may play an additional role after conjugate formation.
12. E. Martz, *Contemp. Top. Immunobiol.* **7**, 301 (1977); H. C. Oettgen, C. Terhorst, L. Cantley, P. Rosoff, *Cell* **40**, 583 (1985); K. O'Flynn, D. C. Linch, P. E. R. Tatham, *Biochem. J.* **219**, 661 (1984).
13. H. Spits, H. Yssel, J. Leeuwenberg, J. E. de Vries, *Eur. J. Immunol.* **15**, 88 (1985); J. Leeuwenberg, H. Spits, W. Tax, P. J. A. Capel, *J. Immunol.* **134**, 3770 (1985); H. Schrezenmeier, R. Kurrle, H. Wagner, B. Fleischer, *Eur. J. Immunol.* **15**, 1019 (1985).
14. H. C. Oettgen, J. Kappler, W. J. M. Tax, C. Terhorst, *J. Biol. Chem.* **259**, 12039 (1984); H. Spits *et al.*, *J. Immunol.* **135**, 1922 (1985).

15. Supported by grants from the Netherlands Cancer Foundation (Koningin Wilhelmina Fonds; NKI 82-4), National Institutes of Health (AI-15066), the American Society (IM 289) and the March of Dimes and the Leukemia Society of America (to C.T.). We thank R. Roozenmond for advice regarding the single cell assay and J. Semeyn and A. Voordouw for technical assistance. We are indebted to M. A. van Halem and S. McGrath for secretarial help.

15 August 1985; accepted 27 December 1985