Recognition of HLA-A2 by Cytotoxic T Lymphocytes After DNA Transfer into Human and Murine Cells

Abstract. *A gene coding for the major histocompatibility antigen HLA-A2 was transferred into human HLA-A2 negative M1 cells and murine L cells. Following transfection, these cells expressed molecules at the cell surface that are biochemically indistinguishable from HLA-A2 antigens on the human cell line JY from which the HLA-A2 gene was isolated. The M1A2 cells were recognized and lysed by a cytolytic T-cell clone specific for HLA-A2. The transfected L cells which express HLA-A2 in association with human β_2-microglobulin were not lysed by this T-cell clone. The specific cytolysis of M1A2 cells could be inhibited by monoclonal antibodies to HLA-A2, and monoclonal antibodies to T3, T8, and LFA-1 on cytotoxic T lymphocytes. These results suggest that killing by allospecific T cells requires HLA-A2 antigens as well as other species-specific structures on the target cell surface.*

Cytolytic T lymphocytes (CTL), which have a critical role in the immune response against viral infections, also appear to be involved in the rejection of allografts. The specificity of cytolytic T cells is defined by T-cell receptors that interact with antigens and products of the major histocompatibility complex (MHC) expressed on target cells. The T-cell receptor on human T cells consists of an α chain and a β chain of 45 to 50 kilodaltons (kD) and 37 to 45 kD, respectively (*1*). The T-cell receptor on human T cells is associated with the T3 complex (*2*), which consists of three nonvariable polypeptides, termed γ (25 kD), δ (20 kD), and ϵ (20 kD) (*3*). Monoclonal antibodies to the T-cell receptor–T3 complex inhibit the activity of antigen-specific CTL (*4*). Monoclonal antibodies to T3 (anti-T3) block the cytolytic reactivity at a stage after the formation of stable conjugates between CTL and target cells (*4*).

Other T-cell surface antigens, the accessory molecules T4, T8, T11, and LFA-1, also appear to be involved in lympholysis mediated by T cells. In contrast to anti-T3, monoclonal antibodies to these other cell surface antigens block conjugate formation between effector and target cells (*5*). These data indicate that adhesion requires structures on the surface of CTL different from the T-cell receptor, which bind to "ligand" molecules present on the target cells.

A cloned genomic DNA fragment coding for the HLA-A2 antigen (isolated from JY cells) (*6*) was transfected into the human HLA-A2$^-$ fibroblast cell line M1 and murine L cells in order to study the requirements for the specific interaction between CTL and target cells. The M1 cell line is a subclone of a transformed fibroblast cell line derived from a patient with *Xeroderma pigmentosum* and is efficient in the uptake and expression of foreign DNA (*7*). Cloned genomic DNA coding for the HLA-A2 antigen and pSV2neo, a plasmid containing the resistance gene for neomycin, were transfected into M1 and L cells (legend to Fig. 1). The colonies were analyzed by indirect immunofluorescence, and 40 percent of the cells reacted with a monoclonal antibody specific for HLA-A2. L-cell clones expressing HLA-A2 are abbreviated as LA2; M1 clones expressing HLA-A2 are called M1A2. L-cell clones coexpressing human β_2-microglobulin and HLA-A2 (LA2β2) were also isolated. The murine LA2 and the human M1A2 cells were each cultured in medium containing human serum in order to ensure the association of the HLA-A2 heavy chains with human β_2-microglobulin (*8*).

Analysis of the expression of HLA-A2 antigens by indirect immunofluorescence (Fig. 1) demonstrates that similar amounts of HLA-A2 antigen were expressed on all three transfectants, but at levels that were tenfold less than the amounts expressed on JY cells (data not shown). In contrast, HLA-A2 was absent from the nontransfected L and M1 cells. Next, a human CTL clone, JR-2-16, which had been isolated from a CTL cell line directed against human JY cells was used for cytolysis studies (Table 1). JR-2-16 was shown to be specific for HLA-A2 (*9*) and was able to lyse the M1A2 cells and JY cells, but not the untransfected M1 cells. Although HLA-A2 was expressed on the surface of transfected L cells in association with human β_2-microglobulin, neither the LA2 nor the LA2β2 cells were lysed by human CTL clone JR-2-16 (Table 1). In addition, a mixed population of HLA-A2–specific CTL was able to kill M1A2, but was unable to lyse LA2 or LA2β2 cells (Table 2). The killing activity of JR-2-16 against M1A2 cells was blocked by an antibody to HLA-A2 and by antibodies to the CTL surface structures T8, T3, and LFA-1, which also block the activity of JR-2-16 against JY (*10*).

The cytolytic reaction is thought to occur in two separate stages (*11*): (i) the [Mg^{2+}]-dependent formation of conjugates between target cells and CTL and (ii) a [Ca^{2+}]-dependent programming for lysis and the delivery of the lethal dose. The formation of conjugates can be inhibited by an excess of target cells which is measurable in "cold target inhibition experiments." Unlabeled transfected L cells were unable to block the cytolytic activity of JR-2-16 against labeled M1A2 or JY cells (Table 3). It is therefore possible that the CTL clone could recog-

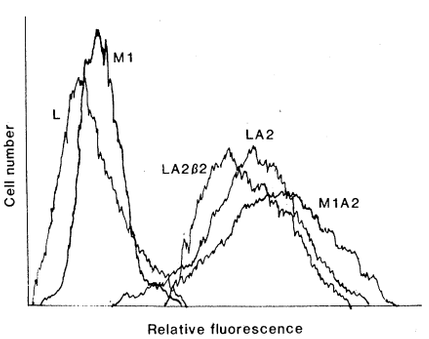

Fig. 1. Reactivity of transfected cell lines with monoclonal antibody to HLA-A2. M1 cells (*6*) and L cells were transfected with a genomic HLA-A2 clone (JYB3.2) (*8*) in phage Charon 4A under control of the metallothionein gene promoter (*14*). The replacement of the normal HLA promoter is mediated via a recombinational event between a plasmid πVx (*15*) containing the promoter for the metallothionein gene fused to the leader sequence of H-2K (*16*) and the leader sequence of HLA-A2 DNA, which is cloned in phage Charon 4A. Phages which have recombined with plasmid as a result of the homologies between leader sequences of H-2 and HLA are selected in a second round of transfection, with the use of a selectable marker on the plasmid. The LA2β2 cell line was derived by transfecting an L-cell line that expresses human β_2-microglobulin (*17*) with the JYB3.2 gene in the absence of the metallothionein gene promoter. Cotransfection of 1×10^6 L cells or 0.5×10^6 M1 cells was performed with 1 μg of phage DNA, 50 ng of pSV2neo (a plasmid containing the gene for resistance against neomycin; Bethesda Research Lab Inc.), and 20 μg of salmon sperm DNA as a carrier. Calcium phosphate precipitation was carried out as described (*18*). The M1 cells were then treated with dimethyl sulfoxide (*7*). Selection of transfected L and M1 cells was performed in Dulbecco's modified Eagle's medium containing fetal bovine serum (10 percent) and neomycin (450 μg/ml; G418) (*19*). Colonies appeared after 3 weeks of culturing. The LA2 and LA2β2 cells which expressed HLA-A2 were isolated by cloning at limiting dilution. For immunofluorescence studies, cells were detached from culture dishes with Hanks buffer containing trypsin (0.05 percent), incubated with antibody 4B to HLA-A2 (*20*), and then incubated with fluoroscein isothiocyanate-labeled goat antibody to mouse immunoglobulin. Finally, cells were sorted or analyzed on an Epics V Cell Sorter (Coulter Instrument).

Table 1. Cytolytic activity of effector cells JR-2-16 against transfected target cells. Target cells were detached from culture dishes with trypsin (0.05 percent) in Hanks salt solution. Labeling with ^{51}Cr and lysis of the target cells in the presence of JR-2-16 effector cells was carried out as described (*9*).

Target cells	Percentage specific lysis at effector:target cell ratios			
	10:1	5:1	2.5:1	1:1
M1A2	40	34	22	22
LA2	1	3	3	3
LA2β2	2	2	2	3
M1	6	6	4	4
L	2	3	2	2
JY	76	54	48	28

Table 2. M1A2 cells as targets for human CTL generated in a mixed lymphocyte culture. Human peripheral blood lymphocytes were stimulated three times with JY cells (irradiated with 6000 rads) on days 1, 7, and 14. The assay was performed on day 20 (*9*).

Target cells	Percentage specific lysis at effector:target ratios	
	30:1	10:1
M1A2	45	34
M1	8	3
JY	80	67
LA2	3	0
LA2β2	2	4

nize the transfected murine cells but not adhere sufficiently to cause lysis of transfected L cells or inhibition of lysis of labeled M1A2 cells.

In order to investigate whether structural differences between HLA-A2 expressed on murine L cells and those expressed on human M1A2 cells could be responsible for the failure of JR-2-16 to kill and recognize the LA2 and LA2β2 cells, the HLA-A2 molecules from these cells were analyzed by sodium docyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing. The molecules precipitated from cell surface-iodine-labeled M1A2, LA2, LA2β2, and JY appeared on SDS-PAGE as identical heterodimers of 45 kD (HLA heavy chain) and 12 kD (β_2-microglobulin), as shown in Fig. 2a. Furthermore, the amount of HLA-A2 protein precipitated from the L cells that expressed HLA was comparable to that precipitated from M1A2. This observation suggests that LA2 and M1A2 expressed similar amounts of HLA-A2. HLA-A2 antigens, isolated from M1A2 and LA2 cells, showed an identical charge heterogeneity to those isolated from JY cells on isoelectric focusing gels (Fig. 2b). The data obtained from SDS-PAGE and isoelectric focusing analysis demonstrated that the structure of HLA-A2 molecules expressed on the cell surface of LA2, LA2β2, and M1 cells was identical to that found on JY cells.

Here we have demonstrated that transfer of an HLA-A2 gene into a human fibroblast cell line resulted in the expression of HLA-A2 proteins on the surfaces of these cells. M1A2 cells were recognized and lysed specifically by the CTL clone JR-2-16 which is directed at HLA-A2. In contrast, murine L cells transfected with the same genomic DNA expressed HLA-A2 molecules in association with human β_2-microglobulin but were not lysed by the CTL clone JR-2-16. Our data indicate that the failure of the human CTL clone to recognize the transfected L cells is not caused by qualitative or quantitative differences in HLA-A2 expression since the cell surface density as well as the primary structure of HLA-A2 on LA2, LA2β_2, and M1A2 was the same. It has been argued that the association of human HLA heavy chains with murine β_2-microglobulin could prevent the recognition of HLA antigens (*12*). However, since the L cells were cultured in human serum (*8*), and we used an L-cell line that expresses human β_2-microglobulin for transfection with HLA-A2 genes, most HLA heavy chains were associated with human β_2-microglobulin on the cell surface. The lack of sensitivity of the LA2 and LA2β2 cells to the activity of JR-2-16 is not due to nonspecific resistance to lysis of these cells since the L cells transfected with HLA-A2 can be lysed by mouse CTL cultures directed against H-2K/D^k (*12*).

Table 3. Cold target inhibition of cytolytic activity by clone JR-2-16 against ^{51}Cr-labeled M1A2 cells. Varying numbers of unlabeled target cells that served as inhibitor cells were mixed with a fixed number of effector cells (JR-2-16) in 96-well Falcon plates. Next, ^{51}Cr-labeled M1A2 cells (2×10^3) were added, and the plates were centrifuged for 3 minutes at 150*g* and incubated for 4 hours at 37°C. After this incubation period, 100 μl of supernatant was collected and the radioactivity was counted in a gamma counter. In the absence of cold target cells, 28 percent specific lysis was found.

Unlabeled cells	Percentage specific lysis at ratios of unlabeled to labeled cells			
	50:1	25:1	12:1	6:1
M1A2	11	15	17	21
LA2	28	27	27	24
LA2β2	25	26	24	24
M1	28	25	25	23
L	23	26	27	26
JY	3	7	10	14

The inability of the LA2 and LA2β2 cells to serve as target cells for JR-2-16 is most likely due to differences in cell surface structures between human and murine cells. Two explanations can be given for the lack of recognition of HLA-A2 on L cells by JR-2-16. First, the T-cell antigen receptor on JR-2-16 might recognize HLA-A2 plus an unknown

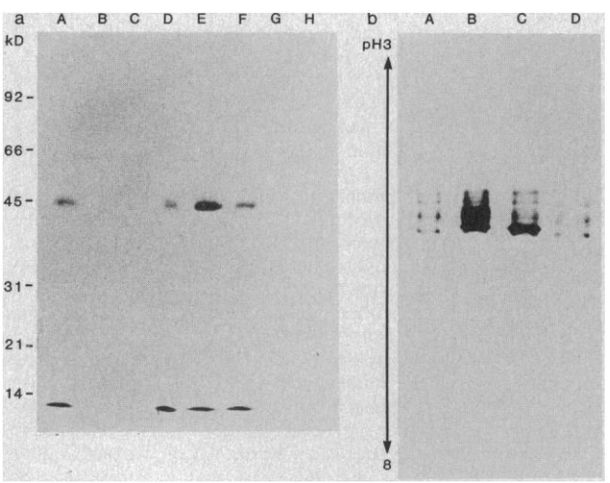

Fig. 2. Analysis of HLA-A2 molecules expressed on L cells and M1A2 cells by SDS-PAGE and isoelectric focusing. (a) SDS-PAGE analysis of molecules precipitated by antibody 4B to HLA-A2 (*20*) from M1, M1A2, L, LA2, and LA2β2 cells. Cells were labeled with ^{125}I, and precipitations were carried out as described (*21*). Similar amounts of radioactivity were precipitated from LA2, LA2β2, and M1A2 cells. Part of the immunoprecipitated material was separated on a polyacrylamide gel (10 percent) (*21*) under reducing conditions. Lane A, LA2 cells, 4B antibody; lane B, LA2 cells, normal mouse serum (NMS) control; lane C, LA2β2 cells, NMS control; lane D, LA2β2 cells, 4B antibody; lane E, JY cells, 4B antibody; lane F, M1A2 cells, 4B antibody; lane G, M1A2 cells, NMS control; lane H, JY cells, NMS control. (b) Isoelectric focusing of HLA-A2 antigens precipitated from LA2, LA2β2, JY, and M1A2 cells. The precipitations which had been used for SDS-PAGE were analyzed on vertical slab isoelectric focusing gels (*22*) with LKB ampholytes *p*I 3.5 to 10 in NP-40 (0.5 percent), and 8*M* urea. Samples were run from anode to cathode. To measure the *p*H gradient, gel slices were incubated in water. Lane A, LA2 cells; lane B, LA2β2 cells; lane C, JY cells; lane D, M1A2 cells.

antigen X, which would be present on JY cells and M1 cells but not on the murine cells. Matzinger and Bevan (*13*) have argued that alloreactivity always involves recognition by the T-cell receptor of a cell surface antigen X plus an MHC product. Thus, antigen X must be a structure that is shared by a human B lymphoblastoid cell line and a transformed fibroblast cell line. Although this explanation cannot be ruled out, we would suggest that the structures on human M1A2 cells which bind to the CTL glycoproteins LFA-1, T8, and T11 are absent from (or different on) murine L cells. In other words, a species-specific barrier exists between the CTL clone and the target cell, which does not allow for the appropriate cell adhesion required for the precise interaction of the T-cell receptor with antigens.

Note added in proof: Similar results have recently been obtained by others (*13a*).

MATTHIJS VAN DE RIJN
CARMELO BERNABEU
BRIGITTE ROYER-POKORA
Laboratories of Molecular Immunology and *Biochemical Pharmacology, Dana-Farber Cancer Institute, Boston, Massachusetts 02115*
JOHN WEISS
JOHN G. SEIDMAN
Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115
JAN DE VRIES
HERGEN SPITS
Netherlands Cancer Institute, Amsterdam, The Netherlands
COX TERHORST
Laboratory of Molecular Immunology, Dana-Farber Cancer Institute, and *Department of Pathology, Harvard Medical School*

References and Notes

1. K. Haskins *et al.*, *J. Exp. Med.* **157**, 1149 (1983); J. Kappler *et al.*, *Cell* **35**, 295 (1983); S. C. Meuer *et al.*, *J. Exp. Med.* **157**, 705 (1983).
2. H. C. Oettgen, J. Kappler, C. Terhorst, *J. Biol. Chem.*, in press; S. C. Meuer *et al.*, *Nature (London)* **303**, 808 (1983).
3. J. Borst, M. A. Prendiville, C. Terhorst, *J. Immunol.* **128**, 1560 (1982); *Eur. J. Immunol.* **13**, 576 (1983); J. Borst, S. Alexander, J. Elder, C. Terhorst, *J. Biol. Chem.* **258**, 5135 (1983).
4. T. W. Chang, P. C. Kung, S. P. Guigras, G. Goldstein, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1805 (1981); 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).
5. T. A. Springer *et al.*, *Immunol. Rev.* **68**, 171 (1982); C. Terhorst, in *Antibodies to Receptors: Probes for Receptor Structure and Function*, M. F. Greaves, Ed. (Chapman & Hall, London, 1984), pp. 131–161.
6. P. A. Biro, V. B. Reddy, A. Sood, D. Pereira, S. M. Weissman, in *Recombinant DNA, Proceedings of the Third Cleveland Symposium on Macromolecules* (Elsevier, Amsterdam, 1981), pp. 41–46.
7. B. Royer-Pokora, W. D. Peterson, W. A. Haseltine, *Exp. Cell Res.* **151**, 408 (1984).
8. We have shown that 70 percent of the human HLA-A2 heavy chain expressed on mouse L cells cultured in human serum, was associated with human β_2-microglobulin. This was due to an exchange reaction between murine β_2-microglobulin on the cell surface and human β_2-microglobulin in the serum [C. Bernabeu, M. v. d. Rijn, P. G. Lerch, C. P. Terhorst, *Nature (London)* **308**, 642 (1984)].
9. H. Spits, H. IJssel, C. Terhorst, J. E. de Vries, *J. Immunol.* **128**, 95 (1982); H. Spits, G. Keizer, J. Borst, C. Terhorst, A. Hekman, J. E. de Vries, *Hybridoma* **2**, 423 (1983).
10. Inhibition of the cytolytic activity of clone JR-2-16 against M1A2 cells by monoclonal antibodies was measured as described (*9*). Percentage inhibition at a dilution of 1:200 of ascites fluid was: 93 (anti-T3), 54 (anti-T8), 31 (anti-LFA1), 0 (anti-T1), 0 (anti-HLA-DR), 80 (anti-HLC-2) and 0 (anti-HLA-B7). The same monoclonal antibodies also block the activity of several CTL clones against JY (*9*).
11. E. Martz, *Contemp. Top. Immunobiol.* **7**, 301 (1977).
12. C. Bernabeu *et al.*, *J. Immunol.* **131**, 2032 (1983).
13. P. Matzinger and M. J. Bevan, *Cell. Immunol.* **29**, 1 (1977).
13a. J. A. Barbosa *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
14. Separate studies showed that the presence of the metallothionein promoter gives rise to a $CdSO_4$-inducible amplification of the expression of HLA molecules in L cells. However, in some cases, transfectants without the metallothionein promoter expressed higher levels of HLA molecules than transfectants with the promoter. This indicated that the overall HLA expression on the cell surface is governed by additional factors, such as the availability of intracellular β_2-microglobulin (M. v. d. Rijn and C. Terhorst, unpublished results).
15. T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning. A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982), pp. 353–363.
16. J. Weiss and J. G. Seidman, unpublished results.
17. P. Kavathas and L. A. Herzenberg, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 524 (1983).
18. M. Wigler *et al.*, *ibid.* **76**, 1373 (1979).
19. The neomycin was a gift from G. Daniel of the Schering Corporation, Raritan, N.J.
20. The antibody to HLA-A2 (4B) was a gift from B. Dupont, Memorial Sloan Kettering Institute.
21. M. v. d. Rijn, P. G. Lerch, R. W. Knowles, C. Terhorst, *J. Immunol.* **131**, 680 (1983); U. K. Laemmli, *Nature (London)* **227**, 680 (1970).
22. A. v. Agthoven and C. Terhorst, *J. Immunol.* **128**, 426 (1982).
23. Supported by NIH grants AI-15066 and AI 19148, American Cancer Society grant IM289, and a grant from the March of Dimes; a postdoctoral fellowship from the American Cancer Society (J.W.); and a scholarship from the Leukemia Society of America (C.T.). We thank Drs. Hildegunde Ertl and Robert Finberg for critical review of the manuscript.

16 July 1984; accepted 11 September 1984

β-Adrenergic Mechanism of Insulin-Induced Adrenocorticotropin Release from the Anterior Pituitary

Abstract. *Intraperitoneal administration of insulin to control rats and to rats with pituitary stalk transections or with lesions of the median eminence resulted in increased plasma adrenocorticotropin (ACTH) levels. The insulin-induced stimulation of ACTH release was blocked in both the control and lesioned animals by prior treatment with either the β-adrenergic antagonist propranolol or the glucocorticoid analog dexamethasone. The direct application of insulin to primary cultures of the anterior pituitary did not evoke ACTH release or affect the maximal ability of corticotropin-releasing factor or epinephrine to stimulate ACTH secretion. The results suggest that insulin stimulates ACTH release by a mechanism in which catecholamines of peripheral origin act directly on the anterior pituitary.*

Insulin is vital in maintaining the normal metabolic state of the body. It is released from the pancreas and reduces circulating glucose by enhancing the uptake of glucose into cells. The hypoglycemia induced by insulin is stressful and causes a rise in plasma adrenocorticotropin (ACTH) and cortisol (*1*). To stimulate ACTH secretion, insulin may act (i) centrally to effect the release of corticotropin-releasing factor (CRF), (ii) by direct stimulation of the anterior pituitary, or (iii) at a peripheral site. We showed that peripheral administration of the β-adrenergic agonist isoproterenol results in the release of ACTH from the adenohypophysis in rats whose pituitary is separated from the brain either by transection of the pituitary stalk or by lesioning of the median eminence (*2*). The effect of isoproterenol on ACTH release is blocked by propranolol, a β-receptor antagonist, and by dexamethasone, an agent that blocks the synthesis and release of ACTH from the anterior pituitary (*3*). These results indicate that catecholamines can release ACTH by direct stimulation of β-adrenergic receptors on the anterior pituitary.

Insulin stress causes a marked increase in plasma epinephrine (*4*), and administration of epinephrine to rats increases ACTH release through a β-adrenergic mechanism (*5*). We now report that insulin-induced hypoglycemia, a physiologic stimulus, releases ACTH by a peripheral β-adrenergic mechanism.

When rats were injected with insulin, a threefold rise in plasma ACTH immunoreactivity was observed (Fig. 1). To examine whether a β-adrenergic mechanism was involved in the insulin-induced release of ACTH, we injected propranolol prior to the insulin administration. Propranolol blocked the rise in plasma ACTH induced by insulin. Propranolol did not affect basal ACTH levels in nonstressed rats.

ACTH can be released from either the anterior or intermediate lobe of the pituitary. Because glucocorticoids selectively block ACTH release from the anterior