CLONED CYTOTOXIC T LYMPHOCYTES THAT RECOGNIZE AN I-A REGION PRODUCT IN THE CONTEXT OF A CLASS I ANTIGEN

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The antigen recognition structure of T cells (T cell receptor) appears designed to recognize antigens in the context of MHC gene products on the cell surface. Minor (non-MHC) transplantation antigens in allogeneic responses and virus-induced antigens on the surface of virus-infected cells are accordingly recognized in the context of the MHC products. On the other hand, it is not clear whether or not MHC antigens themselves are subject to such restriction, and it remains possible that cells recognizing one MHC antigen in the context of another are included among responding T cell populations in allogeneic responses. A major portion of class II (or I region gene products)–specific allogeneic CTL are Lyt-2^+,L3T4^-, the phenotype thought to be characteristic of class I–recognizing T cells (1–3 and Shinohara, N., manuscript in preparation). Therefore, it is tempting to speculate that such populations of CTL might include cells recognizing class II in the context of class I antigens. Although class II–specific CTL have been reported not to be restricted by class I antigens (4, 5), it seems possible that cells specific for one MHC gene product in the context of another have been overlooked because of the limitations in studying bulk lymphocyte populations. In this report, cloned CTLs that recognize the combination of an *I-A*^{k} subregion gene product and H-2K^b antigen are described.

Materials and Methods

Mice. Adult mice of both sexes were used. All mouse strains and F_1s used in the experiments were produced in our own animal colony.

Antibodies. mAbs used in this study were 20-8-4 (anti-H-2K^b) (6), 14-4-4 (anti-I-E^k) (7), and 10-2-16 (anti-I-A^k) (8). Ascites of mAbs were produced as previously described (6).

Development of Cloned CTL. Cell cultures were carried out in DMEM supplemented with nonessential amino acids, sodium pyruvate, 2-ME, 10% FCS, and antibiotics. Before cloning, a bulk in vitro CTL line, QBR anti-MBR, was established by repeated weekly stimulations of B10.QBR splenic lymphocytes with irradiated B10.MBR lymphocytes in medium supplemented with 5% of 48-h culture supernatant of Con A–stimulated rat spleen cells (Con A sup). Cloned CTL were derived through limiting dilution of this bulk line at 10 cells/well in the presence of 10% rat Con A sup. The frequency of the wells with continuous cell growth was 21% (38 wells); 20 lines were established. All 20 lines showed killing activity and were typed as Lyt-2^+,L3T4^-. Cloned CTLs were maintained in the presence of 10% Con A sup with weekly simulation by irradiated B10.QBR spleen

TABLE I

H-2 Complex of the Congeneic Mouse Strains Used In This Series of Experiments

Strain	Haplotype	Origin of the H-2 complex				
		K	I-A	I-E	D	*Qa-Tla**
B10.QBR	bq4	b	b	b	q	q
B10.MBR	bq1	b	k	k	q	m
B10.AKM	m	k	k	k	q	m
C57BL/10 (B10)	b	b	b	b	b	b
B10.A	a	k	k	k	d	a
B10.A (3R)	i3	b	b	k	d	a
B10.A (4R)	h4	k	k	b	b	b
B10.YBR	i17	b	b	b	d	a

The cloned CTL QM3, QM7, and QM11 were derived from B10.QBR anti-B10.MBR bulk alloreactive line. The genetic difference between the responder and the stimulator is confined to the I region. However, there is an additional genetic difference to the right of the D region (*Qa-Tla*) in this combination.

* The names of the farthest traceable haplotypes are used in this paper to indicate the origin of this large segment of the chromosome. This was done solely for the sake of clarity and simplicity.

cells. The details of the cloned CTLs and the bulk CTL lines will be published elsewhere (Shinohara, N., and D. H. Sachs, manuscript in preparation).

CTL Assay. Cell-mediated lympholysis (CML) was carried out as previously described (1). For blocking experiments, effector and target cells were plated in wells containing 10 μl of appropriately diluted antibody and CML assay was then performed in the usual fashion. Experiments were performed in triplicates unless otherwise specified.

Target Blasts. LPS blasts were prepared by culturing spleen cells of the appropriate strains in the presence of 50 μg/ml of *Escherichia coli* LPS for 2 d. Thy-1$^-$ LPS-blast was induced by stimulating the Thy-1-depleted B10.MBR spleen cells with LPS. Ia$^-$ Con A blast was induced by stimulating Ia-depleted B10.MBR spleen cells, supplemented with irradiated syngeneic spleen cells, with 1 μg/ml of Con A for 48 h.

Results

Cloned CTLs were obtained through limiting dilution of a bulk line of B10.QBR lymphocytes maintained in vitro by repeated stimulation with irradiated B10.MBR cells. Since the original bulk line showed activities specific for I-A^k, I-E^k, and Qa type antigens while negative on H-2^b targets (Shinohara, N., and D. H. Sachs, manuscript in preparation), killing activity of the isolated lines was tested on LPS blasts of the B10.MBR (total reactivity), B10.A(3R) (I-E- and Qa-specific killing), B10.A(4R) (I-A-specific killing), and B10.YBR (Qa-specific killing) (Table I). Among 20 isolated lines, 10 lines showed killing activity only on B10.MBR targets (Table II). They were tested on a panel of strains to study their precise genetic specificities. Table III shows the reactivities of QM3 and QM7, cloned CTLs exhibiting such specificity. As a control, a line with defined specificity for I-A^k (QM11) was used. Both QM3 and QM7 were typed as Lyt-2$^+$,L3T4$^-$. As shown in Table III, QM3 and QM7 killed LPS blasts of the B10.MBR but not any other targets, including those expressing I^k gene products such as B10.A and B10.AKM. The lack of reactivity of these lines on the

TABLE II

Genetic Specificity of QBR Anti-MBR CTL Clones

Cloned CTL	Percent specific ^{51}Cr release using target LPS blast:					Specificity
	MBR (AEQ)*	3R (EQ)	4R (A)	YBR (Q)	B10 (−)	
QM1	32.4	27.9	−9.1	35.8	3.1	Q
QM2	50.3	45.5	−11.9	59.6	0.2	Q
QM3	47.4	−2.6	−7.0	−3.5	−5.0	?
QM4	46.2	49.6	−13.0	36.0	−1.4	Q
QM5	46.6	45.1	−6.3	−4.3	−2.3	E
QM6	33.1	−8.7	−0.8	−1.3	−0.4	?
QM7	29.5	−8.6	−1.6	−3.9	−5.9	?
QM8	28.3	18.4	−15.9	40.7	−3.6	Q
QM9	46.7	33.7	−16.5	46.6	0.8	Q
QM10	75.0	95.1	−10.6	80.6	−8.5	Q
QM11	69.8	−12.5	60.2	0.6	−5.4	A
QM12	69.2	73.1	−2.4	60.7	−8.7	Q
QM13	28.7	−12.5	−4.3	−1.2	−0.3	?
QM14	27.2	−16.8	1.8	−5.2	−1.7	?
QM15	22.6	0.2	−6.7	1.1	−2.0	?
QM16	52.9	65.2	−3.2	54.6	−8.8	Q
QM17	17.1	−13.6	−5.0	−4.6	−2.7	?
QM18	72.4	78.6	1.3	70.5	−10.8	Q
QM19	72.0	96.9	−8.7	71.7	−5.0	Q
QM20	13.0	−0.8	0.0	2.8	−2.8	?

This table is a summary of screening experiments carried out on clones obtained from a single set of limiting dilution of QBR anti-MBR bulk line plated at 10 cells/well. Experiments were done in singlicates. The specificities of the clones were later confirmed in fully controlled experiments. Results are expressed as percent specific ^{51}Cr release. Negative values indicate lower Cr release than spontaneous release in the experimental well. When spontaneous release is high, as in the case of LPS blasts (30–40%), coexistence of nonkilling viable cells in the well reduces background release from target. Although this effect is known as protective effect, the exact mechanism underlying this phenomenon is not known.

* Expected reactivity of QBR anti-MBR clones. A, I-A^k; E, I-E^k; Q, Qa-Tla region antigens.

B10.AKM target was particularly surprising, since this strain is the donor of the right side of the recombinant H-2 complex of B10.MBR (9), and should therefore possess all genetic differences that B10.QBR T cells can recognize on B10.MBR.

We envision four possible explanations of these results: (*a*) the existence of a cryptic mutation among the genes of the B10.MBR generated during the derivation of this recombinant strain; (*b*) an intragenic recombination in the *K* or *I-A* structural gene of the *H-2*^{bql} haplotype such that the product polypeptide chain differs from either parental chain; (*c*) the possibility that the observed result was due to complementation between two polymorphic structural genes of a two-polypeptide chain protein as has been documented for the I-E and I-A molecules (10, 11); and (*d*) the possibility that QM3 sees one MHC antigen in the context of another, one of which is located to the right of the recombination point in *H-2*^{bql} and the other to the left.

TABLE III
Genetic Specificity of the Cloned CTLs QM3 and QM7

Target LPS blast	E/T ratio	Percent ^{51}Cr release from target using cloned CTL:		
		QM3	QM7	QM11*
B10.MBR	4:1	59.3 ± 1.9	49.0 ± 1.6	78.7 ± 1.7
	2:1	50.0 ± 2.3	37.7 ± 1.5	75.5 ± 3.7
B10.AKM	4:1	−9.7 ± 0.6	−7.1 ± 1.2	65.9 ± 2.7
	2:1	−6.4 ± 1.1	−9.6 ± 0.6	76.0 ± 2.1
B10	4:1	−11.1 ± 2.1	−0.5 ± 0.3	5.7 ± 1.3
	2:1	−8.5 ± 1.4	−5.6 ± 2.7	2.4 ± 0.9
B10.A	4:1	−7.6 ± 2.0	0.0 ± 0.5	63.5 ± 2.5
	2:1	−4.8 ± 0.8	−2.4 ± 0.4	63.2 ± 1.3
B10.A (4R)	4:1	−17.0 ± 2.3	−6.9 ± 0.7	62.4 ± 1.0
	2:1	−13.7 ± 1.9	−0.2 ± 3.4	66.1 ± 2.9
(B10 × B10.AKM) F_1	4:1	26.6 ± 0.6	14.0 ± 1.6	67.9 ± 2.9
	2:1	27.4 ± 1.0	14.8 ± 0.4	56.5 ± 5.5
(B10.A [4R] × B10) F_1	4:1	36.2 ± 2.7	13.9 ± 3.3	59.4 ± 2.2
	2:1	31.5 ± 1.7	15.7 ± 0.9	58.5 ± 1.7
(B10.A [5R] × B10) F_1	4:1	−2.6 ± 0.8	2.2 ± 1.1	1.2 ± 1.6
	2:1	−1.4 ± 1.7	−1.4 ± 0.3	−0.9 ± 2.4

The cloned CTLs were obtained from a B10.QBR anti-B10.MBR bulk line.
* QM11 specific for $I\text{-}A^k$ was used as a positive control.

To assess these possibilities, killing activities of QM3 and QM7 were tested on targets of various F_1 animals. As seen in Table III, the LPS blasts of (B10 × B10.AKM)F_1 and (B10.A[4R] × B10)F_1 were killed by QM3 and QM7, while none of the parental strains was affected. This result is not easily explained by the first two models envisioned above, and suggests rather that two complementing independent genetic components are responsible for the determinant. The difference between B10.MBR and B10.AKM localizes one of the contributing genes to the left of the recombination point in $H\text{-}2^{bql}$, and the other gene to the right of this point. The successful reconstitution of the target antigen in (B10.A[4R] × B10)F_1 localizes the latter gene within the $I\text{-}A^k$ subregion (Table III). It also makes involvement of the I-E antigen less likely since this F_1 animal should not express the I-E molecule on the cell surface.

To investigate further the third and fourth possibilities, we attempted to block the killing by QM3 and QM7 with mAbs specific for the candidate molecules. Table IV shows that the killing of MBR targets by QM3 was inhibitable by an anti-H-$2K^b$ mAb, suggesting the involvement of H-$2K^b$ molecules. This result argues against the third possibility, since only the heavy chain of the class I molecule is encoded by the gene within the MHC, and the other chain should

TABLE IV

Blocking of Killing by CTL Lines by mAbs to MHC Gene Products

CTL line	Specificity	E/T ratio	Blocking antibody			
			None	Anti-K^b	Anti-I-A^k	Anti-I-E^k
QM3	?	2:1	39.2 ± 3.5*	16.3 ± 0.3	31.4 ± 1.6	34.7 ± 7.2
QM7	?	5:1	39.4 ± 3.3	8.0 ± 1.6	31.4 ± 1.3	35.1 ± 3.7
QM11	I-A^k	2:1	65.7 ± 1.1	67.7 ± 6.6	12.8 ± 1.4	62.3 ± 5.4

Ascites of the following mAbs were added to CML wells at a final dilution of 1:200: 20-8-4 (anti-K^b), 10-2-16 (anti-I-A^k), and 14-4-4 (anti-I-E^k). This preparation of the anti-I-A antibody always showed a slight inhibitory effect on QM3 and QM7, whereas the effect on QM11 was always obvious. The specificity of such slight inhibition was not clear, since inhibition at such a level was also seen even on killing of Ia^- targets by class I–specific CTLs.

* % specific ^{51}Cr release ± SE.

TABLE V

Failure of H-2K^b Mutants To Complement QM3 Target Antigen

Target LPS blast	E/T ratio	Cloned CTL		
		QM3	QM11	Bm10-37*
B10.MBR	4:1	45.5 ± 1.4‡	51.4 ± 1.3	36.7 ± 0.9
	2:1	34.2 ± 2.7	31.0 ± 0.2	18.9 ± 1.6
B10	4:1	−8.5 ± 0.3	−1.4 ± 2.4	47.0 ± 2.0
	2:1	−11.5 ± 3.1	−5.4 ± 0.8	34.6 ± 1.8
C3H	4:1	−5.7 ± 1.4	51.8 ± 2.7	−3.5 ± 0.4
	2:1	−5.8 ± 1.2	37.2 ± 0.6	−4.2 ± 1.4
(C3H × B6) F_1	4:1	31.0 ± 2.1	46.9 ± 1.5	28.9 ± 0.3
	2:1	25.4 ± 1.2	28.7 ± 0.4	20.4 ± 1.3
(C3H × B6.CH-2^{bm1}) F_1	4:1	−7.3 ± 2.0	42.5 ± 1.9	−4.5 ± 1.3
	2:1	−8.9 ± 0.5	30.5 ± 1.6	−1.0 ± 0.3
(C3H × B6.CH-2^{bm3}) F_1	4:1	−5.9 ± 2.1	42.5 ± 1.0	−4.5 ± 2.5
	2:1	−2.6 ± 0.7	30.5 ± 1.2	−1.0 ± 0.8
(C3H × B6.CH-2^{bm6}) F_1	4:1	53.4 ± 1.8	42.3 ± 1.7	15.7 ± 0.6
	2:1	37.7 ± 1.1	27.5 ± 0.9	7.0 ± 0.4
(C3H × B6.CH-2^{bm8}) F_1	4:1	0.2 ± 0.2	42.5 ± 1.7	17.5 ± 0.6
	2:1	2.0 ± 1.3	27.2 ± 0.2	13.4 ± 1.1
(C3H × B6.CH-2^{bm10}) F_1	4:1	43.7 ± 1.7	51.6 ± 2.1	−1.4 ± 2.0
	2:1	37.3 ± 0.8	35.0 ± 1.0	4.5 ± 0.3

* Clone Bm10-37 is a B6.CH-2^{bm10} anti-B6 CTL clone (13), and was used as a positive control for wild type H-2K^b.

‡ % specific ^{51}Cr release ± SE.

be identical among H-2 congeneic strains of the same background. The anti-I-A mAb did not show specific inhibitory effects on the killing. This result could either suggest that the I-A^k molecule is not involved in the specificity or that it

TABLE VI
Killing of QM3 and QM11 on Ia$^+$ and Ia$^-$ Targets

CTL line	Specificity	E/T	Target B10.MBR blast	
			Thy-1$^-$ LPS blast	Ia$^-$ Con A blast
QM3	?	4:1	37.3 ± 3.4‡	8.5 ± 0.7
		2:1	36.8 ± 3.8	8.7 ± 0.3
QM7	?	4:1	33.6 ± 0.7	6.0 ± 0.4
		2:1	33.3 ± 1.7	2.4 ± 1.4
QM11	I-A	4:1	72.6 ± 1.6	6.2 ± 0.7
		2:1	67.5 ± 2.1	6.0 ± 1.7
	Cytotoxicity*			
	Complement alone		12.3 ± 1.0	17.2 ± 0.8
	Anti-Thy-1 + C		9.8 ± 1.1	75.0 ± 2.0
	Anti-I-A^k + C		81.3 ± 1.1	9.6 ± 1.6
	Anti-I-E^k + C		70.9 ± 4.8	6.1 ± 0.4
	Anti-K^b + C		75.7 ± 2.2	81.3 ± 1.2

* Complement-dependent cytotoxicity assay was carried out on the same preparations of labeled targets using indicated mAbs.
‡ % specific ^{51}Cr release ± SE.

is involved as an antigen restricted by K^b, since the blocking of T cell recognition of MHC + X by antibodies to X is known to be extremely difficult (12). To study further the involvement of the K^b molecule in generating the QM3 target antigen, QM3 was tested on LPS blasts made from spleen cells of F$_1$ hybrids between C3H/HeJ (I-A^k) and H-2K^b mutants. As a control in this experiment, Bm10-37, a B6.CH-2^{bm10} anti-H-2K^b CTL clone (13) was used in addition to QM11 (anti-I-A^k). As shown in Table V, the target antigen for QM3 was reconstituted in the F$_1$ between C3H and B10, which carries the wild type H-2K^b. H-2^{bm6} and H-2^{bm10} were also capable of complementing the target antigen. However, three K^b mutants, i.e., H-2^{bm1}, H-2^{bm4}, and H-2^{bm8}, failed to complement the antigenic determinant in F$_1$ hybrids. These mutants differ from the wild type strain solely at the *H-2K^b* locus (14), confirming the involvement of the H-2K^b molecule.

Since the nature of the partner gene mapped to the *I-A^k* subregion was not clear, possible correlations between the expression of class II antigens on target cells and the susceptibility of the target to the killing by QM3 were studied next. Thy-1$^-$ LPS blasts and Ia$^-$ Con A blasts of B10.MBR were prepared and tested for their susceptibility to lysis by these lines and their expression of surface Ia antigens. As shown in Table VI, this experiment indicated a correlation between these two parameters. However, the plateau level of specific killing of the Ia$^+$ targets by QM3 was significantly lower than the percent Ia$^+$ cells as measured by C′-mediated lysis. This result probably reflects heterogeneity of Ia$^+$ cells in terms of sensitivity to lysis by these CTLs, possibly because of differences in the density of the I-A antigen on the surface. However, the possibility remains that an I-A region product other than the I-A molecule is the relevant target.

Discussion

The data in this report suggest that cloned CTLs, such as QM3 and QM7, see the combination of H-2K^b and a product of a gene present within the *I-A*^{k} subregion. The contribution of the K^b molecule to this specificity was clearly indicated by effective blocking of the killing by anti-K^b mAb and by failure of certain K^b mutants to complement the target specificity. However the nature of the partner gene mapped to the I-A subregion remains uncertain. Theoretically, there are three possible candidates: (*a*) the I-A^k (Aα + Aβ) molecule; (*b*) the β-chain of the I-E molecule; and (*c*) product(s) of other genes yet to be defined within this subregion. At present, the most likely candidate for an I-A subregion gene product is the I-A^k antigen. The failure of the anti-Ia mAbs to block this killing would suggest that the Ia antigen plays the role of an X antigen rather than that of an MHC antigen in the restricted recognition of self + X. In agreement with this interpretation is the fact that H-2K^b is the self MHC antigen for the QBR cells and I-A^k is not. Thus it appears that QM3 sees I-A^k in the context of the H-2K^b antigen. To distinguish this possibility from others, experiments involving the use of I-A^k transfectants are currently in progress.

The plateau level of killing of Ia$^+$ cells by QM3 and QM7 was often significantly lower than that by I-A^k-specific CTL, such as QM11, particularly when the target was heterozygous for *I-A*^{k} (Tables III and VI). Such incomplete killing of Ia$^+$ targets might be explained by low avidity, which could result in failure to kill low Ia expressors. It is also possible that these CTLs recognized processed forms of the I-A^k antigen, whose density on the cell surface could be significantly lower.

There are two major classes of MHC gene products known to serve as restricting elements for the recognition of antigens by T cells, i.e., class I and class II antigens. Class I antigens are preferentially recognized by CTL when they recognize foreign antigens on the cell surface. On the other hand, class II antigens play a major role as the presenter of soluble antigens to helper type T cells. Corresponding to the two major classes of MHC products, T cell populations consist of two major subsets that can be distinguished by differences in their expression of accessory interaction molecules. A fairly good correlation has been found between the expression of the Lyt-2 and L3T4 antigens on the surface of T cells and the type of the MHC antigens recognized (15, 16). Thus, in general, T cells recognizing class I antigens belong to the Lyt-2$^+$,L3T4$^-$ subset and those recognizing class II antigens belong to the Lyt-2$^-$,L3T4$^+$ subset. One major exception to this rule has been the CTLs that recognize allogeneic class II antigens. It has been shown that a major portion of such CTL activity was attributable to the function of the Lyt-2$^+$,L3T4$^-$ T cells (1–3, Shinohara, N., and Sachs, D. H., manuscript in preparation). One of the possible explanations for this exception is that these cells see class II antigens in the context of the class I antigen. Our results with the QM3 CTL line show that such cells indeed exist. On the other hand, we have also obtained Lyt-2$^+$,L3T4$^-$ class II–specific CTL lines (QM11) that appear to be capable of recognizing class II antigens without class I antigens. Therefore, both kinds of CTL probably contribute to the Lyt-2$^+$,L3T4$^-$ component of the anti–class II CTL response. In this sense, it will be interesting to determine whether there is a difference between these

restricted and nonrestricted class II–specific CTL lines in their functional dependency on the Lyt-2 molecules, a question under investigation.

Summary

Cloned CTLs QM3 and QM7 isolated from a bulk CTL line B10.QBR anti-B10.MBR recognized a combination of the H-2K^b molecule and an *I-A*^{k} subregion gene product. Such a combinatorial specificity was revealed by complementation of the target antigen in F_1 animals between two negative parental strains carrying *H-2K*^{b} and *I-A*^{k}, respectively. We confirmed the involvement of the H-2K^b molecule by blocking killing with anti-K^b mAb and failure of certain mutant *H-2K*^{b} genes to complement with *I-A*^{k} to generate the determinant in F_1 animals. Although the nature of the *I-A*^{k} subregion gene product is not definitive, there was a correlation between the expression of Ia antigens on the cell surface and susceptibility of the cells to lysis by these CTLs, suggesting that it is the classical I-A^k class II antigen.

The authors thank Dr. M. Kojima for helpful discussions.

Received for publication 13 November 1985 and in revised form 30 December 1985.

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