MOUSE ALLOANTIBODIES CAPABLE OF BLOCKING CYTOTOXIC T-CELL FUNCTION

I. Relationship between the Antigen Reactive with Blocking Antibodies and the *Lyt-2* Locus*

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Thymus-derived lymphocytes (T cells) have been shown to perform a variety of immunological functions in an antigen-specific manner (1). To explain such immunological specificity on a chemical basis, it is necessary to postulate the existence of a class of molecules on the T-cell surface which have specific affinity to relevant antigens by virtue of their structural diversity, i.e., T-cell receptors. Available information on the nature of T-cell receptors has been sparse and controversial. Nevertheless, accumulating evidence, mainly obtained by idiotypic analyses, suggests a role of Ig heavy chain-linked $Igh-V^2$ gene products in the antigen-combining sites of T-cell receptors, although conventional immunoglobulin determinants have not been demonstrated (2-4).

Antibodies to constant region determinants of immunoglobulins are known to block binding of antigen by B cells (5). Thus, if T-cell receptors also have constant regions, antibodies to such determinants might interfere with achievement of T-cell effector functions by blocking receptor-antigen interactions. Of course, antibodies to other cell surface molecules might also exert similar inhibitory effects on effector cell functions.

The effector function of cytotoxic T cells in allogeneic cell-mediated lymphocytotoxicity (CML)[1] reactions have previously been shown to be insensitive to treatment with a variety of antibodies in the absence of complement (6). Attempts to block allogeneic CML by alloantibodies reactive with killer cells have been uniformly unsuccessful, indicating that coating killer cells with antibodies is generally insufficient to affect the effector function. There have been two positive reports indicating inhibition of CML by xenogeneic antisera at the killer cell level in the absence of complement (7, 8), although the target molecules responsible for such inhibition or gene(s) encoding such molecules were not characterized.

If alloantibodies with such inhibitory activity could be raised, they would undoubtedly provide a more useful tool than xenoantibodies for analysis of the nature and genetics of molecules involved in cytotoxic T-cell effector function. Therefore, attempts were made to raise mouse alloantisera capable of inhibiting allogeneic CML in the

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absence of complement. Hyperimmune anti-lymphocyte sera raised in various strain combinations of mice were tested for their ability to inhibit allogeneic CML using killer-target combinations chosen to assess the effect on killer cells. Although most of our attempts were unsuccessful, we succeeded in raising mouse alloantisera in one strain combination which had the requisite properties. In this paper we describe the properties of these sera and an analysis of the linkage relationships of genes coding for the antigens on the cytotoxic T cells responsible for the observed inhibitory effect.

Materials and Methods

Mice. Adult mice, 8–10 wk old, of both sexes were used. Mice of strains AKR/N, BALB/cN, C57BL/6N (B6), C3H/HeN, and DBA/2N were obtained through the National Institutes of Health Small Animal Section. Strains A/J, C57BL/10 (B10), B10.BR, B10.D2, C3H/HeJ, C3H.SW, and CBA/J were produced in our animal colony. BALB.B mice were kindly provided by Dr. M. Potter, National Cancer Institute, and C3H.SW/HzlGlB (CWB) mice were kindly supplied by Doctors L. Herzenberg, Stanford University, and M. Bosma, Fox Chase Institute. B6.Ly2.1 mice were produced in our colony from stock kindly supplied by Dr. E. A. Boyse, Sloan-Kettering Institute.

Immunization. Recipient mice were immunized with a mixture of thymus, spleen, and lymph node cells of normal donor mice, or with cells sensitized repeatedly with allogeneic cells in vitro. Immunizations consisted of 2–5 × 10⁷ cells in 0.1 ml injected i.p. at 1- to 2-wk intervals. For the first immunization, the cell suspension was emulsified in an equal volume of complete Freund's adjuvant, and subsequent immunizations were administered without adjuvant. 7–8 d after each immunization, mice were bled from the tails. Sera from individual mice were pooled, unless otherwise mentioned, decomplemented (56°C for 30 min), and were screened for CML-inhibiting activity.

Target Cells. Normal spleen cells and tumor cells were used as target cells for CML assays. K46 BALB/c tumor cell line (9) was kindly provided by Dr. J. Kim and maintained in culture. 100 µCi of ⁵¹Cr in 0.1 ml was added to 1 ml of cell suspension containing 2 × 10⁷ cells. The mixture was incubated at 37°C for 1 h and washed three times with 15 ml of medium.

CML Assay. 4 million splenic responder cells were cultured with 2 × 10⁶ irradiated splenic stimulator cells in 2 ml of Eagle's minimal essential medium supplemented with glutamine, nonessential amino acids, sodium pyruvate, 0.05 mM 2-mercaptoethanol, antibiotics, 10 mM Hepes, and 10% heat-inactivated fetal calf serum in humidified 1% CO₂ in air at 37°C for 5 d. When a large number of cells was required, 1.4 × 10⁸ responders and 7 × 10⁷ stimulator cells were cultured in 70 ml of medium in a 250-ml plastic tissue culture flask. Cytotoxic activity was assessed in microtiter plates containing 2 × 10⁴ ⁵¹Cr-labeled target cells in 0.1 ml and graded numbers of attacker cells in 0.1 ml per well in triplicates. Cytotoxic activity of each killer population was titrated in every experiment. The plates were centrifuged at 20 g for 2 min at room temperature and incubated for 4 h at 37°C. Cultures were terminated by centrifugation at 800 g for 10 min at 4°C. Supernates were collected using the Titertek Supernatant Collection System (Flow Laboratories, Inc., Rockville, Md.), and were counted in a Searle gamma counter (Searle Diagnostics Inc., subsid. of G. D. Searle & Co., Des Plaines, Ill.). Maximum releasable ⁵¹Cr was measured by incubating targets in 2.5% Triton X-100 (New England Nuclear, Boston, Mass.). Specific percent lysis was calculated as:

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release}} \times 100.$$

Blocking of CML by Antisera. 20 µl of decomplemented serum was placed in microtiter wells in triplicate and 100 µl of cell suspension containing 1.6 × 10⁵ attackers were added. After shaking the plate, ⁵¹Cr-labeled target cells in 100 µl were added and the CML assay was performed as described above. Fetal calf serum used in all experiments was decomplemented (56°C for 30 min).

Complement-mediated Cytotoxicity Assay. Trypan blue cytotoxicity tests were performed as previously described (10). Briefly, 25 µl of serially diluted serum and 25 µl of cell suspensions

TABLE I

Summary of Attempts to Raise CML-blocking Mouse Alloantibodies*

Strain combination	Genetic‡ difference	Maximal§ cytotox-icity	Inhibition of CML
BALB/c α-B6	H-2 + non-H-2	>90% 1:2,042	−, ±‖
A/J α-B10.D2	"	>90% 1:256	−, ±
CBA/J α-B10	"	>90% 1:8,000	−
B10.D2 α-B10.BR	H-2	>90% 1:128	−
C3H/HeJ α-C3H.SW	"	>90% 1:5,120	−, ±
BALB/c α-B10.D2	Non-H-2	>90% 1:128	−
DBA/2 α-BALB/c	"	50%1:512	−
DBA/2 α-B10.D2	"	40%1:256	−
A/J α-B10.A	"	>90% 1:64	−
C3H/HeJ α-CBA/J	"	60%1:128	−
CBA/J α-C3H/HeJ	"	70%1:64	−
CBA/J α-B10.BR	"	>90% 1:512	−, ±
C3H/HeJ α-B10.BR	"	>90% 1:1,024	−, + +¶
C3H/HeN α-B10.BR	"	>90% 1:512	−, + +
CB-20 α-BALB/c	Igh-C	<10%	−
BALB/c α-CB-20	"	<10%	−
(PL/J × B6.PL-Thy1^a)F$_1$ α-B6	Thy-1	>90% 1:256**	−
BALB/c α-(B6 α-BALB/c) cells‡‡	H-2 + non-H-2	>90% 1:512	−
B6 α-(BALB/c α-B6) cells‡‡	"	>90% 1:256	−
(DBA/2 × B6)F$_1$ α-(B6 α-DBA/2) cells‡‡	None	−

* Animals of each group received 8–18 immunizations. Decomplemented pooled sera collected after each immunization were tested for their ability to inhibit CML in the absence of complement. Killer-target combinations were chosen so that the antisera should react only with the killer population. For details, see Materials and Methods.

‡ Genetic differences between the donors and the recipients.

§ Complement-dependent cytotoxicity of the serum on donor spleen cells which showed the highest cytotoxic activity among the sera obtained within the immunization group. The left column shows the maximum percent of spleen cells killed by the serum. The right column indicates reciprocal of the maximal dilution of the serum required to kill more than one-half of the maximum killing.

‖ Inhibition with marginal significance or with poor reproducibility.

¶ Some sera showed significant and reproducible inhibition.

** Cytotoxicity on thymocytes.

‡‡ Immunized with in vitro sensitized cells.

were mixed and incubated for 15 min at 37°C. Wells were washed and rabbit complement was added, followed by another 30-min incubation at 37°C. Cell death was determined microscopically by trypan blue uptake.

Results

Hyperimmune mouse alloantisera were raised in various strain combinations including H-2 and non-H-2 incompatible pairs. Some animals were immunized with cells which had been sensitized to allogeneic cells three times in vitro. These sera were tested for their ability to inhibit allogeneic CML when added to CML cultures in the absence of complement, using strain combinations chosen to assess effects on the killer cells. Over 150 sera raised in 20 different combinations were screened and results are summarized in Table I. Most of these sera failed to show any significant or reproducible inhibitory effect, although many of them proved to have fairly high-titered antibodies reactive with 100% of donor spleen cells in complement-dependent cyto-

TABLE II

Inhibition of CML by a C3H Anti-B10.BR Serum*

Inhibitor	B6 α-BALB on BALB	B10 α-C3H on C3H		B10 α-BALB on K46	
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
None	20.8 ± 2.4‡	11.9 ± 0.6	17.3 ± 1.1	33.9 ± 3.8	46.5 ± 0.7
NMS§	21.3 ± 1.7	11.4 ± 1.9	19.1 ± 5.0	27.2 ± 0.7	45.1 ± 3.3
N18-1 (C3H α-B10.BR)	8.2 ± 2.0	0.2 ± 0.4	8.9 ± 1.0	14.5 ± 0.7	21.4 ± 1.5

(Killer-target combination spans B6 α-BALB, B10 α-C3H, B10 α-BALB)

* For details see Materials and Methods.
‡ Percent specific release ± SD, in the presence of the indicated serum.
§ Normal mouse serum.

toxicity assays. These results suggested that coating of killer cells with antibodies was not sufficient to interfere with their killing function. However, among the C3H anti-B10.BR sera tested, some were found to have significant inhibitory effects on CML. Reproducibility of the inhibitory effect of one of these sera (N18-1) on B6 and B10 killers is shown in Table II. In experiments 2 and 3, C3H spleen cells were used as targets so that antibodies should not react with target cells. In experiments 1, 4, and 5, the target cells used (K46, a BALB/c tumor) were reactive to this non-H-2 antiserum. However, the antibodies in this serum reactive with the target cells proved not to be responsible for the inhibition observed, as will be shown in the following experiments.

To study whether the observed inhibitory effect of this serum was attributable to alloantibodies reactive with the killer population or was of a nonspecific nature independent of their antibody activity, the effects of the serum on killer cells of the donor and recipient strains were examined. Because this serum (C3H anti-B10.BR) should not contain anti-MHC antibodies, all H-2 congenic strains on the B10 background would be expected to show reactivity to this serum similar to that observed for B10.BR. Therefore, C3H and B10 cells were sensitized to each other in vitro and the inhibitory effect of this serum was assessed on CML of the two reciprocal combinations of killers and targets. As shown in Table III, presence of this serum in the CML culture caused significant reduction of specific lysis of target cells in the combination of B10 killers and C3H targets, whereas it did not have a significant effect on C3H anti-B10 CML. In sharp contrast to this serum, the C3H anti-C3H.SW ($H-2^k$ anti-$H-2^b$) serum inhibited CML only when target cells were reactive to this serum. This pattern of inhibition is characteristic of anti-H-2 antibodies, as has been well documented by many investigators (11, 12). This anti-H-2^b serum had unusually high-titered cytotoxic anti-H-2 antibodies (1:5,000 ~1:10,000) and also fairly high-titered anti-Ia antibodies. When B10 and C3H killers, directed to a third strain (BALB/c, $H-2^d$), were tested on the same target cell preparation, again, killing by B10 killers was significantly reduced by the C3H anti-B10.BR serum and C3H killers were insensitive. These results indicated that the inhibitory activity of this serum on CML was not of a nonspecific nature and this effect was achieved by antibodies reacting with the killer population rather than with target cells. It should be also

436 BLOCKING CYTOTOXIC T-CELL FUNCTION

TABLE III

*Specificity of CML-inhibiting Antibodies in the C3H Anti-B10.BR Serum**

Inhibitor	Killer-target combination			
	B10 anti-C3H on C3H	C3H anti-B10 on B10	B10 anti-BALB/c on K46	C3H anti-BALB/c on K46
None	17.3 ± 1.1‡	36.2 ± 1.3	47.1 ± 2.0	51.9 ± 1.7
NMS§	19.1 ± 5.0	35.3 ± 7.2	44.9 ± 1.4	52.4 ± 0.7
N18-1 (C3H α-B10.BR)	8.9 ± 1.0	38.9 ± 7.2	16.2 ± 0.1	40.9 ± 0.2
B-65 (C3H α-C3H.SW)‖	14.0 ± 1.3	−4.6 ± 0.5		

* For details see Materials and Methods.
‡ Percent specific release ± SD, in the presence of indicated serum.
§ Normal C3H serum.
‖ $H-2^k$ anti-$H-2^b$ serum. B10:$H-2^b$, C3H:$H-2^k$.

noted that specificity of killer cells (i.e., H-2 type of target cells recognized by the killers) did not seem to affect sensitivity to the inhibitory effect of this serum.

In an attempt to obtain an insight into the genetic basis of the inhibitory antibodies, sensitivity of CML effector cells of various mouse strains to the inhibitory effect of this antiserum was examined (Table IV). In experiment 1, all killer cells were sensitized to $H-2^b$ cells and tested on C3H.SW spleen cells. In experiment 2, all cells were sensitized to $H-2^d$ cells and tested on K46 cells. Killer cells of A/J, A.BY, BALB/c, BALB.B, B10, B10.BR, B10.D2, and B6 strains were significantly inhibitable by these sera whereas those of AKR, CBA/J, C3H/HeJ, C3H/HeN, C3H.SW, and DBA/2 strains were insensitive, and the distinction between the sensitive and insensitive strains was unambiguous. Complement-dependent cytotoxicity of this serum on spleen cells of representative strains is depicted in Fig. 1. As anticipated from the complexity of the genetic differences between the donor and the recipient, this serum (C3H anti-B10.BR) was toxic to cells of all strains except for those on the C3H background. In particular, it should be noted that the serum was toxic to 100% of spleen cells from AKR and DBA/2 strains with titers comparable to those on B10.BR and BALB/c cells, respectively. This finding again corroborates the notion that simply coating killer cells with antibodies is insufficient to inhibit their killing function. The results of scoring various strains for sensitivity to the CML-inhibiting effect of these sera were compared with the strain distribution of other known genetic markers. A salient correlation was found between this marker and Lyt-2 phenotypes (Table IV).

To study further the possible relationship between the antigen(s) responsible for the inhibitory effect and Lyt-2-linked genes as well as Igh-C-linked genes, killer cells from Lyt-2 congenic and Ig congenic strains were tested. Among B6 ($Lyt-2^b$, $Igh-C^b$, B6.Ly2.1 ($Lyt-2^a$, $Igh-C^b$), C3H.SW ($Lyt2^a$, $Igh-C^a$), CWB ($Lyt-2^a$, $Igh-C^b$), B10.BR ($Lyt-2^b$, $Igh-C^b$), and C3H/HeJ ($Lyt-2^a$, $Igh-C^a$) strains, only B6 and B10.BR strains were significantly sensitive to the inhibitory effect of several different preparations of C3H anti-B10.BR sera (Table V). Complement-dependent cytotoxicity of serum L♂#3 was tested on Ficoll-Hypaque-separated killer populations used in experiment 4. As shown in Fig. 2, this serum killed >98% of in vitro sensitized killer cells of B6, B6.Ly2.1, and B10.BR strains with almost identical titers. Yet, B6.Ly2.1 killers were totally insensitive to the CML-inhibitory effect of this serum (Table V), symbolizing

TABLE IV

Effect of C3H Anti-B10.BR Sera on Killer Cells of Various Strains*

Killer strain‡	Exp. 1 inhibitor			Exp. 2 inhibitor			Sensitivity§	Lyt-2
	None	NMS	N18-1	None	NMS	N18-4		
AKR/N	8.5||	8.1	9.5	7.0	9.2	9.9	−	1
A/J	8.9	10.1	0.4				+	2
A.BY				8.2	9.1	4.3	+	2
BALB/c	13.0	10.8	2.3				+	2
BALB.B				11.7	7.4	−0.8	+	2
B10				18.0	16.2	−1.5	+	2
B10.D2	8.5	5.6	1.7				+	2
B10.BR				13.8	13.0	3.7	+	2
B6				20.8	21.3	8.2	+	2
CBA/J	8.6	6.8	7.7	17.9	18.2	19.3	−	1
C3H/HeJ				13.7	12.8	13.1	−	1
C3H/HeN	8.1	6.0	8.2	21.6	19.0	19.2	−	1
C3H.SW				18.8	21.7	19.8	−	1
DBA/2	10.5	13.0	9.6				−	1

* For details see Materials and Methods.
‡ Normal splenic lymphocytes of indicated strains were sensitized in vitro to C3H.SW cells (Exp. 1) or to BALB/c cells (Exp. 2) and tested on C3H.SW spleen cells (Exp. 1) or K46 tumor cells (Exp. 2).
§ Sensitivity of killer cells to the inhibitory effect of the C3H anti-B10.BR serum.
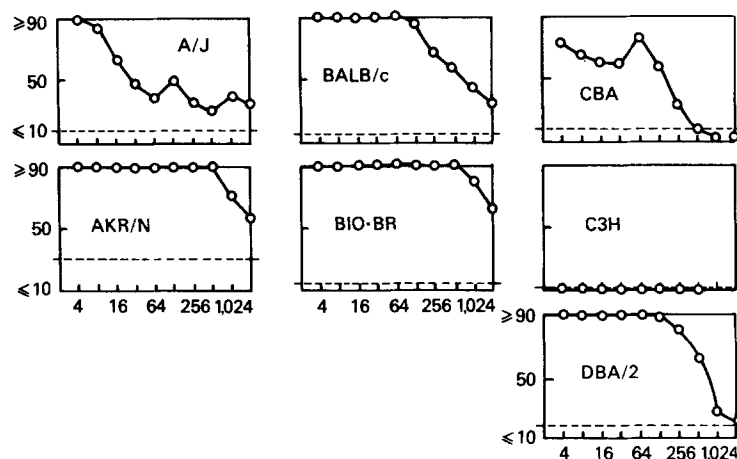\|\| Percent specific release in the presence of the indicated serum.

FIG. 1. Complement-dependent cytotoxicity of serum N18-4 on spleen cells of various strains. Ordinate indicates percent of spleen cells killed. Abscissa indicates reciprocal of serum dilutions. Broken lines indicate complement background.

the discrepancy between cytotoxic activity and CML-inhibiting activity of these antisera. These results suggest that the CML-inhibiting antibodies in these complex sera are solely directed toward molecules encoded by a single genetic locus linked to or identical with Lyt-2.

The experiments shown thus far, established the genetic linkage or identity of the gene(s) encoding the molecules reactive with the CML-blocking antibodies and the Lyt-2 locus. However, because of the complexity of the C3H anti-B10.BR sera, it was

TABLE V

Inhibitory Effect of C3H Anti-B10.BR Sera on Killer Cells of Congenic Strains*

Specificity of killers[†]	Target	Inhibitor[§]	Killer strain					
			B6	B6.Ly2.1	C3H.SW	CWB	B10.BR	C3H
Exp. 1								
		None	23.5 ± 3.2‖	23.0 ± 1.4	18.3 ± 4.2	13.9 ± 1.7		
α-H-2^d	K46	nl C3H	22.8 ± 2.0	25.1 ± 0.3	21.7 ± 5.1	19.4 ± 6.5		
		N18-1	12.3 ± 3.8	24.3 ± 2.8	19.8 ± 1.9	17.8 ± 0.3		
Exp. 2								
		None	25.8 ± 5.0	17.4 ± 0.5				
α-H-2^k	C3H	nl C3H	20.5 ± 2.0	18.3 ± 5.5				
	spleen	N18-4	11.9 ± 5.3	21.4 ± 0.7				
Exp. 3								
		None	14.2 ± 1.8	12.3 ± 2.0				
α-H-2^k	C3H	nl C3H¶	6.5 ± 1.8	5.3 ± 0.9				
	spleen	N18-4	4.1 ± 1.7	12.1 ± 0.3				
Exp. 4								
		None	26.3 ± 1.3	20.6 ± 0.5	45.9 ± 1.7	24.4 ± 0.5	20.6 ± 1.0	22.7 ± 1.3
α-H-2^d	K46	nl C3H	25.5 ± 0.8	18.1 ± 0.7	41.9 ± 1.1	23.6 ± 2.3	20.1 ± 2.8	19.7 ± 1.4
		N23-5	11.7 ± 0.7	22.1 ± 0.1	43.3 ± 5.1	30.7 ± 0.4	8.5 ± 0.7	24.1 ± 0.9
		Lδ#3	14.8 ± 1.2	21.8 ± 1.9	42.7 ± 2.2	25.0 ± 0.3	10.3 ± 1.1	20.5 ± 1.7

* For details see Materials and Methods.

† α-H-2^d: cells were sensitized to B10.D2 cells. α-H-2^k: cells were sensitized to C3H cells.

§ N18-1, N18-4 and Lδ#3: C3H/HeJ anti-B10.BR. N23-5: C3H/HeN anti-B10.BR. After the initial discovery of the inhibitory serum (N18-1), sera from individual mice were screened for CML-inhibiting activity, and only strongly positive sera were pooled (N18-4, N23-5). The immune sera from mouse Lδ#3 showed extraordinarily high specific inhibitory activity, therefore the sera from this single mouse were pooled separately. Serum Lδ#3 was used at 1:8 dilution in this experiment.

‖ Percent specific release in the presence of the indicated serum ± SD.

¶ This batch of normal C3H serum showed nonspecific inhibitory effect on CML. It also inhibited C3H killer cell function. However, the specific inhibition by serum N18-4 was clearly distinguishable as the data show.
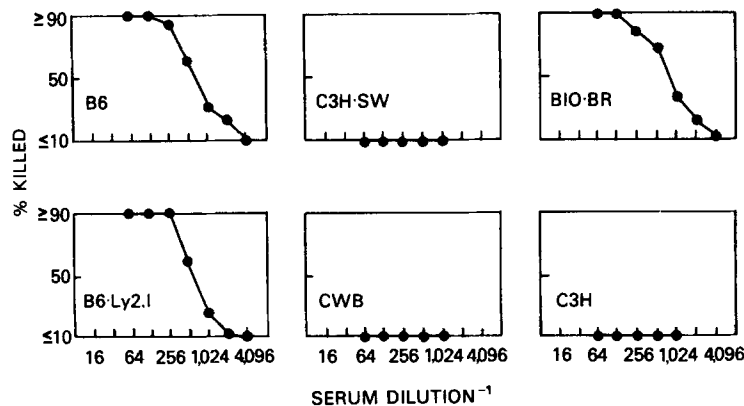

FIG. 2. Cytotoxicity of serum Lδ#3 (C3H anti-B10.BR) on in vitro sensitized killer cell populations. Day 5 in vitro sensitized killer cells used in Exp. 4 of Table V were tested for reactivity to serum Lδ#3 by a complement-dependent cytotoxicity assay. Ordinate: percent dead cells; abscissa: serum dilution^{-1}.

not possible to study the correlation between anti-Lyt-2 antibody activity and CML-blocking activity of these sera. Therefore, an attempt was made to raise CML-blocking sera in an Ly2 congenic combination (i.e., conventional anti-Ly2.2 antisera). Four (C3H × B6.Ly2.1)F$_1$ mice were immunized with a mixture of thymocytes, spleen cells, and lymph node cells from normal B6 mice. After 10 immunizations, two animals started to produce detectable amounts of CML-inhibiting antibodies, whereas

TABLE VI

*Comparison of Anti-Lyt-2 Cytotoxic Antibody Activity and CML-blocking
Activity of Anti-Lyt-2 Sera**

(C3H × B6-Ly2.1)F₁ anti-B6 animal number	Anti-Lyt-2‡ activity	Blocking of CML	
		B10 anti-BALB on K46	C3H.SW anti-BALB on K46
No.			
—	—	36.0 ± 0.2§	10.6 ± 0.5
596	1:40	34.7 ± 0.8	11.9 ± 0.5
597	1:1280	30.0 ± 0.6	12.2 ± 0.6
598	1:320	20.1 ± 0.6	11.4 ± 0.4
599	1:320	8.8 ± 0.5	8.6 ± 1.0

* Four (C3H × B6.Ly2.1)F₁ mice were immunized with a mixture of thymus, spleen, and lymph node cells from normal B6 mice. Results shown here are for sera collected 7 d after the 12th immunization of each animal.

‡ Complement-mediated cytotoxicity on B6 thymocytes. All four sera killed 80–90% of B6 thymocytes. None showed detectable cytotoxic activity on B6.Ly2.1 thymocytes.

§ Percent specific release in the presence of the indicated serum.

the other two did not. Individual sera obtained after 12 immunizations were examined for their CML-blocking activities and their anti-Lyt-2 antibody activity was determined by complement-mediated cytotoxicity on B6 thymocytes (Table VI). Sera from animals 596 and 597 did not show appreciable CML-blocking activities. The other two sera (particularly 599) caused significant specific reduction of target cell killing by B6 killer cells when added to CML culture. All four sera killed 80–90% of B6 thymocytes in the presence of rabbit complement with different titers. However, there was no apparent correlation between cytotoxicity on thymocytes and CML-blocking activity of the sera. For example, serum 597 showed poor CML-blocking activity, whereas it showed relatively high cytotoxic activity on B6 thymocytes. Serum 599, on the other hand, produced potent CML-blocking, although its cytotoxic activity was not as high as that of serum 597. This discrepancy could be a result of differences in the immunoglobulin class of the dominating antibodies in individual sera, or could indicate that CML-blocking activity is not a result of antibodies specific for conventional Lyt-2 molecules, but rather a result of antibodies directed to as yet undefined molecules encoded by *Lyt-2*-linked genes.

Discussion

Allogeneic killer cells are generally resistant to treatment with allo- and xenoantisera in the absence of complement (6). Among the few positive reports of such treatments is that of Kimura (7), who reported that a rabbit xenoantiserum raised against in vivo sensitized mouse alloreactive cells inhibited the allogeneic CML of the same strain combination as used for immunization. The inhibitory activity of this antiserum was attributed to possible anti-idiotypic antibodies reactive with the antigen combining site of the relevant T-cell receptors (7), but the nature or genetics of the putative receptor were not elucidated. Recently, Redelman and Trefts obtained goat anti-rabbit xenoantibodies capable of inhibiting rabbit anti-mouse CML (8). In this laboratory, several xenogeneic anti-mouse lymphocyte sera were found to contain

antibodies which inhibited mouse allogeneic CML in the absence of complement, and at least one component of such inhibitory activity was attributable to antibodies reacting with killer cells (N. Shinohara, unpublished observations). However, the use of xenogeneic antisera introduced numerous complexities making an analysis of molecules and mechanisms responsible for the observed inhibitory effect extremely difficult.

There have previously been no successful reports on attempts to block the effector function of allogeneic killer cells with alloantibodies in the absence of complement. Similarly, most of our attempts to raise such alloantibodies failed (Table I). Even antisera reactive with T cells with extraordinarily high titers did not affect allogeneic killing. Thus, because the coating of killer cells with antibodies to most cell surface alloantigens does not affect their function, our observations strongly suggest a functional significance of the molecules reactive with CML-blocking alloantibodies.

Among our attempts at alloimmunization, successful results have so far been obtained only in the combination C3H anti-B10.BR. Even in this combination, not all immune animals produced CML-inhibiting antibodies. Although two-thirds of the immunized animals started to produce detectable amounts of inhibitory antibodies after >10 immunizations, very few mice (10–15%) produced a sufficient amount of inhibitory antibodies to allow analytical experiments. The earliest production of inhibitory antibodies was observed after four to six immunizations in exceptional animals which became good producers later. Considering this experience, it seems likely that CML-inhibiting activities may have been lost by pooling immune sera before screening in some cases. Thus, it is possible that in combinations other than C3H anti-B10.BR, CML-inhibiting antibodies could be raised if individual animals were screened.

The inhibitory effect we have studied is attributable to the reactivity of antibodies with a killer cell population rather than with target cells, because killer cells of the donor strain background are sensitive to the inhibitory activity, but those of recipient background are not. Killer cells of the sensitive strains are inhibited by the antisera irrespective of H-2 types of the targets they recognize. In addition, these non-H-2 antisera do not interfere with target cell lysis even when they react with target cells (Tables III-V).

The strain distribution of sensitivity of killer cells to the inhibitory effect of the C3H anti-B10.BR antisera is very well correlated with that of the $Lyt-2^b$ allele. Furthermore, the B6.Ly2.1 strain, which differs from the B6 strain at a chromosomal segment including the $Lyt-2$ locus, is insensitive to the inhibitory effect of these complex sera. The genetic specificity of this antibody activity is reinforced by the fact that CML-blocking antibodies could be raised in an $Lyt-2$ congenic pair (Table VI). No apparent correlation was seen between complement-dependent cytotoxicity of antisera on spleen cells or on in vitro sensitized killer cell populations and inhibitory activity of the sera on killer cells of the same strain. These data indicate that the CML-inhibiting activity of these antisera is dependent only on their reaction with products of genes linked to or identical with the $Lyt-2$ locus.

The identity of the molecules responsible for the antibody-mediated inhibition of CML still remains to be studied. Earlier reports by other investigators have suggested that anti-Lyt-2 antibodies do not inhibit CML (6, 13). The discrepancy between the present observation and those observations might imply that the molecules reactive

with the inhibitory antibodies are not Lyt-2 molecules but rather distinct molecules encoded by *Lyt-2*-linked genes. The discrepancy between anti-Lyt-2 cytotoxic antibody activity and CML-blocking activity in the sera raised in the *Lyt-2* congenic combination we have studied also casts doubt on the identity of the molecules reactive with CML-blocking antibodies. However, this discrepancy may be explained by differences in the immunoglobulin class of the dominating antibodies in individual sera, a possibility we are presently investigating. It is important to note that if antibodies to products of a gene closely linked to Lyt-2 were responsible for the blocking observed, it is quite likely that such antibodies would be included as contaminants in conventional anti-Lyt-2 (and perhaps anti-Lyt-3) sera. Studies using monoclonal anti-Lyt-2 hybridoma antibody are also in progress to try to answer this question (N. Shinohara, U. Hammerling, and D. H. Sachs, manuscript in preparation).

At present, we can only speculate on the possible mechanisms of the inhibition of CML we have observed. The most trivial possibility might be agglutination of killer cells by antibodies. Massive agglutination could perhaps prevent killer cells from contacting target cells. Although this possibility has not been formally ruled out, it does not seem likely, because exposing killer cells to high-titered antibodies of other specificities did not inhibit their killing function (Tables I, III–V, Figs. 1 and 2).

A second possibility is alteration of the cell surface by antibodies which somehow results in the inability of killer cells to achieve normal function. Because many other antibodies reactive with T cells failed to cause inhibition, this model requires a certain peculiar nature of the molecules reactive with the inhibitory antibodies. Such a relationship could be a close physical association of the *Lyt-2*-related determinants with some functional molecule necessary for killing. Even without physical association of the two kinds of molecules, such specific interference might occur, as has been exemplified in blocking of B-cell Fc receptors by anti-Ia antibodies (14).

A third possibility is that the inhibitory antibodies react with functional molecules other than antigen receptors. After interacting with the antigen on target cells, killer cells may deliver a killing message to the target cells through mediators either expressed on their surfaces or released locally. If the antibodies reacted with such molecules, killing might not take place. The Lyt-2 antigen has been shown to be a marker for a subpopulation of T cells with certain functions including killer and suppressor cells (15–17). If Lyt-2 molecules were the mediators of killing, this might explain the correlation between this surface marker and the function of cells bearing the molecules. However, this explanation would not account for the fact that many immature peripheral T cells and thymocytes also bear Lyt-1, 2, and 3 (18, 19).

Perhaps the most attractive possibility is that the observed inhibition reflects interaction of anti-receptor antibodies and antigen-recognition structures of killer T cells. In this model, the anti-receptor antibodies would be directed to a constant portion of the receptor, because they inhibit the function of killer cells with various different specificities, i.e., anti-H-2^d, anti-H-2^k and anti-H-2^b. Of course, this apparent lack of killer specificity could be explained by the presence of multiple anti-idiotypic antibodies in the serum and absorption studies would be required to distinguish between these possibilities. If Lyt-2 molecules were the antigen-receptor molecules, this would provide a possible explanation for the presence of Lyt-2 molecules on immature T cells and thymocytes. Recent investigations on the ontogeny of the T-cell repertoire indicate that precursor T cells differentiate and are selected in the thymus

so that the repertoire of mature T cells is restricted in terms of selfreactivity (20). If this is true, precursor T cells should express their clonal marker, i.e., receptors, on their surface so as to be subjected to such selection before full differentiation.

One of the difficulties with this model is the fact that certain populations of T cells lack the Lyt-2 marker (14–16). These include helper cells and I-region-specific mixed leukocyte reaction (MLR)-reactive cells. One would thus have to postulate the existence of two separate sets of genes coding for T-cell receptors, one for Lyt-23$^-$ cells and another for Lyt-23$^+$ cells. Idiotypes of T-cell receptors have been intensively studied recently (reviewed in references 2 and 3), and these studies have suggested that at least a part of the antigen-combining portions of T-cell receptors is encoded by genes linked to the immunoglobulin heavy chain allotype locus. The T cells involved in these studies were helper cells and MLR-reactive cells, both of which functions are predominantly attributed to Lyt-23$^-$ cells in the mouse (15, 16). So far, no genetic relationships of receptors of Lyt-1$^-$23$^+$ cells to Ig heavy chain genes have been demonstrated. It is tempting to speculate that different subsets of T cells could use different sets of genes for their receptors, Ig heavy chain-linked genes for the Lyt-1$^+$23$^-$ population and Lyt-2-linked genes, perhaps κ-light chain-linked genes (21, 22), for Lyt-23$^+$ cells. In this regard, we intend to examine the effects of our alloantisera on other functional T-cell subsets. If similar blocking activities are found, it will be of interest to determine the genetic relationships of the relevant targets.

Summary

In an attempt to produce alloantibodies to cytotoxic T-cell receptors, hyperimmune anti-lymphocyte antisera have been raised in mice of various strain combinations, and have been tested for their ability to block allogeneic cell-mediated lymphocyto-toxicity (CML) in the absence of complement at the T killer cell level. Most of the sera failed to show any significant and reproducible inhibitory effects. However, among C3H anti-B10.BR antisera, some sera were found to be capable of significantly inhibiting CML. This effect was attributable to antibodies reacting with the killer population rather than the target cells, because the sera inhibited B10 anti-C3H CML but not C3H anti-B10 CML. Among mouse strains tested, A/J, BALB/c, B10, and B6 strains were sensitive to the inhibitory effect of the sera whereas AKR, CBA, C3H, and DBA/2 strains were insensitive. This sensitivity of killer cells to the inhibitory effect correlated well with the strain distribution of the Lyt-2.2 antigen. In the presence of complement, these same sera were toxic to 100% of spleen cells of AKR, BALB/c, B10, and DBA/2 strains, with comparable cytotoxic titers. Thus, the inhibitory activity of the sera could not be explained by nonspecific effects of high-titered antibodies. To study the relationship between the antigen(s) responsible for the blocking effect and Lyt-2-linked genes, killer cells from Lyt-2 congenic strains were tested and conventional anti-Lyt-2.2 antisera were raised in an appropriate congenic strain combination. Killer cells from B6, but not from B6.Ly2.1 animals, were significantly sensitive to the blocking effects of the inhibitory C3H anti-B10.BR sera. The conventional anti-Lyt.2.2 sera did produce CML blocking, although there was no apparent correlation between such blocking and the anti-Lyt-2.2 cytotoxic titer. These results thus indicate that the target molecules responsible for blocking of killer cells are encoded or regulated by genes that are closely linked to or identical with Lyt-2.

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