THE CONTROL OF SPECIFICITY OF CYTOTOXIC T LYMPHOCYTES BY THE MAJOR HISTOCOMPATIBILITY COMPLEX (AG-B) IN RATS AND IDENTIFICATION OF A NEW ALLOANTIGEN SYSTEM SHOWING NO AG-B RESTRICTION*

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A variety of studies in the mouse have demonstrated the importance of the major histocompatibility complex (MHC)[1] in the induction and expression of immune responses of thymus-dependent (T) lymphocytes (1). Cytotoxic T lymphocytes (CTL) stimulated with chemically modified cells (2), with virus-infected cells (3), or with cells bearing minor allogeneic histocompatibility antigens (4), lyse most effectively those target cell populations that express both the relevant modifying antigen and the same MHC *K* and/or *D* region gene products as the original stimulating cell population.

The precise mechanism of MHC restriction of CTL lytic activity remains unclear. Nevertheless, the phenomenon has direct implications for the biological role of this gene complex, an issue that has long been the subject of considerable speculation (1, 5, 6). Therefore, it is important to establish the generality of MHC involvement in T-cell recognition for other species.

The present studies examined the MHC control of lytic activity in rat T-lymphocyte populations stimulated with (*a*) cells differing only with respect to minor non-Ag-B-encoded alloantigens, (*b*) influenza virus-infected syngeneic cells, and (*c*) cells from allogeneic strains previously thought to be MHC compatible. The results in the first two systems extend the generality of MHC control of CTL specificity to the rat. However, discrepant results were found in the third; when lymphocyte populations from Lewis (L; Ag-B1) donors presensitized to weak alloantigens of the Fischer 344 strain (F; Ag-B1) were restimulated in culture with Fischer cells, extensive lytic activity was found against

* Supported by U.S. Public Health Service grants CA-15822, CA-09140, AI-10961, and grant no. 851-A-4 from the National Multiple Sclerosis Society.

[1] *Abbreviations used in this paper:* ADCC, antibody-dependent cell-mediated cytotoxicity; Ag-B, major histocompatibility complex of the rat; Con A, concanavalin A; CT, cytotoxic T-cell-defined; CTL, cytotoxic T lymphocyte; F, Fischer strain; FCS, fetal calf serum; H, influenza virus hemagglutinin antigen; HA, virus hemagglutinating unit; *H-2*, major histocompatibility complex of the mouse; HPBS, Hanks' phosphate-buffered saline; L, Lewis strain; LNC, lymph node cells; N2(Ag-B1), an L x L/DA backcross rat homozygous for AgB1 at the MHC; MHC, major histocompatibility complex; MLC, mixed lymphocyte culture; N, influenza virus neuraminidase antigen; PBL, peripheral blood lymphocytes; T, thymus-dependent; TDL, thoracic duct lymphocyte.

Fischer strain target cells as expected, but there was also considerable lysis of target cells from several other strains regardless of Ag-B haplotype. Further genetic analysis indicated that the Lewis and Fischer rat strains, considered to be MHC identical by several criteria—serological (7), mixed lymphocyte cultures (MLC); (8), graft vs. host reactions (9), and the ease of tolerance induction and immunosuppression (10)—actually differ with respect to antigens determined by genes that map with the MHC. These weak Ag-B-linked antigens can be detected only with cytotoxic T-lymphocyte populations (hence, "CT" antigens), and their recognition by CTL is apparently not restricted to MHC compatible interactions.

Materials and Methods

Rats. Rat strains used in this study were from the following sources: (*a*) August 990 (A990; Ag-B1), Black Hooded (BH; Ag-B1), Brown Norway (BN; Ag-B3), DA (Ag-B4), August 28807 (Aug; Ag-B5), and backcross rats were bred and maintained in our own colony at the University of Pennsylvania. (*b*) Fischer 344 (F; Ag-B1), Lewis (L; Ag-B1), Wistar Furth (WF; Ag-B2), Maxx (Ag-B3), and ACI (Ag-B4) rats were purchased from Microbiological Associates, Bethesda, Md. (*c*) AGUS (Ag-B1), August-Oxford (AO; Ag-B2), Hooded Oxford (HO; Ag-B5), the recombinant HO.DA(1R), and the congenic HO.B2 developed by W. Ford were kindly sent by Doctors J. C. Howard and G. Butcher from the animal colony at the Institute for Animal Physiology, Agricultural Research Council, Babraham, Cambridge, Eng. (*d*) Albany (Alb; Ag-B6), and the congenics BN.B2, BN.B4, and Aug.B3 were kindly provided by Dr. D. Götze from the colony at the Wistar Institute, Philadelphia. (*e*) Wild rats captured in the vicinity of the Philadelphia International Airport were provided by Dr. D. Gasser.

Lymphocyte Populations

(*a*) PERIPHERAL BLOOD LYMPHOCYTES (PBL). PBL were prepared for culture by sedimentation in a citrate-dextran solution according to the method of Wilson (11). PBL were prepared for cytotoxicity assays by a modification of the procedure of Davidson and Parish (12); briefly, citrated blood was centrifuged (1,000 *g* for 1 min), the platelet-rich supernate was discarded, and the blood cell pellet was resuspended in Hanks' phosphate-buffered saline (HPBS) and layered over a mixture of 12 parts 14% Ficoll (Pharmacia Fine Chemicals, Piscataway, N. J.) and 5 parts 32.8% Isopaque (Gallard Schlesinger Chemical Mfg. Corp., Carl Place, N. Y.). These gradients were centrifuged (2,000 *g* for 15 min), and the lymphocyte-rich layer was removed from the interface and washed in HPBS.

(*b*) THORACIC DUCT LYMPHOCYTES (TDL). Rats were cannulated as previously described (11) and then restrained in Bollman cages and infused continuously with saline via a femoral vein cannula at a rate of approximately 3 ml/h. TDL were collected over ice into flasks containing 5 ml of HPBS and 100 U of Heparin and then washed in HPBS.

(*c*) LYMPH NODE CELLS (LNC) AND SPLEEN CELLS. LNC and spleen cell suspensions used for in vivo immunizations were prepared according to the method described by Billingham (13). LNC were prepared for culture by gently teasing lymph nodes in HPBS with a pair of watchmaker forceps. Clumps were allowed to settle for 3 min and the remaining single cell suspension was washed twice in HPBS.

Viruses. Influenza type-A virus stocks were grown in embryonated eggs and stored frozen as allantoic fluid containing between 1,200 and 3,000 hemagglutinating (HA) U/ml (14). The strains involved in this study, PR8 (A/PR/8/34 [HON1]) and HK (A/Hong Kong/8/68-X-31 [H3N2]), were obtained originally from Dr. S. Fazekas de St. Groth, Division of Animal Genetics, Sydney, Australia, and Dr. R. G. Webster, St. Jude Children's Research Hospital, Memphis, Tenn., respectively.

Generation of CTL

(*a*) AGAINST MINOR HISTOCOMPATIBILITY ANTIGENS. Standard immunization procedure involved two sets of bilateral skin grafts spaced at least 3 wk apart followed by injection of 50–100 $\times$ 10^6 allogeneic spleen and LNC approximately 1 mo later (15). One-half the immunization dose was

injected i.p. and the rest was distributed among five subcutaneous sites. Certain animals were immunized with lymphoid cells only or skin alone. All donors and recipients were sex-matched. 3–5 wk after the last immunization 20×10^6 immune LNC or TDL and 10×10^6 irradiated (1,700 rads) stimulating LNC were cultured together in Marbrook vessels (Bio-Research Glass, Inc., Vineland, N. J.) following procedures described elsewhere (16).

(*b*) AGAINST INFLUENZA VIRUS-INFECTED CELLS. Rats were primed by i.p. injection of approximately 1,000 HA U of PR8 virus diluted 1:2 in phosphate-buffered saline. 4 wk later, these immune rats were boosted by inoculation of 1,000 HA U of HK virus both i.v. and i.p. TDL were collected over a 24-h period starting 3 days after the second set of injections. These TDL were used directly in the cytotoxic assay.

CTL Assays. CTL activity against minor histocompatibility antigens was assessed by a modification of the microtiter ^{51}Cr-release assay described by Lightbody (17). Target populations consisted of lymphocytes cultured for 3 days at a final concentration 2×10^6 cells/ml in 2 ml of culture media containing 10 μg/ml of concanavalin A (Con A). Approximately 5×10^6 of these Con A blast cells were incubated for 1 h in 0.5 ml culture medium supplemented with 20% fetal calf serum (FCS), 1 mM Hepes buffer (assay medium), and 100 μCi sodium ^{51}Cr-chromate (200–500 Ci/g Cr; New England Nuclear, Boston, Mass.). The effector cells were washed once (150 *g* for 8 min) in assay medium, and diluted to appropriate concentrations. 0.1 ml of the effector cell suspensions, 0.1 ml of cetrimide (hexadecyltrimethylammonium bromide, 5 mg/ml saline; Fisher Scientific Co., Pittsburgh, Pa.), or 0.1 ml of assay medium alone was added to triplicate wells of V-bottom microtiter plates (Linbro, New Haven, Conn.). 5×10^3 viable target cells were then added to each well in a volume of 0.05 ml. The plates were covered with lids, centrifuged (40 *g* for 4 min), and incubated at 37°C for 4–6 h. After incubation, the plates were spun again (1,000 *g* for 10 min), and aliquots transferred to disposable glass tubes for counting in a γ-spectrometer. The maximum level of releasable ^{51}Cr was determined from the cetrimide wells, and the spontaneous release values were determined from the wells with medium alone. Percent-specific lysis was calculated as:

$$\% \text{ specific lysis} = \left(\frac{\frac{\text{cpm}}{\text{experimental}}}{\frac{\text{cpm}}{\text{maximum}}} - \frac{\frac{\text{cpm}}{\text{spontaneous}}}{\frac{\text{cpm}}{\text{maximum}}} \right) \times 100.$$

CTL activity against virus-infected target cells was assayed as described previously (18). Target cells consisted of SV40 transformed fibroblasts which were ^{51}Cr-labeled overnight as monolayers, trypsinized (0.25%), pelleted, resuspended to a concentration of 4×10^6 cells/ml, and infected with 200 HA U/ml of virus. Effector cell TDL populations were washed twice and adjusted to 30×10^6 cells/ml and twofold dilutions thereof. 0.1 ml of cetrimide, medium, or effector cells was dispensed into each well of flat-bottom microtiter plates (Linbro) followed by 15×10^3 target cells in a volume of 0.1 ml. The plates were incubated for 14 h at 37°C. Maximum and spontaneous release were determined as described above.

Cold Target Competition. 0.1-ml aliquots of assay medium containing the appropriate dilutions of effector cells were pipetted into 35-mm Petri dishes (Falcon Plastics, Div. BioQuest, Oxnard, Calif.). 1×10^4 ^{51}Cr-labeled Con A blast cells and 30×10^4 cold competitors were added in 0.05 ml aliquots. The dishes were incubated and rocked at 37°C for 4 h. The contents of the dishes were transferred to glass tubes which were spun at 300 *g* for 10 min, at which time the supernates from these tubes were poured into a second set of tubes for counting.

Antibody-Mediated Cytotoxicity. Heterologous anti-B cell antiserum was prepared in rabbits by repeated i.v. injections of throacic duct cells from B rat donors and selective absorption on thymocytes (19). An anti-T antiserum was kindly provided by Dr. J. C. Howard. It was also a heterologous antiserum raised in a single rabbit by immunization with thoracic duct cells pretreated with anti-B cell antiserum and complement and aborbed with lymphoid tissue from rats.

Another source of anti-T cell antiserum was a homologous serum prepared by immunizing L rats with F lymphoid cells. This alloantiserum recognizes a determinant, Pta, present only on peripheral T cells of rats (20).

Analytical antibody-mediated cytotoxicity assays were performed in Linbro round-bottom microtiter plates using 25 μl microdiluters (Cooke Laboratory Products, Alexandria, Va.). 25 μl

of cells at 5×10^6 cells/ml was added to 25 μl of two-fold dilutions of the antisera followed by 25 μl of complement diluted 1:4 in medium. The plates were incubated for 1 h at 37°C, and viability was then assessed by trypan blue exclusion.

For preparative anti-B or anti-T treatment 20×10^6 cells were incubated for 1 h at 37°C in a final volume of 1.0 ml of medium containing the appropriate dilution of antiserum and 0.1 ml of complement.

Hemagglutination Assays. Hemagglutination assays were performed in duplicate in round-bottom microtiter plates. Red cells were collected in citrate, washed three times in normal saline, and resuspended to a concentration of 1% in saline. 25 μl of this suspension was added to 50 μl of L anti-DA alloantiserum diluted 10-fold in phosphate-buffered saline which contained 5% Ficoll. The plates were agitated and allowed to settle for 4 h.

Results

CTL Ativity against Non-Ag-B-Linked Minor Histocompatibility Antigens. L rats were immunized to DA strain non-Ag-B-linked minor histocompatibility antigens with skin grafts from four individual Ag-B1 homozygous (L x L/DA) backcross donors (N2[Ag-B1]). It was assumed that minor histocompatibility antigens of DA origin segregated randomly among the backcross population, and that immunization of L rats with tissue from more than one backcross animal would increase the likelihood of effective CTL priming.

Immune L and normal L LNC were stimulated in vitro with DA LNC or with LNC from a pool of N2(Ag-B1) animals. Their subsequent cytotoxic activity was assessed on ^{51}Cr-labeled L, DA, and individual N2(Ag-B1) target cell populations. Table I shows the results of a typical experiment. Normal L LNC stimulated with DA LNC were cytotoxic for DA targets but not for the N2(Ag-B1) targets, confirming the serological and MLC typing of the N2 animals. Normal L LNC stimulated with a pool of N2(Ag-B1) cells failed to develop CTL activity against any target. However, immune L LNC stimulated with the pooled N2(Ag-B1) cells were highly cytotoxic for certain N2(Ag-B1) targets, but were only minimally cytotoxic for DA target cells bearing the same minor alloantigens.

CTL Activity against Influenza-Infected Target Cells. DA, F, and L rats were primed with PR8 and challenged 4 wk later with HK virus. These two influenza A strains express different hemagglutinin (H) and neuraminidase (N) determinants but share common internal matrix and ribonucleoprotein components. Rats were challenged with a different virus from that used for priming, as experiments with mice (18, 21) indicated that re-exposure to PR8 would give no response (probably because of neutralization of the virus inoculum by circulating antibody). The cytolytic capacity of immune and normal TDL populations was assayed on HK-infected, PR8-infected, and uninfected F(Ag-B1), L(Ag-B1), and DA(Ag-B4) SV40-transformed fibroblasts. The results of a typical experiment (Fig. 1) demonstrate that virus-immune TDL populations effectively lyse only Ag-B-compatible, virus-infected target cells. DA CTL lysed both the HK- and PR8-infected DA targets. Similarly, the L and F CTL lysed only virus-infected F and L targets.

The Specificity of L Anti-F Cytotoxic Cells. In view of the importance of the MHC on the lytic specificity of CTL described above, it was expected that cytotoxic cells raised within an Ag-B compatible rat strain combination (a L anti-F response, for example) would also display this associative recognition

TABLE I

CTL Activity Generated in Normal and Immune L Lymphocyte Populations against DA Strain Minor Alloantigens

Responders	Stimulators	Target	% Specific lysis* at CTL:target ratios	
			100:1	33:1
Normal L	DA	DA	18.3 ± 2.0	12.1 ± 0.8
		L	0.0 ± 0.9	0.0 ± 1.8
		N2, no. 25	3.4 ± 5.6	2.2 ± 1.4
		N2, no. 28	2.2 ± 1.2	2.3 ± 1.3
		N2, no. 32	1.5 ± 4.1	−1.9 ± 0.9
Normal L	N2 pool‡	DA	0.9 ± 1.1	0.9 ± 1.0
		L	−3.7 ± 1.6	−0.8 ± 0.9
		N2, no. 25	−2.7 ± 0.9	3.6 ± 2.2
		N2, no. 28	1.6 ± 1.9	0.6 ± 0.7
		N2, no. 32	−1.9 ± 0.7	−0.6 ± 0.2
L anti-N2, no. 1§	N2 pool	DA	9.9 ± 3.9	5.2 ± 3.3
		L	−0.8 ± 0.9	0.6 ± 1.0
		N2, no. 25	7.3 ± 1.1	5.0 ± 1.3
		N2, no. 28	40.3 ± 1.7	17.5 ± 1.2
		N2, no. 32	21.0 ± 3.6	10.6 ± 2.4
L anti-N2, no. 2	N2 pool	DA	2.0 ± 1.7	4.0 ± 1.2
		L	−4.4 ± 1.1	0.8 ± 0.3
		N2, no. 25	38.9 ± 7.0	17.3 ± 4.7
		N2, no. 28	48.8 ± 6.3	33.5 ± 1.8
		N2, no. 32	16.4 ± 1.4	12.2 ± 0.9

* Percent-specific levels calculated as described in Materials and Methods ± the percent standard error. Maximum releasable counts ranged from 400–600 cpm, spontaneous release values ranged from 10–21% of maximum.

‡ The N2 pool consisted of equal proportions of LNC from the individual Ag-B1(N2) rats nos. 25, 28, and 32.

§ L anti-N2, no. 1 and no. 2 had each received skin grafts from a different set of Ag-B1(N2) rats.

and thus would (*a*) be highly effective against the specific (F) target cells; (*b*) lyse some, but not necessarily all, Ag-B identical target cells, indicating the presence of these strains of the relevant minor determinant; and (*c*) display little or no lytic activity against target cells of a different MHC haplotype.

Because the generation of cytotoxic activity against minor histocompatibility antigens generally requires prior in vivo sensitization of the responding lymphocyte population (4) and because it was known that lymphocytes from L donors cannot be stimulated to proliferate in mixed lymphocyte cultures by F cells, it was expected that presensitization would also be a prerequisite for the generation of cytotoxicity in the L-F combination. L rats were therefore immunized against F determinants as discussed in Materials and Methods. Immune LNC were restimulated in vitro with cells from L/F F_1 donors and cytotoxic activity assessed in a ^{51}Cr-release assay.

As shown in Fig. 2, cultures of lymphocytes from immune, but not from normal L strain donors, stimulated with L/F cells generated killer activity against the specific (F) strain targets. Contrary to expectations, however, these killer cells were also highly effective against target cells from Ag-B incompatible strains. The cross-reactive lysis of these targets could not be attributed to

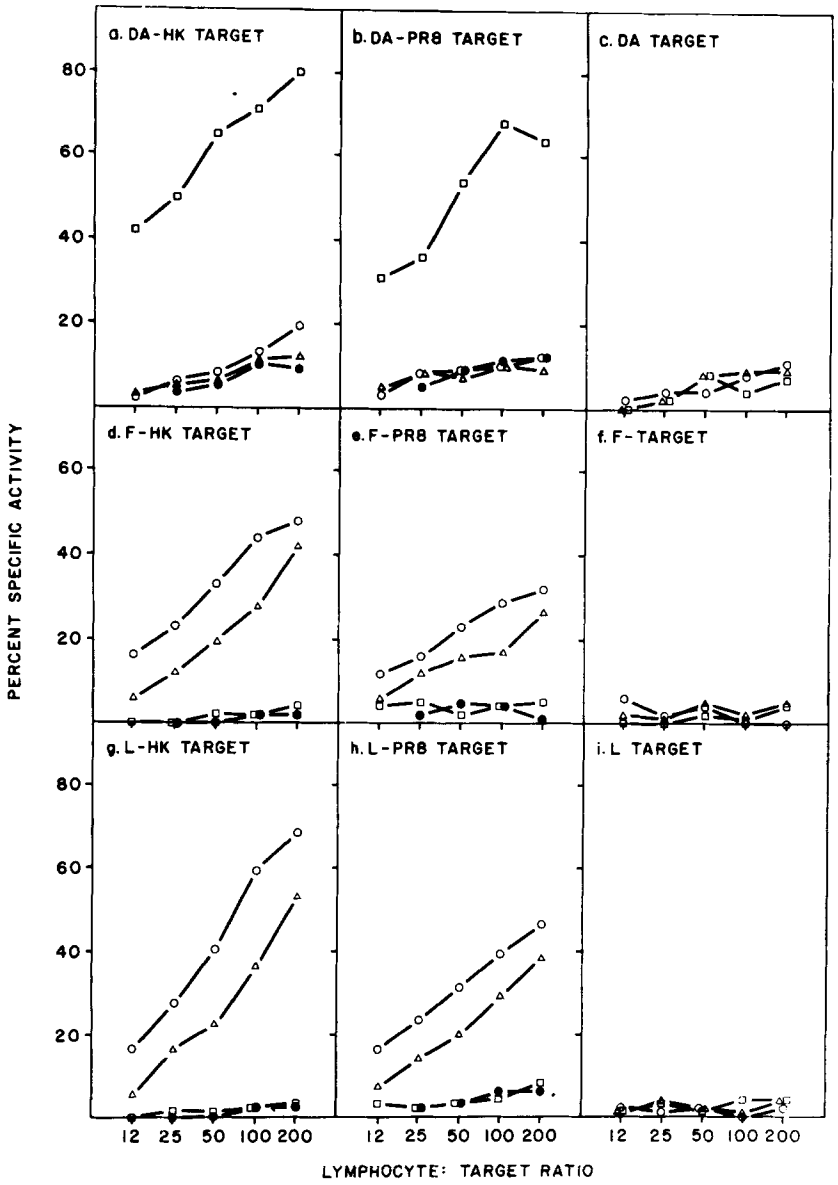

FIG. 1. Cytotoxic T-cell activity against influenza-infected cell surface determinants. Rats were injected i.p. with approximately 1,000 HA U of PR8 influenza virus and then challenged 4 wk later with approximately 2,000 HA U of HK influenza virus. Thoracic duct cells from immune F (○), immune L (△), immune DA (□), or normal L (●) rats were collected from 3–4 days after the second immunization, and their cytotoxic activity was assayed on (a) DA-HK, (b) DA-PR8, (c) normal DA, (d) F-HK, (e) F-PR8, (f) normal F, (g) L-HK, (h) L-PR8, and (i) normal L fibroblast target populations. Maximum release values ± the standard deviation for these target cells were 1,700 ± 10, 2,144 ± 59, 2,060 ± 69, 3,282 ± 92, 3,090 ± 73, 3,773 ± 30, 4,364 ± 91, 3,005 ± 71, and 3,302 ± 44, respectively. Spontaneous release values ranged from 21–25% of maximum release. Percent-specific activity is calculated according to the formula:

$$\left(\frac{\text{cpm experimental} - \text{cpm spontaneous}}{\text{cpm maximum} - \text{cpm spontaneous}}\right) \times 100.$$

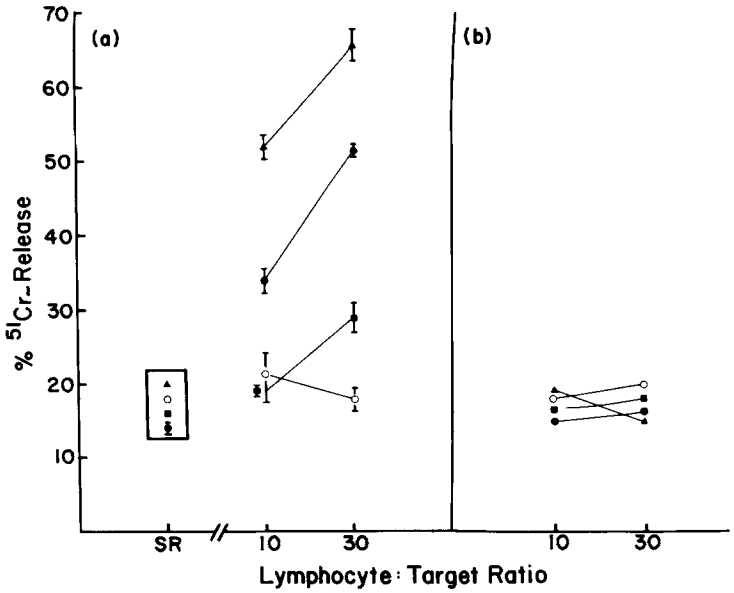

FIG. 2. The cytotoxic response generated in vitro from sensitized and normal L LNC stimulated by F alloantigens. 20 × 10^6 LNC from rats that had either been (*a*) immunized to F by two sets of bilateral skin grafts (2 and 3 mo before assay) and subsequently injected with F lymphoid cells (1 mo before assay), or (*b*) not immunized, were cultured with 6 × 10^6 irradiated (1,700 R) L/F LNC for 6 days in Marbrook vessels and then assayed on ^{51}Cr-labeled Con A blast cells from F (▲), BN (●), WF (■), L (○) donors. The data are presented in terms of percent-specific lysis ± the standard error. In this experiment the maximum release values, F, BN, WF, and L targets were 385.5 ± 13.2, 352.2 ± 13.7, 415 ± 76.0, and 374.7 ± 27.2, respectively.

nonspecific adherence of the killer and target populations by residual mitogen (22) because L Con A blast cells, a target cell population syngeneic to the killer cell population, were not lysed.

15 strains with 6 different Ag-B haplotypes were used for a more extensive analysis of the specificity of L anti-F killer cells. Two basic findings emerge from the experiments summarized in Table II: (*a*) L anti-F cytotoxic cells are highly effective against all other target cells of the Ag-B1 haplotype with the possible exception of AGUS; and (*b*) they consistently lyse, to varying degrees, target cells of other Ag-B haplotypes and target cells from wild rats (Table III). From these results it is possible to establish a heirarchy of lytic susceptibility based on the relative levels of ^{51}Cr-release at a given killer to target ratio; L anti-F killer cells are most effctive against Ag-B1 and Ag-B4 targets, somewhat less effective against Ag-B3 and Ag-B6 targets, and least effective against Ag-B2 and Ag-B5 targets.

Characteristics of the L Anti-F Killer Cell Precursor. To eliminate the possibility that the extensive cross-reactive lysis reflected the operation of an antibody-dependent cell-mediated cytotoxic process (ADCC; 23), 20 × 10^6 immune LNC were pretreated for 1 h at 37°C with either rabbit anti-rat T-lymphocyte antiserum and complement, rabbit anti-rat B-lymphocyte antiserum and complement, or complement alone. This treatment resulted in the specific lysis of 42% of the anti-B treated population, and 56% of the anti-T

TABLE II

The Specificity of L Anti-F Cytotoxic Cells

CTL	Experiment no.	^{51}Cr-labeled Con A target cells															
		Ag-B1					Ag-B2		Ag-B3		Ag-B4		Ag-B5		Ag-B6	
		A990	AGUS	BH	F	L	AO	WF	BN	Maxx	ACI	DA	Aug	HO	BUF	ALB
L anti-F*	401				45‡	0		13	37							
	402	45		47	46	1		8	39		45	48	15			
	407				34	2		30	51			65	21		49	
	411				30	0		7	30		35	44	2			
	416	58		47	70	11		18	21	37		75	18		51	
	428	55		53	51	0		38	52	41	59	62	16		43	35
	435	53	4	40	51	0	48	20	21	14	40	37	10	9	29	34
	436	53	26	52	62	3	22	10	19	13		40		8	31	
	437	70	45	59	65	6						55	0			
	457				45	1						50	0			
	460				50		29					46	18	11		

* 20 × 10^6 Lymphocytes from L rats previously sensitized to F alloantigens cultured in Marbrook vessels for 6 days with 6–10 × 10^6 irradiated (1,700 R) L/F or F stimulator cells.

‡ Percent-specific lysis at a lymphocyte:target ratio of approximately 20:1.

TABLE III

The Ability of L Anti-F Cytotoxic Cells to Recognize Determinants Expressed on Target Cells Derived from Wild Rats

CTL	^{51}Cr-labeled Con A target cells								
	Wild rat					F	L	WF	DA
	no. 34	no. 36	no. 64	no. 65	no. 67				
L anti-F	39*	23	53	52	32	56	6	36	55

* Percent-specific lysis at a lymphocyte:target ratio of 40:1.

treated population. The remaining viable cells were cultured as usual with L/F LNC. As shown in Fig. 3, cytotoxicity was eliminated by pretreatment with anti-T but not by pretreatment with anti-B antiserum. Because MacLennan (23) has shown that ADCC effector cells are not found in TDL, immune TDL were also tested for their ability to differentiate into L anti-F killer cells. On a cell-for-cell basis, TDL generated levels of cytotoxicity equivalent to those of LNC cultures. Moreover, the L anti-F killer cell activity of TDL populations was also susceptible to anti-T antiserum and insensitive to anti-B antiserum (data not shown). These results indicate that the killing activity in this system involves T lymphocytes, rather than ADCC mechanism.

Identity of the Determinants Recognized by L Anti-F CTL on F and DA Populations. If most or all of the antigens of F stimulators that induce CTL activity in L lymphocyte populations are also present on DA cells as the data in Table II would indicate, then (*a*) unlabeled ("cold") DA target cells should compete as well as cold F target cells for lysis of labeled F targets by L anti-F CTL; and (*b*) DA cells should be able to substitute for F stimulator cells in cultures which generate CTL activity against F targets. Figs. 4 and 5 show the results of two experiments designed to test these predictions.

CTL activity of L anti-F CTL on labeled F targets was inhibited equally well by DA and F cold Con A blast cells (Fig. 4), indicating that L CTL detect very

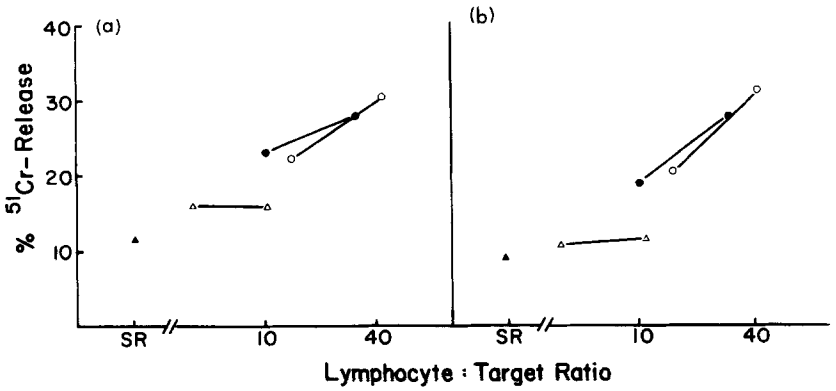

FIG. 3. Susceptibility of L anti-F cytotoxic cell precursors to anti-T cell antiserum and anti-B cell antiserum. 20 × 10^6 LNC from L rats preimmunized to F alloantigens were pretreated with rabbit anti-rat T-cell antiserum and C′ (△), rabbit anti-rat B-cell antiserum and C′ (●), or C′ alone (○). The remaining viable cells were then cultured with 6 × 10^6 irradiated (1,700 rads)L/F LNC for 6 days at which time their cytotoxic activity was assayed on F (a) or DA (b) Con A target cells.

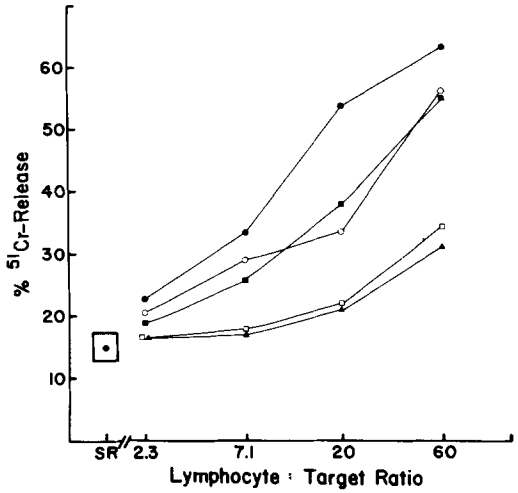

FIG. 4. Inhibition of ^{51}Cr-release from ^{51}Cr-labeled F Con A target cells by unlabeled DA and F Con A blast cells. The cytotoxic activity of L anti-F CTL was assayed on ^{51}Cr-labeled F Con A target cells alone (●), or in the presence of a 30-fold excess of unlabeled L (○), WF (■), DA (□), or F (▲)-unlabeled Con A blast cells.

similar, if not identical, determinant(s) on the F and DA targets. The slight inhibitory effect of the cold syngeneic target L was presumably due to steric hindrance. In this particular experiment, the WF ^{51}Cr-labeled targets were only lysed to a minmal extent and cold WF targets did not appear to have a significant inhibitory effect.

(L/F)F_1 and (L/DA)F_1 cells were equally effective in their ability to restimulate L cells primed in vivo to F alloantigens (Fig. 5). The cytotoxic cells generated in these cultures were tested on a panel of six targets, and they demonstrated identical ^{51}Cr-release patterns on the various target cells. The F

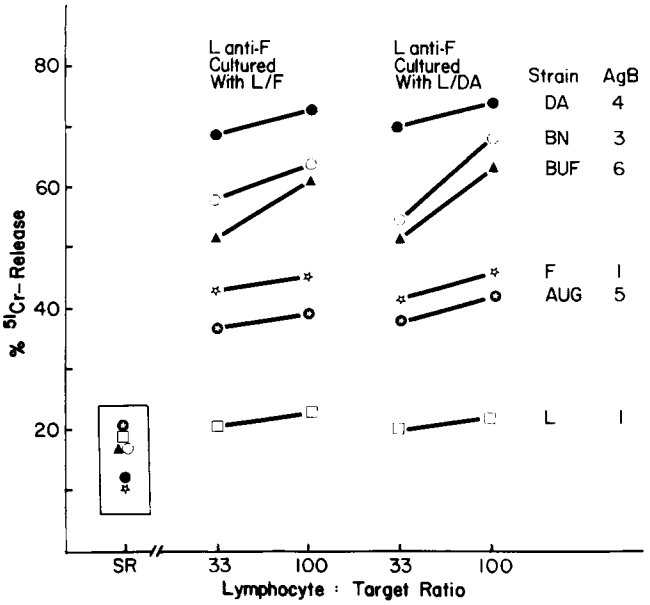

FIG. 5. The specificity of the cytotoxic response generated from F-sensitized L LNC after restimulation (in vitro) with either F or DA cells. 20 × 10^6 sensitized L LNC were cultured for 6 days with either 6 × 10^6 irradiated F (a) or DA (b) LNC, at which time cytotoxic activity was assayed on DA (●), BN (○), F (☆), BUF (▲), Aug (✪), and L (□) ^{51}Cr-labeled Con A target cells.

Con A target cells in this particular experiment had not developed their normal blastoid morphology, a possible explanation for the lower than average (see Table II) level of ^{51}Cr-release from this target.

Backcross Analysis of L Anti-F Cytotoxicity. To test the hypothesis that target cell lysis in the L anti-F combination is not Ag-B haplotype restricted, and that L anti-F CTL can recognize multiple minor histocompatibility antigens encoded by genes that segregate independently, a backcross analysis was undertaken. Cells from a panel of 20 L × L/DA backcross rats were examined for the following characteristics: (*a*) susceptibility to L anti-F CTL, (*b*) ability to respond to L/DA stimulator cells in an analytic MLC, (*c*) ability to stimulate responses by L cell populations in an analytical MLC, and (*d*) hemagglutination by L anti-DA alloantiserum. The results are presented in Table IV and Fig. 6 a and can be summarized as follows: (*a*) Cells from 9/20 backcross donors were lysed indicating that a single locus determined the relevant antigen(s) recognized by L anti-F CTL. (*b*) Lytic susceptibility by L anti-F CTL segregated precisely with the expression of DA MHC gene products as detected by the ability to stimulate L lymphocytes and respond to L/DA stimulator cells in MLC and by hemagglutination by L anti-DA antiserum. Examination of an additional 20 backcross animals confirmed this linkage (Fig. 6 b).

To estimate the number of loci involved in the recognition of the specific (F) target antigens, a panel of L × L/F backcross animals was similarly tested (Fig. 6 c). Approximately 50% (9 out of 17) of this backcross population showed significant ^{51}Cr-release, again a finding consistent with the recognition of target antigens encoded by a single locus.

TABLE IV

Linkage Relationships of the Ag-B Locus and the Locus Encoding the Determinants Recognized by L Anti-F CTL

Assayed population	^{51}Cr-release by L anti-F CTL*	Response to L/DA in MLC	Ability to stimulate L in MLC	Hemagglutination by L anti-DA alloantisera‡
L no. 1	48§	+	−	−
L no. 2	39	+	−	
L/DA no. 1	11	−	+	+
L/DA no. 2	2	−	+	
(L x L/DA)N2				
no. 2	39	−	+	+
no. 3	5	+	−	−
no. 4	47	−	+	+
no. 5	8	+	−	−
no. 6	12	+	−	−
no. 7	48	−	+	+
no. 8	37	−	+	+
no. 9	7	+	−	−
no. 10	3	+	−	−
no. 11	8	+	−	−
no. 12	3	+	−	−
no. 13	47	−	+	+
no. 14	43	−	+	+
no. 15	39	−	+	+
no. 16	6	+	−	−
no. 17	41	−	+	+
no. 18	8	+	−	−
no. 19	6	+	−	−
no. 20	0	+	−	−
no. 21	45	−	+	+

* Percent-specific lysis at a lymphocyte:target ratio of 20:1.

‡ Assays kindly performed by Dr. K. Mitchell.

§ Specific release ≥ 12% is considered positive.

Susceptibility of Congenic Rat Strains to L Anti-F CTL. As demonstrated above (see Table II) target cell populations derived from various rat strains are differentially susceptible to lysis by L anti-F CTL, as defined by the percent-specific lysis at a given killer:target ratio. Several Ag-B congenic rat strains exist that have been derived from strains that differ in terms of L anti-F CTL lytic susceptibility. The lytic susceptibility of target cells from these congenic strains was compared to that of target cells from the strains donating the MHC and background genes of the congenic line. As shown in Table V, the level of ^{51}Cr-release of the congenic strain target cells at a given lymphocyte:target ratio correlated with the MHC haplotype.

Specificity of CTL Generated between Other MHC-Identical Strain Combinations. Other intra-A-B1 strain combinations were examined to determine whether or not the cross-reactivity demonstrated by L anti-F CTL was unique to one particular strain combination. The results of these experiments are compiled in Table VI. Eight out of eight Ag-B1 anti-Ag-B1 CTL populations recognized determinants expressed on MHC nonidentical strains. Examination

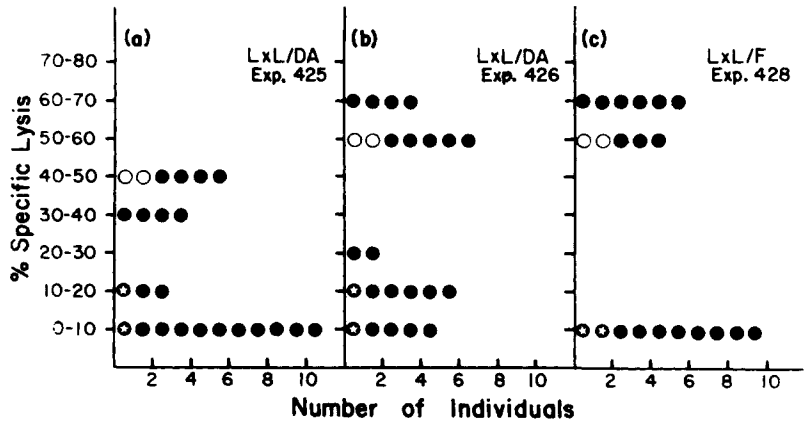

FIG. 6. Distribution of backcross animals according to the relative lytic susceptibility of their target cells to L anti-F CTL. In three separate experiments, L anti-F CTL were assayed on ^{51}Cr-labeled Con A target cells of parental (●), F_1 (○), or backcross origin (●). Each symbol derived from one individual rat at a lymphocyte target ratio of 20:1.

TABLE V

The Specificity of L Anti-F Cytotoxic Cells on Target Cell from Congenic Strains

CTL	Experiment no.	^{51}Cr-labeled target cells									
		Ag-B2				Ag-3		Ag-B4		Ag-B5	
		WF	BN.WF	AO	HO.AO	BN	Aug.BN	DA	BN.DA	HO	AUG
L anti-F	428	38 ± 3.7*	32 ± 3.0			52 ± 1.3		62 ± 1.5	65 ± 0.8		
	442‡	8 × 1.6	6 ± 0.5			8 ± 2.5		30 ± 2.3	33 ± 3.0		
	432	14 ± 1.5	6 ± 2.3			24 ± 0.4		56 ± 3.5	42 ± 1.6		
	444	18 ± 21	22 ± 4.2			26 ± 1.8		57 ± 2.2	57 ± 1.8		
		16 ± 2.9	20 ± 3.9			36 ± 0.8		52 ± 1.3			
	435			48 ± 5.2	42 ± 4.6					9 ± 0.7	
	460			27 ± 1.0	29 ± 2.9					11 ± 1.0	
	432					24 ± 0.4	20.0 ± 1.0				9 ± 0.8

* Percent-specific lysis ± standard error at a lymphocyte:target ratio of 20:1.

‡ Targets were normal PBL; in all other cases, Con A blast cells served as targets.

of the cross-reactivity patterns on targets of Ag-B1 origin suggested the existence of at least four distinct antigenic groupings, whereas an examination of the cross-reactivity on the Ag-B incompatible targets indicated that these groupings could be further subdivided. For example, L anti-F, L anti-A990, and L anti-BH defined the first grouping, yet only L anti-F CTL appeared to cross-react extensively on Ag-B3.

F anti-L CTL also cross-reacted extensively on target cells of allogeneic Ag-B haplotypes, however LNC from DA/F F_1 rats preimmunized to L determinants differentiated into CTL which recognized only L and AGUS targets. Reactivity against other strains was eliminated by incorporation of the DA haplotype into the responder genome.

Characterization of the Determinant Recognized by L Anti-F CTL. Different lymphocyte classes were examined as possible targets for L anti-F CTL. In two separate experiments, 20 × 10^6 normal LNC were treated with either anti-B cell antiserum + C′, anti-T cell antiserum + C′, or C′ alone. The remaining viable cells were ^{51}Cr-labeled. Dead cells were removed from the suspension by

TABLE VI
The Specificity of Ag-B1 Anti-Ag-B1 Cytotoxic T Lymphocytes

CTL	Experiment no.	^{51}Cr-labeled Con A target cells													
		Ag-B1					Ag-B2		Ag-B3		Ag-B4		Ag-B5		Ag-B6	
		A990	AGUS	BH	F	L	AO	WF	BN	MAXX	ACI	DA	AUG	HO	BUF	ALB
L anti-F*	416	58‡		47	70	7		18	21	37		75	18		51	
L anti-A990	416	64			65	9		16				76	15			
	419	40		30	31	0		5	7	7	33	35	12		12	14
L anti-BH	436	37	27	55	54	3	17	7	14	7		41		10	30	
F anti-L	416	10		16	2	45		17	3	16		16	9		31	
	419	23		11	0	59		36	23	30	43	34	21		44	39
	428	8		4	1	70		36	13	15	44	49	38		47	52
A990 anti-L	416	3		14	10	63		15	12	31		39	22		35	
BH anti-L	419	18		0	0	35		15	1	14	23	19	16		17	18
	436	3	31	0	0	31	7	12	1	7		7		5	10	
F anti-A990	416	62			2	39		5				13	22			
	419	53		32	3	42		12	13	24	33	31	24		16	18
A990 anti-F	416	5		31	56	17		2	9	12		49	4		8	
DA/F anti-L	436	3	58	3	3	53	17	4	9	5	1	0	6	0	9	11

* LNC from L rats previously sensitized to F alloantigens cultured with 6–10 × 10^6 irradiated (1,700 rads) F stimulator cells.
‡ Percent-specific lysis at a lymphocyte:target ratio of approximately 20:1.

Ficoll-Isopaque centrifugation, and the viable cells were washed three times. These normal lymphocytes were then used as targets in a typical cytotoxic assay. The results, presented in Table VII, indicate that T cells and B cells are equally susceptible to lysis by L anti-F CTL.

In an attempt to identify the chromosomal region that encodes the determinants detected by L anti-F CTL, we used the rat recombinant HO.DA(1R). HO.DA(1R) expresses the MHC conventional serological determinants of the DA (Ag-B4) parent and the MLC phenotype and Ia-like serological determinants of the HO(Ag-B5) parent, an apparent separation (in mouse terminology) between the *I* and *K, D* regions (24). DA is highly susceptible to lysis by L anti-F CTL, whereas HO cross-reacts only slightly. LNC from L and L/Aug (Ag-B1/Ag-B5)F_1 rats preimmunized to F determinants were cultured with F stimulators, and the resulting CTL were assayed against DA, HO, and HO.DA(1R) Con A-induced blast cells. As indicated in Table VIII, L anti-F CTL lysed HO.DA(1R) more effectively than HO, but not as well as DA. Furthermore, L/Aug anti-F CTL (*a*) did not cause any significant ^{51}Cr-release on the HO target, (*b*) did lyse DA targets were effectively, and (*c*) were cytotoxic for recombinant targets at an intermediate level.

Discussion

Whether or not the regulatory influence of MHC gene products on lytic T-cell interactions previously demonstrated in the mouse (2–4) and recently demonstrated in man (25) repesents a general phenomenon pertaining to T-cell specificity of all species is an important issue. In systems where responder and stimulating rat lymphocyte populations express genotypically identical Ag-B gene products (Fig. 1, Table I, reference 26), CTL were effective only against target cells that express both the modifying determinant(s) and the MHC gene

TABLE VII
Relative Lytic Susceptibility of Con A Blast Cells, T Cells, and B Cells to L Anti-F Cytotoxic T Cells

CTL	Experiment no.	^{51}Cr-labeled Cells			
		Con A blasts	Normal lymphocytes	T lymphocytes	B lymphocytes
L anti-F	460	82 ± 4.1*	43.6 ± 5.2	39.9 ± 6.2	40.6 ± 5.7
L/Aug anti-F	461	52.5 ± 2.2	20.1 ± 4.3	30.2 ± 5.4	28.9 ± 5.3

* Percent-specific lysis at a lymphocyte:target ratio of 20:1 ± 1 standard error.

TABLE VIII
Relative Lytic Susceptibility of DA, HO, and HO.DA(1R) Target Cells to L Anti-F CTL

CTL	Experiment no.	^{51}Cr-labeled Con A target cells		
		DA	HO	IR
L anti-F	460	61*	21	33
		69	34	56
		62	21	45
	461	31	7	14
L/Aug anti-F	460	54	4	31

* Percent-specific lysis at lymphocyte:target ratio of 20:1.

products of the original stimulating cell population, a finding that extends the generality of MHC restriction of lytic T-cell specificity to another species.

However, an apparent exception to this general principle was observed with CTL generated in rat strain combinations previously thought to be MHC identical. The relevant target antigens, which we propose to term CT antigens because they can be detected at present only with cytotoxic T cells, are encoded by genes linked to the MHC locus of this species. In contrast to the MHC-restricted lysis of target cells bearing non-MHC alloantigens, lysis of cells expressing CT alloantigens does not require that stimulator and target cells share previously defined MHC gene products.

The existence of such CT determinants was first demonstrated by the finding that L anti-F CTL killed target cells of six different MHC haplotypes to varying degrees. In particular, third party target cells of the Ag-B4 haplotype (DA and ACI) were lysed as efficiently as the specific Ag-B1(F) target population, indicating that DA and ACI share most, if not all, the relevant CT antigens with F. Two other findings confirmed this suggestion: DA and F cells were equally effective in (*a*) inhibiting the lysis of F targets in cold competition experiments and (*b*) restimulating identical patterns of cross-reactive lytic activity.

The T-cell identity of anti-CT effectors cells in this system was indicated by the sensitivity of the killer cell precursors to anti-T cell serum and their resistance to anti-B cell serum. Also, it was shown that on a cell-for-cell basis TDL were equally capable of differentiating into cross-reactive CTL as LNC. This finding tends to discount ADCC as the lytic mechanism involved because ADCC effector cells are known to be absent from TDL populations (23).

Genetic analysis clearly indicated that the expression of CT antigens in this system is controlled by genes of a single chromosomal region linked to the Ag-B complex. Target cells from approximately 50% of backcross donors from L × L/F matings (Fig. 6c) and from L × L/DA matings (Fig. 6a, b) were susceptible to lysis by L anti-F CTL. In the L × L/DA panels, lytic susceptibility segregated precisely with the expression of serologically and MLC-defined Ag-B gene products of the DA haplotype (Table IV).

Additional evidence for the linkage of CT genes to the MHC locus is demonstrated in the experiments with MHC congenic rat strains. The lytic hierarchy of target cells from various strains to L anti-F CTL—B1 and B4 targets > B3 and B6 > B2 and B5 (Table II)—extended to target cells from congenic rat strains (Table V). Thus, within these combinations the extent of lysis depended on the MHC haplotype of the target cells and not on the remainder of the genetic background. Implicit in this interpretation is the assumption that recognition by T cells of CT determinants, like all other MHC antigens, is not controlled by an MHC "restriction" locus.

Alternatively, it might be suggested that (*a*) CT antigens are encoded by genes located outside the MHC, (*b*) recognition of these antigens is restricted by the MHC, and (*c*) strains showing cross-reactive lysis actually share these restricting MHC gene products. This possibility seems unlikely for two reasons. First, if L anti-F activity is regulated by an MHC gene product, such a restriction locus must be considered distinct from the locus which controls T-cell lysis directed against non-MHC alloantigens (Table I), virus-infected cells (Fig. 1), and TNP-modified cells (26), in this species; such a locus would not correlate with the expression of serologically defined MHC gene products. Secondly, according to the classic restriction model, the extent of lysis depends on two variables: (*1*) the expression of an appropriate restricting MHC gene product, and (*2*) the expression of the relevant weak alloantigenic determinants. To explain the results of the congenic target cell experiments, it must be postulated that two strains of different backgrounds must, in each case, share the same minor antigens as well as the known MHC haplotype, a situation that seems highly improbable.

The observation that L anti-F CTL lyse target cells from only 50% of the individuals from a L × L/F backcross panel (Fig. 6 c) suggests single locus control of the relevant antigens, as indicated above, but it also raises an important question concerning minor H differences between these two strains. Either the L and F strains differ only by the MHC-linked CT antigens or immune responses to other minor H antigens encoded outside the MHC are preempted by the response to CT antigens. In this context, it is of particular interest that DA/F anti-L CTL are highly lytic only for L and AGUS targets, showing no activity on other target cells either of the Ag-B1 or of other haplotypes (Table VI).

The complexity of the CT antigenic system is indicated by the extensive cross-reactivity demonstrated by CTL generated in other intra-Ag-B1 strain combinations. Although it remains to be formally demonstrated that the genes encoding these determinants are linked to the MHC with strain combinations other than L anti-F, the lack of restriction suggests that this will be the case upon completion of proper genetic analysis. Such "weak" MHC differences

could account for the rejection of tissue grafts previously attributed to the effects of minor non-Ag-B histocompatibility antigens in these Ag-B1 strain combinations (27, 28). A similar MHC-linked weak antigenic difference between two Ag-B4 strains, DA and AVN, has been reported by Holan (29) with a macrophage adherence inhibition assay.

There are two obvious similarities between *I* region determinants of the mouse and CT antigens of the rat—linkage to MHC and the lack of a restricting influence by gene products of the serologically defined MHC subregions (30, 31). At this point, it would be premature to equate these two antigen systems, especially because *I* region differences induce primary MLC responses, whereas CT antigens apparently do not. Furthermore, anti-*I* region sera that detect predominantly B-cell surface antigens are readily produced in mouse strain combinations differing by *H-2 I* (32), whereas attempts to raise anti-CT antibody have so far been unsuccessful.

The intra-Ag-B locus recombinant, HO.DA(1R), recently described by Butcher and Howard (24) expresses the DA serological phenotype and the HO MLC phenotype. The 1R strain was employed in the current studies in an attempt to map more precisely the locus responsible for the expression of CT antigens. L anti-F and L/Aug anti-F CTL were assayed on target cells derived from DA, HO, and 1R rats. The lytic susceptibility of the targets fell into the following sequence: DA > 1R > HO, a finding that suggests either that L anti-F CTL recognize CT antigens encoded by several genes dispersed along the Ag-B complex, or that 1R expresses the same CT determinants as DA but at a lower density on the target cell surface (Dr. J. C. Howard, personal communication).

A possible explanation for the extensive variations found mainly in Ag-B1 strains can be extrapolated from examination of the mutant mouse lines. Klein noted (33) that the *H-2K*^{b} allele appears to be particularly able to accommodate mutational events. A similar situation may also apply to the Ag-B1 haplotype, especially if it can be shown that the various Ag-B1 anti-Ag-B1 CTL populations recognize CT determinants predominantly. The widespread distribution of the Ag-B1 haplotype in strains of diverse origin (7, 34–36) suggests that the incorporation of the Ag-B1 allele into the genome may confer a selective advantage on subsequent progeny. Whether CT determinants represent minor antigenic differences in the molecular configuration of the MHC serologically defined gene products (i.e. "public-like" specificities) or whether they are a separate class of cell surface molecules remains an important question.

Summary

The regulatory influence of the rat major histocompatibility complex (MHC) (Ag-B complex) on the specificity of cytotoxic T lymphocytes was investigated. It was shown that the effector cells were specific for the original Ag-B phenotype in rat systems in which the responder and stimulator cell populations were unquestionably MHC identical but expressed different minor alloantigens or viral antigens. However, combined in vivo immunization and restimulation in culture of lymphocytes from rat strains previously thought to be MHC compatible resulted in the generation of cytotoxic T lymphocytes which effectively lyse not only target cells from the specific stimulating strains but also,

to varying degrees, target cells from third party strains regardless of their Ag-B haplotypes. Genetic analysis indicates that expression of these cytotoxic T-cell-defined ("CT") antigens, found on both T and B lymphocytes, detectable thus far only with cytotoxic lymphocytes, is controlled by a single locus which segregates in backcross populations with the rat MHC. Discrepancies between the nature of CT antigens of the rat Ag-B and *I*-region specificities of the mouse *H-2* are discussed.

The authors wish to thank Mrs. M. Solomon for developing the fibroblast cell lines, Ms. Shirley McCloud and Mr. Mike Merva for their excellent technical assistance, Ms. Karen Nowell for preparation of this manuscript, and Mrs. Dianne Wilson for preparation of the figures. The authors especially appreciate the helpful criticisms of this study by Dr. J. C. Howard.

Received for publication 15 July 1977.

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