EXPRESSION OF T-CELL DIFFERENTIATION ANTIGENS ON EFFECTOR CELLS IN CELL-MEDIATED CYTOTOXICITY IN VITRO

Evidence for Functional Heterogeneity Related to the Surface Phenotype of T cells*

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Thymus-derived lymphocytes (T cells) participate in cell-mediated immune reactions represented by delayed hypersensitivity (1), graft-vs-host reactivity (2), and cell-mediated cytotoxicity (CMC)[1] (3); and they act as "helpers" in the production of antibody (4) and in the regulation of immune responses (5). Effector ("killer") cells concerned in CMC reactions against allogeneic (3), xenogeneic (6), or syngeneic (7) tumor cells in vitro are characteristically T cells.

Lymphoid cells from nonimmune animals can act as killer cells in the presence of antibody against target cells (8), but these antibody-dependent killer cells, which bear Fc and C3 receptors (9), evidently are not T cells. Using a syngeneic Moloney sarcoma system, some investigators report the killer cells to be thymus dependent (10, 11) and others not (12). In CMC against a human bladder carcinoma (13), non-T cells have been said to play a major role. Thus while there is agreement that T cells can acquire killer activity, this is not necessarily an exclusive function of T cells as defined by current criteria.

Using Thy-1 (θ) antisera prepared in congenic strains we have confirmed that killer cells from the peritoneal cavity of hyperimmune alloimmunized mice are Thy-1$^+$.[2] However, the killer population is said to be heterogeneous and to change

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[1] *Abbreviations used in this paper:* BALB, BALB/c; B6, C57BL/6; CMC, cell-mediated cytotoxicity; EBSS, Earle's balanced salt solution; FBS, fetal bovine serum; NAPC, nonadherent peritoneal cells; NMS, normal mouse serum; NRS, normal rabbit serum; NTA, natural thymocytotoxic autoantibody; NTA-RA, NTA-reactive antigen; PC, peritoneal cells.

[2] Shiku, H., M. A. Bean, L. J. Old, and H. F. Oettgen. 1975. Cytotoxic reactions of murine lymphoid cells studeid with a [^{3}H]proline microcytotoxicity test. *J. Natl. Cancer Inst.* In press.

its physical properties (14, 15) with time after immunization; also, distinguishable subtypes of T cells cooperate in vitro in generating the CMC killer population (16). From this latter standpoint particularly we have been concerned with characterizing the CMC killer population, represented in our hands by the nonadherent peritoneal cells (NAPC) of immune mice, in terms of their surface exhibition of antigens other than Thy-1 that constitute hallmarks of the T cell (Table I). In view of growing evidence that T cells are diverse in function (16–19) we have looked for evidence that they may exhibit concordant diversity in exhibition of these other surface markers, notably those of the Ly series, which might serve to identify T-cell subsets with different functions. This present report is concerned exclusively with the CMC function of T cells.

Our technique for measuring CMC was the [^{3}H]proline microassay which we have recently developed (20). In this test, adherent target cells are prelabeled with [^{3}H]proline, and

TABLE I

T-Cell Surface Antigens

Antigen system (old notation) :(reference)	Genetic locus (chromosome)	All mice express:	Normal cells on which these specificities are expressed
Thy-1(θ):(36,37)	9	Thy-1.1 or Thy-1.2	Thymocytes and other T cells, brain, and epidermal cells
Ly-1 (Ly-A):(35)	19	Ly-1.1 or Ly-1.2	Thymocytes and other T cells
Ly-2 (Ly-B):(35)	6 closely linked	Ly-2.1 or Ly-2.2	
Ly-3 (Ly-C):(32)		Ly-3.1 or Ly-3.2	
TL (38)	17	no TL, TL.2 or TL.1,2,3	Thymocytes only
NTA-RA*:(27)	—	NTA-RA	Thymocytes and other T cells, and brain
MSLA‡:(39)	—	MSLA	Thymocytes and other T cells

* Natural thymocytotoxic autoantibody-reactive antigen.

‡ Mouse specific lymphocyte antigen.

radioactivity remaining is measured after CMC has taken place and the monolayer has been washed free of effector cells and nonviable target cells. This assay therefore primarily monitors the destruction (measured as detachment) of target cells, in contrast to the Takasugi and Klein assay (21) which is influenced by target cell proliferation as well as destruction. This [^{3}H]proline assay has been valuable in detecting CMC against alloantigens or tumor antigens or target cells (references 20 and 22, and footnote 2).

Materials and Methods

Mice (Tables II and III). For clarity the formal nomenclature of congenic mouse strains (all of which in this study were derived at Memorial Sloan-Kettering Cancer Center, New York) has been simplified from the standard designations listed by Klein (23) as follows: *B6/Ly-1.1* for C57BL/6-Ly-1^a/Boy; *B6/Ly-2.1* for C57BL/6-Ly-2^a/Boy; *B6/Ly-2.1 Ly-3.1* for C57BL/6-Ly-2^{a}Ly-3^a/Boy; *A/Thy-1.1 for A-Thy-1^a*/Boy; *AKR/H-2^b* for AKR.B6/1/Boy; *B6/H-2^k* for C57BL/6-H-2^k/Boy; *A/TL$^-$* for A-T1a^b/Boy.

Antisera. See Table III.

Complement (C)-Dependent Cytotoxicity Assay (Table III). Modified from Gorer and O'Gorman

TABLE II

Mice Used, and Their T-Cell Antigen Phenotypes

Mouse strain	Thy-1	Ly-1	Ly-2	Ly-3	TL
B6	2	2	2	2	Negative
B6/Ly-1.1*	2	1	2	2	Negative
B6/Ly-2.1*	2	2	1	2	Negative
B6/Ly-2.1 Ly-3.1*	2	2	1	1	Negative
A	2	2	2	2	1,2,3
A/Thy-1.1*	1	2	2	2	1,2,3

* Congenic strains.

TABLE III

Antisera

Serum: anti-	Production	Cytotoxicity assay titer*		Dilution for CMC assay
		On thymocytes	On immune NAPC	
Thy-1.1	(B6 × A)F_1 anti-A/Thy-1.1‡ thymocytes	2,500	160	20
Thy-1.2	(A/Thy-1.1 × AKR/H-2^b‡) F_1 anti-A spontaneous leukemia ASL1	10,240	320	20
Ly-1.1	(BALB × B6)F_1 anti-B6/Ly-1.1‡thymocytes	2,560	2,560	20
Ly-1.2§	C3H anti-CE thymocytes	160	80 ~ 160	20
Ly-2.1	B6/H-2^k ‡ anti-CE thymocytes	1,600	320	20
Ly-2.2	(C3H × B6/Ly-2.1‡)F_1 anti-B6 leukemia ERLD	640	80	20
Ly-3.2§	C58 anti-CE thymocytes	160	40	15
TL.1, 2, 3§	(A/TL^- ‡ × B6)F_1 anti-ASL1	5,120	negative	20
NTA-RA‖	Old untreated NZB mice	256 ~ 512	20	5
MSLA	Rabbit anti-ASL1 absorbed in vivo in A mice	160	160	15

* Reciprocal of dilution giving 50% of maximal percentage lysis.

‡ Congenic strains.

§ Absorbed with syngeneic thymocytes to remove autoantibody.

‖ Kindly provided by Doctors R. C. Mellors and T. Yoshiki, Hospital for Special Surgery, Cornell University Medical College, New York.

(24, 25): 0.05 ml vol of (*a*) cells (5 × 10^6/ml), (*b*) antiserum (serially diluted), and (*c*) C; incubated together in 10 × 15-mm glass tubes for 45 min at 37°C; proportion of dead cells determined by adding 0.1 ml of 0.16% freshly prepared trypan blue to each tube. In early experiments selected rabbit serum (diluted 1/15) was used as the C source; later, rabbit serum absorbed with mouse cells in the presence of EDTA (26) was preferred (diluted 1/6); for anti-MSLA tests, selected guinea pig serum (diluted 1/3) was used instead. In titrating the natural thymocytotoxic autoantibody (NTA) of NZB mice we washed the presensitized cells before adding C, because this enhances the titer (27).

Cultured Target Monolayer Cells. Cultures from A and C57BL/6 (B6) mouse embryos, and from two BALB/c (BALB) methylcholanthrene-induced sarcomas (Meth A or Meth 113, which gave similar results), were used as target cells between their 4th to 10th passages in vitro, as described.[2]

[^3H]Proline CMC Microassay (references 20 and 22, and footnote 2). Briefly, 1,000 [^{3}H]proline-

labeled target cells were seeded in each well of a Microplate II (no. 3040, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). On the following day, 1×10^5 effector cells (NAPC), obtained and pretreated as described below, were added. After incubation (humidified 5% CO_2 in air at 37°C for 12 to 20 h) unattached dead target cells and effector cells were washed away with 37°C phosphate-buffered saline with 5% fetal bovine serum (FBS). After processing,[2] the residual radioactivity (counts per minute) of cells attached to each well was measured, indicating the numbers of viable target cells remaining. Protocol: four replicates per sample; every effector suspension was tested on syngeneic control target monolayer(s) as well as on the cognate target monolayer(s). The "percent CMC" is calculated as $[1 - (a)/(b)] \times 100$, where (a) = counts per minute of target monolayer exposed to NAPC from "immune" donors, and (b) = counts per minute "standard," i.e., of same target monolayer treated with NAPC from "nonimmune" donors.

Immunization of Donor Mice for CMC Assays. Mice were immunized weekly four times by intraperitoneal (i.p.) inoculations of 5×10^6 allogeneic spleen cells; peritoneal cells (PC) were harvested 3 days later.[2] Nonimmune PC were obtained from mice inoculated with syngeneic spleen cells according to the same schedule. In certain experiments (Table VIII) PC were harvested 5 days after a single i.p. inoculation of 100×10^6 allogeneic spleen cells.[2] The donor to target cell combinations used all involved *H-2* incompatibility and are apparent from the abbreviated notations in the table ("*B6 anti-BALB*", etc.).

Detection of T-Cell Surface Antigens on Killer Cells by Pretreatment of NAPC with Selected T-Cell Antisera. NAPC from immune and nonimmune donors (see preceding section) were washed twice in Earle's balanced salt solution (EBSS) with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 2.5% FBS (56°C/30 min); resuspended in CMC-assay-medium and incubated ($3–5 \times 10^6$ cells/ml) in T-60 glass flasks (5% CO_2 in air) at 37°C for 1 h. The CMC-assay-medium was Eagle's minimal essential medium (MEM) supplemented with 2 mM glutamine, 1% nonessential amino acids, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 10% FBS (56°C/30 min), and 5×10^{-5} M 2-mercaptoethanol. Unattached cells were transferred to new flasks and incubated for a further hour under the same conditions. The unattached cells (NAPC) were washed once and resuspended (5×10^6 cells/ml) in EBSS with supplements as above and 5×10^{-5} M 2-mercaptoethanol which helps to maintain functional lymphocyte activity (28). Aliquots of the cells were then exposed to three or more selected antisera under conditions similar to the standard C-dependent cytotoxicity assay (see above); i.e. one volume of cell suspension, added to one volume of antiserum (dilutions shown in Table III and VII), and one volume of C; C preparation as described for C-dependent cytotoxicity assay (above); incubated in siliconized glass tubes, with occasional shaking, for 45 min, in 5% CO_2 in air. Each tube was then sampled for the viability counts (trypan blue) which constitute the data for "% cells lysed" given in Table X. Meanwhile the remainders of the NAPC suspensions were washed twice and resuspended (1×10^6 viable cells/ml) in CMC-assay-medium; 0.1 ml was added to each well of the Microtest plate II already seeded with [^{3}H]proline prelabeled target cells ([^{3}H]proline CMC microassay, see above). The "percent reduction of CMC" (Tables IV–VIII, and X) resulting from pretreatment of the NAPC population was calculated as $[1 - (a)/(b)] \times 100$, where (a) = "*percent CMC*" (see above) of NAPC pretreated with test antiserum and C, and (b) = percent CMC of NAPC pretreated with control serum plus C. The "*percent inhibition of CMC*" (Table IX) is calculated in an identical fashion but refers to the change in CMC of effector cells in the continued presence of antisera without C. The control serum was sometimes normal mouse (NMS) or normal rabbit serum (NRS) but more usually and whenever possible the "reciprocal" immune serum, illustrated by anti-Ly-1.1 in place of the cognate anti-Ly-1.2 in Table VI.

Results

Pretreatment of Immune NAPC Population with Various T-Cell Antisera; Effect on Subsequent CMC Capacity

ANTI-Thy-1. Table IV shows in detail two experiments (others are summarized in Table X) in which elimination of Thy-1$^+$ cells by Thy-1 antiserum and C abolished all CMC. The main evidence for specificity is the control in which use of reciprocal Thy-1 antiserum (anti-Thy-1.1 in place of anti-Thy-1.2) gave only a

slight reduction of CMC as compared with the "diluent only" control (e.g., cpm 1,069 compared with 889 in exp. 1 in Table IV) such as might be expected from slight toxicity from the addition of the C source above. The last entry in Table IV is "bystander control"showing that lysis of irrelevant cells (Thy-1.1 thymocytes by anti-Thy-1.1) did not nonspecifically depress the CMC of the Thy-1.2 effector cells. The percent NAPC lysed by Thy-1 antiserum during pretreatment in these experiments is shown in Table X.

ANTI-MSLA, ANTI-TL, AND NTA. Results with these three systems are illustrated in detail in Table V (summary of all results in Table X). Pretreatment with

TABLE IV

Thy-1 Antigen on Killer Cells

Cells (NAPC)	Pretreatment*	Exp. 1 (B6 anti-BALB system)			Exp. 2 (A anti-B6 system)		
		cpm (target cells)	CMC	Reduction of CMC	cpm (target cells)	CMC	Reduction of CMC
			%	%		%	%
Nonimmune	Diluent only	2,639	0		3,991	0	
Immune	Diluent only	889	66		326	92	
Immune	Anti-Thy-1.2	2,646	0	100	4,024	−1	101
Immune	Anti-Thy-1.1	1,069	59	Standard	390	90	Standard
Immune, plus Thy-1.1 thymocytes‡	Anti-Thy-1.1	1,074	59	0	418	89	1

* With C also in the case of NAPC being exposed to antiserum; this applies to pre-treatment in experiments shown in all other tables except IX.

‡ Control for "bystander effects:" Lysis of extraneous Thy-1.1 thymocytes by Thy-1.1 antiserum did not interfere with CMC of Thy-1.2 NAPC.

TABLE V

MSLA, TL, and NTA-RA on Killer Cells

Cells (NAPC)	Pre-treatment	Exp. 1			Exp. 2		
		cpm (target cells)	CMC	Reduction of CMC	cpm (target cells)	CMC	Reduction of CMC
			%	%		%	%
B6 anti-BALB system							
MSLA system							
Nonimmune	Diluent only	6,804	0		4,708	0	
Immune	Diluent only	901	87		1,278	73	
Immune	Anti-MSLA	6,331	7	92	4,054	14	81
Immune	NRS	1,087	84	Standard	1,271	73	Standard
A anti-B6 system							
TL system							
Nonimmune	Diluent only	3,991	0		2,492	0	
Immune	Diluent only	326	92		398	84	
Immune	Anti-TL1,2,3	415	90	2	727	71	9
Immune	NMS	356	91	Standard	557	78	Standard
B6 anti-BALB system							
NTA-RA (NZB) system							
Nonimmune	Diluent only	2,803	0		2,552	0	
Immune	Diluent only	1,313	53		302	88	
Immune	NTA	2,353	16	63	1,675	34	60
Immune	NMS	1,556	44	Standard	371	85	Standard

anti-TL gave no suppression of CMC; NTA gave incomplete suppression; and anti-MSLA gave stronger suppression, approaching the level of suppression by anti-Thy-1.

ANTI-Ly-1, ANTI-Ly-2, AND ANTI-Ly-3. Representative results are illustrated in detail in Table VI, and again the results of all experiments are summarized in Table X, where the proportions of cells lysed by the antisera are also shown. The salient feature of these results is that the proportion of cells lysed by the various antisera during pretreatment is not concordant with the effect on suppression of CMC. Two extremes are illustrated in Table VI: (*a*) no CMC suppression by

TABLE VI

Ly Antigens on Killer Cells

Cells (NAPC)	Pretreatment	Exp. 1			Exp. 2		
		cpm (target cells)	CMC	Reduction of CMC	cpm (target cells)	CMC	Reduction of CMC
			%	%		%	%
B6 anti-BALB system							
Ly-1 system							
Nonimmune	Diluent only	16,277	0		4,977	0	
Immune	Diluent only	2,697	83		1,213	76	
Immune	Anti-Ly-1.2	2,938	82	−2	1,617	67	13
Immune	Anti-Ly-1.1	3,300	80	Standard	1,096	78	Standard
B6 anti-BALB system							
Ly-2 system							
Non-immune	Diluent only	2,203	0		7,501	0	
Immune	Diluent only	528	76		1,072	86	
Immune	Anti-Ly-2.2	1,552	30	60	5,749	23	74
Immune	Anti-Ly-2.1	565	74	Standard	801	89	Standard
B6 anti-BALB system							
Ly-3 system							
Nonimmune	Diluent only	2,826	0		7,501	0	
Immune	Diluent only	330	88		1,072	86	
Immune	Anti-Ly-3.2	2,108	25	70	7,044	6	93
Immune	NMS	409	85	Standard	1,255	83	Standard

anti-Ly-1.2, although 74% (mean) of Thy-1$^+$ cells had been lysed (Table X), as compared with (*b*) 70% CMC suppression by anti-Ly-3.2 (Table VI) which lysed a smaller proportion of the T cells (54%; Table X). It must be emphasized that such results were obtained repeatedly with concurrent CMC tests carried out on aliquots of the same NAPC preparation pretreated with each of these two antisera, so the danger of variation from experiment to experiment is not a factor that need be considered.

Use of Thy-1 and Ly Congenic Mouse Strains (Table II) in Further Exploration of the Killer Cell Phenotype (Table VII). The special value of congenic strains differing for T-cell-specific surface markers in the context of this study is that

TABLE VII

Use of Thy-1 and Ly Congenic Strains in Further Study of the Phenotype of the Killer Cells

Cells (NAPC)	Pretreatment	cpm (target cells)	CMC	Reduction of CMC
			%	%
A/Thy-1.1 (congenic) anti-B6 system				
Nonimmune	Diluent only	4,571	0	
Immune	Diluent only	327	93	
Immune	Anti-Thy-1.1	3,860	16	83
Immune	Anti-Thy-1.2	304	93	Standard
B6/Ly-1.1 (congenic) anti-BALB system				
Nonimmune	Diluent only	4,718	0	
Immune	Diluent only	1,541	67	
Immune	Anti-Ly-1.1 (1/20)*	3,216	32	48
Immune	Anti-Ly-1.1 (1/160)	3,229	32	48
Immune	Anti-Ly-1.1 (1/320)	2,507	47	23
Immune	Anti-Ly-1.1 (1/640)	1,920	59	3
Immune	Anti-Ly-1.2 (1/20)	1,832	61	Standard
B6/Ly-2.1 (congenic) anti-BALB system				
Nonimmune	Diluent only	18,224	0	
Immune	Diluent only	5,884	68	
Immune	Anti-Ly-2.1	7,342	60	15
Immune	Anti-Ly-2.2	5,360	71	Standard
B6/Ly-2.1 Ly-3.1 (congenic) anti-BALB system				
Nonimmune	Diluent only	4,198	0	
Immune	Diluent only	429	90	
Immune	Anti-Ly-2.1	841	80	8
Immune	Anti-Ly-2.2	551	87	Standard
Immune	NMS	552	87	0
Immune	Anti-Ly-3.2	442	89	Standard

* Dilution of antisera.

comparative tests on the alternative allelic product in each system can be carried out on a uniform genetic background, e.g., anti-Ly-1.2 acting on B6 killer cells (Ly-1.2) as compared with anti-Ly-1.1 acting on B6/Ly-1.1 killer cells (congenic with B6). In the case of Thy-1, this "reciprocal system" (anti-Thy-1.1 acting on A/Thy-1.1 cells) gave essentially the same results, i.e. virtually complete suppression of CMC (Table VII), as anti-Thy-1.2 acting on A strain cells (Table IV).

Results from the Ly congenic lines are especially important because one might infer from data based only on B6 killer cells (Table VI) that the phenotype Ly-1↓:Ly-2/Ly-3↑ is typical of all mouse NAPC killer cells. In fact the comparable results with the Ly congenic cells raise doubts on this point because CMC suppression by anti-Ly-1.1 was considerable and CMC suppression by anti-Ly-2.1 was only slight (Table VII), in contrast with the Ly-1.2 and Ly-2.2 alternatives (Table VI). However, regarding the CMC suppression by an-

ti-Ly-1.1 it is noteworthy that the cytotoxic titer of this antiserum is exceptionally high (1/2,560; Table III) although its titer in regard to CMC suppression is much lower. Therefore at critical dilutions there is a high cytolytic effect with little or no CMC suppression. (These discrepancies are further considered below; see Discussion).

A few general comments: It is unfortunate that anti-Ly-3.1 serum was not available at the time of these experiments. However, the completely negative result with Ly-3.2 antiserum (on Ly-3.1 cells) is worth emphasis as an excellent control for serological specificity.

Results with Singly Immunized NAPC Donors (Table VIII). We wished to determine whether the surface antigen phenotype of killer cells generated at an

TABLE VIII

Thy-1 and Ly Antigens Phenotype of Killer Cells from Singly Immunized Mice

Cells (NAPC)	Pretreatment	cpm (target cells)	CMC	Reduction of CMC
			%	%
B6 anti-BALB system				
Nonimmune	Diluent only	3282	0	
Immune	Diluent only	1641	50	
Immune	Anti-Thy-1.2	2582	21	67
Immune	Anti-Thy-1.1	1171	64	Standard
Immune	Anti-Ly-1.2	1157	65	−7
Immune	Anti-Ly-1.1	1269	61	Standard
Immune	Anti-Ly-2.2	2362	28	56
Immune	Anti-Ly-2.1	1199	63	Standard
Immune	Anti-Ly-3.2	2574	21	66
Immune	NMS	1189	64	Standard
B6/Ly-1.1 (congenic) anti-BALB system				
Nonimmune	Diluent only	1122	0	
Immune	Diluent only	612	45	
Immune	Anti-Ly-1.1	937	16	67
Immune	Anti-Ly-1.2	558	50	Standard

early stage of immunization would differ from that of hyperimmune donors, i.e. whether there might be some qualitative change in CMC-active T cells with progressive immunization, of the sort that characterizes the progression of antibody responses (29). We knew from our own experience that CMC activity of PC as a whole is augmented by repeated immunization.[2] But Table VIII gives no evidence for a qualitative change, because the comparative CMC suppression by a panel of Ly sera was the same in singly immunized donors as in multiply immunized donors (compare Table VIII with Tables VI and VII).

Lack of CMC Inhibition by T-Cell Antisera in the Absence of C (Table IX). If it were the case that any of the T-cell surface components identified by T-cell antisera mediated some essential event in CMC, such as recognition of antigen, it might be expected that the cognate antisera would block the CMC of viable killer cells without the need for ensuing C-dependent lysis. We found no evidence for this because pretreatment of NAPC with any of the several T-cell antisera (or

TABLE IX

CMC is not Inhibited by Exposure of Killer Population to Various Alloantisera in the Absence of C

Cells* (NAPC)	Antiserum	3 h‡			15 h‡		
		cpm (target cells)	CMC	Inhibition of CMC	cpm (target cells)	CMC	Inhibition of CMC
			%	%		%	%
Nonimmune	Diluent only	4,188			3,746		
Immune	Diluent only	818	80		397	89	
Immune	Anti-*H-2*^{b} (1)§	540	87	−5	424	89	0
Immune	Anti-*H-2*^{b} (2)§	663	84	−1	441	88	1
Immune	Anti-Thy-1.2	751	83	1	329	91	−2
Immune	Anti-Ly-1.2	723	82	1	392	89	0
Immune	Anti-Ly-2.2	843	80	4	406	89	0
Immune	Anti-Ly-3.2	508	88	−6	278	93	−4
Immune	NMS	710	83	Standard	404	89	Standard

* B6 anti-BALB system; preincubated (10^6 cells/ml), for 1 h at 4°C with antiserum 1/5; cells transferred to wells of CMC assay plate without washing (therefore final concentration of antibody during CMC assay was 1/10).

‡ Incubation period for CMC assay.

§ (1) DBA/2 anti-C57BL leukemia EL4 and (2) *B6/H-2*^{k} anti-C57BL leukemia EL4.

H-2 antisera) listed in Table IX, in the absence of C, produced no CMC suppression during either a 3 or 15 h CMC assay period, despite the fact that the concentrations of antisera used were higher than standard and that the antisera remained in the medium throughout the CMC assay.

Discussion

The results are summarized in Table X, to which we refer in the following discussion. Certain features need little comment. The ability of some T-cell-specific cytotoxic antisera (to Thy-1.1, Thy-1.2, and MSLA) to abolish virtually all CMC confirms that T cells are essential to this function of the immune NAPC population, regardless of whether some cells other than T cells may also be involved. The entirely negative results with anti-TL might be anticipated from the fact that TL is expressed exclusively on thymocytes and not on functional peripheral T cells. The intermediate suppression of CMC activity by NTA is not particularly informative because this is not a strictly defined antigenic system in the sense of Thy-1, TL, and the Ly series.

Undoubtedly greater interest attaches to the different degrees of CMC suppression observed with various Ly antisera, because of the possibility that functional heterogeneity of the T-cell population, arising from progressive, alternative, or divergent differentiation, might be reflected by different profiles of surface exhibition of Ly antigens. Our study provides some pointers in that direction.

Such an interpretation of results with the Ly antisera depends on comparing:

TABLE X
*Summary**

Sera: anti-	Immune NAPC lysed during incubation with antibody and C		Reduction of CMC (%): mean (individual tests)
	Mean % (range)	As % Thy-1$^+$ cells	
Thy-1.1	45 (45)		83 (83)
Thy-1.2	39 (25–48)		100 (101,100,100)
Ly-1.1	33 (26–40)	85	58 (48,69)
Ly-1.2	29 (22–33)	74	4 (−4, −3,11,13)
Ly-2.1	8 (6–12)	20	11 (8,13,15)
Ly-2.2	23 (10–31)	60	63 (48,60,60,74,75)
Ly-3.2	21 (12–28)	54	77 (63,70,80,93)
TL.1,2,3	<5	<10	5 (2,9)
NTA-RA	39 (34–43)	100	62 (60,63)
MSLA	36 (33–41)	92	86 (81,92)

* Based on Tables IV to VIII and other experiments using the same protocols.

(*a*) the proportion of T cells lysed by a particular Ly antiserum, with (*b*) the CMC capacity of the residual population. We shall make the reasonable assumption that all Ly-bearing cells belong to the T-cell (Thy-1$^+$) lineage. On this basis it is apparent that the proportion of T cells lysed by a given Ly antiserum does not regularly correspond with the degree to which CMC capacity is lost (Fig. 1). The extremes are represented by: (*a*) Ly-1.2 antiserum—74% T cells destroyed without loss of CMC capacity, and (*b*) Ly-3.2-antiserum—only 54% T cells destroyed, but with 77% loss of CMC. This implies that most of the 26% CMC-competent cells remaining after anti-Ly-1.2 lysis are susceptible to lysis by Ly-3.2 antiserum. Hence the T-cell members of the NAPC population must be heterogeneous, and the NAPC CMC-effector cell population of B6 mice has the phenotype Ly-1.2↓:Ly-2.2/Ly-3.2↑.[3]

Can it be inferred from this that the killer population phenotype characteristic of all mice is Ly-1↓:Ly-2/Ly-3↑? On the present data, which were deliberately restricted to repeated CMC tests with a single dilution of each antiserum (to keep the study within manageable limits) it is not possible to give a definite answer. But it seems likely that Ly classification will become increasingly important in the study of T-cell subsets, so it may be timely to comment further on the Ly systems in this context,[4] and to speculate further about the interpretation of our

[3] Sullivan, Berke and Amos mention similar findings on Ly-1.2 and Ly-2.2 in a footnote to their report in Transplantation **16**:388, 1973.

[4] Having undertaken in this discussion to deal briefly in a general way with the Ly systems, we add the following points about them to complete the account. In addition to the new Ly-5 system noted above, other unanalyzed serological reactions on lymphocytes point to the existence of yet other T-cell Ly systems that remain to be delineated. So the Ly class of T-cell antigens, i.e. of antigens represented exclusively and invariably on mouse T cells, is evidently extensive, and involves several distinct genetic loci. It is a peculiarity of Ly systems that immunization between congenic Ly strains sometimes completely fails to give rise to the Ly antibody sought; in each case in Table III (where antiserum preparation is summarized) when the immunization protocol shown does not involve use of

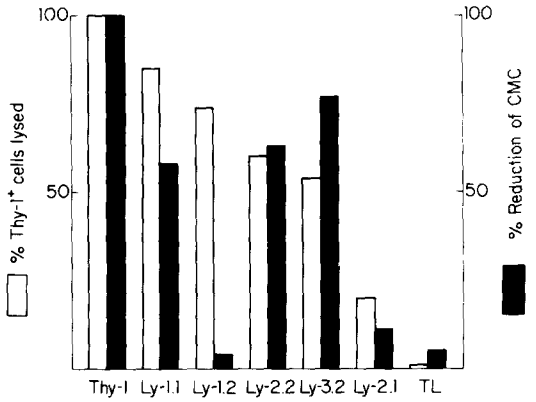

Fig. 1. Summary: Discordance of cytolytic and CMC suppressive capacity of different T-cell antisera (based on Table X).

initial study. Undoubtedly the issues will automatically become much clearer when results of corresponding studies, using the same Ly antisera but different T-cell functional criteria, become available.

To simplify matters we can regard Ly-2 and Ly-3 as a single system (Ly-2/Ly-3) on the grounds that although they must refer to different mutable sites they are so closely linked that no crossing-over has been observed among several hundred segregants; moreover there is mutual interference between Ly-2 and Ly-3 antibodies competing for sites on the thymocyte surface (32); therefore Ly-2 and Ly-3 antigens must be closely adjacent, perhaps even on the same molecule. In keeping with this, the results with Ly-2.2 and Ly-3.2 antisera (Table X and Fig. 1) were very similar. (A third Ly system, Ly-5, is already well-defined[5] but has not so far been employed in any functional study.)

First, these two systems show a possibly noteworthy difference in regard to the sensitivity of peripheral T cells to lysis by the respective antisera (thymocytes by contrast are virtually all sensitive to lysis by all Ly antisera under optimal conditions). In the case of Ly-1 antisera, the end point titers against both cell types (thymocytes and peripheral T cells) are roughly the same, but with Ly-2/Ly-3 antisera the titers against peripheral T cells are considerably lower than against thymocytes (Table III, and see confirmatory data in reference 33). Therefore by this criterion alone the two systems appear to differ.

The reason for concluding that the CMC cell may characteristically be low in Ly-1 antigen is that 74% of T cells can be lysed by anti-Ly-1.2 serum with

congenic partners, this is due to the failure of "congenic immunization" to give rise to the particular Ly antibody. Of several possible explanations, control by *Ir* genes or inadvertent induction of chimerism or some other variety of tolerance are less likely, on evidence available, than some requirement for additional antigenicity of the sort described by Schierman and McBride (30) in immunization with chicken red blood cells (double incompatibility giving rise to antibody where single incompatibility will not). A second peculiarity of immunizations involving only Ly differences, whether in the congenic situation or not, is that large amounts of thymocyte autoantibody are often formed (31). This is more characteristic of some Ly immunizations than that of others (Table III).

[5] Komuro, K., K. Itakura, E. A. Boyse, and M. John. 1974. Ly-5; a new T-lymphocyte antigen system. *Immunogenetics*. In press.

virtually no lowering of CMC (Table X and Fig. 1). But on the other hand, anti-Ly-1.1 used against B6/Ly-1.1 congenic cells eliminated 85% of T cells with substantial reduction of CMC (58%). So either there are no general profiles of representation of Ly systems on T cells of different functions, or else the substantial CMC suppression by anti-Ly-1.1, in contrast to anti-Ly-1.2, has another explanation. In favor of the latter view is that anti-Ly-1.1 lysed a high population of T cells (85%), and that it had the highest titer of all Ly antiserum. As our protocol called for use of the Ly-1.1 and Ly-1.2 antisera at the same dilution (1:20) it may be that the very high relative concentration of Ly-1.1 antibody resulted in lysis of cells bearing only small amounts of Ly-1.1, or that a contaminant non-Ly-1 antibody was involved; the use of a congenic system for preparing Ly-1.1 antiserum does not necessarily exclude this because the Ly congenic strains differ for loci linked to Ly which are responsible for histoincompatibility, although these have not been shown to induce cytotoxic antibody (34). Either could account for the higher lytic titer of anti-Ly-1.1 (Table III) in comparison with its titer in CMC suppression (Table VII). In short, it may be more significant under these conditions that the anti-Ly-1.2 was negative in CMC suppression than that the Ly-1.1 antiserum was substantially positive, i.e., the former result cannot be otherwise interpreted but the latter can. This question requires more detailed study.

Secondly, if the killer population is Ly-2/Ly-3 rich, why was anti-Ly-2.1 so weak in CMC suppression? Here we can only point out that for reasons unknown anti-Ly-2.1 lyses a far lower proportion of NAPC than any other Ly antiserum (only 8%), corresponding to its low representation on peripheral T cells according to quantitative absorption (35); yet despite this it was capable of marginal suppression of CMC (11%).

To reiterate our conclusions: The contention that different T-cell subsets among the NAPC population of B6 mice are distinguishable by the different effects of Ly-1.2 and Ly-2.2/Ly-3.2 antisera is adequately substantiated by our data, and the phenotype of the killer cell here is undoubtedly Ly-1.2↓:Ly-2.2/Ly-3.2↑. Whether the phenotype of the NAPC killer cells of the mouse generally will prove to be Ly-1↓:Ly-2/Ly-3↑ is a provocative hypothesis to be studied further.

Summary

The cell-mediated cytotoxicity (CMC) of nonadherent cells from the peritoneal cavity (NAPC) of alloimmunized mice can be measured by the [^{3}H]proline microassay. The exhibition of thymus-derived (T) cell antigens on these killer cells was studied by incubating them with the relevant T-cell antisera and complement (C), under optimal conditions for lysis, before performance of the CMC assay.

Under these conditions, the following T-cell antigens were demonstrable on the killer population in terms of percent reduction in CMC by the respective antisera: (*a*) Thy-1.1 (83%) and Thy-1.2 (100%), (*b*) MSLA (86%), (*c*) NTA-RA (a T-cell antigen recognized by naturally occurring autoantibody of NZB mice) (62%), (*d*) Ly-1.1 (58%), (*e*) Ly-2.1 (11%; considered a marginal result) and

Ly-2.2 (63%), and (*f*) Ly-3.2 (77%). The following were not demonstrable: (*g*) TL, and (*h*) Ly-1.2. (*i*) The antigen Ly-3.1 was not studied.

Omission of C deprived all T-cell antisera tested of their capacity to suppress CMC, indicating that the cell components recognized by such antisera may perform no direct function in CMC. On the assumption that all Ly^+ cells are Thy-1^+, it is clear that the T-cell members of the immune NAPC population must be heterogeneous. This follows from the fact that the proportions of T cells lysed by different Ly antisera did not correspond with ensuing degree of loss of CMC capacity. The extremes were represented by anti-Ly-1.2 (74% Thy-1^+ cells lysed, but no reduction in CMC) and Ly-3.2 (54% Thy-1^+ cells lysed, with 77% reduction in CMC).

From this initial survey it appears that the C57BL/6 mice killer T-cell population active in CMC in vitro is relatively rich in surface antigens of the Ly-2/Ly-3 category and relatively poor in representation of the Ly-1 surface antigens. It remains to be seen whether this killer cell phenotype, poor in Ly-1 and rich in Ly-2/Ly-3, is characteristic of the mouse generally. From these results it appears that subsets of T cells with different immunological functions may exhibit qualitative or quantitative differences in surface antigens specified by different Ly loci; this will be easier to assess in the future when the results of experiments with the same Ly antisera but dealing with T-cell functions other than CMC become available.

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