THE RECEPTOR SPECIFICITY OF ALLOREACTIVE T CELLS

Distinction between Stimulator K, I, and D Region Products and Degeneracy of Third-Party H-2 Recognition by Low-Affinity T Cells*

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It has been widely accepted that the reaction of T cells to allogeneic major histocompatibility complex (MHC)[1] antigens is specific; however, the degree of this specificity is not clear. A major paradox in MHC recognition is the unusually high frequency of precursor T cells capable of developing into cytotoxic effector cells (1–3), or into cells proliferating in the mixed lymphocyte reaction (MLR) (4), after stimulation across a single MHC haplotype difference. Considering the high number of different haplotypes (5), these data imply that most, if not all, cells of the T-cell pool are reactive to one or another of the MHC alloantigens (6). Thus, if T-cell receptors are distributed clonally, it becomes difficult to account for the known T-cell reactions to non-MHC antigens (7). Analysis of cross-reactivities offers one possible solution to this controversy (8). In fact, cytotoxic T lymphocytes (CTL) generated against a set of allogeneic K and/or D region determinants have been shown to cross-kill target cells of independent H-2 haplotypes (9, 10). Furthermore, cross-killing of allogeneic cells by xenoantigen-induced CTL, and cross-reactions of alloantigen-induced CTL with chemically or virally modified syngeneic cells have been reported (11–13). Cross-reactions are not restricted to the Ly-2,3$^+$ subclass of T cells to which CTL belong (14), but they are also demonstrable in I region-induced proliferative responses of (possibly) Ly-1$^+$ T cells (15, 16). These data were interpreted to indicate the generation of T-cell clones reacting against shared and/or cross-reacting MHC determinants present on different allogeneic, xenogeneic, and antigen-modified syngeneic cells (13). However, it is equally possible that a large number of T cells with low-affinity (cross-reactive) receptors become stimulated by allogeneic MHC determinants (8, 17). It is difficult to distinguish between these two possibilities by the analysis of effector

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[1] *Abbreviations used in this paper:* Con A, concanavalin A; CTL, cytotoxic T lymphocyte; FCS, fetal calf serum; FDA, fluorescein diacetate; GVHR, graft-versus-host reaction; HBSS, Hanks' balanced salt solution; LPS, lipopolysaccharide; 2-ME, 2-mercaptoethanol; MHC, major histocompatibility complex; MLC, mixed lymphocyte culture; MLR, mixed lympocyte reaction; N.I.H., National Institutes of Health; NMS, normal mouse serum; PBS, phosphate-buffered saline; R/S, responder/stimulator.

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functions of T cells, because the final outcome with both mechanisms is cross-reactivity.

We have therefore approached this problem at the single cell level by detecting binding of stimulator-derived alloantigens by T blasts activated in the mixed lymphocyte culture (MLC) (18). We have shown previously that the binding phenomenon is H-2 specific, and involves both the Ly-1$^+$ and the Ly-2,3$^+$ subset of T blasts (19, 20). The binding is dependent on the active biosynthesis of a trypsin-sensitive surface structure, with a turnover rate of at least 5–6 h (19, 21). After trypsin treatment and overnight incubation, rebinding of stimulator antigen material by 40–50% of the blasts has been demonstrated; whereas a two- to fivefold smaller fraction of blasts bound unrelated (third party) H-2 antigens (21).

The results presented here strongly suggest that T blasts with a wide range of receptor affinities are generated in primary MLC. At antigen excess, almost all blasts are capable of binding stimulator alloantigens. Under these conditions, the binding of stimulator K, I, and D region products is specific. However, a very poor specificity is exhibited with respect to third party H-2 haplotypes. The binding of stimulator and third-party antigens is H-2 specific, because both are inhibited with the relevant anti-H-2 sera. Furthermore, cold antigen inhibition of binding indicates that the same cell population reacts with both stimulator and third-party antigens, with a higher affinity for the former. In contrast, highly selected long-term MLC blasts do not bind third-party H-2 antigens.

Materials and Methods

Mice. A/J, CBA, C57BL/10 (B10), B10.D2, and SJL mice (The Jackson Laboratory, Bar Harbor, Maine), and (A × B6)F_1 mice (a gift from Dr. C. G. Fathman, Department of Immunology, Mayo Clinic, Rochester, Minn.) were used.

Media. The culture medium for MLC was RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum (FCS) (Flow Laboratories., Inc., Rockville, Md.), L-glutamine, antibiotics, and 2-mercaptoethanol (2-ME) as described previously (18). The same medium without 2-ME was used for mitogen stimulation. For preparation, washing, and incubation of the cells with membrane vesicles, medium with 5% FCS and without 2-ME was used.

MLC. Primary MLCs were set up with nylon-wool column passaged (22) lymph node responder cells (<2% Ig$^+$ [B] cell contamination by direct immunofluorescence). The stimulator cells were x-irradiated (3, 300 rad) or mitomycin-treated spleen cells. Spleen cells (1×10^7/ml) were incubated with 25 μg/ml of mitomycin C (Bristol Labs. of Candiac, Quebec, Canada) (except for the experiment in Table II) at 37°C for 30 min., and washed three times. 4-ml mixtures of 1×10^7 responder and 1×10^7 or 2×10^7 stimulator cells (see Tables and Figs.) were cultured as described (18). The proliferative response was assessed by [^{3}H]thymidine incorporation (9, 18). Stimulation indices (counts per minute in allogeneic per counts per minute in syngeneic cultures) measured on day 4 ranged from 20 to 44. Greater than 99% of blasts from 4-d MLC were T cells as determined by indirect immunofluorescence with rabbit anti-T-cell serum (18).

Long-term B10.D2 vs. B10 cultures were set up with lymph node responder cells according to the method of Fathman et al. (15). The cells were restimulated at 2- to 3-wk intervals for up to 15 mo. The proliferative response was tested in 0.2-ml cultures with graded numbers of responder cells, and 1×10^6 x-irradiated stimulator cells from different strains (15). The peak response occurred on day 3, and was measured in terms of thymidine incorporation. Stimulation indices varied between 109 and 649.

Mitogen Stimulation. 30-ml cultures of spleen cells (3×10^6/ml) were set up with 2 μg/ml of concanavalin A (Con A) (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N.

J.), or with 75 μg/ml of lipopolysaccharide (LPS, *Escherichia coli*; Difco Laboratories, Detroit, Mich.), in Falcon No. 3204 flasks (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.), and incubated at 37°C in a 95% air, 5% CO_2 atmosphere for 3 d.

Preparation and Labeling of Membrane Vesicles. Plasma membrane vesicles were prepared from 3-day LPS blasts by nitrogen cavitation (23). Cells were resuspended at 3×10^7/ml in 0.065 M NaCl, 0.25 mM $MgCl_2$, 0.25 M sucrose, 0.01 M Hepes, pH 7.4, and 10^{-3} M phenylmethylsulfonyl fluoride. The cell suspension was pressurized at 850 lb/in^2 for 10 min in a pressure homogenizer (Parr Instruments Co., Moline, Ill.), released slowly and repressurized for 15 min and released through the nozzle; EDTA was added to a final concentration of 10^{-3} M. The cellular material was centrifuged at 750 *g*, 15 min, to remove nuclei, the supernate was centrifuged at 20,000 *g*, 15 min, to remove lysosomes and mitochondria, and the supernate was again centrifuged at 120,000 *g*, 60 min, to pellet the membrane vesicles. This material contained both plasma membrane and endoplasmic reticulum. Because H-2 determinants are detected only on plasma membrane and not on endoplasmic reticulum (24), separation of plasma membrane was not required for analysis of binding by indirect immunofluorescence. For autoradiography, however, plasma membrane vesicles had to be separated by centrifugation at 300,000 *g* for 5 h over Ficoll (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc.) (1.09 g/cm^3 in 1 mM Hepes, pH 8.2, 1 mM $MgCl_2$) (24), as radiolabeled endoplasmic reticulum was found to bind nonspecifically to MLC blasts (Bruce E. Elliott. Unpublished result.) Vesicle material was labeled with ^{125}I by chloroglycoluril (25). Protein concentrations were determined by the method of Bradford (26).

Alloantisera. Anti-H-2 and anti-Ia sera of restricted specificity were obtained from Dr. J. C. Ray (Transplantation and Immunology Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.). Anti-K^k serum was a gift from Dr. D. C. Shreffler (Department of Genetics, Washington University, School of Medicine, St. Louis, Mo.). Hyperimmune anti-H-2^k and anti-Thy 1.2 sera were raised in this laboratory by Dr. R. S. Kerbel and one of us (Bruce E. Elliott), respectively. Anti-Ly 6.2 antisera (a gift from Dr. R. S. Kerbel) was raised by injecting a DBA/2 tumor MDAY-D2 into (BALB/c × A)F_1 recipients, and has been characterized extensively.[2] Details of all antisera are described in Table I. Antisera were stored in 50-μl aliquots at −70°C until use.

Testing and Absorption of Alloantisera. Antisera used for the detection of stimulator antigens on responder cells were tested on Con A blasts for cross-reactivity with the responder strain, by protein A-rosetting. The rosetting procedure described by Johnson (27) was modified for blasts as follows. 3-d Con A cultures were centrifuged over Ficoll-Urovison (Shering, AG, Berlin, West Germany) (1.09 g/cm^3) (28), washed three times, and the cells were resuspended at 4×10^6/ml in Hanks' balanced salt solution (HBSS) with 5% FCS and 10 mM sodium azide. Cells (25 μl) were incubated with equal volumes of antisera (diluted in HBSS with azide) in 40- × 7-mm round-bottom plastic tubes (Luckham Ltd., Burghess Hill, England) on ice for 30 min. After two washes, 25 μl medium and 25 μl of a 1% suspension of protein A- (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc.) coupled sheep erythrocytes were added, and the mixtures centrifuged at 500 rpm for 10 min. After 1 h of incubation at 37°C, the pellets were gently resuspended and mixed at 1:1 with fluorescein diacetate (FDA, diluted 1:50 in phosphate-buffered saline PBS) (29). Rosettes (more than four erythrocytes per viable blast) were counted under phase contrast-fluorescence double illumination. Background rosettes (after incubation with normal mouse serum) never exceeded 2%.

Antisera exhibiting cross-reactivity (>2% rosettes, when tested on Con A blasts) were absorbed with $2–5 \times 10^7$ Con A blasts (per 100 μl serum) on ice, for 30 min. No cross-reactivity between anti-K^k and anti-D^d sera was detected.

Preparation of MLC Blasts and Exposure to Membrane Vesicles. The cultures were harvested on day 2, 3, or 4, and dead cells were removed by centrifugation over Ficoll-Urovison (28). After three washes, the cells were counted and viability (>95%) was determined by FDA (29). Alloantigen binding was assayed on 4-d MLC blasts recovered as above. For testing the binding of membrane vesicles, blasts obtained from 3-d primary MLC were treated with trypsin to

[2] Kerbel, R. S., P. Frost, and I. F. C. McKenzie. Production of high-titered anti-Ly-6.2 sera: preferential reactivity with activated lymphocyte populations. Submitted for publication.

TABLE I
Alloantisera Used in this Study

Designation	Recipient	Donor	Titer*	Source
D-2	(B10.A[5R] × LP.RIII)	B10	160	N.I.H.‡
D-19	(B10.A × A.CA)	A.SW	>640	N.I.H.
D-23b	B10.D2 × SJL)	B10.A	660	N.I.H.
D-31	(B10 × A)	B10.D2	>640	N.I.H.
Anti-D^d	(B10.AKM × A.SW)	A.TH	1,024 (B10.D2)	N.I.H.
Ia 1.2	(A.BY × B10.HTT)	A.TL	160 (B10.K)	N.I.H.
Ia 7	(B10 × HTI)	B10.A(5R)	320	N.I.H.
Anti-K^k	A.TL	A.AL	>500	Shreffler
Anti-H-2^k	B10.D2	B10.BR	240	Kerbel
Anti-Thy-1.2	AKR	C3H Thymocyte	1,280	Elliott
Anti-Ly-6.2	(BALB/c × A)F_1	MDAY-D2	2×10^5§	Kerbel

* Reciprocal serum titer to yield 50% specific killing in a cytotoxicity test with rabbit complement is shown. Except where indicated in parenthesis, target cells were of donor origin.
‡ N.I.H., National Institutes of Health.
§ Target cells were mitogen-activated cells of the appropriate strain.

remove surface-bound stimulator antigens (21). Preparation of long-term MLC blasts for membrane vesicle binding (on day 2 after restimulation) involved the separation of blasts from surviving stimulator cells by velocity sedimentation (30), before trypsin treatment. Trypsinized cells were incubated in fresh culture medium (at 5×10^5/ml) for an additional period of 5–8 h.

The cells (5×10^5 per group) were resuspended in 0.25 ml medium or medium containing membrane vesicles at various dilutions and incubated at 37°C for 2 h. In previous experiments (19, 21), and in Fig. 1, vesicle concentration was expressed in cell equivalents. This allowed comparison of binding of the same vesicle preparation by responder blasts of different MLC combinations. To compare the binding of different vesicle preparations by blasts from a single MLC combination (such as in Fig. 2), vesicles were quantitated in terms of μg protein per ml. The cells were then centrifuged over Ficoll-Urovison, and washed. Excess antigen was removed from the cell suspensions by centrifugation over a cushion of 100% FCS. After a final wash, cell suspensions were prepared for immunofluorescence or autoradiography. For inhibition of vesicle-binding, membrane vesicles were preincubated with antisera at 0°C for 60 min. An equal volume of cells was added, and the mixture incubated and processed as above.

Detection of Alloantigen-binding by Immunofluorescence. Binding of stimulator antigens was detected by incubation of the responder blasts with antisera directed against different H-2 region products of the stimulators, followed by immunofluorescence, as described (23). In these experiments, a fluorescein isothiocyanate-conjugated $F(ab')_2$ fragment of sheep anti-mouse IgG (N. L. Cappel Labs., Cochranville, Pa.) was used at a final concentration of 0.25 mg Ig per ml to visualize surface-bound alloantibodies. Before use, all antisera were centrifuged at 20,000 *g*, 15 min, to remove large aggregates. Incubations with antisera and washing of the cells were carried out at 4°C in the presence of 10 mM sodium azide. After the final wash, the cells were smeared with a pen nib, air dried, fixed in 100% methanol, and mounted in 50% glycerol-PBS (pH 7.2).

Autoradiography. Binding of radiolabeled vesicles to responder blasts was demonstrated by autoradiography as described (19). Briefly, cells smeared on gelatin-coated, acid-washed slides were fixed in methanol, washed twice in distilled water, and dipped in Kodak NTB-2 emulsion (Eastman Kodak Co., Rochester, N. Y.) three times. The slides were dried overnight in the dark and exposed in light-tight boxes at 4°C for 1 or 2 wk. The slides were then developed with Kodak D-19 developer, fixed, washed, and stained with methyl green pyronine (British Drug Houses, Ltd., Toronto, Canada).

Scoring of Preparations. Immunofluorescent preparations were scored under a Leitz Ortholux II fluorescence microscope with an HBO-100 mercury vapour lamp, and an Opak Fluor Ploem vertical illuminator (E. Leitz GmbH, Wetzlar, Germany). Blasts were characterized as round

cells with sharp, regular contours, and with a diameter of at least twice that of a small lymphocyte in the same preparation. Percentages of Ig^+ blasts were calculated from cell counts obtained by scoring 100–250 blasts per group.

Autoradiograms were viewed under oil immersion at magnification of 1000 ×. Blasts were defined as large cells with prominent nucleoli, and abundant, strongly pyroninophylic cytoplasm. Background grain counts as established in preparations with cells not treated with antigen were 0–1 grains per cell. A cell with more than three grains was considered positive. In the positive preparations, a wide range of grain-count distribution was observed, with an average of 4–10 grains per cell. At least 170 cells per group were scored, and the percentage of positive cells calculated.

Results

Effect of Prolonged Stimulator Cell Survival on Alloantigen-binding by MLC Blasts. Our previous experiments indicated that the alloantigens bound by MLC blasts exist in the form of stimulator-derived membrane vesicles (18–21). Because shedding of plasma membrane vesicles has been observed as a general mechanism for the turnover of various membrane components (31), prolonged stimulator cell survival might increase the amount of antigen available for binding of MLC blasts. We therefore compared the proportion of antigen-binding blasts stimulated in MLC with x-irradiated stimulators or mitomycin-treated stimulators. The latter survive longer, although they are unable to divide as this drug inhibits DNA synthesis (32).

Data from a representative experiment with nylon-wool passaged B10.D2 responders and CBA stimulators are given in Table II. After incubation with anti-stimulator H-2 serum, 53.7% of blasts recovered from 4-d MLC with x-irradiated stimulator cells exhibited a patchy or capped pattern of fluorescence, characteristic of stimulator antigen-binding (18). The proportion of antigen-binding blasts was 80.3 and 90.3% respectively, when stimulator cells treated with different concentrations of mitomycin were used. The proportion of antigen-binding blasts seemed to correlate positively with the fraction of viable small lymphocytes of stimulator origin in the cultures (Table II). The majority of blasts was of responder origin, because they exhibited bright, ring-type fluorescence with anti-responder H-2 serum. Normal mouse serum, and antiserum directed against an unrelated H-2 haplotype did not bind to MLC blasts. The percentage of antigen-binding blasts from cultures in eight separate experiments with mitomycin-treated stimulators ranged from 73 to 96%, as opposed to 35 to 58% in parallel cultures with x-irradiated stimulators. Furthermore, the relative amount of antigen bound per blast was greatly enhanced as estimated by the intensity of immunofluorescence and the size of protein A rosettes formed with anti-stimulator sera (data not shown).

These data suggest that viable mitomycin-treated stimulator cells provide a prolonged and/or better source of antigen, than x-irradiated cells, the majority of the latter dying within 24 h in culture. Furthermore, these results imply that under appropriate conditions almost all blasts generated in MLC are capable of binding stimulator H-2 antigens.

Serological Analysis of Stimulator-derived Antigens Bound by Responder Blasts. The involvement of stimulator H-2 and Ia antigens in the binding phenomenon, and the specificity of binding was studied on blasts recovered from 4-d MLC with mitomycin-treated or x-irradiated stimulator cells (Table III).

Similar percentages of blasts binding stimulator K, I-A (plus I-B), and D region products were detected in MLCs with mitomycin-treated and x-irradiated stimulator

TABLE II

Binding of Stimulator H-2 Antigens by T Blasts from MLR Cultures with Mitomycin-treated or X-irradiated Stimulator Cells

MLR*		Pretreatment of stimulator cells	Antisera‡	H-2 regions detected by antisera	Percentage Ig^+ blasts	Percentage Ig^+ small lymphocytes§
Responder	Stimulator					
					%	%
B10.D2$_{NA}$‖	CBA	Mitomycin (15 μg/ml)	Anti-H-2^k	K^k, I^k, D^k	90.3¶**	51.2
(H-2^d)	(H-2^k)	Mitomycin (15 μg/ml)	D-31	K^d, I^d	87.2‡‡	50.7
(H-2^d)	(H-2^k)	Mitomycin (15 μg/ml)	D-33	K^b, I^b	0.9	5.1
(H-2^d)	(H-2^k)	Mitomycin (15 μg/ml)	NMS§§	—	2.9	5.6
(H-2^d)	(H-2^k)	Mitomycin (50 μg/ml)	Anti-H-2^k	K^k, I^k, D^k	80.3¶‖‖	25.4
(H-2^d)	(H-2^k)	Mitomycin (50 μg/ml)	D-31	K^d, I^d	97.1‡‡	80.8
(H-2^d)	(H-2^k)	Mitomycin (50 μg/ml)	D-33	K^b, I^b	1.0	ND¶¶
(H-2^d)	(H-2^k)	Mitomycin (50 μg/ml)	NMS	—	0	ND
(H-2^d)	(H-2^k)	X-irradiation (3,300 rad)	Anti-H-2^k	K^k, I^k, D^k	53.7¶	2.8
(H-2^d)	(H-2^k)	X-irradiation (3,300 rad)	D-31	K^d, I^d	98.2‡‡	96.2
(H-2^d)	(H-2^k)	X-irradiation (3,300 rad)	D-33	K^b, I^b	0	2.9

* Responder/stimulator (R/S) ratio: 1/2.

‡ Final dilution of antisera was 1/10, except D-31 (1/25). See designations of antisera in Table I.

§ Ring-type fluorescence.

‖ Nylon-wool passaged nonadherent (NA) lymph node cells.

¶ Spotted and capped fluorescence.

** Stimulator blasts, i.e., blasts exhibiting strong ring-type fluorescence with anti-H-2^k antibody (9.0%) were not included in this count.

‡‡ Bright, ring-type fluorescence.

§§ NMS, normal mouse serum (used at 1/10).

‖‖ Stimulator blasts, i.e., blasts exhibiting strong ring-type fluorescence with anti-H-2^k antibody (1.7%) were not included in this count.

¶¶ ND, not done.

cells. A minor but significant fraction of blasts bound antigens encoded by I subregions telomeric to I-B. However, analysis of specificity of binding by using mixtures of antibodies directed against different stimulator H-2 region products revealed striking differences between blast populations activated against x-irradiated and mitomycin-treated cells, respectively. When blasts from MLC with mitomycin-treated stimulators were tested, the proportion of blasts stained with mixtures of antisera in all combinations, was very close to the sum of those stained separately by the individual antibodies in each mixture. Furthermore, an antiserum (D-23b) directed against stimulator K plus I region products detected as many blasts as the mixture of antibodies (anti-K^k and Ia-1.2) that recognized products of these regions separately. Titration of anti-stimulator sera on antigen-binding blasts indicated that antibody-concentrations were not limiting in these experiments (20) (data not shown). Similar results were obtained in three separate experiments using the same strain combination, and in a further four experiments using different responder strains activated against H-2^k stimulators. Blasts binding stimulator K and I-A region products represented the majority of cells generated in all combinations tested, whereas the proportions of blasts binding stimulator E/C and D region products varied with the strain combi-

TABLE III

Binding of Stimulator K, I, and D Region Products by T Blasts from MLR Cultures with Mitomycin-treated or X-irradiated Stimulator Cells

Experiment	MLR*		Antisera‡	H-2 regions recognized by antisera	Percentage Ig^+ blasts from MLC with stimulator cells	
	Responder	Stimulator			Mitomycin-treated§	X-irradiated
					%	
I	SJL_{NA}‖	A/J	D-23b	K^k, $I\text{-}A^k$, $I\text{-}B^k$, $I\text{-}J^k$, $I\text{-}E^k$	71.7	38.5
	($H\text{-}2^a$)	(kkkkkdddd)¶	Anti-K^k	K^k	36.2	38.3
	($H\text{-}2^a$)	(kkkkkdddd)¶	Ia 1.2	$I\text{-}A^k$, $I\text{-}B^k$, $I\text{-}J^k$	38.3	33.0
	($H\text{-}2^a$)	(kkkkkdddd)¶	Anti-D^d	D^d	28.4	22.0
	($H\text{-}2^a$)	(kkkkkdddd)¶	Anti-K^k + Ia 1.2	K^k, $I\text{-}A^k$, $I\text{-}B^k$, $I\text{-}J^k$	72.2	46.7
	($H\text{-}2^a$)	(kkkkkdddd)¶	Ia 1.2 + anti-D^d	$I\text{-}A^k$, $I\text{-}B^k$, $I\text{-}J^k$, D^d	62.6	39.3
	($H\text{-}2^a$)	(kkkkkdddd)¶	Anti-K^k + Ia 1.2 + anti-D^d	K^k, $I\text{-}A^k$, $I\text{-}B^k$, $I\text{-}J^k$, D^d	92.9	51.9
	($H\text{-}2^a$)	(kkkkkdddd)¶	D-19	K^a, I^a, D^a	92.2	97.1
	($H\text{-}2^a$)	(kkkkkdddd)¶	NMS**	—	0	1.8
II	SJL_{NA}	A/J	Anti-K^k	K^k	23.2	ND‡‡
	SJL_{NA}	A/J	Anti-D^d	D^d	26.2	ND
	SJL_{NA}	A/J	Anti-K^k + anti-D^d	K^k, D^d	49.2	ND
	SJL_{NA}	A/J	Anti-K^k + Ia 1.2 + anti-D^d	K^k, $I\text{-}A^k$, $I\text{-}B^k$, $I\text{-}J^k$, D^d	74.3	35.3
	SJL_{NA}	A/J	Ia7	$I\text{-}J^k$, $I\text{-}E^k$, $I\text{-}C^d$, S^d, G^d	7.4	ND
	SJL_{NA}	A/J	D-19	K^a, I^a, D^a	96.3	ND
	SJL_{NA}	A/J	NMS	—	0	1.9

* R/S ratio: 1/2.

‡ Final dilution of antisera was 1/10, except D-19 (1/15). Anti-K^k and Ia 7 (100 ml) were absorbed with 2×10^7 SJL Con A blasts. See designations of antisera in Table I.

§ Mitomycin C was used at a concentration of 25 μg/ml.

‖ Nylon-wool passaged nonadherent (NA) lymph node cells.

¶ Origin of H-2 regions of A/J ($H\text{-}2^a$).

** NMS used at 1/10.

‡‡ ND, not done.

nation (data not shown). Taken together, the observed additive effects suggest that stimulator K, I, and D region products are bound by practically nonoverlapping populations of T blasts, from MLC with mitomycin-treated stimulators.

In contrast, a large degree of overlap was demonstrated between the corresponding blast populations from MLC with x-irradiated stimulators. We have shown previously that this overlap can be eliminated, by presenting the different antigens on separate stimulator cells (20). Thus this overlap possibly reflects the binding of membrane fragments containing more than one type of stimulator H-2 products.

The specificity of antigen-binding by blasts from MLC with mitomycin-treated stimulators suggests that (*a*) most of the antigen is taken up by these cells in a close-to-molecular form, in contrast to MLC with x-irradiated stimulators, and (*b*) the vast majority (92.9% in experiment I, Table III), of these blasts bear receptors with exquisite specificity for stimulator K, I, and D region products, respectively.

Binding of Stimulator and Third-Party Membrane Vesicles by Primary MLC Blasts. The finding that a greatly increased proportion of antigen-binding T blasts generated in primary MLC is detected under conditions in which excess antigen derived from mitomycin-treated stimulators is present, raises the question of specificity and affinity of this cell population with respect to third-party H-2 haplotypes. We therefore tested the binding of stimulator or third-party membrane vesicles over an excess to a limiting concentration range. Membrane vesicles were prepared by nitrogen cavitation from

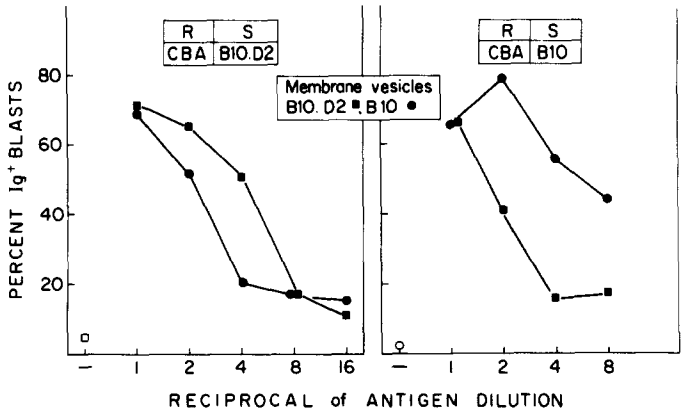

FIG. 1. Effect of antigen concentration on stimulator and third-party vesicle binding by primary MLC blasts. Blasts from 3-d MLC (set up at 1:1 responder/stimulator ratio with x-irradiated stimulators) were treated with trypsin, and incubated for 5 h in fresh medium. (Cell recoveries: 95.8% and 94.3%.) Membrane vesicles were added at different concentrations. Neat (1) vesicle concentration was equivalent to 3.6×10^8 LPS blasts/ml (protein concentrations, B10: 92 μg/ml, B10.D2: 60 μg/ml). Binding of B10.D2 (■) vesicles was detected with a mixture of D-31 and anti-D^d sera, and that of B10 (●) vesicles with D-33 plus D-2 antisera (each at 1/10 final dilution), followed by immunofluorescence. The antibodies recognize K, I, and D region products on the vesicles, and do not cross-react with the responder strain (CBA). Blasts (99.1% from CBA vs. B10.D2, and 100% from CBA vs. B10) were of responder origin, as shown by indirect immunofluorescence using antiserum D-23b (at 1/15 final dilution). Open symbols represent background antigen-binding cells (□, 3.9%; and ○, 1.7%) after trypsin treatment.

LPS-activated blasts which express greater amounts of H-2 and Ia antigen than small lymphocytes (33).

A crisscross experiment with CBA responders, and B10.D2, or B10 stimulator cells is shown in Fig. 1. Blasts were treated with trypsin to remove spontaneously bound stimulator antigens (21). Cells from each MLC were incubated with either B10 or B10.D2 vesicles. The results show that percentage of blasts binding antigens of the stimulating strain increases in proportion to the concentration of the vesicles to a plateau level (between 70 and 80%). The binding of third-party vesicles follows a similar pattern. Surprisingly, at high antigen concentrations both stimulator and third-party vesicles were bound almost to the same extent by the responder blasts, whereas a preferential binding of stimulator vesicles was observed at lower antigen concentrations. Analysis by autoradiography revealed a similar pattern of cross-reactivity in the binding of $H\text{-}2^b$, $H\text{-}2^d$, or $H\text{-}2^s$ vesicles by $H\text{-}2^k$ responders activated against $H\text{-}2^b$ or $H\text{-}2^d$ cells (data not shown).

This finding raises the following possibilities: (*a*) most of the blasts generated in a primary MLC are capable of binding alloantigens of both stimulator and unrelated H-2 haplotypes, with a higher average affinity for the former, or (*b*) the binding observed at high vesicle concentrations is nonspecific.

Inhibition of Membrane Vesicle Binding by Anti-H-2 Antibodies. To determine the specificity of stimulator and third-party vesicle binding by primary MLC blasts, we investigated the inhibition of binding with anti-H-2 antibodies (Table IV). Radiolabeled vesicles of both B10 and B10.D2 haplotype were bound by CBA blasts activated against B10. The binding of B10 and B10.D2 vesicles was effectively inhibited by preincubation with the appropriate antibodies against H-2 and Ia determinants on

TABLE IV

Inhibition with Anti-H-2 Sera of Radiolabeled Stimulator and Third-Party Plasma Membrane Vesicle Binding by Blasts from Primary MLC

MLC*		Antiserum preincubation‡	H-2 regions recognized by antisera	Plasma membrane vesicles§	Percentage labeled blasts‖
Responder	Stimulator				
					%
CBA_{NA}¶	B10	—	—	—	0
($H\text{-}2^k$)	($H\text{-}2^b$)	—	—	B10	49.0
($H\text{-}2^k$)	($H\text{-}2^b$)	D33 + D2	K^b, I^b + D^b	B10	5.9
($H\text{-}2^k$)	($H\text{-}2^b$)	D31 + α-D^d	K^d, I^d + D^d	B10	41.7
($H\text{-}2^k$)	($H\text{-}2^b$)	—	—	B10.D2	39.7
($H\text{-}2^k$)	($H\text{-}2^b$)	D31 + α-D^d	K^d, I^d, D^d	B10.D2	5.2
($H\text{-}2^k$)	($H\text{-}2^b$)	D33 + D2	K^b, I^b, D^b	B10.D2	34.4

* Cultures were set up with x-irradiated stimulators, at a 1:1 R/S ratio (to minimize spontaneous antigen binding).

‡ See Table I for antiserum designations. Anti-D^d was cross-absorbed with B10 spleen + lymph node cells (1×10^8 per 100 μl). Antisera were used at 1/10 final dilution.

§ Vesicles were prepared as described in the text. The final concentration of radiolabeled vesicles was 6.8 μCi/ml (sp act: B10, 0.1 μCi/μg; B10.D2, 0.03 μCi/μg).

‖ Detected by autoradiography after 6-d exposure.

¶ Nylon-wool passaged nonadherent (NA) lymph node cells.

the vesicles. No cross-inhibition was observed when vesicles of one strain were incubated with (noncross-reacting) antibodies against the other.

These results indicate that both stimulator and third-party vesicle binding is H-2 specific, i.e., it occurs via H-2 products on the vesicles. The H-2K, I, and D region specificity of vesicle binding has been demonstrated in a separate series of experiments (Bruce E. Elliott and Zoltan A. Nagy. Manuscript in preparation.).

Antisera Against Non-H-2 Determinants Do Not Inhibit Stimulator and Third-Party Vesicle Binding. Because the data in Table IV do not exclude the possibility of nonspecific steric inhibition, we investigated whether antibodies against non-H-2 vesicle determinants could block the binding of stimulator or third-party vesicles. In the experiment reported here (Table V) Ly-6.2 was chosen as a non-H-2 product because of its strong expression on activated cells.[2] A high-titered anti-Ly-6.2 serum[2] was used to provide a fair comparison with anti-H-2 antibodies. CBA ($H\text{-}2^k$, Ly 6.1) T cells were activated against B10 cells, and the blasts were treated with trypsin to remove surface-bound stimulator antigens. The cells were then mixed with B10 ($H\text{-}2^b$, Ly 6.2) or B10.D2 ($H\text{-}2^d$, Ly 6.2) vesicles which had been preincubated with anti-Ly 6.2 or anti-H-2 sera. Vesicle binding was detected by indirect immunofluorescence via the antibodies used for preincubation. By this approach, the presence of noninhibitory antibodies on the cell-bound vesicles could be confirmed.

The data in Table V demonstrate that vesicle binding is detected by using either anti-H-2 or anti-Ly 6.2 as a first antibody in indirect immunofluorescence. When the vesicles were preincubated with the relevant anti-H-2 sera, a marked (68 and 62%, respectively) inhibition of binding was observed. In contrast, anti-Ly 6.2 serum did not inhibit the binding of either stimulator or third-party vesicles. In fact, the binding of antibody-pretreated vesicles was easier to visualize, because they localized in large,

TABLE V

No Inhibition of Stimulator and Third-Party Vesicle Binding by Primary MLC Blasts with Antisera against Non-H-2 Determinants

MLC*		Preincubation of vesicles with antisera‡	Membrane vesicles§	Incubation of cells with antisera	H-2 regions recognized by antisera	Percentage Ig^+ blasts‖	Percentage inhibition of binding
Responder	Stimulator						
						%	%
CBA_{NA}¶	B10	—	B6	D33	K^b, I^b	63.4	0
CBA_{NA}¶	B10	D33	B6	—	K^b, I^b	19.9	68.6
CBA_{NA}¶	B10	—	B6	α-Ly-6.2	—	54.2	0
CBA_{NA}¶	B10	α-Ly-6.2	B6	—	—	53.3	1.7
CBA_{NA}¶	B10	NMS**	B6	—	—	3.4	—
CBA_{NA}¶	B10	—	—	D33	K^b, I^b	5.0	—
CBA_{NA}¶	B10	—	—	α-Ly-6.2	—	4.5	—
CBA_{NA}¶	B10	—	—	NMS	—	2.3	—
CBA_{NA}¶	B10	—	B10.D2	D31	K^d, I^d	39.9	0
CBA_{NA}¶	B10	D31	B10.D2	—	K^d, I^d	14.8	62.9
CBA_{NA}¶	B10	—	B10.D2	α-Ly-6.2	—	38.1	0
CBA_{NA}¶	B10	α-Ly-6.2	B10.D2	—	—	41.8	−9.7
CBA_{NA}¶	B10	—	B10.D2	D33	K^b, I^b	2.7	—
CBA_{NA}¶	B10	NMS	B10.D2	—	—	1.2	—
CBA_{NA}¶	B10	—	—	D31	K^d, I^d	1.4	—

* Cultures were set up with x-irradiated stimulators of a 1:1 R/S ratio.

‡ See Table I for antiserum designations. Antisera were absorbed with CBA Con A blasts ($20–40 \times 10^6$ per 100 μl). Final dilution was 1/10 for anti-H-2 sera and NMS, and 1/40 for anti-Ly-6.2.

§ Vesicles were used at a final concentration of 70 μg protein per ml.

‖ Detected by indirect immunofluorescence.

¶ Nylon-wool passaged nonadherent (NA) lymph node cells. Responder cells were treated with trypsin (21) and incubated for 8 h in fresh medium (recovery of blasts: 93.5%).

** Normal CBA serum.

strongly fluorescing patches on the blasts. This pattern of localization suggests the uptake of large groups of vesicles cross-linked with antibody. None of the antibodies bound to the cells in the absence of vesicles, nor did cell-bound vesicles absorb irrelevant anti-H-2 antibodies. Similar experiments using anti-Ly 6.2 or anti-Ly 5.1 sera and radiolabeled vesicles yielded the same results (Bruce E. Elliott and Zoltan A. Nagy. Manuscript in preparation.). These data confirm that the binding of both stimulator and third-party vesicles is H-2 specific.

Cold Antigen Inhibition of Radiolabeled Stimulator Membrane Vesicle Binding by Primary MLC Blasts. We have shown previously that the binding of radiolabeled stimulator vesicles by MLC blasts can be inhibited with unlabeled (cold) vesicles of the stimulator haplotype, but not with vesicles of the responder haplotype (19). To determine whether or not recognition structures on the same cells are involved in stimulator and third-party vesicle binding, the inhibition of radiolabeled stimulator vesicle binding with different unlabeled vesicle preparations was studied.

The binding of B10 vesicles to B10-activated CBA blasts was effectively inhibited by both B10 and B10.D2 vesicles (Table VI). However, an at least eightfold-higher concentration of B10.D2 than B10 vesicles was required to cause comparable degrees

TABLE VI

Cold Antigen Inhibition of Radiolabeled Stimulator Membrane Vesicle Binding by Blasts from Primary MLC

MLC*		Cold antigen‡	Cold antigen concentration§	Radiolabeled membrane vesicles‖	Percentage labeled blasts¶
Responder	Stimulator				
					%
CBA_{NA}**	B10	—	—	B10	44.2
($H\text{-}2^k$)	($H\text{-}2^b$)	A.BY ($H\text{-}2^b$)	10	B10	11.5
($H\text{-}2^k$)	($H\text{-}2^b$)	A.BY ($H\text{-}2^b$)	20	B10	7.5
($H\text{-}2^k$)	($H\text{-}2^b$)	A.BY ($H\text{-}2^b$)	80	B10	8.3
($H\text{-}2^k$)	($H\text{-}2^b$)	B10.D2 ($H\text{-}2^d$)	20	B10	36.5
($H\text{-}2^k$)	($H\text{-}2^b$)	B10.D2 ($H\text{-}2^d$)	40	B10	26.6
($H\text{-}2^k$)	($H\text{-}2^b$)	B10.D2 ($H\text{-}2^d$)	80	B10	14.0
($H\text{-}2^k$)	($H\text{-}2^b$)	CBA ($H\text{-}2^k$)	20	B10	41.3
($H\text{-}2^k$)	($H\text{-}2^b$)	CBA ($H\text{-}2^k$)	40	B10	49.6
($H\text{-}2^k$)	($H\text{-}2^b$)	CBA ($H\text{-}2^k$)	80	B10	43.8

* Cultures were set up with x-irradiated stimulator cells, at 1:1 R/S ratio.

‡ Membrane vesicles prepared as described were mixed with radiolabeled B10 vesicles at the final concentrations indicated. Cells were incubated with the mixtures (or with radiolabeled B10 vesicles alone) for 2 h at 37°C.

§ μg protein/ml.

‖ Vesicles were prepared and labeled as described. The concentration of vesicles was 29 μg (1.4 μCi) per ml.

¶ Detected by autoradiography after 14-d exposure.

** Nylon-wool passaged nonadherent (NA) lymph node cells.

of inhibition. Syngeneic (CBA) vesicles did not inhibit the binding of stimulator vesicles at any concentration tested.

These results indicate that stimulator and third-party vesicles compete for binding sites on the same cells and that these sites have a higher average affinity for stimulator than for third-party vesicles.

Comparison of Membrane Vesicle Binding by Long-Term and Primary MLC Blasts. In view of the observed poor specificity of primary MLC blasts with respect to unrelated H-2 haplotypes, we investigated whether or not a long-term selection against a single H-2 haplotype would improve the specificity of alloantigen-binding by T cells.

We therefore studied binding of stimulator and third-party membrane vesicles by B10.D2 responder cells, which had been restimulated with B10 cells up to 30 times over a period of 15 mo. Cell surface phenotype of the blasts, as assessed by indirect immunofluorescence was: $H\text{-}2.31^+$ (97.2%) and $Thy\text{-}1.2^+$ (94.4%). The cells gave a strong proliferative response to B10 and (B6 × A)F_1 stimulators, a marginal response to CBA (3.5–4% of the response to B10), and no significant response to SJL stimulators (Table VII). The specificity of membrane vesicle binding closely correlated with that of the proliferative response. Large proportions of blasts bound B10 and (B6 × A)F_1 vesicles, but no binding of SJL and CBA (very marginal) vesicles was observed at any antigen concentration tested (Fig. 2A). The specificity of this long-term line was confirmed in three successive experiments.

For a direct comparison with the long-term line, vesicle binding by primary MLC blasts from the same strain combination was studied (Fig. 2B). The pattern of cross-reactivity was identical to that shown in Fig. 1. At the highest antigen concentration, both stimulator (B6) and third-party (CBA and SJL) vesicles were bound to almost

TABLE VII

Specific Proliferative Response of Long-Term Primed B10.D2 Responder Cells in MLC with Stimulator Cells of Different H-2 Haplotypes

Responder cells from long-term B10.D2 vs. B10 MLC (cells/well)*	Counts per minute of ^{3}H‡ per well after restimulation§ with:				
	B10.D2 (H-2^d)	B10 (H-2^b)	(B6 × A)F$_1$ (H-2$^{b \times a}$)	CBA (H-2^k)	SJL (H-2^s)
× 10^4	*cpm ± SD*				
5	286 ± 145	185,707 ± 22,634	76,680 ± 7,847	6,593 ± 2,202	480 ± 153
2.5	344 ± 198	133,250 ± 9,173	ND	5,770 ± 1,568	642 ± 381
1.25	289 ± 190	97,051 ± 8,434	ND	3,436 ± 1,171	351 ± 111

* Responder cells were B10.D2 lymph node cells stimulated with B10 spleen cells approximately every 2 wk for 12 mo (25 times).

‡ Cultures were pulsed overnight with [^{3}H]thymidine (2 μCi per well) and harvested on day 3. [^{3}H]-thymidine incorporation is expressed as the mean ^{3}H counts per minute per well ± SD of three wells per group.

§ Stimulators (10^6 cells per well) were 3,300-rad-irradiated spleen cells from the strains indicated.

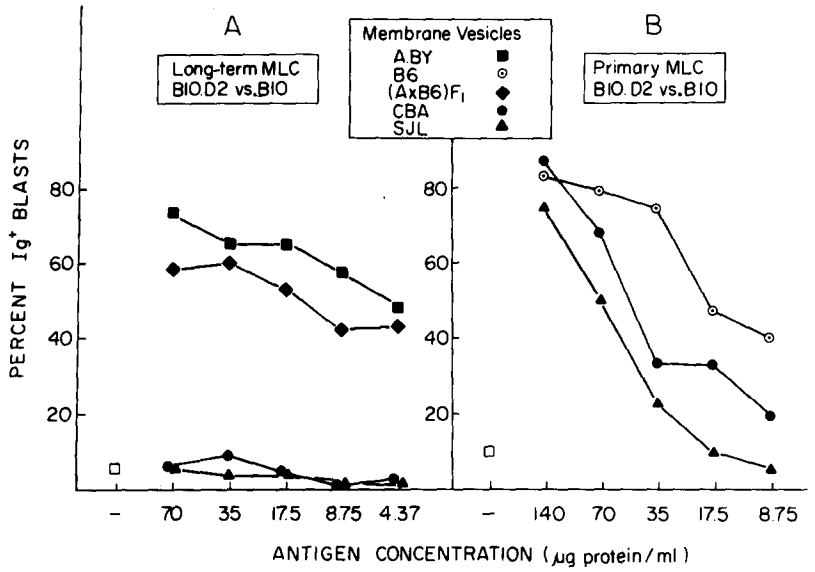

FIG. 2. Binding of stimulator and third-party vesicles by long-term (A) and primary (B) MLC blasts. 2-d MLC blasts from long-term B10.D2 vs. B10 cultures were separated by velocity sedimentation to remove surviving stimulator small lymphocytes. Blasts from both long-term and 3-d primary MLC of the same strain combination were treated with trypsin and incubated for 8 h in fresh medium (blast recoveries 83.3 and 85.7%, respectively). Membrane vesicles were added at different concentrations. Vesicle binding was detected by antisera D-33 (for B10, B6, and [B6 × A] F$_1$), anti-H-2^k (for CBA), and D-19 (for SJL), followed by immunofluorescence. Antisera (100 μl) were preabsorbed with 2 × 10^7 (D-33, anti-H-2^k) or 4 × 10^7 (D-19) B10.D2 Con A blasts. Most MLC blasts carried responder H-2 determinants (91.7% [A], and 96.3% [B] H-2.31$^+$)). Background antigen binding (□) was 5.4% (A) and 9.9% (B) after trypsin treatment.

the same extent; whereas a preferential binding of stimulator vesicles was apparent at limiting vesicle concentrations. Furthermore, a preferential cross-reactivity with CBA as compared to SJL vesicles was observed at all antigen concentrations. The proportion of vesicle-binding blasts from primary MLC decreased rapidly at higher antigen

dilutions; whereas the proportion of long-term MLC blasts binding stimulator vesicles (Fig. 2 A) did not change significantly with the concentration of antigen.

These data confirm that the majority of blasts generated in primary MLC are capable of binding both stimulator and H-2-unrelated vesicles with different affinities. In contrast, long-term, multiply restimulated blasts derived from the same MLC combination exhibit higher affinity and improved specificity of vesicle binding, the latter corresponding to the specificity of proliferative response by this cell line.

Discussion

The binding of stimulator antigens by alloactivated T blasts provides a system to study T-cell specificity at the single cell level. Alloantigen binding occurs both spontaneously in the course of MLR (18, 20), or graft-versus-host reaction (GVHR) (34), and after short-term (2 h) incubation of MLC-activated blasts with stimulator cell material (membrane vesicles or antigens from MLC supernates) (19, 21). We have previously demonstrated specificity of the binding in both systems. However, because ~50% of the blasts from 4-d MLCs did not bind detectable amounts of antigen by any of these experimental approaches, the relation of the binding phenomenon to the proliferative MLR response was unclear.

In this paper we have demonstrated that the detectable proportion of alloantigen-binding T blasts from primary MLC is strictly dependent on antigen concentration (Fig. 1), and can be enhanced to >90% (Tables II and III). These findings suggest that the vast majority of blasts were generated in response to the stimulating antigens (with or without the aid of soluble mediators [35–37]), and thus render the possibility of nonspecific triggering unlikely. Furthermore, these observations can only be reasonably interpreted by assuming that many of the blasts bear low-affinity receptors because antigen binding by these cells is only demonstrable at high antigen concentrations.

Because the experimental systems described in this paper detect alloantigen-binding by almost all T blasts with a possibly wide range of receptor affinities, it was important to determine the specificity of binding under these conditions. The specificity of T cells for stimulator K, I, and D region products was investigated on blasts activated against mitomycin-treated allogeneic cells. Products of the three major H-2 regions have been shown to be bound by nonoverlapping populations of blasts, the sum of which may represent up to 93% of total blasts (Table III). Thus specific distinction of the responder blasts between stimulator H-2 products (20) is retained under these conditions.

Of the Ia specificities bound, determinants encoded by the I-A subregion are predominant (Table III). The I-B subregion, although formally not ruled out, is probably not involved here (38). Similar results have been reported using the GVHR system (34). However, we have also observed a significant binding of stimulator E/C subregion products in some of the strain combinations tested. Studies of this phenomenon are in progress.

The data in Table III demonstrate that the number of antigen-binding blasts is greater, and the specificity for H-2 region products is improved, when mitomycin-treated stimulators are used instead of x-irradiated cells. The reason for this difference is not fully understood. It appears that antigens presented on viable stimulator cells are taken up preferentially by the T blasts (Table II). This phenomenon is reminiscent

of a cell-mediated mitogenic response, in which submitogenic amounts of Con A, when presented on mitomycin-treated syngeneic cells cause T cells to proliferate (39). Soluble stimulator antigens in MLC supernates seem to be less important for the binding, because their concentration usually does not correlate with the degree of antigen-binding by blasts from the same cultures (Zoltan A. Nagy. Unpublished observation.). Nevertheless, the specificity of binding (Table III) strongly suggests that the antigens are taken up in a close-to-molecular form.

The specificity and affinity of MLC blasts with respect to unrelated H-2 haplotypes was investigated by using a plasma membrane vesicle-binding assay, which allows quantitation of the antigen. Vesicles were prepared from LPS blasts known to express large amounts of H-2 and Ia antigens (33). Primary MLC blasts, when tested against limiting concentrations of stimulator and third-party vesicles, exhibited a preferential binding of stimulator vesicles, as expected (Fig. 1) (21). However, at high concentrations, both stimulator and third-party vesicles were bound by almost equal proportions (~70%) of blasts. The binding of both vesicle types has been shown to be specific by inhibition with the relevant anti-H-2 antisera (Table IV). Steric hindrance does not seem to be involved here, because antibodies against non-H-2 antigens of the vesicles do not inhibit binding (Table V) (Bruce E. Elliott and Zoltan A. Nagy. Manuscript in preparation.). Furthermore, the binding of radiolabeled stimulator vesicles was inhibited by both stimulator and third-party vesicles, although an eightfold-higher concentration of the latter was required to achieve the same extent of inhibition (Table VI). The observed cross-reactions cannot be attributed exclusively to clones reacting with determinants shared between the stimulating and third-party strains, unless one postulates that practically all clones were generated by such determinants. Thus these findings suggest that the distinction by most MLC-activated T blasts between stimulator and third-party alloantigens may be imperfect. Receptor specificity is manifested at the cell population level only as a higher average affinity for the stimulating haplotype. It should be pointed out, however, that individual receptor affinities cannot be directly inferred from these data, as the binding of membrane vesicle antigens most likely involves multipoint attachments (40). This would imply that the concentration of vesicles might influence the probability of multiple bond-formation which is required for the firm attachment of vesicles to blasts with weakly interacting receptors. In a recent study, Fathman and Hengartner (41) have compared the specificity of the proliferative response in bulk long-term MLC with that of cloned alloreactive T cells. These workers have also concluded that bulk MLC might allow the generation and expansion of clones with low-affinity (cross-reactive) receptors. However, the frequency of such clones is probably much higher in a primary MLC than in the long-term cultures.

Because our results raised the possibility that all MLC blasts might react at lower affinity with antigens of any given H-2 haplotype, we investigated membrane vesicle-binding by a highly selected long-term MLC cell line. The proliferative response of this cell line is practically monospecific (Table VII). We have shown that these cells do not bind third-party vesicles at any concentration tested (Fig. 2A). Furthermore, the concentration of stimulator vesicles affects only slightly the detectable proportion of antigen-binding blasts. Antigen density on the vesicles (shown by using [B6 × A]F_1 instead of B10 vesicles) does not seem to influence the slope of the binding curve. These observations indicate that long-term MLC cells have a higher average affinity

and more restricted specificity for the stimulating cells than primary MLC cells. High-avidity antigen binding may actually represent a selective advantage for survival in long-term cultures.

The data presented here, and reported by others (9–13, 15, 16) indicate a large degree of heterogeneity among cells generated by a single MHC difference. It becomes therefore, difficult to conceive, how all these specificities can be represented within a cell population carrying the same idiotype (42, 43). There are at least two possible explanations for this. Either all cells reacting to an MHC haplotype share at least one common idiotype, or antibodies against a whole array of idiotypes are contained within a given anti-idiotypic serum. In support of the latter, Binz et al. (44) have shown that specific anti-idiotypic antibodies against Ly-1^+2^- or Ly-1^-2^+ cells can be demonstrated by absorption of anti-idotypic antisera with one or the other cell type.

In conclusion, our data suggest that T cells recognize the products of different H-2 regions more specifically than allelic variants of the H-2 complex antigens. This asymmetry of specificity might be related to the fact that distinction between K, I, and D region products is relevant to T-cell functions (10, 14, 45, 46), whereas alloreactivity lacks any apparent biological significance (13). It appears on the basis of these results that the high frequency of alloreactive T-cell precursors (1–4) is explained at least partially by the generation of large numbers of cells with low-affinity receptors for the stimulating antigens. Low affinity seems to parallel cross-reactivity. Thus primary MLC cell populations are probably higher heterogeneous, and considerable overlaps may exist between populations generated by different sets of MHC alloantigens. This implies that the actual frequency of cells strictly specific for nonshared stimulator determinants (as shown in Fig. 2A) must be minute.

Summary

The specificity of binding of stimulator-derived H-2 antigens by mixed lymphocyte culture (MLC)-activated T blasts was investigated under conditions of antigen excess. We have shown that the detectable proportion of alloantigen-binding blasts from primary MLC is a function of antigen concentration, and can represent up to >90% of total blasts, when the antigen is presented in the appropriate form (on mitomycin-treated viable stimulator cells, or membrane vesicles prepared from lipopolysaccharide blasts), and at nonlimiting concentration. Thus stimulator alloantigen-binding directly parallels the proliferative response and is not restricted to a subpopulation of T blasts. However, the marked dependence of the binding on antigen concentration indicates that cells with a wide range of receptor affinities for the stimulating determinants are involved. In view of this possibility, the specificity of binding by these cells was studied. We have demonstrated that stimulator K, I, and D region products are bound by nonoverlapping subpopulations of blasts, the sum of which may represent 93% of total blasts. Thus, specific distinction by these cells between different H-2 region products is not affected by the putative heterogeneity in terms of receptor affinities. However, specificity with respect to unrelated H-2 haplotypes is strictly dependent on antigen concentration. A preferential binding of stimulator membrane vesicles occurs at limiting concentrations; whereas the majority of blasts bind stimulator and third-party vesicles equally well at high vesicle concentrations. The binding of both vesicle types is specific in that it can be inhibited with the relevant anti-H-2 sera. Furthermore, stimulator and third-party vesicles seem to compete for binding sites on the same cells, as shown by cold antigen inhibition.

From these results, we propose that there is an imperfect distinction between stimulator and third-party H-2 antigens by the majority of primary MLC blasts. In contrast, highly selected long-term MLC blasts do not bind third-party H-2 antigens at any concentration, and seem to have high affinity for the stimulating antigens.

We conclude that large numbers of clones with low-affinity (cross-reactive) receptors are generated in primary MLC, most of which become eliminated during long-term selection. This implies that the frequency of cells strictly specific for nonshared stimulating determinants must be minute.

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