

## SPECIFIC BINDING OF ALLOANTIGENS TO T CELLS ACTIVATED IN THE MIXED LYMPHOCYTE REACTION

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The presence of surface Ig-bearing blasts among cells activated against allogeneic determinants has been shown by several investigators (1-8) with immunofluorescence and cytotoxicity techniques. Some of these Ig-positive cells are B cells, since there is evidence suggesting that not only T but also B cells undergo blast transformation during a mixed lymphocyte reaction (MLR)<sup>1</sup> (1-3). However, most of these Ig-positive blasts have been shown to be T cells (4-7). The origin of surface Ig on activated T blasts is not established unequivocally. Goldschneider and Cogen (5) suggested that rat T cells activated in MLR express surface Ig (detected by immunofluorescence) in response to specific stimulation with antigen. Gorczynski and Rittenberg (7) have detected Ig-positive cells (by antimouse Ig plus complement [C]) among in vitro primed mouse T cells reactive in secondary MLR; these authors have not stated whether surface Ig is actively synthesized or passively acquired by these cells. Pernis et al. (6) have shown that surface Ig on allogeneic activated thoracic duct lymphocytes (T.TDL), prepared by injection of parental thymus cells into lethally irradiated F<sub>1</sub> hybrid mice was passively acquired. Hudson and Sprent (4, 9) have shown that this surface Ig was produced by B cells present in the thymus cell inoculum. T.TDL were shown to be Fc receptor negative in two independent studies with several methods (10, 11).

Our experiments were designed to study the origin and specificity of surface Ig on MLR-responsive mouse T blasts activated in vitro. The results suggest that in MLR, responder-type bursa equivalent-derived (B) cells produce alloantibodies directed against *H-2* determinants of the stimulator cells. Furthermore, it appears that alloantigens derived from the stimulator cells bind to the surface of most responder blasts. This binding seems to be specific and does not require the presence of antibodies against stimulator antigens in the culture. The alloantigens can be detected on the responder cells at the end of the culture period by both IgM and IgG alloantibodies.

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<sup>1</sup> Abbreviations used in this paper:  $\alpha$ , anti; B, bursa equivalent-derived; B6, C57BL/6J; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; MLR, mixed lymphocyte reaction; PBS, phosphate-buffered saline; R, responder; S, stimulator; SI, stimulation index; TRITC, tetramethylrhodamine; T.TDL, allogeneic-activated thoracic duct lymphocytes.

## Materials and Methods

**Mice.** CBA/J (The Jackson Laboratory, Bar Harbor, Maine), C57BL/6J (B6) (Bomholtgaard, Denmark), SJL/J, and (CBA × B6) F<sub>1</sub> (Institut für Biologisch-Medizinische Forschung Ag, Füllinsdorf, Switzerland) were used. The sources of recombinant strains are listed elsewhere (12). 2- to 5-month-old male and female mice were used throughout.

**Media.** The culture medium was HEPES-buffered (10 mM) RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with L-glutamine (2 mM final concentration), streptomycin (100 µg/ml), penicillin (100 IU/ml), 5% human serum, and 2-mercaptoethanol ( $3 \times 10^{-5}$  M final concentration). The medium used for the preparation of cell suspensions and washing of cells was the same except that it did not contain 2-mercaptoethanol. The concentration of human serum was 2.5%.

**Cell Preparation.** Lymph node cells from axillary, cervical, inguinal, and mesenteric nodes were prepared as described previously (13). The cells were counted and viability (78–93%) was determined by exclusion of eosin Y (1% in phosphate-buffered saline [PBS]). Responder cells were enriched for T cells by passing normal lymph node cells over nylon wool columns according to the method of Julius et al. (14). 97–99% of the nonadherent cells were T cells (as tested by anti-Thy 1.2 serum plus guinea pig C); between 50 and 90% of the T cells loaded on the column were recovered. B-cell contamination (Ig<sup>+</sup> by immunofluorescence) was 0.5–2.5%.

**MLR.** Unidirectional MLR cultures were set up: stimulator cells were X irradiated (3,300 R, Phillips RT 305 at 300 kV, 10 mA, and 100 R/min). The responder cells were either untreated, or nylon wool column-separated lymph node cells. The concentration of responder cells was adjusted to  $5 \times 10^6$ /ml, that of stimulator cells to  $1 \times 10^7$ /ml. 2 ml of responder (R) cell suspension was mixed with an equal volume of stimulator (S) cell suspension (R/S ratio, 1:2) in a Falcon 30 ml plastic bottle (no. 3012; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). The bottle was incubated in an upright position in a 5% CO<sub>2</sub>-95% air humidified atmosphere for 4 days. After 3 days, 40 µCi of [<sup>3</sup>H]thymidine (2Ci/nmol; The Radiochemical Centre, Amersham, England) were added and thymidine incorporation was determined on day 4 of culture by processing a 0.2-ml sample from each bottle according to methods described previously (13). The stimulation index (SI) was calculated as the ratio of counts in allogeneic cultures to that in syngeneic cultures.

**Preparation of Blasts and Incubation with Alloantisera.** Blasts were recovered from MLR cultures on day 4 by centrifuging 4 ml of cultured cells over a 2 ml Ficoll-Urovision layer (density 1.077 g/cm<sup>3</sup>) at 600 g for 15 min. Cells (at least 98% viable) were recovered from the interface, and washed three times in RPMI medium plus 5% heat-inactivated fetal calf serum (FCS). An average of 50% of the viable cells obtained from the allogeneic cultures were large- and medium-sized blasts. Blast cells were defined as cells with a diameter at least twice that of a small lymphocyte, with a smaller nuclear to cytoplasmic ratio than small lymphocytes, and with a nonsegmented nucleus (in distinction to nonlymphoid cells). In some experiments, cells (not more than  $2.5 \times 10^5$  per group) were incubated at 4°C for 1 h with 50 µl medium to which 50 µl of the appropriate antiserum diluted in medium without serum had been added.

**Preparation of Alloantisera.** To obtain "early" alloantisera mice were injected intraperitoneally (i.p.) with  $6 \times 10^7$  living allogeneic spleen and lymph node cells in 0.5 ml RPMI medium without FCS. 5 days after the injection the mice were bled and the serum collected. Hyperimmune CBA anti-B6 serum was raised by a first injection (i.p.) of  $2 \times 10^7$  spleen and lymph node cells followed by 10 weekly boosts of  $4 \times 10^6$  cells per mouse (i.p.), and prepared as described above. Mice were bled 7 days after the last injection.

The following hyperimmune anti-H-2 antisera were a generous gift of Dr. D. C. Shreffler, Department of Human Genetics, University of Michigan, Ann Arbor, Mich.: (B10 × A)F<sub>1</sub> anti-A.SW, A.TL anti-A.AL, A.TH anti-A.TL, and A.TL anti-A.TH. All the antisera were heat inactivated (56°C, 30 min), aliquoted, and stored at -70°C until use. Cytotoxic titers and cross-reactivity of antisera were determined by a dye exclusion microcytotoxicity test [described elsewhere (15)], using lymph node target cells from the appropriate strains.

For absorptions two volumes of antiserum were mixed with one volume packed spleen and lymph node cells and incubated for 20 min at 4°C. This was repeated two or three times.

**Fractionation of Antisera.** Antisera were fractionated accordingly to sedimentation velocity by centrifugation through a convex exponential gradient from 10 to 40% sucrose in PBS (mixing chamber volume per gradient, 10 ml). In some experiments a cushion of 60% sucrose was applied to

the bottom of the gradient. Centrifugation was performed in a Spinco model L ultracentrifuge (SW 41 rotor; Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 40,000 rpm for 18 h. Fractions were dialyzed against PBS and reconcentrated to the original volume of antiserum by pressure dialysis and kept frozen ( $-20^{\circ}\text{C}$ ) until use.

**Immunofluorescence.** Cells were washed three times in Hanks' balanced salt solution (Flow Laboratories Ltd., Irvine, Scotland) containing 5% heat-inactivated FCS and 10 mM sodium azide at  $4^{\circ}\text{C}$ , and were kept throughout in this medium. Cells (no more than  $2.5 \times 10^6$  per group) were resuspended carefully in  $50 \mu\text{l}$  of medium to which  $50 \mu\text{l}$  of the appropriate fluorescent reagent was added (at a predetermined dilution), incubated at  $4^{\circ}\text{C}$  for 30 min, and washed once between each incubation. After the final antiserum treatment cells were washed three times and adjusted to  $5 \times 10^5/\text{ml}$ ,  $50 \mu\text{l}$  of which were smeared onto a glass slide by cytocentrifugation. The preparations were air dried, fixed in 100% ethanol, and mounted in 50% glycerol/PBS (pH 7.2). Preparations were scored under a Leitz Orthoplan fluorescence microscope with an Osram HBO-200 mercury vapour lamp and an Opak Fluor Ploem vertical illuminator (E. Leitz GmbH, Wetzlar, Germany).

$\text{Ig}^+\text{T}^+$  blasts were detected by treating cells simultaneously with rabbit IgG antimouse T (16) (a gift from Dr. C. Bron, Institut de Biochimie, Université de Lausanne, Switzerland) detected by (tetramethylrhodamine) [TRITC]-sheep antirabbit Ig, and (fluorescein isothiocyanate) [FITC]-sheep antimouse Ig. An example of an  $\text{Ig}^+\text{T}$  blast is shown in Fig. 1. In some experiments, cells were stained indirectly with rabbit antimouse- $\mu$  detected by (TRITC)-sheep antirabbit Ig; or directly with (FITC)-goat-antimouse- $\mu$ , or (FITC)-goat-antimouse- $\gamma$ .

Immunofluorescent reagents were kindly provided by L. Forni. Sheep antimouse Ig serum was prepared by immunization of a sheep with mouse total Ig (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.). Commercial goat antimouse- $\gamma$  (Melloy Laboratories, Springfield, Va.) was absorbed with Sepharose-coupled  $\text{F}(\text{ab})_2$  fragments of commercially available mouse IgG (Schwarz/Mann Div., Becton, Dickinson & Co.) (17). Goat IgG antimouse- $\mu$  and rabbit antimouse- $\mu$  were prepared by immunization of goats with purified MOPC-104E myeloma protein (IgM) (passed over a G200 column, followed by starch gel electrophoresis), and absorbed with mouse IgG.

IgG fractions from all anti-immunoglobulin sera were purified by ammonium sulfate precipitation and passaged over a DEAE-cellulose column. The IgG fraction was conjugated with FITC or TRITC by the method of Cebra and Goldstein (18). Protein concentrations were measured by spectrophotometry and adjusted to 0.5 mg/ml. Sodium azide (10 mM) was added.

## Results

**The Presence of Surface Ig on T Blasts Activated In Vitro against Allogeneic Cells.** When normal lymph node cells were stimulated with X-irradiated allogeneic lymph node cells in several strain combinations, 24–44.3% of all blasts recovered from the cultures were T blasts with surface Ig as assessed by immunofluorescent double staining. Three representative experiments of this kind are shown in Table I. The proportion of B blasts ( $\text{T-Ig}^+$ ) varied from experiment to experiment (range 1–11%). Very few double-negative blasts were observed. Anti- $\mu$  reagents usually stained more cells than were found to be  $\text{T}^+\text{Ig}^+$ , suggesting that both  $\text{Ig}^+$  T blasts and B blasts were detected by this method. The degree of stimulation expressed either as counts per minute or as SI did not seem to correlate with the percentage of  $\text{Ig}^+$  T blasts per total blasts (nor did it correlate with the percentage of blast cells in the cells recovered; but this is not unexpected, as the Ficoll-Urovison separation step leads to enrichment for blast cells). There were few blasts in the syngeneic cultures and of them 0–7.2% were Ig or  $\mu$ -positive.

When B6-unfractionated lymph node responder cells were activated against strains B10.D2 or B10.A(5R), which are very similar to B6, apart from defined,

TABLE I  
Surface Ig on MLR-Activated T Blasts from Normal Lymph Node Responder Cells

Exp.	Strain combination		$\mu^+$ blast/total blast		T <sup>+</sup> Ig <sup>+</sup> blast/total blast		T <sup>-</sup> Ig <sup>+</sup> blast/total blast		% Total blast	
	R*	S†		%		%		%		SI‡
1	CBA/J	(CBA × B6)F <sub>1</sub>	71/227	31	53/222	24	ND	—	37	12
	CBA/J	CBA/J	4/65	6.2	ND	—	ND	—	—	1
2	A/J	B6	93/220	42.3	77/224	34.4	25/245	10.2	25.1	58.9
	A/J	SJL	119/214	59.6	86/194	44.3	19/176	10.8	20.8	8.3
	A/J	A/J	6/115	5.2 (0-6.2)	3/91	3.3 (1.6-7.2)	ND	—	—	1
3	B6	B10.D2	71/153	46.4	71/222	32.0	4/220	1.8	53.3	43.3
	B6	B10.A(6R)	87/210	41.4	57/183	31.1	9/211	4.3	44.4	20.2
	B6	B6	2/66	3.0	2/132¶	1.5 (0.9-4.8)	ND	—	7.3	1

\* Responder cells, normal lymph node cells.

† Stimulator cells, lymph node cells irradiated with 3300 R.

‡ SI, experimental cpm/cpm in syngeneic control cultures.

|| Pool of four experiments. The range of percent-positive cells is indicated in parentheses.

¶ Pool of two experiments.

serologically detectable differences in the *H-2* complex, 32.0% and 31.1% T<sup>+</sup>Ig<sup>+</sup> blasts were observed. These results suggest that the Ig detected on the surface of T blasts might be antibodies directed against *H-2* determinants of the stimulators.

#### T Blasts from MLR Cultures with B-Depleted Responder Cells

**BINDING OF "EARLY" AND HYPERIMMUNE ALLOANTISERA.** When nylon wool-passaged nonadherent T cells (0.8% Ig<sup>+</sup>) from A/J mice were cultured with B6 or SJL stimulator cells, only 3.2 and 4.4% of the total blasts were T<sup>+</sup>Ig<sup>+</sup> (Table II), respectively. In contrast, unfractionated lymph node responder cells, activated against the same stimulators, yielded 34.4 and 44.3% T<sup>+</sup>Ig<sup>+</sup> blasts.

The ability of T<sup>+</sup>Ig<sup>-</sup> blasts to bind early (5 day) alloantiserum was investigated. After incubation with A/J anti-B6 serum, 39.1% of the total blasts from A/J responder/B6 stimulator cultures were T<sup>+</sup>Ig<sup>+</sup>; whereas A/J anti-SJL serum bound to only 11.6% of blasts (Table II). The latter binding could be due to a residual cross-reactivity of the antiserum detected in a microcytotoxicity test after absorption with B6 cells. Comparable frequencies of Ig<sup>+</sup> cells were obtained with anti- $\mu$  reagents. No binding to T<sup>+</sup>Ig<sup>-</sup> blasts of anti-B6 serum was observed when the anti-B6 activity was removed by absorption with B6 cells.

Conversely, when T blasts from A/J responder/SJL stimulator cultures were incubated with A/J anti-SJL serum, 49.6% T<sup>+</sup>Ig<sup>+</sup> cells were observed; again this activity could be absorbed with SJL cells. Anti-B6 serum stained only 8.1% of A/J cells activated against SJL. A similar experiment with hyperimmune anti-stimulator alloantibodies yielded similar results (unpublished data). Although binding of antistimulator antibodies to the responding T blasts occurred in every experiment, the resulting increase in the frequency of T<sup>+</sup>Ig<sup>+</sup> blasts varied considerably from experiment to experiment (see data presented below).

#### BINDING OF SUCROSE GRADIENT-FRACTIONATED HYPERIMMUNE ALLOANTIBOD-

TABLE II  
*T Blasts Activated in MLR Cultures with B-Depleted Responder Cells: Binding of Alloantibodies to Antigen-Activated T Blasts*

Responder cells	Strain combination		Incubation with anti-serum†	$\mu^+$ blast/total blast		T <sup>+</sup> Ig <sup>+</sup> blast/total blast		T <sup>-</sup> Ig <sup>+</sup> blast/total blast		% Total blast	SI	
	R	S			%	%	%	%				
Normal lymph node cells	A/J	B <sub>6</sub>	—	93/220	42.3	77/224	34.4	25/245	10.2	25.1	58.9	
	A/J	SJL	—	119/214	55.6	86/194	44.3	19/176	10.8	20.8	8.3	
Nonadherent* lymph node cells	A/J	B <sub>6</sub>	—	12/376	3.2	6/116	5.2	1/141	0.7	33.3	50.5	
	"	"	$\alpha$ B <sub>6</sub>	119/209	56.9	102/261	39.1	0/150	0			
	"	"	$\alpha$ B <sub>6</sub> §	6/244	2.5	ND	—	ND	—			
	"	"	$\alpha$ SJL	6/278	2.2	8/69	11.6	0/98	0			
	"	A/J	SJL	—	2/45	6.0	1/45	—	0	0	20.4	4.9
	"	"	"	$\alpha$ B <sub>6</sub>	12/100	12.0	7/86	8.1	0/70	0		
"	"	"	$\alpha$ SJL	34/99	34.3	56/113	49.6	1/72	1.4			
"	"	"	$\alpha$ SJL	5/84	6.0	ND	—	ND	—			

\* Nonadherent lymph node cells (0.8% Ig<sup>+</sup>) passed through nylon wool.

† Serum from A/J mice immunized 5 days before with B<sub>6</sub> or SJL spleen and lymph node cells ( $60 \times 10^6$ ). A/J anti-B<sub>6</sub> serum absorbed twice with SJL plus A/J spleen and lymph node cells. Cytotoxic titer: 1/32 (on B<sub>6</sub> LN cells).

A/J anti-SJL serum absorbed twice with B<sub>6</sub> plus A/J spleen and lymph node cells. Cytotoxic titer: 1/16 (on SJL lymph node cells). All antisera were used at a final dilution of 1/8.

§ Absorbed two times with B<sub>6</sub> spleen and lymph node cells.

|| Absorbed two times with SJL spleen and lymph node cells.

IES TO MLR-ACTIVATED T BLASTS. Since Ig may bind to T blasts either as free antibody or as antigen-antibody complexes, the ability of T blasts to bind specific antibody from 7S and 19S fractions of hyperimmune alloantisera separated by sucrose gradient centrifugation was investigated. When T blasts from MLR cultures of CBA nonadherent responder/B<sub>6</sub> stimulator cells were incubated with the 7S fraction of anti-B<sub>6</sub> serum, 68.0% of total blasts were  $\gamma^+$ T<sup>+</sup> (Table III). Significant specific IgM binding (6.1%) also occurred when CBA blasts were incubated with the pellet of the sucrose gradient (which may contain immune complexes) or the 19S fraction (12.5%). Normal CBA serum did not bind.

*Origin of Blasts from MLR Cultures.* Although it seemed very unlikely that a sufficient number of irradiated stimulator cells would survive until day 4 of MLR cultures to account for these results, the origin of the blasts had to be investigated. Thus, cells from a 4 day MLR culture set up with B-depleted responder cells, were treated with either antistimulator or antiresponder hyperimmune alloantiserum, and the binding of antibodies to T blasts was detected by immunofluorescence (Table IV).

When blasts from A/J responder/B<sub>6</sub> stimulator cultures were incubated with an antiserum specific for the K-region products of A/J responder cells (A.TL anti-A.AL), 94.4% of T blasts showed bright fluorescence over the whole cell with an FITC-coupled anti-Ig reagent. With an anti-B<sub>6</sub> serum, two different patterns of fluorescence could be distinguished: 3.8% of T blasts showed bright fluorescence, similar to that obtained with the anti-K<sup>k</sup> antiserum, and 41.9% of T blasts showed the same type of spotted ring membrane fluorescence observed in the previous experiments with antistimulator antibody (Fig. 1). These data suggest that the majority of blasts is of responder origin.

TABLE III  
Binding of Sucrose-Gradient-Fractionated Alloantibodies to MLR-Activated T Blasts

Strain combination		Incubation with:		$\mu^+$ blast/ total blast	$\gamma^+$ blast/total blast	T <sup>+</sup> Ig <sup>+</sup> blast/ total blast		T <sup>-</sup> Ig <sup>+</sup> blast/ total blast		% Total blast	SI		
R*	S	Antise- rum†	Fr‡			T <sup>+</sup> Ig <sup>+</sup> blast/ total blast	T <sup>-</sup> Ig <sup>+</sup> blast/ total blast	%	%				
CBA/J	B6	—	—	1/257	0.4	0/238	0	5/299	1.7	0/299	0	55.2	76.5
"	"	$\alpha$ B6	—	30/209	14.4	98/217	45.2	83/274	31.0	4/352	1.1		
"	"	$\alpha$ B6	7S	14/226	6.2	149/219	68.0	ND	—	ND	—		
"	"	$\alpha$ B6	19S	27/216	12.5	1/202	0.5	ND	—	ND	—		
"	"	$\alpha$ B6	P	14/231	6.1	2/217	0.9	ND	—	ND	—		
"	"	NS	—	0/219	0	0/226	0	2/199	1.0	1/199	0.5		

\* Responder cells, nylon wool passaged nonadherent lymph node cells (3% Ig<sup>+</sup>).

† Hyperimmune CBA anti-B6 serum absorbed three times with SJL spleen plus lymph node cells: cytotoxic titer, 1/80 (on B6 lymph node cells).

‡ 7S, 19S, and pellet (P) fractions of CBA  $\alpha$ B6 hyperimmune serum separated by sucrose gradient centrifugation. 7S fraction, cytotoxic titer 1/356; 19S fraction, cytotoxic titer 1/32; P (pellet), cytotoxic titer <1/2; (on B6 lymph node cells). All sera and serum fractions were used at a final dilution of 1/20.

|| NS, normal CBA serum.

TABLE IV  
T Blasts Activated in MLR Cultures with B-Depleted Responder Cells: Binding of Antistimulator or Antiresponder Antibodies

Strain combination		Incubation with antise- rum‡ (dilu- tion)	T <sup>+</sup> Ig <sup>+</sup> blast/total blast			T-Ig <sup>+</sup> blast/total blast	% Total blast			
R*	S		Bright	Spotted	Total					
			%	%	%	%				
A <sub>(kkd)</sub> §	B6 <sub>(bbb)</sub> §	— (-)	5/181	2.8	0/181	0	2.8	1/180	0.6	54.7
A <sub>(kkd)</sub>	B6 <sub>(bbb)</sub>	$\alpha$ K <sup>k</sup> (1/20)	170/184	94.4	0/184	0	94.4	ND	—	
A <sub>(kkd)</sub>	B6 <sub>(bbb)</sub>	$\alpha$ B6 (1/50)	9/213	3.8	99/236	41.9	45.7	ND	—	

\* Responder cells, nylon wool-passaged nonadherent lymph node cells.

†  $\alpha$ B6: CBA $\alpha$ B6 absorbed twice with A/J and SJL spleen and lymph node cells.

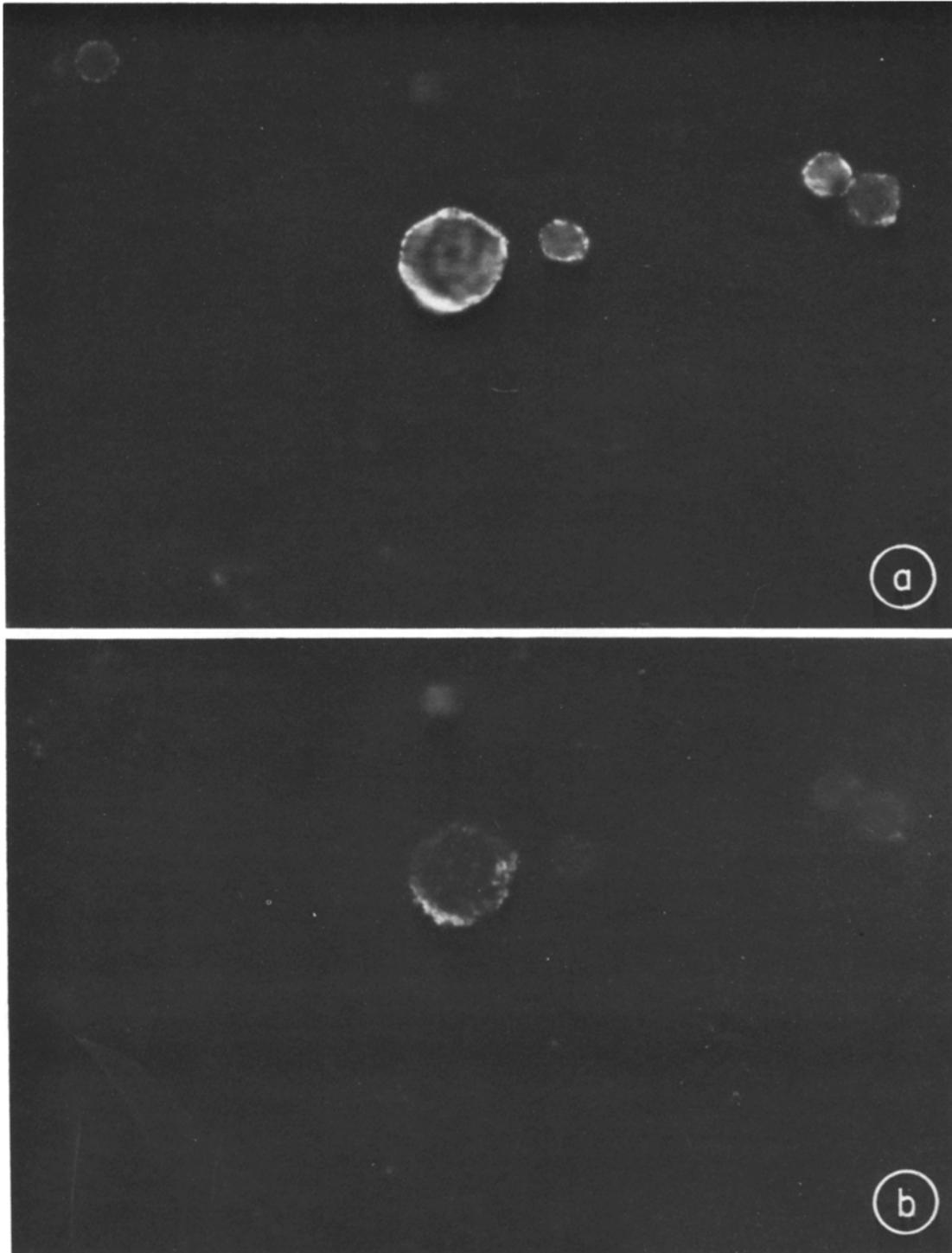
$\alpha$ K<sup>k</sup>: A.TL $\alpha$ A.AL absorbed twice with B6 spleen and lymph node cells.

§ H-2 haplotypes (letters refer to allelic origin of K, I, S and D regions, respectively).

*Specificity of Antigen Binding to MLR-Activated T Blasts.* The results presented so far suggest that in MLR cultures, B cells can produce alloantibodies against the stimulator cells and that such antibodies are passively absorbed via stimulator antigens bound by the responding T cells. If so, the question arises whether one responder T blast binds alloantigens from only one stimulator, this was investigated in the following experiment.

CBA nonadherent lymph node cells were activated against B6, SJL, or a mixture of both stimulators (i.e., the last culture contained twice as many irradiated cells). On day 4, cell populations were incubated with either anti-H-2<sup>s</sup>, anti-B6, or both sera and the percentage of resulting T<sup>+</sup>Ig<sup>+</sup> blasts scored as above.

The results (Fig. 2) show that after stimulation with cells from only one strain, an increasing proportion of responder cells could be stained after treatment with increasing concentrations of the corresponding antistimulator serum. At the highest antibody concentration (1/8), 72 and 80% of responder blasts stimulated with SJL or B6 cells, respectively, were stained. No change in the



**FIG. 1.** B-depleted CBA responders were activated in MLR against SJL stimulators. T blasts were treated with anti-H-2<sup>s</sup> serum (1/20), followed by a double-staining procedure with rabbit anti-T serum [detected by (TRITC)-sheep antirabbit Ig] and (FITC)-sheep antimouse Ig as described in the text. The same cells were viewed under illumination for rhodamine or fluorescein. (a) TRITC-positive blast and small lymphocytes (T cells), (b) FITC-positive blast (Ig<sup>+</sup>).

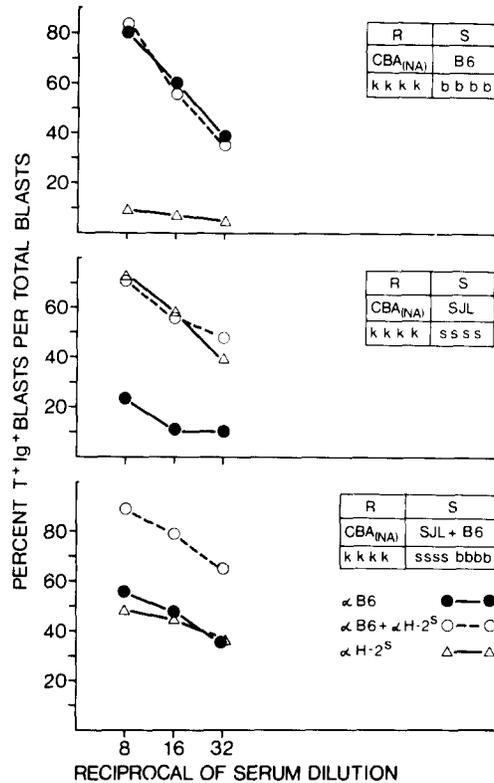


FIG. 2. Specific binding of alloantibody to MLR blasts. B-depleted CBA responders were activated in MLR against B6 or SJL, or B6 plus SJL stimulators. T blasts were treated with  $\alpha$ B6,  $\alpha$ H-2<sup>s</sup>, or  $\alpha$ B6 plus  $\alpha$ H-2<sup>s</sup> sera.

frequency of stained cells occurred when the control antiserum was added; when treated with the control antiserum alone, no stained cells (anti-H-2<sup>s</sup>), or only a very small percentage (anti-B6, see also Table II) were observed. On the other hand, when CBA cells had been activated against both B6 and SJL, between 47 and 55% of the blasts were stained when treated with either antistimulator serum alone at the highest concentration; but when treated with both sera together (each kept at the same concentration), 89% of the doubly stimulated cells were stained. When backgrounds and cross-reaction are considered this amounts to an approximately additive effect of the two antisera. The titration curves obtained in this experiment (Fig. 2) strongly suggest that most or all responder cells carry antigens of only one stimulator on their surface.

*Specificities of Surface-Bound Alloantigens.* B6 nonadherent lymph node cells were stimulated with A.AL (*K<sup>k</sup>I<sup>S</sup>S<sup>k</sup>D<sup>a</sup>*) (Table V) cells. After incubation with anti-I<sup>k</sup> or anti-K<sup>k</sup> sera (at a dilution of 1/50), 52 and 47.3%, respectively, of the total blasts were T<sup>+</sup>Ig<sup>+</sup>. Thus both I-region and K-region products from the stimulator cells were bound to the responder blasts.

After incubation with both sera together (each at the same dilution, i.e., 1/50), the proportion of T<sup>+</sup>Ig<sup>+</sup> blasts increased to 78.9%; this increase in the number of binding cells, compared to that observed when cells were treated separately

TABLE V  
*Binding of Alloantibodies against K- or I-Region Products to T Blasts Activated in MLR Cultures with B-Depleted Responder Cells*

Strain combination		Incubation with antiserum‡	T <sup>+</sup> Ig <sup>+</sup> blast/total blast		T <sup>-</sup> Ig <sup>+</sup> blast/total blast		% Total blast
R*	S			%	%		
B6 <sub>(dobb)</sub> §	A.AL <sub>(kkk)</sub> §	-	3/222	1.4	0/222	0	44.0
"	"	αIa <sup>k</sup>	106/204	52.0	6/204	2.9	
"	"	αK <sup>k</sup>	105/222	47.3	6/222	2.7	
"	"	αIa <sup>k</sup> + αK <sup>k</sup>	180/228	78.9	3/228	1.3	
"	"	αIa <sup>s</sup>	10/226	4.4	4/226	1.8	

\* Responder cells, nylon wool-passaged nonadherent lymph node cells (2.7% Ig<sup>+</sup>).

‡ Antisera: αIa<sup>k</sup>, A.TH anti-A.TL; αK<sup>k</sup>, A.TL anti-A.AL; αIa<sup>s</sup>, A.TL anti-A.TH (absorbed two times with B6 spleen and lymph node cells). Final dilution of all antisera was 1/50.

§ *H-2* haplotypes (letters refer to allelic origin of *K*, *I*, *S*, and *D* regions, respectively).

with each serum, suggests that most (approximately 80%) of the blasts bound antibodies against either *K*-region or *I*-region products but not both.

### Discussion

Our findings demonstrate the presence of immunoglobulin (probably mostly IgM) on T blasts from lymph node responder cells activated in a unidirectional MLR. Other investigators (1-3, 5, 7) have also reported surface Ig on MLR-activated cells, however, indirect procedures were used in which separate cell suspensions were scored for surface Ig and T-cell markers. Because the double-staining procedure used in the present work allowed detection of T markers and surface Ig on the same cell, the proportion of Ig<sup>+</sup> T blasts and B blasts could be determined directly.

The surface Ig detected on MLR-activated T cells is most likely dependent on the presence of B cells present in the cultures: almost no surface Ig was observed on T blasts from MLR cultures with B-depleted responder cells (Table II). Hudson et al. (8, 9) have obtained similar findings: when unfractionated parental thymus cells were injected into irradiated F<sub>1</sub> mice, the activated T cells recovered from the thoracic duct (T.TDL) carried surface IgM. Depletion of B cells from the thymus cell inoculum reduced the percentage of Ig-positive T.TDL. Furthermore, T blasts, activated in recipients differing at the *Mls* locus (serologically unrecognizable), but identical at the *H-2* complex, did not have detectable surface IgM; while Ig-bearing T.TDL appeared in a combination differing only at the *I* and *S* regions of the *H-2* complex (J. Sprent, personal communication). Together with our own findings of Ig-bearing T cells activated in vitro against stimulators very similar to the responders, apart from serologically detectable differences at the *H-2* complex (Table I), these results suggest that in both systems the Ig detected is mainly, or entirely, antibody directed against *H-2*-associated antigens of the stimulator or recipient.

Both "early" and hyperimmune alloantisera directed against the stimulator cells bound specifically to Ig<sup>-</sup> responder T blasts (Table II). When hyperimmune serum was fractionated into 7S and 19S fractions by sucrose gradient centrifuga-

tion, both classes of antibody bound specifically (Table III). These findings suggest that rather than immune complexes, free antibody bound directly to stimulator alloantigens already present on the responder blasts.

When the responder blasts from MLR cultures with only one stimulator were treated with increasing concentrations of antistimulator serum, increasing proportions of T blasts were stained (Fig. 2). Thus, stimulator alloantigens were detected on most or all responder blasts. This finding suggests that during the MLR culture period, there is a high degree of selection for T cells activated specifically against stimulator determinants. Recent experiments (unpublished) in this laboratory indicate that surface-bound stimulator determinants (and antistimulator antibodies) can be removed from MLR-activated responder T blasts by trypsin treatment. After a 2 h recovery period, followed by an incubation at 37°C with the corresponding and control supernates from the MLR cultures, specific binding of stimulator determinants to the trypsin-treated T blasts occurred. There was no evidence for any requirement of alloantibody for this observed antigen binding.

The results reported in Fig. 2, strongly suggest that when one responder cell population is activated against two stimulators, most or all of the resulting blast cells carry antigen derived from only one stimulator on their surface. Both *I*- and *K*-region products of the stimulators can be detected on responder cells. The data in Table V suggest that most cells carry products of only one region. However, there was some indication of significant overlap of the two cell populations (about 20%). This overlap is, in our view, most likely due to, (*a*) a certain small amount of nonspecific sticking of stimulator cell material to the responder cells and/or (*b*) a reflection of the fact that some proportion of the material specifically bound by the responding cells exists in the form of membrane fragments, containing more than one type of antigen. Whether or not the cells binding *K*- or *I*-region products, respectively, belong to distinct subclasses corresponding to the functional subpopulations of T cells distinguishable on the basis of their expression of different Ly antigens, as described by Cantor and Boyse (19) remains to be investigated.

### Summary

Immunoglobulin (Ig) is present on a large fraction of T cells from unfractionated lymphocytes activated by *in vitro* stimulation with *H-2*-incompatible cells (mixed lymphocyte reaction [MLR]).

Removal of bursa equivalent-derived (B) cells from the responder cell population before mixed culture, by filtration through nylon wool columns, reduces the percentage of Ig-bearing responder T blasts to background levels. Thus, Ig on the T blast is probably of B cell origin. A large fraction of T blasts activated in the absence of B cells can be stained with alloantibodies directed against the stimulator cells. This staining occurs with "early" and hyperimmune alloantisera, including the 7S fraction of the latter.

B-depleted responder cells were activated against a mixture of two different stimulator cells and the resulting T blasts stained with different concentrations of sera directed either against one or both stimulator cells. We obtained results which strongly suggest that most or all responder T blasts stain with only one

antistimulator serum. When antisera directed against different segments of the H-2 complex of the stimulator cells were used, it seemed that most responder T cells only bound antibody directed against a single segment.

We propose that T cells activated in MLR carry stimulator alloantigens on their surface, and that this is due to specific antigen binding, not requiring the presence of B-cell-derived antibody. These histocompatibility antigen-binding T blasts can be detected by appropriate antistimulator alloantibodies.

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### References

1. Perkins, W. D., L. C. Robson, and M. R. Schwarz. 1975. Immunoglobulin-positive cells: their appearance in the mixed lymphocyte reaction. *Science (Wash. D. C.)* 188:365.
2. Vischer, T. L. 1972. Immunoglobulin-like surface molecules and theta antigen during the specific and non-specific stimulation of mouse spleen cells *in vitro*. *Clin. Exp. Immunol.* 11:523.
3. Piguet, P-F., and P. Vassalli. 1972. Thymus-independent (B) cell proliferation in spleen cell cultures of mouse radiation chimeras stimulated by phytohemagglutinin or allogeneic cells. *J. Exp. Med.* 136:962.
4. Sprent, J., and L. Hudson. 1973. Surface immunoglobulin on H-2 activated T lymphocytes. *Transplant. Proc.* 5:1731.
5. Goldschneider, I., and R. B. Cogen. 1973. Immunoglobulin molecules on the surface of activated T lymphocytes in the rat. *J. Exp. Med.* 138:163.
6. Pernis, B., J. F. A. P. Miller, L. Forni, and J. Sprent. 1974. Immunoglobulin on activated T cells detected by indirect immunofluorescence. *Cell. Immunol.* 10:476.
7. Gorczyński, R. M., and M. B. Rittenberg. 1975. Analysis of mixed leucocyte cultures (MLC) reactive cells after *in vitro* priming. Changes in avidity of T cell receptors. *Cell. Immunol.* 16:171.
8. Hudson, L., J. Sprent, J. F. A. P. Miller, and J. H. L. Playfair. 1974. B-cell-derived immunoglobulin on activated mouse T lymphocytes. *Nature (London)*. 251:60.
9. Hudson, L., and J. Sprent. 1975. Specific adsorption of IgM antibody onto H-2-activated mouse T lymphocytes. *J. Exp. Med.* 143:444.
10. Krammer, P. H., L. Hudson, and J. Sprent. 1975. Fc-receptors, Ia-antigens, and immunoglobulin on normal and activated mouse T lymphocytes. *J. Exp. Med.* 142:1403.
11. Basten, A., J. F. A. P. Miller, N. L. Warner, R. Abraham, E. Chia, and J. Gamble. 1975. A sub-population of T cells bearing Fc-receptors. *J. Immunol.* 115:1159.
12. Meo, T., G. Vives, A. M. Rijnbeek, V. C. Miggiano, M. Nabholz, and D. C. Shreffler. 1973. A bipartite interpretation and tentative mapping of H-2-associated MLR determinants in the mouse. *Transplant. Proc.* 5:1339.
13. Nabholz, M., J. Vives, H. M. Young, T. Meo, V. Miggiano, A. Rijnbeek, and D. C. Shreffler. 1974. Cell-mediated cell lysis *in vitro*: genetic control of killer cell production and target specificities in the mouse. *Eur. J. Immunol.* 4:378.

14. Julius, M., H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived lymphocytes. *Eur. J. Immunol.* 3:645.
15. Frelinger, J. A., J. E. Niederhuber, C. S. David, and D. C. Shreffler. 1974. Evidence for the expression of Ia (H-2-associated) antigens on thymus-derived lymphocytes. *J. Exp. Med.* 140:1273.
16. Sauser, D., C. Anckers, and C. Bron. 1974. Isolation of mouse thymus-derived lymphocyte surface antigens. *J. Immunol.* 113:617.
17. Nisonoff, A. 1964. Enzymatic digestion of rabbit gamma globulin and antibody, and chromatography of digestion products. *Methods Med. Res.* 10:134.
18. Cebra, J. J., and G. Goldstein. 1965. Chromatographic purification of tetramethylrodamine-immune globulin conjugates and their use in the cellular localization of rabbit gamma-globulin polypeptide chains. *J. Immunol.* 95:230.
19. Cantor, H., and E. A. Boyse. 1975. Functional subclasses of T lymphocytes bearing different Ly antigens. II. Cooperation between subclasses of Ly<sup>+</sup> cells in the generation of killer activity. *J. Exp. Med.* 141:1390.