

GENERATION OF T CELL COLONIES FROM RESPONDER
STRAIN 2 GUINEA PIGS THAT RECOGNIZE THE COPOLYMER
L-GLUTAMIC ACID, L-LYSINE IN ASSOCIATION WITH
NONRESPONDER STRAIN 13 Ia ANTIGENS

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Although it is now generally accepted that I region associated (Ia) antigens function as the products of the major histocompatibility complex (MHC)-linked immune response (*Ir*) genes, the specific mechanism of action and site of expression of Ia antigen function remains controversial (1, 2). The issue can best be summarized as follows: do antigen-presenting cells or macrophages dictate *Ir* gene control by allowing a restricted combination of determinants of conventional antigen and cell surface Ia molecules (presentation or determinant selection model [3-5]), or do macrophages present antigens in an unrestricted manner to T cells that bear receptors whose specificity has been dictated by the products of the *Ir* genes/Ia antigens (hole in the repertoire model [6, 7])? The latter selection of T cell receptors could occur at the genotypic level or during T cell differentiation in the thymus or bone marrow.

In this report we have used the newly developed T cell cloning technology to demonstrate that T cells from nonimmune responder strain 2 guinea pigs can be primed in vitro to the linear copolymer L-glutamic,L-lysine (GL) in association with allogeneic, nonresponder strain 13 macrophages. The implications of these findings vis-à-vis theories of *Ir* gene function will be discussed.

Materials and Methods

In Vitro Priming in Bulk Cultures. Purified T lymphocytes were obtained by passing mesenteric lymph node cells from unprimed strain 2 guinea pigs (Division of Research Services, National Institutes of Health) over nylon wool adherence columns. Peritoneal exudate cells (PEC) obtained from nonimmune strain 13 guinea pigs injected 3-4 d earlier with 25 ml of sterile mineral oil (Marcol 52, Humble Oil and Refining Co., Houston, TX) were used as a source of macrophages without further purification. Irradiated (2,500 rad) PEC (10×10^6 cells/ml) were incubated with GL (100 μ g/ml, 40,000 mol wt; Pilot Chemical Division, New England Nuclear, Boston, MA) at 37°C for 1 h, followed by extensive washing to remove unbound antigen. Priming cultures were established in 24-well plates (Costar, Data Packaging, Cambridge, MA) by mixing strain 2 T cells (5×10^6) with GL-pulsed strain 13 PEC (1×10^6) in 1.5 ml of RPMI 1640 (Biofluids, Rockville, MD) supplemented with L-glutamine (300 μ g/ml), penicillin (100 U/ml), streptomycin (100 μ g/ml), 5-fluorocytosine (1 μ g/ml), 2-mercaptoethanol (5×10^{-5} M), and 5% normal guinea pig serum (NGPS). The priming cultures were maintained for 14 d, and fresh media with 5% NGPS was added to each well every other day.

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Generation of T Cell Colonies on Soft Agar. At the end of the 14-d priming culture, the cells were harvested and resuspended in media with 5% fetal calf serum (FCS) and plated in the upper layer of a double layer of soft agar consisting of 0.35% Noble agar (Difco Laboratories, Detroit, MI), complete media, and 10% FCS. The lower layer of agar had been prepared 24 h previously and consisted of 0.5% agar in complete media containing 10% FCS, GL (300 $\mu\text{g}/\text{ml}$), 20% T cell growth factor (TCGF), and strain 13 PEC (1×10^5 cells/ml). The source of TCGF was crude supernatants of concanavalin A- (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ) stimulated cells prepared as previously described (8) to which was added methyl- α -D-mannopyranoside (20 mg/ml) (Calbiochem-Behring, La Jolla, CA). After 7 d on agar, colonies were picked with a pasteur pipette and split into two flat-bottomed microtiter wells; both wells contained complete media with 5% FCS, 5% TCGF, and strain 13 PEC (1×10^4), but only one of the wells for each colony contained GL (100 $\mu\text{g}/\text{ml}$). All wells were fed twice weekly with fresh strain 13 PEC (1×10^4), complete media with 5% FCS, 25% TCGF, and GL (100 $\mu\text{g}/\text{ml}$) where appropriate. Those colonies demonstrating enhanced growth in the presence of GL were transferred into 24-well plates and fed twice weekly as above until sufficient numbers of cells were available for assay in proliferation assays.

Assay for DNA Synthetic Responses. Cells (1×10^4) from an individual colony were cultured in 96-well flat-bottomed microtiter plates (Costar) with strain 2 or 13 PEC (1×10^5) in the presence or absence of GL (100 $\mu\text{g}/\text{ml}$) in 0.2 ml cultures in complete media with 5% FCS and 5% TCGF; single wells for each parameter were established, and after 72 h of culture each individual well was divided into three new wells and fed with fresh PEC, TCGF, and antigen where appropriate. After an additional 48-h culture, 1 μCi of tritiated thymidine (^3H]TdR) (New England Nuclear) was added to each well, and 18 h later the [^3H]TdR uptake was determined as previously described (8).

Alloantibodies to Guinea Pig Ia Antigens. The preparation and characteristics of strain 13 anti-strain 2 serum (anti-Ia.2,4) and strain 2 anti-strain 13 serum (anti-Ia.1,3,7) have been described in detail previously (9). ^{51}Cr -release assays were performed as described (9) except that rabbit serum (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) was used as a source of complement, and supernatant fluids were collected with a Titertek supernatant collection system (Flow Laboratories, Rockville, MD).

Results

In preliminary experiments we attempted to prime nonimmune T cells from responder strain 2 guinea pigs with GL-pulsed nonresponder strain 13 PEC by coculturing the cells for 2 wk in bulk cultures and then plating the responding cells on a double layer of soft agar containing a source of TCGF, fresh strain 13 PEC, and GL. When 5×10^5 to 5×10^6 cells were plated, 10–50 colonies could be identified and easily picked 7 d later. However, after expansion in liquid culture in the presence of TCGF, strain 13 PEC, and GL, the vast majority of the resultant lines proved to be alloreactive, and no enhancement of the proliferative response to strain 13 PEC was seen when GL was added to the cultures.

To facilitate possible identification of strain 2 T cell colonies that specifically recognized GL in association with strain 13 PEC, the T cell cloning technology described above was modified so that immediately after picking from the agar each colony was divided into two microtiter wells, both of which were fed with fresh irradiated strain 13 PEC and TCGF, but only one of which received GL. Only those colonies that morphologically demonstrated significantly enhanced growth in the presence of GL were then expanded in 24-well plates and tested in proliferation assays. The resultant colonies could be divided into three groups based on their response profile, and examples of each group are shown in Table I. Group I colonies (A24 and A14) were typical alloreactive colonies and demonstrated no enhanced response in the presence of GL. Group II (colonies B7 and A34) demonstrated a

TABLE I
Proliferative Response of Strain 2 T Cell Colonies Primed In Vitro to GL-pulsed Strain 13 PEC

Group	Colony	³ H]TdR incorporation in the presence of			
		Strain 2 PEC		Strain 13 PEC	
		-GL	+GL	-GL	+GL
				<i>cpm</i>	
I	A24	362	292	55,717	42,930
	A14	2,182	738	81,596	77,941
II	B7	860	848	45,949	82,062
	A34	2,714	1,125	20,838	44,791
III	B4	420	603	615	18,162
	C6	1,010	1,178	2,442	10,000
	B6 (experiment I)	2,316	4,762	7,878	23,693
	B6 (experiment II)	969	1,415	2,649	59,375
	B6 (experiment III)	2,880	5,354	5,686	20,410

T cells (1×10^6) from each colony were cultured with strain 2 or 13 PEC (1×10^5) in the presence or absence of 100 μ g/ml of GL. After 72 h, each well was split into three new wells, and the cultures continued for an additional 72 h. ³H]TdR incorporation was determined in the last 18 h of culture and is expressed as total cpm/well; each value is the mean of three determinations.

TABLE II
Effect of Anti-Ia Sera on the GL-specific Proliferative Response of Colony B6

Experiment	Serum	³ H]TdR incorporation
1	NGPS	Δ cpm 4,052
	Anti-Ia.2,4	4,926
	Anti-Ia.1,3,7	0
2	Anti-Ia.2,4	4,316
	Anti-Ia.1,3,7	177

T cells from colony B6 (5×10^5) were cultured for 72 h with strain 13 PEC (1×10^5) in the presence or absence of GL (100 μ g/ml) in 0.2 ml of media containing 5% FCS and 5% NGPS or anti-Ia antisera. ³H]TdR incorporation was determined during the final 18 h of culture and is expressed as Δ cpm/culture (cpm obtained in the presence of GL and strain 13 PEC minus cpm obtained in the presence of strain 13 PEC but in the absence of GL); each value is the mean of three determinations.

significant proliferative response to strain 13 PEC, but a marked enhancement of the response was seen when GL was added to the cultures. Of greatest interest were colonies in group III (colonies B4, C6, and B6), which proliferated only in the presence of GL and strain 13 PEC. In three separate cloning experiments using this expansion protocol, ~5% of the total colonies picked demonstrated GL-specific responses in the presence of strain 13 PEC in short-term proliferation assays. In no case was a response observed to GL in association with syngeneic responder strain 2 PEC.

To further analyze the MHC restriction of these colonies, we examined the effect of alloantisera to Ia antigens on the proliferative response of colony B6 (Table II). A strain 13 anti-strain 2 serum (anti-Ia.2,4) directed against the responding T cells had no effect on the proliferative response, whereas a strain 2 anti-strain 13 serum (anti-Ia.1,3,7) directed toward the antigen presenting PEC markedly inhibited the GL-specific response.

To formally rule out the possibility that colony B6 was a rare colony that arose from the irradiated strain 13 T cells that contaminated the strain 13 PEC population and that responded to GL in association with strain 13 PEC, we analyzed the

TABLE III
Cytotoxic Effect of Anti-Ia Sera on T Cell Colonies

Colony	T cell strain	Percent specific lysis (\pm SEM)	
		Anti-Ia.2,4	Anti-Ia.1,3,7
B6	2	42 \pm 6	4 \pm 1
131	(2 \times 13)F ₁	36 \pm 4	41 \pm 5

Colony B6 was generated as in Table I. Colony 131 was generated from (2 \times 13)F₁ T cells primed in vivo to complete Freund's adjuvant and was restricted in its proliferative response to tuberculin in association with strain 2 PEC (results not shown). ⁵¹Cr-labeled cells (10⁶) were incubated with a 1/15 dilution of the anti-Ia sera in the presence of rabbit complement at 37°C, and the amount of radioactivity present in the supernatant fluids was determined in a gamma counter. Spontaneous release was determined by incubating cells with NGPS and rabbit complement, whereas total release was determined by lysing the labeled cells with Nonidet P-40. Percent lysis was calculated as follows: 100 \times [(experimental release - spontaneous release)/(total release - spontaneous release)].

expression of Ia antigens on the responding T cell population in a cytotoxicity assay. We have previously demonstrated that antigen-specific T cell colonies express and synthesize high levels of Ia antigens (R. B. Clark, T. Malek, J. Chiba, and E. M. Shevach, unpublished observations). As can be seen in Table III, colony B6 was readily lysed by anti-Ia.2,4 serum but not by anti-Ia.1,3,7 serum, in spite of the fact that colony B6 had never been cultured or fed with syngeneic strain 2 PEC. This result confirms that colony B6 is of strain 2 origin. As a control population for the specificity of the reagents we have used, we have also demonstrated in Table III that tuberculin-specific colony 131, which had been generated from (2 \times 13)F₁ T cells primed in vivo to tuberculin, but propagated in vitro only in the presence of strain 2 PEC, was readily lysed by both anti-Ia.2,4 and anti-Ia.1,3,7 sera and therefore expresses Ia antigens of both parental haplotypes.

Discussion

Previous experiments (10) in murine radiation chimera models have suggested that the T cell Ir phenotype is an acquired trait and depends on gaining the ability to recognize responder MHC antigens. Chimera experiments do not distinguish whether nonresponsiveness represents the failure of the T cell population to develop a receptor for nonresponder Ia antigens and the nominal antigen or the failure of the macrophage of the nonresponder to process and present the antigen in an appropriate form. In this report, we were successfully able to identify a number of T cell colonies of responder strain 2 origin that proliferated specifically when challenged with GL in the presence of allogeneic, nonresponder strain 13 macrophages. We were easily able to separate the completely alloreactive T cells from the antigen-specific T cells by cloning the population in soft agar after the priming procedure in liquid cultures. Identification of colonies specific for nonresponder Ia and GL was facilitated by splitting each soft agar colony into two liquid cultures immediately after picking them from the agar and then examining the resultant colonies for differential growth in the presence of the priming antigen.

The ability to prime T cells across an I region barrier is consistent with our previous observations (11) that the requirement for MHC restriction is a result of the environment in which the T cells are primed. We do not dispute the possibility that the thymus plays a critical role in the positive or negative selection of a population of self-

restricted T cells, but clearly T cells with the capability of recognizing foreign antigen "x" in association with allogeneic Ia are present in the normal animal. It is, however, possible that the receptors of a T cell clone that is capable of recognizing foreign antigen x in association with allogeneic Ia would also be capable of reacting with antigen "y" in association with self-Ia.

The simplest interpretation of our data is that macrophages from nonresponder strain 13 guinea pigs are fully capable of presenting GL to a responder T cell population, and that no limitation exists in the macrophage processing, surface display, or association of GL with strain 13 Ia antigens. These data argue strongly against determinant selection models of *Ir* gene function (4, 5). One other interpretation of our data should be considered. It is possible that the strain 2 colonies that respond to GL in association with strain 13 Ia are specific for a rare or minor determinant on this linear copolymer, which is also immunogenic in strain 13 animals, but is not seen at the level of the intact animal because it involves too small a number of clones to generate a response (12).

Our results are most compatible with theories (2, 6, 7) of *Ir* gene function based on the concept of a hole in the repertoire taking place during the induction of self-tolerance. Thus, the failure of the strain 13 guinea pig to respond to GL is secondary to the fact that the complex of GL and strain 13 Ia mimics the complex of strain 13 Ia and some unknown self-antigen, and the clone capable of seeing such a complex is deleted during T cell ontogeny in the bone marrow or thymus. Our results clearly rule out the possibility that the failure of guinea pigs to respond to the GL-strain 13 Ia complex is secondary to the absence of genes coding for receptors for this complex in the germ line of the species. Similar conclusions have recently been reached by Ishii et al. (13).

Summary

T cells from nonimmune responder strain 2 guinea pigs were primed in vitro to the copolymer GL in association with allogeneic, nonresponder strain 13 PEC. T cells that recognized GL in association with strain 13 Ia were separated from alloreactive T cells by cloning the population in soft agar following the priming in liquid culture. The existence of T cells of responder origin that recognize antigen in association with nonresponder macrophages is most consistent with clonal deletion models of *Ir* gene function.

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