ANTIGEN-REACTIVE T CELL CLONES

III. Low Responder Antigen-presenting Cells Function Effectively to Present Antigen to Selected T Cell Clones Derived from (High Responder × Low Responder)F_1 Mice*

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The immune response to certain synthetic polypeptide antigens and native proteins has been shown to be controlled by immune response (Ir) genes, which reside in the murine major histocompatibility complex (MHC)[1] (reviewed in 1). The expression of such Ir gene control has been localized to T cells, B cells, and/or antigen-presenting cells (2–4). Support for the concept of Ir gene expression in antigen-presenting cells depends largely upon the observations that T cells from (high responder × low responder)F_1 animals recognize (respond to) antigen in the context of antigen presentation by high responder antigen-presenting cells. Such immune T cells are not stimulated by antigen presented by antigen-presenting cells of low responder strains (5–7). Based upon these observations, it has been suggested that phenotypic low responsiveness is due to the inability of low responder antigen-presenting cells to process or bind antigen in such a way that it can be effectively recognized by T cells. Alternatively, these results can be interpreted to suggest that there are "holes" in the T cell repertoire in low responder mice (8). This hypothesis is based on the concept that T lymphocytes recognize antigen in conjunction with I region gene products on the cell surface of antigen-presenting cells. The basic postulate is that the association between certain I region gene products and self antigens antigens mimics the association between the same I region product and certain foreign antigens, thus allowing natural tolerance to eliminate responsiveness to such foreign antigens.

Long-term, soluble antigen-reactive, MHC-restricted, proliferating T cells have been shown to be useful tools for analysis of the precise structure of antigen-presenting determinants (9, 10). In this report, we describe our observations on selected T cell clones derived from (high responder × low responder)F_1 [(H-2^a × H-2^b)F_1] mice that were immunized with poly(Tyr,Glu)-poly-D,L,-Ala-poly-Lys [(T,G)-A--L], which recognized (T,G)-A--L in association with antigen-presenting cells from low responder (H-2^a) mice. These data suggest that there is no functional defect in the ability of low responder antigen-presenting cells to "associate" (T,G)-A--L in an immunogenic form with the I-A^k region product.

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[1] *Abbreviations used in this paper:* FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; KLH, keyhole limpet hemocyanin; MHC, major histocompatibility complex; (T,G)-A--L, poly(Tyr,Glu)-poly-D,L,-Ala-poly-Lys.

Materials and Methods

Mice. A/J (A), C57BL/6 (B6), and (B6 × A)F_1 [(B6A)F_1] mice were purchased from The Jackson Laboratory, Bar Harbor, Maine or were bred from Jackson Laboratory stock in the animal facilities at Mayo Clinic. B10.A(3R) and B10.A(4R) mice were generously provided by Dr. Chella David, Mayo Medical School. B10.MBR breeding stock were generously provided by Dr. David Sachs, National Institutes of Health. Adult mice age 6–20 wk were used in all experiments.

Antigens. (T,G)-A--L (lot MC9) was purchased from Miles Laboratories, Inc., Research Products Div., Elkhart, Ind. Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.

Antigen-pulsed Spleen Cells. This was done with a slight modification as described by Yano et al. (11). Briefly, spleen cells were suspended in culture media at 10 × 10^6/ml in Teflon tubes (Curtain-Matheson Scientific, Houston, Tex.) with (T,G)-A--L (200 μg/ml) or KLH (100 μg/ml). After 2 h, cells were irradiated (3,300 rad), washed twice to remove free antigen, and used as antigen-pulsed spleen cells.

Removal of Adherent Cells from (T,G)-A--L-immune Lymph Node Cells by G-10 Sephadex Passage. This was done as described by Ly and Mishell (12) with a slight modification. (T,G)-A--L-immune lymph node cells were suspended in Hanks' balanced salt solution (HBSS) containing 5% fetal calf serum (FCS) at a concentration of 1 × 10^8/ml. 1 ml of cell suspension was applied onto prewashed Sephadex G10 (Pharmacia Fine Chemicals, Piscataway, N. J.) column (30 ml bed vol) and eluted with HBSS containing FCS. The first 20 ml of effluent was collected, washed, and used as adherent cell-depleted (T,G)-A--L-immune lymph node cells. Viable cell recovery after G-10 Sephadex passage ranged from 60 to 80%.

Immunization, Long-Term Culture, and Cloning of (T,G)-A--L-reactive T Cells. These techniques have been described previously (9). Briefly, lymph node cells from regional draining nodes of immunized mice were cultured and propagated by serial restimulation with antigen and filler cells. At various times, aliquots of these cells were stimulated and plated in soft agar from which colonies of antigen-reactive T cells were obtained. After assay of the colonies, limiting dilution cloning technology was used to isolate clones of interest.

Assay of Proliferative Responses. These techniques have been described previously (9). Briefly, 1 × 10^4 cells from the in vitro cultures are stimulated with 200 μg/ml (T,G)-A--L in the presence of 1 × 10^6 irradiated spleen cells in 0.2 ml of culture media. 48 h later, after a 16-h pulse with 2 μCi of tritiated thymidine, the cells were harvested using an automated harvester. Incorporation of tritiated thymidine was measured by standard scintillation counting. Results are expressed as the mean of triplicate cultures; the standard deviation of each mean was <10%.

Results

Cells from Low Responder Strain A Mice Can Present (T,G)-A--L to (Low Responder × High Responder)F_1 T Cells. (T,G)-A--L-reactive T cells (line 2) from (low responder × high responder)F_1 [(B6A)F_1] mice have been maintained in vitro for >14 mo by repeated stimulation with (T,G)-A--L in the presence of syngeneic antigen-presenting cells (irradiated spleen cells). The proliferative response of line 2 to (T,G)-A--L in the presence of antigen-presenting cells from either parental A or B6, or syngeneic (B6A)F_1 mice was assayed at various times after the initiation of long-term culture. As shown in Table I, 1 mo after the initiation of culture, line 2 recognized (T,G)-A--L in association with antigen-presenting cells from high responder B6 and (B6A)F_1 mice, but could not recognize (T,G)-A--L in association with antigen-presenting cells derived from spleens of low responder strain A mice. These results are consistent with the observations reported by others (5–7). However, when the proliferative response to (T,G)-A--L of line 2 was assayed >5 mo after the initiation of culture on the same panel of antigen-presenting cells, we could demonstrate that (T,G)-A--L was effectively presented by antigen-presenting cells of low responder strain A mice. The recognition

TABLE I

Antigen Presentation by Cells from Low Responder Mice

Cell lines or clones	A		B6		A + B6*		$(B6A)F_1$	
	Medium	(T,G)-A--L	Medium	(T,G)-A--L	Medium	(T,G)-A--L	Medium	(T,G)-A--L
				[^{3}H]TdR uptake (cpm)				
Line 2 (1 mo)‡	437	398	782	2,371	582	2,944	446	3,614
Line 2 (5 mo)	499	26,671	2,173	6,798	3,416	21,425	1,438	23,395
Line 2 (8 mo)	180	13,181	264	1,676	NT§	NT	264	13,088
F.11‖	780	13,065	2,473	3,150	3,868	10,443	375	11,413
A,14	150	6,287	335	450	485	4,349	218	4,426

1×10^4 $(B6A)F_1$-derived (T,G)-A--L-reactive long-term-cultured T cells (line 2) or clones derived from line 2 were stimulated with 200 μg/ml of (T,G)-A--L in the presence of 1×10^6 antigen-presenting cells from strain A, B6, or $(B6A)F_1$ mice. Proliferative responses were measured on day 2.

* 1:1 mixture of A and B6 cells.

‡ Age of cell lines at the time of assay.

§ Not tested.

‖ Clones derived from line 2 after 5 mo of in vitro culture.

of antigen in the presence of strain A antigen-presenting cells was as efficient as the recognition of antigen in the presence of syngeneic $(B6A)F_1$ antigen-presenting cells. Also illustrated by these data is the fact that high responder antigen-presenting cells (B6) had a markedly decreased ability to present (T,G)-A--L to line 2 T cells at this time. The reason for this time-dependent decrease in the frequency of cells responding to antigen presentation by high responder B6 cells is not clear.

Spleen Cells from Low Responder Strain A Mice Pulsed with (T,G)-A--L Can Present Antigen to (T,G)-A--L-reactive T Cells. The long-term (T,G)-A--L-reactive $(B6A)F_1$ derived T cells (line 2) were maintained in vitro by repeated stimulation with fresh antigen and syngeneic irradiated spleen cells every 14 d. It is possible that there exist residual syngeneic antigen-presenting cells in such a long-term bulk population. To exclude the possibility of antigen presentation by such residual antigen-presenting cells in the assay culture, antigen-pulsing experiments were carried out. Spleen cells were pulsed with antigen as described in Materials and Methods. As demonstrated by the data in Table II, (T,G)-A--L-pulsed spleen cells from low responder strain A as well as from high responder strain B6 or $(B6A)F_1$ mice could effectively stimulate line 2, which had been maintained for >14 mo in culture. Cells pulsed with an unrelated antigen, KLH, did not support the proliferation of this long-term line. As control, the reactivities of clone 2a.2, which was derived from line 2 at ~2 mo of culture and is restricted to recognition of antigen in the context of $I\text{-}A^b$ antigen-presenting cells (10), can recognize pulsed antigen-presenting cells from strain B6 as well as from $(B6A)F_1$ mice, but cannot recognize the pulsed antigen-presenting cells from strain A mice (Table II). Thus, the possibility of carry-over of free antigen in the antigen-pulsed cell populations or the release of carry-over antigen in culture is unlikely to be due to the inability of the pulsed strain A spleen cells to stimulate clone 2a.2. Antigen-pulsed strain A spleen cells were ineffective in stimulating clone 2a.2 even in the presence of unpulsed irradiated B6 or $(B6A)F_1$ spleen cells (data not presented). These data suggest that antigen presentation by residual antigen-presenting cells contained in the responding T cell populations is not a likely explanation of the results presented in this paper.

Clones of (T,G)-A--L-reactive T Cells Can Recognize Antigen in Association with Antigen-presenting Determinants of Low Responder Strain A Antigen-presenting Cells. To prove that

TABLE II

Ability of Antigen-pulsed Low Responder Spleen Cells to Stimulate T Cells

Lines or clones	Antigen	Antigen-presenting cells		
		A	B6	(B6A)F_1
Line 2 (14 mo)*	Medium	222	899	382
	(T,G)-A--L	13,470	7,185	15,994
	KLH pulsed	153	435	138
	(T,G)-A--L pulsed	4,491	5,699	14,554
2a.2	Medium	1,062	1,378	1,641
	(T,G)-A--L	1,229	6,662	6,576
	KLH pulsed	875	1,384	1,251
	(T,G)-A--L pulsed	1,302	5,067	4,549

1×10^4 cells from (T,G)-A--L-reactive long-term-cultured T cells derived from (B6A)F_1 mice (line 2) and clone 2a.2 (restricted by $A^b_\alpha A^b_\beta$, see text) were stimulated with 200 μg/ml of (T,G)-A--L in the presence of 1×10^6 antigen-presenting cells (3,300 rad irradiated spleen cells) from strain A, B6, or (B6A)F_1 mice or they were stimulated by 1×10^6 (T,G)-A--L or KLH-pulsed 3,300 rad irradiated spleen cels. Proliferative responses were measured on day 2.

* Age of cell lines at the time of assay.

there exist clones of (T,G)-A--L-reactive T cells from (B6A)F_1 mice, which can recognize (T,G)-A--L in association with antigen-presenting cells of low responder strain A, T cell clones were isolated from line 2 after 5 mo of in vitro culture by soft agar cloning techniques, followed by limiting dilutional cloning (10). Limiting dilution cloning was carried out with 0.3 T cells/well in the presence of (T,G)-A--L, rat concanavalin A supernate, and filler cells from either strain (B6A)F_1 or A mice (10). Clones were expanded by serial stimulation with (T,G)-A--L on fresh filler cells from the same strain as used for cloning. As shown in Table I, most of the clones isolated from line 2 recognized (T,G)-A--L in association with A and (B6A)F_1 antigen-presenting cells and not in association with B6 antigen-presenting cells. These results suggest that there is an effective presentation of (T,G)-A--L in association with I-A^k antigen-presenting determinants on low responder strain A antigen-presenting cells.

One of the possible reasons that line 2 can recognize antigen presented by antigen-presenting cells of low responder mice after such long-term culture might be that the culture conditions have in some way preferentially supported the growth and expansion of antigen-reactive clones having receptors with low affintiy for (T,G)-A--L. Such clones might either be relatively rare in vivo or would not be triggered because of the presence of high affinity clones, which would efficiently remove the antigen. Alternatively, such clones might have recognized nonimmunodominant epitopes of (T,G)-A--L and, as mentioned above, might have been supported through growth and expansion by unknown mechanisms. To test the first possibility and to see whether clones presented (T,G)-A--L by low responder antigen-presenting cells have receptors with low affinity for (T,G)-A--L, antigen dose-response curves of clone F-11 (restricted by $A^k_\alpha A^k_\beta$) and clone 2a.2 (restricted by $A^b_\alpha A^b_\beta$) were compared. Clone F.11 and clone 2a.2 were stimulated with various concentrations of (T,G)-A--L in the presence of antigen-presenting cells from (B6A)F_1 mice. As shown in Table III, the response to antigen at all concentrations tested by these two clones are almost identical. Although the number of clones tested is limited, these results suggest that clones that recognize

TABLE III

Dose-Response Studies of (T,G)-A--L-reactive Clones

	Antigen concentration (μg/ml)							
	400	200	100	50	20	10	5	0
Clone 2a.2	9,764*	9,621	6,518	5,420	4,931	3,173	2,468	1,939
Clone F.11	14,794	14,097	10,166	7,102	4,496	2,359	1,792	1,202

* 1×10^4 cloned cells (either clone 2a.2 or clone F.11) were stimulated with various amounts of (T,G)-A--L in the presence of 1×10^6 antigen-presenting cells (3,300 rad irradiated spleen cells) from(B6A)F_1 mice. Proliferative responses were measured on day 2 as counts per minute. Clone 2a.2 is restricted by $A^b_\alpha A^b_\beta$, whereas clone F.11 is restricted by $A^k_\alpha A^k_\beta$ (see text).

TABLE IV

Genetic Mapping of Low Responder Antigen-presenting Determinants

Antigen-presenting cells	H-2 haplotype*								A.12	
									Medium	(T,G)-A--L
A	k	k	k	k	k	d	d	d	150	6,287
B6	b	b	b	b	b	b	b	b	335	450
(B6A)F_1	k	k	k	k	k	d	d	d	218	4,426
	b	b	b	b	b	b	b	b		
B10.A(3R)	b	b	b	b	k	d	d	d	179	309
B10.A(4R)	k	k	d	d	d	d	d	d	300	5,277
B10.MBR	b	k	k	k	k	k	k	q	160	5,620

Clone A.12 was derived from line 2 in Table I after 5 mo of in vivo culture. 1×10^4 cells from clone A.12 were stimulated with (T,G)-A--L in the presence of 1×10^6 antigen-presenting cells from various strains of mice. The proliferative response was measured on day 2.

* H-2 haplotypes (KABJECSD) of the antigen-presenting cells are shown for ease of presentation.

(T,G)-A--L in the context of low responder antigen-presenting determinants have receptors of similar affinity for (T,G)-A--L.

Genetic Mapping of Antigen-presenting Determinants on Low Responder Strain A Mice. That the antigen-presenting determinants on low responder strain A antigen-presenting cells that present (T,G)-A--L to line 2 are controlled by the I-A subregion of the H-2 complex is demonstrated by data contained in Table IV. Thus, B10.A(4R) and B10.MBR cells can present (T,G)-A--L to clone A.12, but B10.A(3R) cells cannot.

Inability of Low Responder Strain A Antigen-presenting Cells to Present Antigen to Fresh Immune (T,G)-A--L-reactive T Cells Is Not Due to Suppressor Cells. One of the possible reasons for the inability of strain A antigen-presenting cells to present antigen to T cells from (B6A)F_1 mice immunized with (T,G)-A--L might be the existence of suppressor cells that somehow prevent recognition or proliferation after recognition of antigen, in association with strain A antigen-presenting cells. To explore this possibility, fresh (T,G)-A--L immune lymph node cells were prepared from (B6A)F_1 mice immunized with (T,G)-A--L and passed through Sephadex G-10 columns as described (12). Various numbers of such adherent cell-depleted fresh (T,G)-A--L immune cells were mixed with (T,G)-A--L-reactive T cells from line 2, which had been in culture for 14 mo. These cells were co-cultured in the presence of optimal amounts of (T,G)-A--L in the presence of antigen-presenting cells from strain A, B6 or (B6A)F_1 mice. The data presented in Table V suggest that within the limit of cell numbers tested,

TABLE V

Adherent Cell-depleted Lymph Node Cells from (T,G)-A--L-primed (B6A)F1 Mice Do Not Suppress the Recognition of (T,G)-A--L Long-Term-Cultured (T,G)-A--L-reactive T cells from (B6A)F1 Mice that Recognize (T,G)-A--L in Association with Strain A Antigen-presenting Cells

Line 2 (14 mo)	Fresh (T,G)-A--L-immunized lymph node cells (adherent cell depleted)	A		B6		$(B6A)F_1$	
		Medium	(T,G)-A--L	Medium	(T,G)-A--L	Medium	(T,G)-A--L
		[^{3}H]TdR uptake (cpm)					
1×10^4	0	151	11,790	1,737	10,120	264	14,477
1×10^4	0.75×10^5	579	15,896	2,374	12,210	844	19,263
1×10^4	1.5×10^5	997	18,034	3,276	15,789	1,470	24,447
1×10^4	3×10^5	2,293	26,715	5,685	27,741	3,067	30,610

Fresh (T,G)-A--L-immunized lymph node cells were obtained from regional draining nodes of B6A mice that had been immunized with (T,G)-A--L at the base of the tail 7 d before the assay. Cells were passed through a Sephadex G-10 column to remove the adherent cell population. Varying numbers of adherent cell-depleted fresh (T,G)-A--L-immunized lymph node cells were admixed with 1×10^4 cells from line 2 (after 14 mo of continuous in vitro growth) and stimulated with (T,G)-A--L in the presence of antigen-presenting cells from strain A, B6, or $(B6A)F_1$ mice. The proliferative responses were measured on day 2.

adherent cell-depleted fresh (T,G)-A--L-immune $(B6A)F_1$ cells did not suppress the proliferative response of line 2 to (T,G)-A--L in the presence of low responder strain A antigen-presenting cells. The increase of tritiated thymidine incorporation seen when fresh (T,G)-A--L immune lymph node cells are admixed with line 2 T cells in the presence of (T,G)-A--L might be explained in one of several ways. The most likely interpretation from our viewpoint is that the interaction of line 2 T cells with antigen results in the liberation of growth supporting factors, which nonspecifically recruit other T cells from the immune lymph node and allow their proliferation (13). The increased tritiated thymidine incorporation in the media control reflects the basal level of tritiated thymidine incorporation in the immune lymph node cells in the absence of added line 2 T cells. Fresh (T,G)-A--L-immune $(B6A)F_1$ cells not passed through Sephadex G-10 columns did not show any suppressive activity (data not presented). Although not conclusive, these results might provisionally exclude the existence of suppressor cells in immune lymph node cells of (high responder × low responder)F_1 mice immunized with (T,G)-A--L, which would suppress the ability of such immune T cells to proliferate in response to (T,G)-A--L in association with strain A antigen-presenting cells.

Discussion

The results reported here have shown that cells from low responder strain A mice can present (T,G)-A--L to long-term-cultured (T,G)-A--L-reactive T cell lines and clones derived from (high responder × low responder)F_1 [$(B6A)F_1$] mice. The possibility of carryover of syngeneic $(B6A)F_1$ antigen-presenting cells in the proliferative assay is unlikely because spleen cells pulsed with antigen could stimulate long-term-cultured line 2. Moreover, certain clones derived from such long-term T cell lines were carried on filler cells from strain A for >3 mo before the assay. These data suggest that there is no functional defect in the ability of (T,G)-A--L to associate with

antigen-presenting determinants on low responder antigen-presenting cells. The reason certain clones of (T,G)-A--L-reactive T cells that recognize (T,G)-A--L in association with low responder antigen-presenting determinants can become a predominant cell type in long-term cultures is not clear. It should be noted that all clones isolated at an earlier stage of culture (2 mo) from such (high responder × low responder)F_1 (T,G)-A--L-reactive lines showed restriction specificities only in accordance with high responder phenotype (10), i.e., they could recognize (T,G)-A--L only in association with antigen-presenting determinants of B6 and/or (B6A)F_1 antigen-presenting cells. There are several possible explanations for the presence of clones of T cells in long-term cultures of (high responder × low responder)F_1 mice immune to (T,G)-A--L that recognize (T,G)-A--L in association with low responder antigen-presenting cells. One possible explanation is that low frequency clones reactive with nonimmunodominant epitopes contained in (T,G)-A--L might be allowed by the culture conditions. Recognition of nonimmunodominant epitopes by low responder mice has been supported by previous studies on the role of antigenic structures and cellular interactions (14). The second possibility might be the emergence of (T,G)-A--L-reactive clones with low affinity for (T,G)-A--L. Data presented in Table III would provisionally exclude this as being the explanation for the emergence of at least one of the clones that we have identified. These data suggest that the recognition of (T,G)-A--L by clone F.11, which is restricted in recognition by low responder I-A^k-presenting determinants, recognizes (T,G)-A--L, as well as does clone 2a.a, which is restricted by the I-A^b high responder I-A product. That such clones exist has been shown by the data in this paper. The reason they do not dominate or cannot be recognized in vivo is not clear. T cells from (high responder × low responder)F_1 mice taken directly from immunized animals could not recognize (T,G)-A--L in association with antigen-presenting cells of low responder mice, as has been shown by others (5–7). One of the possible explanations for the inability of fresh (high responder × low responder)F_1 (T,G)-A--L-immune T cells to recognize antigen in association with low responder antigen-presenting cells is that there exist suppressor cells in such immunized cell populations that do not allow proliferative responses of F_1 T cells to antigen in association with low responder antigen-presenting cells. Indeed, suppressor cells and factors derived from such cells have been demonstrated to exist in nonresponder mice after immunization in several Ir gene-controlled systems (15, 16). However, the data in Table V would suggest that we have not been able to demonstrate suppressor cell activity from freshly immune F_1 lymph node cells, which will suppress the proliferation of long-term lines of (T,G)-A--L-immune T cells that can recognize antigen associated with low responder strain A antigen-presenting cells. Earlier data using not only (T,G)-A--L, but other Ir gene-controlled systems, suggested that simply altering the route of immunization would allow effective antigen recognition (17). The results presented in this report do, however, suggest that effective antigen recognition of (T,G)-A--L in strain A mice does not reside in the inability of antigen-presenting cells to exhibit antigen in the appropriate association with I-A^k antigen-presenting determinants. Thus, although it is not clear from these studies where the defect resulting in low responder status resides, these data clearly demonstrate that low responder I region restriction determinants can effectively restrict the recognition of (T,G)-A--L by immune T cells.

Summary

Long-term-cultured poly(Tyr,Glu)-poly-D,L,-Ala-poly-Lys [(T,G)-A--L]-reactive T cells and clones derived from (high responder × low responder)F_1 [(C57BL/6 ×

A/J)F_1] mice were shown to recognize (T,G)-A--L presented by cells from low responder strain A/J mice. The antigen-presenting determinant(s) that allowed recognition of (T,G)-A--L by such T cell clones was controlled by the I-A subregion of the major histocompatibility complex. These results suggest that there is no functional defect in the ability of low responder Ir gene products (I-A antigens) to associate with (T,G)-A--L for effective recognition by T cells. Although these results might tentatively be interpreted to suggest that Ir gene-controlled low responsiveness is due to the inability of the T cell to recognize the association between (T,G)-A--L and low responder I-A gene products, it is similarly possible that there might be a defect in the functional capabilities of low responder antigen-presenting cells to effectively process (T,G)-A--L into immunodominant epitopes.

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