GENE COMPLEMENTATION

Neither *Ir*-GLϕ Gene Need Be Present in the Proliferative T Cell to Generate an Immune Response to Poly(Glu^{55}Lys^{36}Phe^{9})$_n$

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The T cell proliferative response to the synthetic polypeptide poly(Glu^{55}Lys^{36}Phe^{9})$_n$ (GLϕ)[1] has been shown to be controlled by two separate immune response (*Ir*) genes, one mapping in the *I-A* subregion, the other in the *I-E/C* subregion (1). Thus, this system presented the possibility of analyzing in more detail the *Ir* gene control of the cell interactions required to generate a T cell proliferative response. It was conceivable that one gene product was expressed in the antigen-presenting cell (APC), the other in the T lymphocyte, and that both genes were required for a successful interaction. However, experiments involving radiation chimeras of the sort nonresponder parent A (P_a) bone marrow plus nonresponder parent B (P_b) bone marrow transferred into lethally irradiated complementing responder ($P_a \times P_b$)F_1 recipients [$P_a + P_b \rightarrow (P_a \times P_b)F_1$] demonstrated that at least one cell type had to express both gene products to function (2). Further experiments involving GLϕ presentation by nonimmune spleen cells to primed T lymphocytes from a complementing responder F_1 demonstrated that neither nonresponder parent (P_a or P_b) possessed cells that could present GLϕ, even if both types of spleen cells were added together, whereas ($P_a \times P_b$)F_1 spleen cells could present GLϕ (2). These results suggested that the APC was one cell type, which had to express both *Ir*-GLϕ gene products to generate an immune response to GLϕ.

In this paper, we examine the requirement for *Ir* gene expression in the T lymphocyte. Knowing that the APC had to express both *Ir*-GLϕ gene products, we transferred $P_a + P_b \rightarrow (P_a \times P_b)F_1$ chimeric spleen cells, which lack F_1 APC, into acutely irradiated ($P_a \times P_b$)F_1 mice along with T cell-depleted F_1 bone marrow as a source of responder APC. These mice responded to GLϕ, which demonstrates that both *Ir*-GLϕ genes do not have to be present in the T lymphocyte to generate a proliferative response to GLϕ.

Materials and Methods

Animals. C57BL/10(B10), B10.A, and B10.A(5R) strains were purchased from The Jackson Laboratory, Bar Harbor, Maine. B10.A(4R), B10.A(18R), (B10.A × B10)F_1 [(A × B)F_1],

[1] *Abbreviations used in this paper:* APC, antigen-presenting cell(s); ATS, anti-thymocyte serum; Con A, concanavalin A; DNP, dinitrophenylated; GLϕ, poly(Glu^{55}Lys^{36}Phe^{9})$_n$; *Ir*, immune response; MHC, major histocompatibility complex; OVA, ovalbumin; P_a, nonresponder parent A; P_b, nonresponder parent B; PETLES, peritoneal exudate T lymphocyte-enriched cells; PPD, purified protein derivative of *Mycobacterium tuberculosis*; RαMB, rabbit anti-mouse brain antiserum; (T,G)-A--L, poly(Tyr,Glu)-poly D,L-Ala--poly Lys; TNP, trinitrophenyl.

(B10.A[5R] × B10.A)F_1 [(5R × A)F_1], and (B10.A × B10.A[18R])F_1 [(A × 18R)F_1] strains were bred in our own colony (Laboratory of Immunology, National Institutes of Health, Bethesda, Md.). Dr. Jack Stimpfling (McLaughlin Research Institute, Great Falls, Mont.) kindly provided some of the B10.A(18R) and (B10.A × B10.A[18R])F_1 animals.

Radiation-induced Bone Marrow Chimeras. Mice that had been given neomycin (2 g/liter) (The Upjohn Co., Kalamazoo, Mich.) and bacitracin (1 g/liter) (kindly provided by Dr. J. Small, Veterinary Resources Branch, National Institutes of Health) in their drinking water for 1 wk were exposed to 900–950 R either at 126 R/min from a heavily filtered x-ray source, or 40 R/min from a cobalt source. 6–24 h after irradiation, animals were reconstituted with 10^7 bone marrow cells administered by tail vein injection. The bone marrow had been harvested from animals treated with 0.6 cm^3 of a 1:10 dilution of anti-thymocyte serum (ATS) (lot 3-9225, Microbiological Associates, Walkersville, Md.) intraperitoneally 3 and 1 d before sacrifice and 5 mg of cortisone acetate (The Upjohn Co.) intraperitoneally 2 d before sacrifice. The harvested bone marrow was then treated in vitro by a two-step cytotoxicity method with 1 cm^3 of a 1:40 dilution of rabbit anti-mouse brain antiserum (RαMB) per 10^8 cells for 30 min at room temperature, followed by excess guinea pig complement (4 ml of a 1:3 dilution). The RαMB was shown not to contain appreciable anti-stem-cell activity in a [^{125}I]iododeoxyuridine spleen uptake assay (3). Bone marrow treated in this fashion had no demonstrable responsiveness to concanavalin A (Con A) or allogeneic stimulator cells. Bone marrow recipients had no detectable Con A-responsive cells in their spleens until day 15 post-reconstitution. Reconstituted, irradiated mice were kept 12 wk before use, maintained on autoclaved food and bedding, and acidified water. Individual animals were H-2 typed in a two-step microcytotoxicity assay on the day of experimentation. All chimeras had >90% of their spleen cells of donor origin, and, in the case of $P_a + P_b \rightarrow F_1$ animals, each parental type constituted between 30 and 70% of the spleen cells.

T Cell Proliferation Assay. T cells were purified from thioglycolate-induced peritoneal exudate cells 14 d after immunization by passage over nylon-wool columns, as previously described (4). Peritoneal exudate T lymphocyte-enriched cells (PETLES) were cultured in Click's medium that contained 10% fetal calf serum in round-bottom 96-well microtiter plates at 1–2 × 10^5 cells/well in 0.2 cm^3 vol along with soluble antigen or antigen-pulsed APC (5). Stimulation was assessed at day 5 by measuring the incorporation of [^{3}H]thymidine. The data are expressed as arithmetic mean counts per minute ± SEM, or as counts per minute change, the difference between the mean antigen-stimulated and medium control cultures. The Student's t test was used to ascertain significant differences.

Antigens and Immunizations. Dinitrophenylated (DNP)-ovalbumin (OVA) (DNP-OVA), which contained an average of seven DNP groups per molecule of OVA, was prepared as previously described (5) and used for immunization at 10 μg/mouse and in culture at a concentration of 30 μg/ml. Purified protein derivative of *Mycobacterium tuberculosis* (PPD) (Connaught Medical Research Laboratory, Willowdale, Ontario) was used in culture at 20 μg/ml. Pigeon cytochrome *c* was used for immunization at 40 μg/mouse. Tobacco hornworm moth cytochrome *c* cyanogen bromide cleavage fragment 81–103 was used in culture at 10 μg/ml because this antigen elicits a heteroclitic response from T cells immunized with pigeon cytochrome *c* (6). Both cytochrome preparations were the gift of Dr. M. Ultee and Dr. E. Margoliash, Northwestern University, Evanston, Ill. The branched-chain synthetic amino acid polymer poly(Tyr,Glu)-poly D,L-Ala--poly Lys [(T,G)-A--L] (lot MC6), originally purchased from Miles-Yeda (Rehovot, Israel) was the generous gift of Dr. Howard Dickler and Dr. Alfred Singer, Immunology Branch, National Cancer Institute, National Institutes of Health. It was used for immunization at 50 μg/mouse and in culture at 100 μg/ml. GLϕ was purchased from Miles-Yeda and was the generous gift of Dr. Alan Rosenthal (Merck Sharpe & Dohme, Rahway, N. J.). It was used at 30 μg/mouse for immunization and 100 μg/ml in culture. All immunizations were carried out in the hind footpads by injecting 0.1 cm^3 of an emulsion that contained a 1:1 mix of antigens in normal saline and complete Freund's adjuvant that contained 1 mg/ml of *M. tuberculosis* strain H37Ra.

Acute Transfer Experiments. Because the PETLES assay measures secondary immune responses, priming must be done in an environment that provides responder APC. In $F_1 \rightarrow$ parent chimeras, responder APC were found in the spleen and peritoneal cavity; thus, no transfer was

necessary. However, in parent → F_1 chimeras, responder presenting cells were provided for priming by transferring 10^7 T cell-depleted F_1 bone marrow cells together with 5×10^7 chimeric spleen cells intravenously into lethally irradiated (900–950 R) F_1 hosts. These animals were immunized on the day of adoptive transfer in the hind footpads with an emulsion of antigens and complete Freund's adjuvant. T cell proliferation was assayed in a PETLES population 2 wk later. Some recipients had been thymectomized at 4–6 mo of age and were used 6 wk after thymectomy.

Results

Importance of Complete T Cell Depletion of Donor Marrow when Creating Radiation Chimeras. Our initial studies of the T lymphocyte proliferative response to GLϕ in F_1 → P_a radiation chimeras suggested that there was little or no host restriction. As shown in Fig. 1, when donor marrow was treated once with a commercially available AKR anti-C3H ascites (anti-Thy-1) and guinea pig complement, F_1 → P_a chimeras rapidly developed a detectable proliferative response to PPD (4 wk) and pigeon cytochrome *c* (6 wk), antigens to which both the donor and host are responders. Surprisingly, these chimeras also rapidly developed a strong response to GLϕ (4 wk),

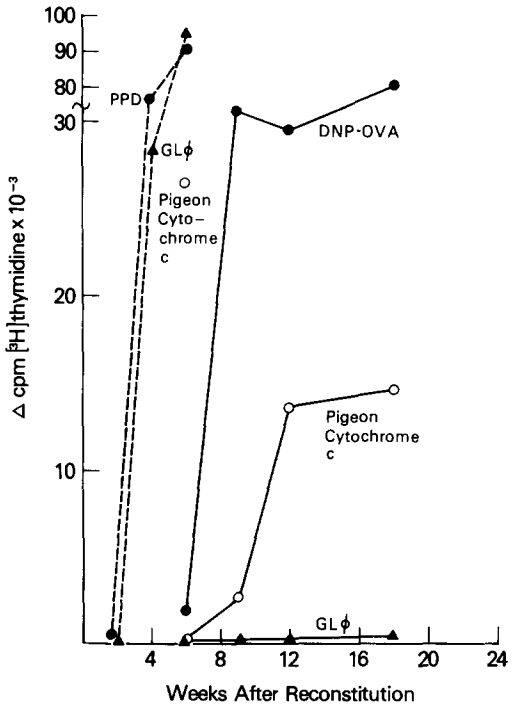

FIG. 1. Antigen-specific T cell proliferation (Δcpm) is plotted against weeks after bone marrow reconstitution of lethally irradiated mice. All chimeras are F_1 → P_a in which the donor is a responder to DNP-OVA, PPD, GLϕ, and pigeon cytochrome *c* and the recipient is a responder to DNP-OVA, PPD, and pigeon cytochrome *c* but a nonresponder to GLϕ. The group of chimeras represented by the dashed lines (assayed 2–6 wk post-reconstitution) were given bone marrow treated once with commercial anti-Thy-1.2. Antigen-specific T cells appeared 4–6 wk after reconstitution and were of donor Ir phenotype. The group of chimeras represented by the solid lines (assayed 6–18 wk post-reconstitution) were given bone marrow rigorously depleted of T cells by ATS, cortisone, and RαMB treatment (Materials and Methods). Antigen-specific T cells emerged at 9–12 wk and were of host Ir phenotype.

an antigen to which the donor, but not the host, is a responder. Such animals retained the same pattern of responsiveness for up to 15 mo. To test the possibility that residual post-thymic T cells in the donor marrow (7) rapidly expanded in the irradiated host to dominate the peripheral T cell pool, anti-Thy-1-treated bone marrow was transferred into adult-thymectomized, lethally irradiated hosts. When some of these chimeras developed functional T cells of donor Ir phenotype 6 wk after reconstitution (data not shown), more rigorous techniques of T cell depletion of donor bone marrow were pursued.

At least two types of T lymphocytes are known to contaminate bone marrow cell preparations: one is the blood-borne, recirculating, long-lived T cell that is sensitive to treatment with ATS (8); the second is the early post-thymic cell that is resistant to ATS but sensitive to cortisone treatment (9). With this knowledge in mind, we empirically devised a T cell-depletion regimen by employing both ATS and cortisone treatment in vivo followed by RαMB treatment of the marrow cells in vitro (Materials and Methods). Such exhaustively depleted marrow from (5R × A)F_1 mice was used to reconstitute lethally irradiated B10.A mice and their T cells assayed at varying times after reconstitution (Fig. 1).

In contrast to the chimeras created with only anti-Thy-1-treated marrow, the chimeras created with exhaustively T cell-depleted bone marrow showed no responses to any of the antigens tested at 6 wk. By 9 wk after reconstitution, the (5R × A) → A T cells gave a large proliferative response to the potent antigen DNP-OVA, a barely detectable response to pigeon cytochrome *c*, to which the B10.A host is a responder, and no response to GLϕ, to which the B10.A host is a nonresponder. By 12 wk, the chimeras appeared to be completely reconstituted as indicated by the full response to the relatively weak immunogen, pigeon cytochrome *c*. Strikingly, no response to GLϕ was evident, even as late as 18 wk after reconstitution. Thus, (5R × A)F_1 stem cells, which in an isogeneic environment would develop into GLϕ responder T cells, failed to so develop when they matured in a nonresponder environment, provided that the bone marrow was rigorously depleted of mature T cells before transfer.

These Ir-restricted, F_1 → P chimeras were also H-2 restricted in their response to antigens such as DNP-OVA to which either parental haplotype is a responder. As shown in Fig. 1, (5R × A)F_1 → B10.A chimeras responded well to soluble DNP-OVA. However, when the antigen was presented to the chimeric T cells on either B10.A or B10.A(5R) nonimmune spleen cells, only the B10.A spleen cells were capable of eliciting a significant proliferative response (Table I). The inability of the chimeric T cells to recognize DNP-OVA in association with B10.A(5R) spleen cells was not caused by a failure to prime such cells as a result of the absence of the appropriate presenting cells in the host. As shown in Table I, chimeric spleen cells were capable of presenting both GLϕ and DNP-OVA to immune B10.A(5R) T cells, thus demonstrating the presence of functional B10.A(5R) restriction elements in these animals. Thus, the failure of the genotypic (5R × A)F_1 T cells to recognize DNP-OVA in association with B10.A(5R) APC suggested that the T cells had become restricted during their development in the B10.A host to recognition of only B10.A major histocompatibility complex (MHC) products.

T Cells from P_a + P_b → F_1 Chimeras Behave Like F_1 T Cells when Primed in the Presence of Sufficient F_1 APC. B10.A and B10.A(18R) marrow rigorously depleted of T cells were given in equal amounts to lethally irradiated (900–950 R) (A × 18R)F_1 mice.

TABLE I

The Ability of Parental, F_1, and F_1 → Parent Chimeric Spleen Cells to Present Antigen to Parental and F_1 → Parent Chimeric T Cells

APC	5R × A → A chimeric T cells (PETLES)			B10.A T cells (PETLES)			B10.A(5R) T cells (PETLES)				
	Medium	DNP-OVA	Δcpm	Medium	DNP-OVA	Δcpm	Medium	DNP-OVA	Δcpm	GLϕ	Δcpm
Continuous antigen	5,009 ± 300	85,211 ± 8,100	80,202	1,946 ± 400	73,544 ± 5,000	71,598	3,183 ± 1,000	66,495 ± 3,200	63,312	54,911 ± 4,000	51,728
B10.A spleen*	5,542 ± 600	53,628 ± 3,300	48,086	2,881 ± 300	42,697 ± 3,900	40,086	11,299 ± 1,400	14,071 ± 3,100	2,772	11,925 ± 1,500	626
B10.A(5R) spleen*	5,617 ± 500	8,163 ± 400	2,546	9,132 ± 800	13,018 ± 2,100	3,886	3,547 ± 600	61,857 ± 5,800	58,310	22,618 ± 1,100	19,071
(5R × A)F_1 spleen*	—	—	—	7,856 ± 700	29,668 ± 1,800	21,812	6,816 ± 1,100	42,169 ± 5,100	35,353	16,733 ± 2,100	9,917
5R × A → A spleen*	—	—	—	8,319 ± 1,000	31,405 ± 3,700	23,086	6,422 ± 700	39,423 ± 3,900	33,001	17,371 ± 1,400	10,949

* In these experiments, 10^5 antigen-pulsed or nonpulsed (medium) B10.A, B10.A(5R), (5R × A)F_1, or (5R × A)F_1 → A chimeric spleen cells were added to 1–2 × 10^5 (5R × A)F_1 → A, B10.A, or B10.A(5R) PETLES in each well. 4 d later, [^{3}H]thymidine was added to each well, and the cultures were harvested 16–18 h later. Δcpm represents antigen-specific proliferation and is the difference in [^{3}H]thymidine incorporation between cultures receiving pulsed and nonpulsed cells. Statistically significant responses are underlined.

TABLE II

A + 18R → (A × 18R)F_1 Chimeric T Cells Respond to GLϕ when Primed in the Presence of Sufficient Responder APC

Experiment	Source of PETLES	Proliferative response (cpm ± SEM) to				
		Medium	DNP-OVA	GLϕ	(T,G)-A--L	Pigeon cytochrome *c*
1	A + 18R → A × 18R	6,219 ± 802	55,046 ± 4,128	5,059 ± 735	43,502 ± 846	15,471 ± 1,276
2	(A + 18R → A × 18R) spleen → A × 18R	501 ± 87	27,234 ± 2,982	2,467 ± 181	17,411 ± 1,070	12,656 ± 1,249
3a	(A + 18R → A × 18R) spleen → A × 18R	1,802 ± 206	23,851 ± 2,242	3,928 ± 277	16,133 ± 695	9,574 ± 612
3b	(A + 18R → A × 18R) spleen + -T (A × 18R) BM → A × 18R	889 ± 112	60,172 ± 4,511	42,669 ± 2,984	45,613 ± 3,629	21,613 ± 1,417
3c	-T (A × 18R) BM → A × 18R	619 ± 184	762 ± 100	518 ± 117		
4a	(A + 18R → A × 18R) spleen + -T (A × 18R) BM → A × 18R	464 ± 222	21,040 ± 1,459	33,596 ± 1,784	21,451 ± 2,660	18,836 ± 409
4b	18R spleen + -T (A × 18R) BM → A × 18R	3,187 ± 850	43,784 ± 5,202	3,616 ± 291	35,688 ± 2,192	4,408 ± 644
5	(A + 18R → A × 18R) spleen + -T (A × 18R) BM → THYMX (A × 18R)	542 ± 64	87,260 ± 953	20,668 ± 4,371	35,927 ± 2,525	10,395 ± 3,009

The designation "-T" means T cell depleted. "BM" is bone marrow. "THYMX" refers to adult thymectomized mice. Underlined values are significantly different from the medium control by Student's *t* test. Lethally irradiated (A × 18R)F_1 animals were reconstituted with a 1:1 mixture of T cell-depleted parental bone marrow. 3 mo later these chimeric animals were immunized, and their T cell proliferative responses were measured (Exp. 1). Nonimmune chimeric spleen cells were transferred to irradiated F_1 mice either alone (Exps. 2 and 3a) or along with T cell-depleted F_1 bone marrow (Exps. 3b and 4a). In one case the recipient was thymectomized (Exp. 5). T cell-depleted bone marrow alone (Exp. 3c) or together with nonresponder parental spleen (Exp. 4b) were transferred to irradiated F_1 as control experiments.

When these A + 18R → (A × 18R)F_1 chimeras were immunized 12 wk or more after reconstitution (Table II, Exp. 1) good proliferative responses were observed to DNP-OVA, an antigen to which both parents can respond, (T,G)-A--L, an antigen to which only the 18R(I^b) can respond, and pigeon cytochrome *c*, an antigen to which only the B10.A(I^a) can respond. However, no response to GLϕ was seen. This confirmed our previous findings that the chimeras behave like a mixture of the parental haplotypes and that the GLϕ response required the presence of at least one F_1 cell type (2). Thus, rigorous depletion of T cells from the donor marrow did not alter these conclusions.

In A + 18R → (A × 18R)F_1 chimeras, the T lymphocytes have developed in a responder F_1 environment, but the APC, which derive from the donor bone marrow (see below), are of nonresponder parental origin. Our previous studies demonstrated the requirement for responder F_1 presenting cells to generate a GLϕ proliferative response (2). In an effort to provide them, chimeric spleen cells were transferred into an acutely irradiated (A × 18R)F_1, the adoptive recipient immunized immediately, and the PETLES response assayed 2 wk later in the presence of F_1 APC in culture. As shown in Table II (Exps. 2 and 3a), this adoptive transfer resulted in a very small response to GLϕ. However, compared with the large responses to DNP-OVA, (T,G)-A--L, and especially the weaker antigen, pigeon cytochrome *c*, the response to GLϕ had to be considered marginal at best. However, when the phenotype of the splenic APC of the adoptive recipients was assayed 2 wk after transfer, no cells capable of presenting GLϕ to immune F_1 T cells were found (data not shown). This result suggested that the turnover of the APC in the spleen of lethally irradiated mice must be more rapid than 2 wk and raised the possibility that the failure of the chimeric T cells to respond well to GLϕ when transferred into the acutely irradiated second host was because of an inadequate number of responder APC for priming and not because of an intrinsic *Ir* gene defect in the T cell.

To examine this question, spleen cells and peritoneal washings obtained by lavage of the peritoneal cavity from normal and irradiated B10 mice were compared for their ability to present DNP-OVA to syngeneic immune T cells. Mice were irradiated with 900–950 R at varying times before assay. The results are depicted graphically in Fig. 2 with the left panel showing presentation by spleen cells and the right showing presentation by cells in peritoneal washings. The figure shows that as early as 2 h after 900–950 R, a decrease in the ability of spleen cells to present antigen was observed, and no antigen presentation above allogeneic controls was detectable by day 4 after irradiation in all eight experiments in which it has been tested. Mixing irradiated and normal spleen populations did not inhibit antigen presentation, thus ruling out nonspecific suppression as an explanation. In animals not reconstituted by hemopoietic stem cells, assaying spleens for APC as late as day 8 after irradiation revealed no return of antigen presenting activity, which suggested that the disappearance was not transient.

Fig. 3 reveals the effect of reconstituting irradiated mice with T cell-depleted bone marrow on the reappearance of APC activity. Three types of radiation chimeras were created: B10 → B10.A(3R), B10.A(3R) → B10, and B10.A → B10. Both B10 and B10.A(3R) animals possess $I\text{-}A^b$ alleles, and, therefore, spleen cells from both strains of mice should present DNP-OVA to immune T cells recognizing $I\text{-}A^b$. In Fig. 3 (panel A), the spleen cells from B10 → B10.A(3R) and B10.A(3R) → B10 chimeras 4 d after irradiation and reconstitution were compared with B10, B10.A(3R), and

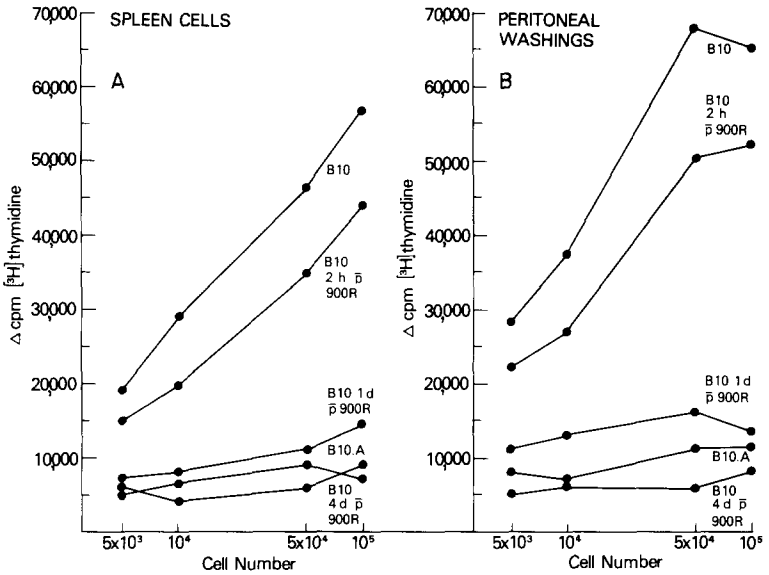

FIG. 2. Antigen-specific T cell proliferation (Δcpm) of DNP-OVA-immune PETLES from B10 mice in response to DNP-OVA-pulsed spleen cells in panel A, and in response to DNP-OVA-pulsed peritoneal washings in panel B are plotted against the log of cell number. Spleen and peritoneal cells from B10 animals at various times after ($\bar{p}$) receiving 900 R are compared with normal syngeneic and allogeneic controls for their ability to present antigen. 2 h after 900 R, both spleen and peritoneal cells were about one-half as efficient at antigen presentation as unirradiated cells, and, by 24 h after 900 R, antigen presentation was only slightly better than allogeneic cells.

allogenic B10.A spleens for their ability to present DNP-OVA to B10.A(3R)-immune PETLES. Both types of chimeras and B10 and B10.A(3R) spleen cells all initiated a proliferative response to DNP-OVA, whereas the allogeneic B10.A spleen cells did not. This suggests that the injection of a proliferating stem-cell pool into the irradiated mice reversed the loss of presenting activity in the spleen at 4 d after irradiation. In Fig. 3 (panel B), the ability of the B10.A(3R) → B10 and B10 → B10.A(3R) chimeric spleen cells to present GLϕ to B10.A(3R) PETLES was compared with B10 and B10.A(3R) spleen cells. B10.A(3R) spleen cells possess both *Ir*-GLϕ genes (one mapping in *I-A*^{b} the other in *I-E*^{k}/*C*^{d}) and, unlike the low-responder B10 cells, will present GLϕ to immune responder T cells. Thus, B10 → B10.A(3R) and B10.A(3R) → B10 chimeras should enable us to determine unambiguously whether the APC in the spleen of irradiated mice is repopulated by radioresistant host APC from another site or by donor bone marrow stem cells. The data show that the B10.A(3R) → B10 spleen cells could present GLϕ and therefore were of donor marrow origin. The B10 → B10.A(3R) spleen cells behaved like B10 cells (panel B), being unable to present GLϕ, although they did present DNP-OVA well (panel A); this demonstrated that the hosts APC do not survive to repopulate the spleen.

B10.A → B10 chimeras were created to determine whether the results with B10 → B10.A(3R) and B10.A(3R) → B10 chimeras were a result of the fact that donors and recipients were *I-A* compatible. If rapid APC reconstitution from donor marrow was a result of an *I-A* region-dependent cell trafficking, B10.A → B10 antigen-pulsed spleen cells might not generate proliferative responses in B10.A T cells. In Fig. 3

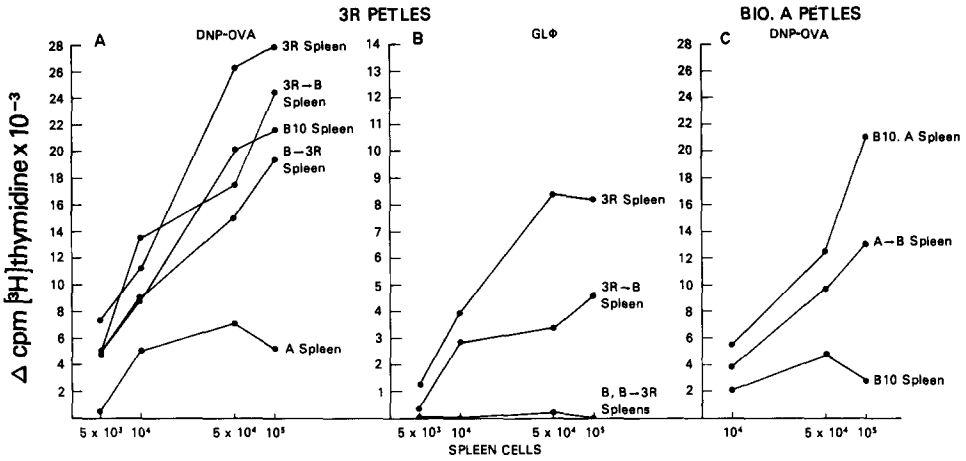

FIG. 3. In panel A, the proliferative response (Δcpm) of B10.A(3R) DNP-OVA-immune PETLES to DNP-OVA-pulsed spleen cells are plotted against cell number. Antigen-pulsed spleen cells from B10, B10.A(3R), and B10.A are compared with spleen cells from B10.A(3R) → B10 and B10 → B10.A(3R) chimeras 4 d after reconstitution. The chimeras were nearly as effective as the parental B10.A(3R) and B10 at presenting DNP-OVA. In panel B, the proliferative response of GLϕ-immune PETLES to GLϕ-pulsed spleen cells are plotted against cell number. B10.A(3R) spleen cells presented GLϕ, as did B10.A(3R) → B10 chimeric spleen cells, whereas B10 and B10 → B10.A(3R) did not. In the chimeras, the presenting cell phenotype was that of the donor bone marrow. In panel C, the ability of B10.A → B10 chimeric spleen cells to present DNP-OVA to B10.A DNP-OVA-immune PETLES is shown. Again, the chimeric spleen cells were of donor phenotype.

(panel C), this issue is resolved by the data that show that B10.A → B10 chimeric spleen cells successfully presented DNP-OVA to B10.A T cells 4 d after reconstitution, although they failed to present to B10.A(3R) T cells (data not shown). Thus, even in allogeneic chimeras, the experiments suggested that the APC in the spleen and peritoneal cavity disappear within 4 d after lethal irradiation and are rapidly replaced by APC from the reconstituting bone marrow.

This rapid turnover of peripheral APC could have been responsible for the apparent low responsiveness to GLϕ of A + 18R → (A × 18R)F_1 chimeric T cells in the adoptive host. If adequate numbers of F_1 presenting cells were not present in the priming environment, then secondary in vitro responses (as in the PETLES assay) might not have been detectable. Therefore, to eliminate this potential reason for low responsiveness, we repeated the adoptive transfer of A + 18R → (A × 18R)F_1 chimeric spleen cells into irradiated (A × 18R)F_1 animals and added T cell-depleted (A × 18R)F_1 bone marrow as a source of responder APC. The results of this experiment are shown in Table II (exp. 3). The presence of the T cell-depleted F_1 bone marrow in the adoptive host enabled the chimeric T cells to manifest a proliferative response to GLϕ (Table II, line 3b) in addition to responding to DNP-OVA, (T,G)-A--L, and pigeon cytochrome *c* as seen before. To assure that the T cell-depleted F_1 bone marrow cells were not providing the T cells that were responding to the GLϕ, the bone marrow was transferred alone into irradiated F_1 mice. As shown in Table II (line 3c), no antigen-responsive T cells were detected. These results suggest that both *Ir*-GLϕ genes do not have to be expressed in the T cell to generate an immune response to GLϕ, provided that the T cells mature in a responder environment and that they are

primed to the antigen in a host with sufficient responder APC.

Although the T cell-depleted F_1 bone marrow seemed not to be a source of responding T cells, it was possible that in the presence of mature spleen cells from the chimera that F_1 stem cells could more rapidly differentiate into functional T cells. To rule out this possibility, nonresponder B10.A(18R) spleen cells were transferred into irradiated (A × 18R)F_1 mice along with T cell-depleted F_1 bone marrow. As shown in Table II (line 4b), the adoptively transferred mature B10.A(18R) splenic T cells retained their MHC-dictated pattern of responsiveness showing proliferation to DNP-OVA and (T,G)-A--L but no response to GLϕ and pigeon cytochrome *c*. Finally, to unequivocally rule out the possibility of an F_1 stem cell rapidly differentiating into a GLϕ-responder T cell, we performed adult thymectomy on (A × 18R)F_1 animals and used them as adoptive hosts 6 wk later. A + 18R → (A × 18R)F_1 chimeric spleen cells plus T cell-depleted F_1 bone marrow transferred into thymectomized, lethally irradiated F_1 animals showed responsiveness to all four antigens, DNP-OVA, (T,G)-A--L, pigeon cytochrome *c*, and GLϕ as shown in Table II (Exp. 5). Therefore, the adoptive host's thymus does not seem to play a role in the appearance of GLϕ responsiveness in these animals.

Thus, A + 18R → (A × 18R)F_1 chimeras behaved as a mixture of $H\text{-}2^a$ and $H\text{-}2^b$ cells until they were primed to antigen in an environment providing adequate F_1 APC. Once this requirement was met, a phenotypic alteration could be detected in these parental T cells that had matured in an F_1 environment. They appeared to have learned to respond to antigen in the context of F_1 H-2 restriction elements.

A Two T Cell Model for Gene Complementation. The development of GLϕ-responsive T cells in A + 18R → (A × 18R)F_1 chimerals could mean that T cells possessing a responder allele at either *I-A* or *I-E/C* can develop the recognition structure for the unique F_1 restriction element when they mature in an F_1 environment. However, an alternative explanation for the data suggested to us by Dr. Alfred Singer is that tolerance induction in the chimera allows the $H\text{-}2^a$ and $H\text{-}2^b$ donor T cells to interact without a mixed-lymphocyte reaction. In the F_1 GLϕ responder, one T cell specific for $I\text{-}A^b$ plus GLϕ and one T cell specific for $I\text{-}E^k/C^d$ plus GLϕ might interact to make an immune response only when GLϕ is presented on an F_1 ($I\text{-}A^b$, $I\text{-}E^k/C^d$) APC, which brings the two T cells together. Similarly, in the chimera with $H\text{-}2^a$ and $H\text{-}2^b$ T cells tolerant to each other, the GLϕ response is revealed when F_1 APC are provided. To test this hypothesis, we made (A × B)F_1 → B10.A chimeras that were restricted to $H\text{-}2^a$ responses (DNP-OVA and pigeon cytochrome *c* responders) and (A × B) → B10 chimeras that were restricted to $H\text{-}2^b$ responses [DNP-OVA and (T,G)-A--L responders]. Neither type of chimera responded to GLϕ (see Table IV). If two interacting T cells are required for a GLϕ response, one specific for $I\text{-}A^b$ plus GLϕ and one specific for $I\text{-}E^k/C^d$ plus GLϕ, then (A × B)F_1 → B chimeric T cells could provide the former and (A × B)F_1 → A chimeric T cells the latter. Therefore, we mixed (A × B)F_1 → A and (A × B)F_1 → B chimeric spleen cells, administered them intravenously to irradiated (A × B)F_1 mice, immunized them in the footpads, and assayed their PETLES 2 wk later. The results of this experiment are shown in the last line of Table III. The proliferation to DNP-OVA, (T,G)-A--L, and pigeon cytochrome *c* revealed the successful generation of a mixture of $H\text{-}2^a$ and $H\text{-}2^b$ specific T cells; however, no response to GLϕ was observed. This was strong evidence against a two T cell model for gene complementation in the GLϕ response and supported the interpretation that

TABLE III

Responder Ir-GLϕ Alleles Do Not Function in Separate Subsets of T Cells

Source of PETLES	Proliferative response (cpm ± SEM) to				
	Medium	DNP-OVA	GLϕ	(T,G)-A--L	Pigeon cytochrome *c*
A × B → A	2,231 ± 243	69,682 ± 5,449	2,181 ± 196	2,466 ± 290	23,448 ± 931
A × B → B	1,586 ± 329	51,227 ± 4,820	1,721 ± 83	39,754 ± 3,469	1,231 ± 296
(A × B → A) + (A × B → B) → A × B	3,561 ± 104	52,771 ± 3,414	4,639 ± 507	57,753 ± 1,613	31,478 ± 1,247

Underlined values are statistically significantly different from the medium control. (A × B)F_1 bone marrow was given to lethally irradiated parental mice, and their antigen responsiveness was tested in the PETLES assay 3 mo later. Spleen cells from A × B → A and A × B → B chimeras were mixed and given to lethally irradiated (A × B)F_1 mice. These mice were immunized, and their PETLES were assayed 2 wk later.

TABLE IV

Neither Ir-GLϕ Responder Allele Need Be Present in the T Cell

Line	Source of PETLES	Proliferative response (cpm ± SEM) to				
		Medium	DNP-OVA	GLϕ	(T,G)-A--L	Pigeon cytochrome *c*
a	4R → A × 18R	1,796 ± 211	51,819 ± 4,484	1,664 ± 199	2,009 ± 312	1,982 ± 368
b	(4R → A × 18R) spleen + -T (A × 18R) BM → A × 18R	519 ± 97	34,890 ± 4,053	27,268 ± 3,775	28,588 ± 807	12,224 ± 1,439
c	-T (A × 18R) BM → A × 18R	800 ± 100	800 ± 100	700 ± 100		

The designation "-T" means T cell depleted. "BM" means bone marrow. Underlined values are significantly different from the medium control by Student's *t* test. B10.A(4R) bone marrow was used to reconstitute lethally irradiated (A × 18R)F_1 mice, and these animals had their T cell responses measured by PETLES assay (line a) or donated spleen cells to acutely irradiated adoptive (A × 18R)F_1 hosts that also received T cell-depleted F_1 bone marrow (line b). Some adoptive hosts received T cell-depleted bone marrow only (line c). The adoptive F_1 hosts were immunized on the day of transfer, and their T cells were assayed 2 wk later.

in $P_a + P_b \rightarrow F_1$ chimeras a population of T cells is generated whose repertoire has been expanded to recognize F_1-specific structures on APC.

The results of this experiment also bear on the mechanism by which $F_1 \rightarrow P$ chimeras develop T cells restricted to host haplotype-specific interactions. It could be argued that the restriction of $F_1 \rightarrow P_a$ to *H-2*^{a} phenotype responses is a manisfestation of suppression of all *H-2*^{b}-reactive clones. If suppression were the explanation for the acquired H-2 restriction, then each subpopulation of T cells in ($F_1 \rightarrow$ A) and ($F_1 \rightarrow$ B) mice should have suppressed the other in the mixing experiment and no antigen-specific proliferation should have been seen. The fact that T cells from these animals behaved like mixtures of *H-2*^{a} and *H-2*^{b} T cells makes suppression a most unlikely explanation for thymic restriction.

Neither Responder Allele Need Be Present in the T Cell. The GLϕ responsiveness of A + 18R → (A × 18R)F_1 T cells suggested that both gene products did not have to be expressed in the T cell. However, because each parental T cell possesses one of the *Ir*-GLϕ genes, it was possible that a responder T cell had to express one or the other *Ir*-GLϕ gene product. To test this possibility we turned to the B10.A(4R) strain that possesses neither responder *Ir*-GLϕ allele. In addition this strain is a nonresponder to pigeon cytochrome *c* and (T,G)-A--L. T cell-depleted B10.A(4R) bone marrow cells were transferred to lethally irradiated (A × 18R)F_1 recipients, and the chimeras were immunized 3 mo later. As shown in Table IV, 4R → (A × 18R)F_1 chimeras, which have 4R-type APC, showed a proliferative response to DNP-OVA but did not proliferate in response to any antigens to which 4R is a nonresponder (Table IV, line a). However, when 4R → (A × 18R)F_1 chimeric spleen cells were transferred into

irradiated (A × 18R)F_1 mice along with T cell-depleted F_1 bone marrow (Table IV, line b) good proliferative responses to DNP-OVA, (T,G)-A--L, pigeon cytochrome *c*, and GLϕ were seen. F_1 bone marrow alone did not produce responsiveness to any of these antigens (Table IV, line c). Thus, the B10.A(4R) cells acquired the ability to manifest responses in both one- and two-gene controlled systems by maturing in a responder environment. It is clear from the results of this experiment that low responsiveness is not an intrinsic property of T cells bearing low-responder alleles, but is a phenotype that can be altered. Thus, for GLϕ, neither *Ir* gene need be present in the T cell to mount a proliferative response.

Discussion

The mechanism of H-2 restriction and *Ir* gene control has been under intensive investigation in many laboratories. Recently, experiments done with animals manipulated such that they contain cells of differing genotypes (chimeras) have shed light on the process by which T cell precursors acquire self recognition. Zinkernagel (7) demonstrated that cytotoxic cells of (P_a × P_b)F_1 genotype that had matured in a parental (P_a) environment were restricted to lysing virus-infected targets displaying K^a or D^a. Despite their genotype, no cells with anti-K^b or anti-D^b plus virus specificity could be demonstrated, and control experiments (10) suggested that suppression could not explain the failure to detect such cells. Furthermore, genotypic P_a cytotoxic cells that had developed in a (P_a × P_b)F_1 environment acquired specificity to lyse virus-infected targets displaying K^b and/or D^b in addition to K^a and D^a targets; however, this could only be demonstrated when the chimeric cells were sensitized in an irradiated F_1 host (7). Thymic transplant experiments suggested that the thymus was responsible for altering the phenotype of the maturing T cells (7, 11, 12). These seminal observations have been extended to systems measuring transplantation across minor histocompatibility barriers (12), male-specific killing (13), delayed-type hypersensitivity (14), helper T activity (12, 15–20), trinitrophenyl (TNP)-modified target cytotoxicity (21, 22), and, in this report, T cell proliferation.

The simultaneous alteration of H-2 restriction and Ir phenotype of helper T cells by the developmental environment has been reported in systems measuring antibody responses. Kappler and Marrack (17) found that (A × B)F_1 → A (low responder) chimeric helper T cells could not support a secondary TNP-(T,G)-A--L-specific plaque-forming cell response and were unable to cooperate with B parent's macrophages and B cells. The loss of responsiveness paralleled the loss of capacity to interact with H-2 products of the responder Ir genotype. The site of this Ir restriction of the T cell was shown to be the thymus by Hedrick and Watson (23) for a secondary antibody response to calf skin collagen in F_1 → nonresponder thymus chimeras. For chimeras of the type A(low-responder) → (A × B)F_1 Kappler and Marrack (17) as well as Hodes et al. (24) found that P_a chimeric T cells could help P_b macrophages and B cells to produce a secondary or primary anti-TNP-(T,G)-A--L response. But these P_a chimeric T cells could not convert P_a macrophages to responder phenotype (24). Thus, the T cell phenotype was altered by relaxing its genetic restriction to interact with cells expressing $H\text{-}2^b$ gene products. Similar results have been obtained in cytotoxic systems (7, 13, 22, 25).

We have extended these observations to the proliferative T cell and have used chimeric animals to examine the mechanism of gene complementation in antigen

responses under dual *Ir* gene control. Similar to other workers who used one-gene systems, we have found that responder $(P_a \times P_b)F_1$ genotype T cells maturing in a nonresponder P_a environment are nonresponders to antigens to which parent P_b genotype cells should respond (Table III). Furthermore, the non-Ir-controlled responses of the chimeric T cells to antigens such as DNP-OVA are only through interactions with host-MHC-bearing APC (Table I). Thus, $F_1 \rightarrow P_a$ chimeric T cells lost from their repertoire the capacity to interact with P_b APC. For dual *Ir* gene-controlled responses, T cell development in either parental environment led to the loss of GLϕ responsiveness (Table III). Even mixtures of the two types of chimeric cells, $F_1 \rightarrow A$ and $F_1 \rightarrow B$, could not overcome this defect (Table III). It would appear from these experiments that in all cases T cells must mature in a high-responder environment to respond to the antigen. For GLϕ, the high-responder environment is only that of the F_1.

The nonresponsiveness to GLϕ of $A + B \rightarrow (A \times B)F_1$ chimeric T cells primed in the chimera demonstrated that in addition to having T cells mature in a responder environment at least one cell type participating in the proliferative response had to express both *Ir*-GLϕ genes, i.e., come from a responder donor (Table II) (2). When these chimeric T cells were primed in an environment that provided responder APC, the T cells were capable of proliferating in response to GLϕ, which showed that both *Ir*-GLϕ genes have to be present in the APC but not in the T lymphocyte (Table II). The finding that $B10.A(4R) \rightarrow (A \times B)F_1$ chimeric T cells responded to GLϕ when primed with responder APC showed that neither *Ir*-GLϕ gene need be present in the T cell (Table IV). Therefore, both complementing *Ir*-GLϕ genes must be expressed in the APC and neither need be in the T cell as long as it has developed in an environment in which both genes are present. These data support the concept emerging from the two-dimensional gel studies of Jones et al. (26), the Ia-sequencing studies of Cook et al. (27) and Silver (28), and our studies (29) of complementation for APC function by strains bearing $Ia.7^+$ *I-E/C* gene products, that gene complementation involves the pairing of an *I-E/C*-encoded α-chain with an *I-A*-encoded β-chain to form a single two-chain-restricting element. Thus, complementation occurs at the level of a single cell (APC) by post-translational assembly of the two gene products, not by cooperation between two cells each expressing one responder allele.

An acutely irradiated F_1-adoptive host has been shown to be an adequate source of F_1 APC for helper T cell priming by Sprent (15) although not by Waldmann et al. (30). For priming the proliferative T cell, whose secondary response is assayed at least 14 d after priming, this simple adoptive transfer was found not to be adequate. The rapid disappearance of peripheral APC required the addition of T cell-depleted bone marrow to provide sufficient presenting cells to prime the proliferating T lymphocyte. It is possible that the success of the adoptive host in providing APC for priming the helper T cell achieved by some workers represents a kinetic difference in requirements for priming different T cell subsets. Alternatively, it may be necessary to reexamine conclusions obtained from such experiments in light of the possibility that the only source of functional APC may be in the donor cell population.

Our findings on the rapid turnover of peripheral APC after irradiation raise some additional questions on the nature and function of this cell type. For example, we have no explanation for the finding that the splenic and peritoneal APC are radiosensitive in vivo but function well after in vitro irradiation (5). Furthermore, the

splenic APC may be different from cells with the same function in the liver (Küpffer cells) (31), skin (Langerhans cells) (32), and thymus (33). The turnover of the latter two types of APC after radiation has recently been shown to be slower than that of the splenic APC (34, 35) (D. L. Longo and R. H. Schwartz. Manuscript in preparation.). Differences in rates of turnover of the cell in different sites may be important. In particular, our recent discovery of the slow turnover of the APC in the thymus is interesting because a parsimonious theory of H-2 restriction could be advanced if the thymic APC could be demonstrated to play an important role in the development of self-recognition in the thymus.

The results of others (7, 13, 17, 22–25) in one-gene Ir-controlled systems taken together with the data presented in this paper on one-gene and complementing two-gene Ir-controlled systems lead to the conclusion that at least one class of *Ir* genes is expressed in the APC. An Ir-controlled response can be initiated by these cells in any T cell capable of interacting with this *Ir* gene product. These responder T cells can be genotypic responders or genotypic nonresponders that have matured in a responder environment. If the T cells matured in a nonresponder environment, they were unable to be stimulated to make Ir-controlled responses because they appear not to have acquired the ability to interact with responder gene products on the APC. To date, *Ir* gene control and H-2 restriction of immune responses have not been separable. Thus, the *Ir* gene product and the H-2-restricting element may be the same structure (7, 36).

Summary

The cellular requirements for immune response (*Ir*) gene expression in a T cell proliferative response under dual *Ir* gene control were examined with radiation-induced bone marrow chimeras. The response to poly(Glu^{55}Lys^{36}Phe^{9})$_n$ (GLϕ) requires two responder alleles that in the [B10.A × B10.A(18R)]F$_1$ map in *I-A^b* and *I-E^k/C^d*. Chimeras in which a mixture of the nonresponder B10.A parental cells (which possess only *I-E^k/C^d*) and the nonresponder B10.A(18R) parental cells (which possess only *I-A^b*) were allowed to mature in a responder F$_1$ environment did not respond to GLϕ, which suggests that at least one cell participating in the response needed to possess both responder alleles to function. When T cells from such A + 18R → F$_1$ chimeras were primed in the presence of responder antigen-presenting cells (APC), the chimeric T cells responded to GLϕ, which suggests that both responder alleles must be expressed in the APC but not necessarily in the T cell. Interestingly, acutely irradiated F$_1$ animals were found not to be an adequate source of responder APC for priming the proliferating T cell because of the rapid turnover of peripheral APC after irradiation. In adoptive transfer experiments, T cell-depleted bone marrow had to be used as a source of responder APC.

When bone marrow cells from (B10.A × B10)F$_1$ responder animals were allowed to mature in a low-responder B10 or B10.A parental environment, neither chimera, F$_1$ → A or F$_1$ → B, could respond to GLϕ. This demonstrated that the presence of high-responder APC, which derive from the donor bone marrow, was not sufficient to generate a GLϕ response. It appears that in addition it is essential for the T lymphocytes to mature in a high-responder environment. Finally, B10.A(4R) T cells, which possess neither *Ir*-GLϕ responder allele, could be educated to mount a GLϕ-proliferative response provided that they matured in a responder environment and

were primed with APC expressing both responder alleles. Therefore, the gene products of the complementing *Ir*-GLϕ responder alleles appear to function as a single restriction element at the level of the APC. T cells that do not possess responder alleles are not intrinsically defective, because they could be made phenotypic responders if they developed in an environment in which responder major histocompatibility complex (MHC) products were learned as self and if antigen was presented to them by APC expressing responder MHC products.

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