GENE COMPLEMENTATION

Neither Ir-GL¢ Gene Need

Be Present in the Proliferative T Cell to

Generate an Immune Response to Poly(Glu55Lys36Phe9)n

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The T cell proliferative response to the synthetic polypeptide poly(Glu55Lys36Phe9)n (GL¢) 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 (Pα) bone marrow plus nonresponder parent B (Pβ) bone marrow transferred into lethally irradiated complementing responder (Pα × Pβ)F1 recipients [Pα + Pβ → (Pα × Pβ)F1] demonstrated that at least one cell type had to express both gene products to function (2). Further experiments involving GL¢ presentation by nonimmune spleen cells to primed T lymphocytes from a complementing responder F1 demonstrated that neither nonresponder parent (Pα or Pβ) possessed cells that could present GL¢, even if both types of spleen cells were added together, whereas (Pα × Pβ)F1 spleen cells could present GL¢ (2). These results suggested that the APC was one cell type, which had to express both Ir-GL¢ gene products to generate an immune response to GL¢.

In this paper, we examine the requirement for Ir gene expression in the T lymphocyte. Knowing that the APC had to express both Ir-GL¢ gene products, we transferred Pα + Pβ → (Pα × Pβ)F1 chimeric spleen cells, which lack F1 APC, into acutely irradiated (Pα × Pβ)F1 mice along with T cell-depleted F1 bone marrow as a source of responder APC. These mice responded to GL¢, which demonstrates that both Ir-GL¢ genes do not have to be present in the T lymphocyte to generate a proliferative response to GL¢.

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)F1 [(A × B)F1],...
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 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 of a 1:40 dilution of rabbit anti-mouse brain antiserum (RaMB) per 10$^7$ cells for 30 rain at room temperature, followed by excess guinea pig complement (4 ml of a 1:3 dilution). The RaMB was shown not to contain appreciable anti-stem-cell activity in a $[^{3}H]$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 δ,ε-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 Sharp & 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 $\rightarrow$ $F_1$ chimeras, responder presenting cells were provided for priming by transferring $10^7$ T cell-depleted $F_1$ bone marrow cells together with $5 \times 10^7$ 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 GI.4b in $F_1 \rightarrow P_a$ 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, $F_1 \rightarrow P_a$ 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),

![Figure 1: Antigen-specific T cell proliferation (Δcpm) is plotted against weeks after bone marrow reconstitution of lethally irradiated mice. All chimeras are $F_1 \rightarrow P_a$ 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 RaMB treatment (Materials and Methods). Antigen-specific T cells emerged at 9–12 wk and were of host Ir phenotype.](image-url)
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 RaMB treatment of the marrow cells in vitro (Materials
and Methods). Such exhaustively depleted marrow from (5R × A)F1 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)F1 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, F1 → 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)F1 → 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 demon-
strating the presence of functional B10.A(5R) restriction elements in these animals.
Thus, the failure of the genotypic (5R × A)F1 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 Pa + Pb → F1 Chimeras Behave Like F1 T Cells when Primed in the Presence
of Sufficient F1 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)F1 mice.
TABLE I
The Ability of Parental, F1, and F1 → Parent Chimeric Spleen Cells to Present Antigen to Parental and F1 → 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 |
| Continuous antigen | 5.009 ± 0.000 | 82.211 ± 8.100 | 90.202 | 1.946 ± 0.000 | 73.544 ± 5.000 | 71.588 | 3.183 ± 1.000 | 66.495 ± 3.300 | 61.312 | 54.911 ± 4.000 | 51.728 |
| B10.A spleen* | 3.542 ± 0.000 | 53.628 ± 5.300 | 48.086 | 2.881 ± 0.000 | 42.697 ± 3.900 | 40.086 | 1.799 ± 1.400 | 14.071 ± 3.100 | 7.272 | 11.925 ± 1.500 | 6.386 |
| B10.A(5R) spleen* | 5.617 ± 0.000 | 8.163 ± 4.000 | 7.246 | 9.132 ± 0.000 | 13.018 ± 2.100 | 3.886 | 3.347 ± 0.600 | 61.857 ± 5.800 | 58.910 | 22.618 ± 1.100 | 19.071 |
| (5R × A)F1 spleen* | — | — | — | 7.896 ± 0.000 | 29.968 ± 1.000 | 21.812 | 6.816 ± 1.000 | 42.169 ± 5.100 | 35.353 | 16.733 ± 2.100 | 9.917 |
| 5R × A → A spleen* | — | — | — | 8.191 ± 1.000 | 31.405 ± 3.700 | 23.006 | 6.422 ± 0.700 | 39.423 ± 3.000 | 31.001 | 17.371 ± 1.600 | 10.949 |

*In these experiments, 10⁵ antigen-pulsed or unpulsed (medium) B10.A, B10.A(5R), (5R × A)F1, or (5R × A)F1 → A chimeric spleen cells were added to 1.2 × 10⁶ (5R × A)F1 → A, B10.A, or B10.A(5R) PETLES in each well. 4 d later, [3H]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 [3H]thymidine incorporation between cultures receiving pulsed and unpulsed cells. Statistically significant responses are underlined.

TABLE II
A + 18R → (A × 18R)F1 Chimeric T Cells Respond to GLp when Primed in the Presence of Sufficient Responder APC

| Experiment | Source of PETLES | Proliferative response (cpm ± SFM) to |
|-----------|----------------|------------------|
|           | Medium | DNP-OVA | GLp | (TG)-A-I- | Pigeon extracellular | GLp |
| 1 | A + 18R → A × 18R | 6.219 ± 0.002 | 43.046 ± 4.128 | 50.09 ± 7.63 | 65.06 ± 4.80 | 14.77 ± 1.276 |
| 2 | A + 18R → A × 18R | 8.051 ± 1.07 | 77.213 ± 2.982 | 61.17 ± 1.07 | 51.41 ± 2.982 | 17.65 ± 1.276 |
| 3 | A + 18R → A × 18R | 8.902 ± 0.066 | 77.213 ± 2.982 | 61.17 ± 1.07 | 51.41 ± 2.982 | 17.65 ± 1.276 |
| 4 | A + 18R → A × 18R | 26.672 ± 0.012 | 60.172 ± 4.311 | 52.669 ± 2.982 | 45.623 ± 3.659 | 21.877 ± 1.417 |
| 5 | T(A × 18R) BM → A × 18R | 6.079 ± 0.104 | 76.02 ± 1.104 | 61.17 ± 1.07 | 51.41 ± 2.982 | 17.65 ± 1.276 |
| 6 | T(A × 18R) BM → A × 18R | 46.01 ± 0.022 | 77.213 ± 2.982 | 61.17 ± 1.07 | 51.41 ± 2.982 | 17.65 ± 1.276 |
| 7 | A + 18R → A × 18R | 3.187 ± 0.050 | 43.74 ± 5.202 | 3.818 ± 2.91 | 37.089 ± 2.912 | 4.489 ± 6.44 |
| 8 | A + 18R → A × 18R | 54.10 ± 0.032 | 87.20 ± 5.933 | 20.668 ± 4.37 | 25.927 ± 2.32 | 10.375 ± 3.09 |

The designation "−T" means T cell depleted. "BM" is bone marrow. "THYMX" refers to adult thymectomized mice. Underlined values are significantly different from the middle control by Student's t test. Lethally irradiated (A × 18R)F1 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). Nonspecific chimeric spleen cells were transferred to irradiated F1 mice, either alone (Exp. 2 and 3a) or along with T cell-depleted F1 bone marrow (Exp. 3b and 4a). In one case the recipient was thymectomized (Exp. 5). T cell-depleted bone marrow alone (Exp. 3c) or together with nonsponder parental spleen (Exp. 4b) were transferred to irradiated F1 as control experiments.
When these A + 18R → (A × 18R)F1 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(F) can respond, and pigeon cytochrome c, an antigen to which only the B10.A(F) 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 F1 cell type (2). Thus, rigorous depletion of T cells from the donor marrow did not alter these conclusions.

In A + 18R → (A × 18R)F1 chimeras, the T lymphocytes have developed in a responder F1 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 F1 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)F1, the adoptive recipient immunized immediately, and the PETLES response assayed 2 wk later in the presence of F1 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 F1 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-Aβ alleles, and, therefore, spleen cells from both strains of mice should present DNP-OVA to immune T cells recognizing I-Aβ. 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
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)-immune PETLES was compared with B10 and B10.A(3R) spleen cells. B10.A(3R) spleen cells possess both I-GLφ genes (one mapping in I-A<sup>B</sup>, the other in I-E<sup>B</sup>/C<sup>E</sup>) 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
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PETLES BIO. A PETLES

28 A DNP-OVA GL@ C

DNP-QVA 3R

Spllen 14 B 28

13 26 26

3R--B

~ 24 //Spleen 12 24

144x653

B10 Spleen X 20 B--3R 10 20

144x602

18 Spleen ! /~ 18

127x370

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)F1 chimeric T cells in the adoptive host. If adequate numbers of F1 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)F1 chimeric spleen cells into irradiated (A × 18R)F1 animals and added T cell-depleted (A × 18R)F1 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 F1 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 F1 bone marrow cells were not providing the T cells that were responding to the GLφ, the bone marrow was transferred alone into irradiated F1 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

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

Although the T cell-depleted F1 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 F1 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)F1 mice along with T cell-depleted F1 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 GL4 and pigeon cytochrome c. Finally, to unequivocally rule out the possibility of an F1 stem cell rapidly differentiating into a GL4-responder T cell, we performed adult thymectomy on (A × 18R)F1 animals and used them as adoptive hosts 6 wk later. A + 18R → (A × 18R)F1 chimeric spleen cells plus T cell-depleted F1 bone marrow transferred into thymectomized, lethally irradiated F1 animals showed responsiveness to all four antigens, DNP-OVA, (T,G)-A--L, pigeon cytochrome c, and GL4 as shown in Table II (Exp. 5). Therefore, the adoptive host’s thymus does not seem to play a role in the appearance of GL4 responsiveness in these animals.

Thus, A + 18R → (A × 18R)F1 chimeras behaved as a mixture of H-2a and H-2b cells until they were primed to antigen in an environment providing adequate F1 APC. Once this requirement was met, a phenotypic alteration could be detected in these parental T cells that had matured in an F1 environment. They appeared to have learned to respond to antigen in the context of F1 H-2 restriction elements.

A Two T Cell Model for Gene Complementation. The development of GL4-responsive T cells in A + 18R → (A × 18R)F1 chimeras 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 F1 restriction element when they mature in an F1 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-2a and H-2b donor T cells to interact without a mixed-lymphocyte reaction. In the F1 GL4 responder, one T cell specific for I-A^b plus GL4 and one T cell specific for I-E^d/C^d plus GL4 might interact to make an immune response only when GL4 is presented on an F1 (I-A^b, I-E^d/C^d) APC, which brings the two T cells together. Similarly, in the chimera with H-2a and H-2b T cells tolerant to each other, the GL4 response is revealed when F1 APC are provided. To test this hypothesis, we made (A × B)F1 → B10.A chimeras that were restricted to H-2a responses (DNP-OVA and pigeon cytochrome c responders) and (A × B) → B10 chimeras that were restricted to H-2b responses [DNP-OVA and (T,G)-A--L responders]. Neither type of chimera responded to GL4 (see Table IV). If two interacting T cells are required for a GL4 response, one specific for I-A^b plus GL4 and one specific for I-E^d/C^d plus GL4, then (A × B)F1 → B chimeric T cells could provide the former and (A × B)F1 → A chimeric T cells the latter. Therefore, we mixed (A × B)F1 → A and (A × B)F1 → B chimeric spleen cells, administered them intravenously to irradiated (A × B)F1 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-2a and H-2b specific T cells; however, no response to GL4 was observed. This was strong evidence against a two T cell model for gene complementation in the GL4 response and supported the interpretation that
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 $H^{-2^d}$ phenotype responses is a manifestation of suppression of all $H^{-2^d}$-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 $H^{-2^d}$ 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 $A + 18R \rightarrow (A \times 18R)F_1$ 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)F1 recipients, and the chimeras were immunized 3 mo later. As shown in Table IV, 4R \rightarrow (A \times 18R)F1 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 \rightarrow (A \times 18R)F1 chimeric spleen cells were transferred into
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irradiated (A × 18R)F1 mice along with T cell-depleted F1 bone marrow (Table IV, line b) good proliferative responses to DNP-OVA, (T,G)-A--L, pigeon cytochrome c, and GLα were seen. F1 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 GLα, 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 (Pα × Pβ)F1 genotype that had matured in a parental (Pα) environment were restricted to lysing virus-infected targets displaying Kβ or Dα. Despite their genotype, no cells with anti-Kβ or anti-Dβ plus virus specificity could be demonstrated, and control experiments (10) suggested that suppression could not explain the failure to detect such cells. Furthermore, genotypic Pα cytotoxic cells that had developed in a (Pα × Pβ)F1 environment acquired specificity to lyse virus-infected targets displaying Kα and/or Dα in addition to Kβ and Dβ targets; however, this could only be demonstrated when the chimeric cells were sensitized in an irradiated F1 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)F1 → 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 F1 → nonresponder thymus chimeras. For chimeras of the type A(low-responder) → (A × B)F1 Kappler and Marrack (17) as well as Hodes et al. (24) found that Pα chimeric T cells could help Pα macrophages and B cells to produce a secondary or primary anti-TNP-(T,G)-A--L response. But these Pα chimeric T cells could not convert Pα macrophages to responder phenotype (24). Thus, the T cell phenotype was altered by relaxing its genetic restriction to interact with cells expressing H-2β 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 ÷ 1-E/C gene products, that gene complementation involves the pairing of an I-E/C-encoded \(\alpha\)-chain with an I-A-encoded \(\beta\)-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 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(Glu55Lys36Pheg)n (GL~) requires two responder alleles that in the [B10.A × B10.A(18R)]F1 map in I-Ab and I-Eb/Cd. Chimeras in which a mixture of the nonresponder B10.A parental cells (which possess only I-Eb/Cd) and the nonresponder B10.A(18R) parental cells (which possess only I-Ab) were allowed to mature in a responder F1 environment did not respond to GL~(n), 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 → F1 chimeras were primed in the presence of responder antigen-presenting cells (APC), the chimeric T cells responded to GL~, which suggests that both responder alleles must be expressed in the APC but not necessarily in the T cell. Interestingly, acutely irradiated F1 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 × B100)F1 responder animals were allowed to mature in a low-responder B10 or B10.A parental environment, neither chimera, F1 → A or F1 → B, could respond to GL~. This demonstrated that the presence of high-responder APC, which derive from the donor bone marrow, was not sufficient to generate a GL~ 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-GL~ responder allele, could be educated to mount a GL~ 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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