GENETIC REGULATION OF DELAYED-TYPE HYPERSENSITIVITY RESPONSES TO poly(LTyr,LGlu)-poly(DLAla)--poly(LLys)

II. Evidence for a T-T-Cell Collaboration in Delayed-Type Hypersensitivity Responses and for a T-Cell Defect at the Efferent Phase in Nonresponder H-2^k Mice*

BY GIDEON STRASSMANN, ZELIG ESHHAR,‡ AND EDNA MOZES

From the Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel

Immune responses to the synthetic polypeptide poly(LTyr,LGlu)-poly(DLAla)--poly(LLys) [(T,G)-A--L][1] (1), such as humoral responses (2), delayed-type hypersensitivity responses (DTH) (3), and antigen-dependent T-cell proliferation, (4) are regulated by genes that are located in the major histocompatibility complex of the mouse (H-2). Thus, mice possessing the H-2^b haplotype are responders and mice of the H-$2^{a,d,f,k,s}$ haplotypes are nonresponders. In a previous study (3), we have demonstrated the existence of two types of nonresponder mouse strains: (*a*) H-2^a and H-2^k haplotypes; educated T cells of these strains could mediate DTH responses to (T,G)-A--L in the appropriate (responder × nonresponder)F_1 mice but not in syngeneic recipients, and (*b*) H-2^f and H-2^s haplotypes; T cells of these strains could not be activated to mediate DTH responses in any recipient. These studies suggested that the defect in DTH responses to (T,G)-A--L in H-2^a and H-2^k mice is not expressed on their T cells or on their antigen-presenting cells in the activation phase.

In this study we have analyzed the efferent phase of the DTH response to (T,G)-A--L to identify the cell type which expresses the genetic defect. Here we have demonstrated that, for efficient DTH responses, T-T-cell collaboration is required. In addition, we have located the genetic defect of nonresponder (H-2^k and H-2^a) mice in the efferent stage of the immune process, namely, in a second T-cell population that is needed for the manifestation of specific DTH reactions to (T,G)-A--L.

Materials and Methods

Animals. C57BL/6, C57BL/6 nu/nu, C3H.SW, C3H/DiSn, C57BL/10.A (B10.A), (C3H/DiSn × C3H.SW)F_1, (C3H/DiSn × C57BL/6 Ly 1^a)F_1, and (B10.A × B10)F_1 mice of 2–3 mo

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[1] *Abbreviations used in this paper*: "B" mice, 4-wk-old C3H.SW mice that were thymectomized and, 6 wk later, irradiated and supplemented with 20 × 10^6 bone marrow cells after treatment with anti-Thy-1.2 serum and complement; DTH, delayed-type hypersensitivity; ^{125}IUdR, [^{125}I]5-iodo-2′-deoxyuridine; L, left; R, right; (T,G)-A--L, poly(LTyr,LGlu)-poly(DLAla)--poly(LLys).

of age were used. Inbred mice were obtained from the Experimental Animal Unit of The Weizmann Institute of Science, Rehovot, Israel, and F_1 hybrids were bred at the Department of Chemical Immunology, The Weizmann Institute of Science.

C3H.SW "B" mice were prepared as follows: mice of 4 wk of age were thymectomized, 6 wk later, they were irradiated and supplemented with 20×10^6 bone marrow cells after treatment with anti-Thy-1.2 serum and complement ("B" mice). Mice were used 4–6 wk later.

Antigen. The multichain synthetic polypeptide (T,G)-A--L was synthesized and characterized as previously described (5).

In Vivo Generation of Educated T Cells. Thymocytes (10^8) were injected intravenously into syngeneic recipients irradiated with 800 rad (cobalt). Recipients were immunized with 20 μg of (T,G)-A--L in complete Freund's adjuvant (CFA) (H37 RA; Difco Laboratories, Detroit, Mich.) intraperitoneally. Spleens containing the T-educated cells were removed 7 d later and the single cell suspensions prepared were transferred into naive recipients.

Measurement of DTH Responses. Viable educated cells (25×10^6) were irradiated (1,200 rad) before transfer into naive recipients. DTH activity was determined according to Vadas et al. (6). 16 h after cell transfer, recipients were challenged by an injection of 10 μl of 2 mg/ml antigen intradermally in the right ear. 10 h after challenge, mice received 0.1 ml of a 1.0-mM solution of 5-fluorodeoxyuridine and 30 min later, 2 μCi of [^{125}I]5-iodo-2′-deoxyuridine (^{125}IUdR). Mice were killed 24 h after challenge, ears were cut and the amount of radioactivity was determined in a gamma counter (Packard Instrument Co., Downers Grove, Ill.). The results are expressed as the ratio of radioactivity in the right (R) ear to that of the left (L) ear (R/L ^{125}IUdR uptake). Positive DTH response was considered when the R/L ^{125}IUdR uptake was >1.2. The results are expressed as the arithmetic mean of all mice in the group ± SE. *P* values were calculated by the Student's *t* test.

Cell Fractionation. Adherent cells were obtained by incubating normal spleen cells suspended in minimal essential medium + 5% fetal calf serum for 90 min at 37°C on plastic Petri dishes (Nunc, Denmark). Nonadherent cells were obtained by two washes of the above-mentioned incubated cells. T cells-enriched population were prepared by passing nonadherent cell suspensions on a nylon-wool column according to Julius et al. (7)

Antisera Production. Anti-Lyt-2.2 was obtained from (C3H/eB × C57BL/6-Ly-2^b)F_1 mice injected with C57BL/6 thymocytes and was kindly provided by Dr. Peter Lonai of the Weizmann Institute of Science. Anti-Lyt-1.1 was obtained from (C57BL/6 × BALB/c)F_1 hybrid mice injected with C57BL/6-Ly-1^a thymocytes. Anti-Lyt-2.1 was obtained from C57BL/6-H-2^k mice injected with CE/J thymocytes and was kindly provides by Dr. Alfa Peled of the Weizmann Institute of Science.

Treatment of Cells with Antisera. To 1 ml of cells at a concentration of 30×10^6/ml, 1 ml of antiserum was added to a final concentration of 1:30 for anti-Lyt-1.1 and anti-Lyt-2.1 and 1:10 for anti-Lyt-2.2. The cells were left at 4°C for 20 min, washed, and resuspended in rabbit complement of low cytotoxicity (Cederlane Laboratory Ltd., London, Ontario, Canada) used at 1:10 dilution for 45 min at 37°C.

Results

T-T-Cell Collaboration in DTH Responses to (T,G)-A--L. In a previous study (3), we have shown that the inability of H-2^k- and H-2^a-activated T cells to mediate DTH responses to (T,G)-A--L is a result of a defect in the efferent phase of this reaction. To localize the defect in nonresponder mice, we first investigated the possible need for a second T cell in the manifestation of DTH responses. (T,G)-A--L-specific educated cells of responder origin were transferred into either normal or T cell-depleted recipients. The educated cells were irradiated before transfer into the various recipients, as we have previously shown that irradiation of these cells is required to eliminate suppressor activity (8). Data presented in Table I demonstrate that significant DTH responses were observed when C57BL/6 educated cells were transferred into normal recipients but not into either homozygous, congenitally nude (nu/nu), or neonatal, thymectomized mice of the same H-2 haplotype. We repeated the experiments using

TABLE I

The Inability of Educated T Cells to Mediate DTH Responses in T Cell-depleted Syngeneic Mice

Educated cell donor*	Recipient strain	No. of responders per group	R/L ^{125}IUdR uptake ± SE‡
C57BL/6	C57BL/6	7/8	1.67 ± 0.14
C57BL/6	C57BL/6 nu/nu	0/4	1.04 ± 0.02
C57BL/6	C57BL/6 neonatal thymectomized	0/6	0.94 ± 0.08

* 25×10^6 educated cells were irradiated (1,200 rad) before transfer into naive recipients.
‡ Significant difference from C57BL/6 neonatal thymectomized group: $P < 0.01$.

TABLE II

*The Requirement for a Second Population of T Cells for the Manifestation of DTH Responses to (T,G)-A--L**

Educated cell donor	Recipient strain	Additional cells transferred‡	No. of responders per group	R/L ^{125}IUdR uptake ± SE§
C3H.SW	C3H.SW	—	6/6	1.74 ± 0.05‖
C3H.SW	C3H.SW "B" mice	—	1/6	1.08 ± 0.07
C3H.SW	C3H.SW "B" mice	15×10^6 effluent from nylon-wool column	5/6	1.75 ± 0.15‖
C3H.SW	C3H.SW "B" mice	12×10^6 adherent cells**	0/5	0.96 ± 0.07
C3H.SW	C3H.SW "B" mice	20×10^6 nonadherent cells after treatment with anti-Thy-1.2 serum and complement	1/6	0.95 ± 0.08
C3H.SW	C3H.SW "B" mice	20×10^6 nonadherent cells after treatment with normal mouse serum and complement	5/6	1.49 ± 0.12¶

* 25×10^6 educated cells were irradiated (1,200 rad) and transferred with different additional cells to "B" mice or normal syngeneic mice.
‡ Non-antigen-stimulated spleen cells.
§ Significant difference between two groups in each section:
‖ $P < 0.002$.
¶ $P < 0.01$.
** Plastic-adherent cells.

"B" mice. As shown in the upper part of Table II, such mice were also unable to manifest DTH responses when injected with educated, irradiated T cells. Reconstitution of "B" mice with 15×10^6 nylon-wool-enriched normal T cells before transfer of activated cells led to significant DTH responses that were comparable to those obtained in syngeneic, normal recipients of educated cells (Table II). No DTH responses were elicited by the reconstitution of "B" mice with 12×10^6 normal adherent cells. Treatment of nonadherent spleen cells with anti-Thy-1.2 serum and complement before the reconstitution of "B" mice, obliterated the response (Table II). These data highly suggest that for the manifestation of DTH responses to (T,G)-A--L, the presence of a second, normal T-cell population is required in addition to antigen-activated T cells.

T-Cell Defect in the Efferent Phase of DTH Responses to (T,G)-A--L in Nonresponder H-2^k Mice. As we have previously reported (3) and as shown in the upper part of Table

TABLE III

*The Ability of (C3H/DiSn × C3H.SW)F_1 T Cells to Collaborate with C3H/DiSn Nonresponder Educated T Cells for the Manifestation of DTH Responses in C3H/DiSn Recipients**

Group	Educated cell donor	Recipient strain	Additional cells transferred‡	No. of responders per group	No. of Exp.	R/L ^{125}IUdR uptake ± SE§
A	C3H/DiSn	C3H/DiSn	—	2/17	3	1.02 ± 0.05
B	C3H/DiSn	(C3H/DiSn × C3H.SW)F_1	—	23/27	3	1.46 ± 0.06‖
C	C3H/DiSn	C3H/DiSn	12×10^6 adherent cells	0/5	1	1.04 ± 0.05
D	C3H/DiSn	C3H/DiSn	20×10^6 nonadherent cells	4/6	1	1.36 ± 0.09‖
E	C3H/DiSn	C3H/DiSn	15×10^6 nonadherent cells effluent from nylon-wool column	15/18	3	1.41 ± 0.06‖
F	C3H/DiSn	C3H/DiSn	20×10^6 nonadherent cells treated with anti-Thy-1.2 serum and complement	1/9	2	1.04 ± 0.04
G	C3H/DiSn	C3H/DiSn	20×10^6 nonadherent cells treated with normal mouse serum and complement	6/8	2	1.38 ± 0.11¶
H	C3H/DiSn	C3H/DiSn	15×10^6 nonadherent cells effluent from nylon-wool column, irradiated (1,000 rad)	2/11	2	1.05 ± 0.08
I	C3H/DiSn	C3H/DiSn	15×10^6 nonadherent cells effluent from nylon-wool column, irradiated (500 rad)	1/5	1	0.99 ± 0.07

* 25×10^6 viable irradiated, educated cells were transferred together with different F_1 cells into C3H/DiSn naive recipients.
‡ Normal (C3H/DiSn × C3H.SW)F_1 spleen cells. Separation into adherent and nonadherent cells was performed on plastic culture plates.
§ Significant differences were determined between groups B from A; D and E from C; G from F:
‖ $P < 0.01$.
¶ $0.01 < P < 0.02$.

TABLE IV

The Ability of (B10.A × B10)F_1 T Cells to Collaborate with B10.A Nonresponder Educated T Cells for the Manifestation of DTH Responses in B10.A Recipients

Educated cell donor	Recipient strain	Additional cells transferred*	No. of responders per group	R/L ^{125}IUdR uptake ± SE‡
B10.A	B10.A	—	1/5	0.95 ± 0.07
B10.A	(B10.A × B.10)F_1	—	7/8	1.45 ± 0.11§
B10.A	B10.A	15×10^6 cells effluent from nylon-wool column	5/6	1.37 ± 0.10‖
B10.A	B10.A	15×10^6 cells effluent from nylon-wool column, irradiated (500 rad)	0/4	1.05 ± 0.07

* Normal (B10.A × B10)F_1 spleen cells.
‡ Significant difference between the two experimental groups to the first group:
§ $P < 0.01$.
‖ $0.02 < P < 0.05$.

II, (T,G)-A--L-educated T cells of H-2^k origin could mediate DTH responses to (T,G)-A--L in appropriate (responder × nonresponder)F_1 mice but not in syngeneic recipients. Thus, there is no defect in the activation phase to (T,G)-A--L in nonresponder H-2^k mice. To identify which cell population is expressing the defect in this cellular response to (T,G)-A--L, we have tested the ability of adherent, nonadherent, and nylon-wool-enriched cells of normal (H-2^k × H-2^b)F_1 spleen cells to correct the defect of naive H-2^k recipients when transferred together with syngeneic, educated, irradiated

TABLE V

Lyt Phenotype of the Educated T Cells that Mediate DTH to (T,G)-A--L

Treatment of cells before transfer*	No. of responders per group	R/L ^{125} IUdR uptake ± SE‡
Anti-Lyt-1.1 and complement	1/5	1.10 ± 0.11
Anti-Lyt-2.1 and complement	5/5	1.67 ± 0.18§
Complement alone	4/5	1.45 ± 0.08

* After treatment with the various reagents, 25 × 10^6 viable educated cells of C3H/DiSn were transferred into (C3H.SW × C3H/DiSn)F_1 naive recipients.
‡ Significant difference between the two upper groups:
§ $P < 0.01$.

cells. As can be seen in Table III, nonadherent cells and nylon-wool-enriched T cells, but not adherent cells, could collaborate with irradiated, educated cells for the manifestation of DTH responses in C3H/DiSn nonresponder naive recipients. More nonadherent cells (20 × 10^6) than adherent cells (12 × 10^6) were transferred because the nonadherent cell population contained B cells as well as T cells. The additional cells transferred were sensitive to treatment with anti-Thy-1.2 serum and complement. (Table III, group F). These results indicate that the cells expressing the defect in H-2^k nonresponder mice are of the T lineage. Irradiated (1,000 or 500 rad) nylon-wool-enriched T cells could not collaborate with educated, irradiated cells for the manifestation of DTH responses in normal H-2^k mice (Table III, groups H and I), indicating that the second T-cell population required for DTH responses is radiosensitive.

Similar results were obtained using the B10.A (H-2^a) nonresponder mouse strain (Table IV). 25 × 10^6 educated, irradiated B10.A cells failed to elicit DTH response in syngeneic recipients. However, 15 × 10^6 nylon-wool-enriched, normal (B10 × B10.A)F_1 cells but not 500-rad-irradiated cells did reconstitute the response.

Lyt Phenotype of the T Cells that Participate in DTH Responses to (T,G)-A--L. Lyt-specific antisera have been useful in the characterization of T cell subpopulations required for various functions. Therefore, we have utilized these antisera to determine the Lyt phenotypes of the two T cells which participate in the (T,G)-A--L-specific DTH reaction. As shown in Table V, treatment of C3H/DiSn educated T cells with anti-Lyt-1.1 sera and complement abolished their ability to mount DTH responses in (C3H.SW × C3H/DiSn)F_1 naive recipients. Anti-Lyt-2.1 sera and complement, used as a control, did not affect the function of the educated T cells. Thus, the educated T cells mediating the DTH responses to (T,G)-A--L are of the Lyt-1^+ phenotype. Table VI demonstrates the characterization of the second T cell required for DTH responses. Treatment of normal (C3H/DiSn × C57BL/6-Ly-1^a)F_1 nonadherent splenic cells with anti-Lyt-1.1 serum and complement abolished the ability of such cells to collaborate with educated cells and to manifest DTH responses in H-2^k recipients, whereas treatment with either anti-Lyt-1.1 alone or with normal mouse serum and complement did not affect this ability. Furthermore, treatment of normal (B.10 × B10.A)F_1 nonadherent splenic cells with anti-Lyt-2.2 serum and complement abrogated the DTH responses. Because there is no segregation between the Lyt 2 and the Lyt 3 antigens, these data suggest that the second T cell required for the manifestation of DTH to (T,G)-A--L is of the Lyt $1^+,2^+,3^+$ phenotype. It should be noted that titrations were performed with the anti-Lyt sera used in the experiments described in

TABLE VI

Lyt Phenotype of the Second T Cell Involved in DTH Responses to (T,G)-A--L

Origin of the second T cells transferred*	Treatment of cells before transfer	No. of experiment	No. of responders per group	R/L ^{125}IUdR uptake ± SE‡
(C3H/DiSn × C57BL/6-Ly1^a)F$_1$	Anti-Lyt-1.1 serum and complement	2	1/12	1.07 ± 0.04
(C3H/DiSn × C57BL/6-Ly1^a)F$_1$	Anti-Lyt-1.1 serum alone	1	6/6	1.41 ± 0.07§
(C3H/DiSn × C57BL/6-Ly1^a)F$_1$	Normal mouse serum and complement	1	6/7	1.42 ± 0.10§
(B10.A × B10)F$_1$‖	Anti-Lyt-2.2 serum and complement	2	1/11	1.07 ± 0.05
(B10.A × B10)F$_1$	Normal mouse serum and complement	2	11/13	1.60 ± 0.08§

* After treatment with corresponding reagents, 20 × 10^6 viable nonadherent F$_1$ splenic cells were mixed with 25 × 10^6 irradiated, educated C3H/DiSn cells and injected intravenously into each C3H/DiSn recipient.

‡ Significant differences between groups treated with anti-Lyt serum and complement to the control groups:

§ $P < 0.01$.

‖ B10.A shares with C3H/DiSn the K end of the I region of the H-2 complex. Therefore (B10.A × B10)F$_1$ nonadherent cells which carry the Lyt 2.2 and Lyt 1.2 phenotypes were chosen as a source of the second T cell.

Tables V and VI and the antisera proved to be specific for the cell combinations used in our experiments.

Discussion

DTH responses result from a complex of intercellular interactions. T cells mediating DTH are activated by antigen presented on macrophages adjacent to H-2 gene products (9, 10). Upon restimulation with antigen, activated T lymphocytes release lymphokines which recruit infiltrating cells to the injected site (11). In this report, we have demonstrated the participation of a second, normal T-cell population in the above-described process. Thus, when H-2^b- (responder strain) educated, irradiated cells were transferred into syngeneic mice depleted of T cells, no DTH responses to (T,G)-A--L were observed (Tables I and V). The nonresponsiveness of "B" mice was restored after receiving effluent cells that did not bind to nylon-wool column but not adherent cells. These results and the sensitivity of the second cell population to low doses of irradiation (in vitro), to anti-Thy-1.2, and to anti-Lyt treatment (Table V) strongly suggest that the second cell that participates in the DTH reaction to (T,G)-A--L is of the T lineage. To our knowledge, this is the first demonstration that two distinct T-cell subpopulations are required for the manifestation of DTH responses. Synergism between two distinct T-cell populations has been reported for various T-cell functions such as rejection of syngeneic SV-40-induced sarcoma in mice (12), cell-mediated cytotoxicity (13, 14), and helper activity (15). As both anti-Lyt-1 and anti-Lyt-2 antibodies obliterated the ability of the second cell type to cooperate with the (T,G)-A--L-educated T cells, and if we assume that one additional cell type is required for the T-T-cell cooperation, it is suggested that this cell is of the Lyt 1$^+$,2$^+$,3$^+$ phenotype. T cells which mediate DTH responses were found to express the Lyt 1$^+$

phenotype (16, 17). In our system, the (T,G)-A--L-educated T cells are also of this phenotype (Table V). Thus, it appears that educated Lyt 1^+ cells cooperate with Lyt $1^+,2^+,3^+$ cells in DTH reaction specific to (T,G)-A--L. Although Lyt $1^+,2^+,3^+$ cells represent ~50% of peripheral T lymphocytes (18), the functions of these cells have not been as clearly identified. Such cells appear to be precursors of T-cell subpopulations (19). We suggest here a functional effect in the DTH response mediated by Lyt $1^+,2^+,3^+$ cells as a result of interaction with Lyt 1^+ cells. A similar interaction between antigen activated Lyt 1^+ helper cells and a second, nonimmune set of T cells (Lyt $1^+,2^+,3^+$ Qa 1^+) was reported to result in a suppressive activity which plays a role in regulating immune responses (20).

In a preceding paper (3) we have shown that the defect in the inability of nonresponder (H-2^k and H-2^a) mice to mediate DTH responses is in the efferent phase of this reaction. Thus, H-2^k and H-2^a T cells could be activated with (T,G)-A--L to mediate DTH responses in the appropriate F_1 responder strain but not in syngeneic recipients. In the present study we identified and characterized the cell which expresses the genetic defect in these mouse strains as a radiosensitive T cell with the Lyt $1^+,2^+,3^+$ phenotype.

A defect at the level of the antigen-presenting cell has been suggested by Miller (10) who studied DTH reactions to the copolymer L-glutamic $acid^{60}$-L-$alanine^{30}$-L-$tyrosine^{10}$, designated GAT (10), the immune response to which is also genetically controlled. Indeed, when the genetic defect in the ability of H-2^s mice to mount DTH responses to (T,G)-A--L was studied, we have observed that neither T cells of H-2^s origin nor of the responder (H-2^b × H-2^s)F_1 origin could be activated on antigen-pulsed adherent cells of the H-2^s strain, suggesting also a defect in the latter, nonresponder mice at the antigen-presenting cell (G. Strassmann, Z. Eshhar, and E. Mozes. Manuscript in preparation.). On the other hand, we have shown here that in the group of nonresponders, which includes mice of the H-2^k and H-2^a haplotypes (3), the genetic defect in the ability to mediate DTH responses to (T,G)-A--L is expressed on the level of a non-antigen-stimulated, radiosensitive T cell. The successful collaboration of this T cell with an antigen-activated, radioresistant T cell is required for the manifestation of the DTH reaction.

Summary

The intercellular interactions and the site of the genetic defect in delayed-type hypersensitivity (DTH) response to poly(LTyr,LGlu)-poly(DLAla)--poly(LLys) [(T,G)-A--L] has been studied in a system where the T-cell education phase was separated from the efferent phase. In this cellular response, T-T-cell collaboration is required, because T cell-depleted mice were unable to manifest DTH responses after they were transferred with educated and irradiated T cells. Reconstitution of adult thymectomized mice that were irradiated and supplemented with bone marrow cells after treatment with anti-Thy-1.2 serum and complement, with T cells but not with accessory cells gave rise to significant responses. Educated, radioresistant cells required the presence of normal radiosensitive T cells for successful DTH responses to (T,G)-A--L. The genetic defect of nonresponder H-2^k and H-2^a mice has been located in the above-mentioned, second T-cell population that participates in the efferent phase of this immune reaction. Further characterization revealed that the educated cells are of the Lyt1^+ phenotype and that the second normal T cells are expressing the Lyt

$1^+,2^+,3^+$ phenotype. Thus, the genetic defect of H-2^k and H-2^a mice in the DTH response to (T,G)-A--L is expressed on the non-antigen-stimulated Lyt $1^+,2^+,3^+$ T cells.

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