Brief Definitive Report

GENETIC CONTROL OF SPECIFIC IMMUNE SUPPRESSION

IV. Responsiveness to the Random Copolymer L-Glutamic Acid^{50}-L-Tyrosine^{50} Induced in BALB/c Mice by Cyclophosphamide*

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Previous reports from our laboratory have demonstrated the stimulation of specific suppressor T cells in genetic nonresponder mice after immunization with the terpolymer of L-glutamic acid, L-alanine, and L-tyrosine (GAT) (1, 2) and with the copolymer of L-glutamic acid and L-tyrosine (GT) (3–5). These findings raise two important questions: (*a*) do the specific suppressor T cells inhibit an antibody response which would otherwise develop in nonresponder mice; and, (*b*) can specific helper T-cell activity be detected in these animals. Responsiveness appears to be completely dominant over suppression in (responder × suppressor)F_1 hybrids, therefore, we have been unable to detect suppressor cells in these hybrids after conventional immunization with GAT (2). However, using special conditions of antigen administration, GAT helper activity could be demonstrated in nonresponder DBA/1 ("suppressor") mice. Thus, GAT-specific helper activity was not detected in these nonresponder animals after immunization with GAT irrespective of the adjuvant used, but could be stimulated by macrophage-bound GAT or by GAT complexed with methylated bovine serum albumin GAT-MBSA (6).

In the current report we have taken advantage of the fact that suppressor T-cell activity is more sensitive to cyclophosphamide treatment than T-cell helper activity (7) to demonstrate the presence of GT-specific helper activity in "nonresponder" BALB/c mice. We describe: (*a*) the dose of cyclophosphamide and conditions of treatment which inhibits the well-documented stimulation of specific suppressor T cells in BALB/c mice injected with GT previous to immunization with GT-MBSA, and (*b*) the ability of cyclophosphamide to permit the development of primary PFC responses to GT in these "nonresponder" mice.

These cyclophosphamide-induced responses are not characterized by the high levels of antibody detected in genetic responder animals.

Materials and Methods

Mice. The mice were purchased from The Health Research Laboratories, Buffalo, N. Y., or were bred in our animal facilities.

Antigens. The antigens and hemolytic plaque assay used in these experiments are the same as those described in the companion paper (5).

Immunization and Treatment with Cyclophosphamide. Cyclophosphamide (Cytoxan) was purchased from Mead-Johnson Laboratories, Evansville, Ind. The drug was administered intraperitoneally in the dose of 200 mg/kg. To investigate the effect of the drug on the suppressive activity of

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GT, BALB/c mice were injected intraperitoneally with cyclophosphamide followed 2 days later with 100 μg GT in a mixture of magnesium and aluminum hydroxides (Maalox, William H. Rorer, Inc., Fort Washington, Pa.) or with Maalox alone. 10 days later, the mice were immunized intraperitoneally with 10 μg of GT as GT-MBSA emulsified in an equal volume of complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, Mich.). To study the effect of cyclophosphamide on responsiveness to GT, other groups of BALB/c mice were injected with the drug or with physiological saline intraperitoneally (Group V and VI of Table I) and 12 days later immunized with 100 μg GT in CFA.

GT-Specific Suppressor Extract and Control Maalox Extract. GT-specific suppressor extract was prepared by ultransonification and ultracentrifugation of thymocytes and spleen cells from BALB/c mice immunized 3 days previously with 100 μg GT in Maalox, as described previously (8, 9). As control, an extract referred to as Maalox extract was prepared similarly from cells of animals injected with Maalox alone. The details of the preparations and the properties of the GT-specific suppressor factor on the primary PFC response of BALB/c to GT-MBSA will be described separately.[1] The GT-specific suppressor extract behaved like the GAT-specific suppressor factor(s) previously described by this laboratory (9).

Antigen-Binding Assay. The humoral response to GT was measured by antigen-binding assay employing the homologous GT copolymer. GT was iodinated by the chloramine-T method with carrier-free ^{125}I (New England Nuclear, Boston, Mass.) and separated from inorganic iodide by passage over 0.5 × 25-cm columns of Sephadex G-25F (Pharmacia Fine Chemicals, Piscataway, N. J.). Serum samples diluted 1:5 with phosphate-buffered saline were assayed by modified Farr assay which has been described previously (10).

Results and Discussion

As shown in Table I, 12 days were allowed to elapse between treatment with cyclophosphamide and immunization with GT-MBSA to permit recovery of B-cell function. The comparable responses of Group I and III indicate that cyclophosphamide in the dose and time used did not affect the primary response to GT-MBSA when assayed 7 days later. The suppressive effects normally induced by GT preimmunization, however, were completely abolished by this treatment (compare Groups I and II and II and IV, respectively). This effect may be interpreted to reflect the inhibitory activity of the drug on the stimulation of GT-specific suppressor T cells.

But the most interesting result is the development of GT-specific primary IgG responses in animals treated with cyclophosphamide 12 days earlier (Groups V and VI). The response to GT immunization after this treatment were unequivocal and did not differ significantly from the responses of control mice to GT-MBSA (Groups I and VI); however, no specific IgM responses have been detected in animals treated with cyclophosphamide.

The antigen-binding values of the sera from animals treated with cyclophosphamide presented in Table II show that the responses to GT in these animals are seen early after primary immunization and do not progress in spite of secondary challenge. On the whole, these responses are considerably weaker than are observed in genetic responder (C3H × GT Swiss responder)F_1 mice not treated with cyclophosphamide. The reason for the limited anti-GT responses observed may be the weak responsiveness of the BALB/c animals or the recovery of suppressor cell activity after the cyclophosphamide treatment or both. Responsiveness to GT is only observed in some Swiss mice (3). The data in Table II on the responses of (C3H × GT Swiss responder)F_1 mice, which segregate into

[1] Debré, P., C. Waltenbaugh, and B. Benacerraf. Manuscript in preparation.

TABLE I
Effect of Cyclophosphamide on GT-Specific Suppression in BALB/c Mice

Group	No. animals per group	Day 0	Day 2	Day 12	IgG-Specific PFC/ spleen	P value
					Arith. mean ± SE	
I	9	Saline	Maalox	GT-MBSA*	9,800 ± 1,256	<0.0001
II	11	Saline	GT‡	GT-MBSA	2,390 ± 893	
III	12	Cyclophosphamide§	Maalox	GT-MBSA	12,864 ± 2,220	NS
IV	12	Cyclophosphamide	GT	GT-MBSA	14,050 ± 2,807	
V	10	Saline	–	GT‖	605 ± 525	<0.0003
VI	18	Cyclophosphamide	–	GT	6,705 ± 1,071	

* 10 μg GT as GT-MBSA in CFA intraperitoneally.
‡ 100 μg GT in Maalox intraperitoneally.
§ 200 mg of cyclophosphamide/kg intraperitoneally.
‖ 100 μg GT in CFA intraperitoneally.

TABLE II
Effect of Cyclophosphamide on GT Antibody Responses in BALB/c Mice

Strain	Immunization schedule			GT binding ± SE (no. of mice)	
	Day 0	Day 2	Day 11	Day 11	Day 23
				%	
BALB/c	–	GT*	GT	−1.9 ± 2.3 (5)	1.5 ± 3.9 (5)
BALB/c	Cyclophosphamide‡	GT	GT	11.3 ± 1.1 (15)	11.6 ± 3.6 (9)
(C3H × GT Swiss responder)F_1	–	GT	–	–	35.4 ± 6.5 (7)
(C3H × GT Swiss responder)F_1	–	GT	–	–	4.1 ± 2.7 (7)

* GT 100 μg in CFA. BALB/c Day 11 binding values were significantly different $P > 0.0001$.
‡ Cyclophosphamide 200 mg/kg.

TABLE III
Effect of Cyclophosphamide on Specific Suppression by GT Extract in BALB/c Mice

Group	No. animals per group	Day 0	Day 9	Day 12 GT-MBSA‡	IgG-Specific PFC/ spleen	Suppression	P value
					Arith. mean ± SE	%	
I	5	Cyclophosphamide*	Maalox	–	6,775 ± 1,418		
II	5	Cyclophosphamide	GT	–	5,970 ± 2,583	12	NS
III	5	Saline	–	Maalox extract	6,540 ± 842		
IV	5	Saline	–	GT extract	560 ± 290	91	<0.0001
V	7	Cyclophosphamide	–	Maalox extract	5,300 ± 1,075		
VI	7	Cyclophosphamide	–	GT extract	2,021 ± 679	62	<0.02

Same legends as Table I.
* 200 mg of cyclophosphamide/kg intraperitoneally.
‡ On day 12 all mice were given the indicated extract plus 10 μg GT as GT-MBSA in CFA, i.p.

equal numbers of responders and nonresponders, demonstrate that the parental Swiss responder mice were heterozygous for the GT gene(s).

The demonstration of the suppressor activity for the GT-MBSA responses of extracts prepared from thymus and spleen cells of GT-primed mice[1] raised the question whether treatment with cyclophosphamide inhibits the stimulation of suppressor activity or renders the cells of the immune spleen insensitive to the suppressive activity of the GT-specific "suppressor factor." This issue was explored in the experiments in Table III.

BALB/c mice were injected with 0.5 ml of 1/2 dilution of extracts prepared from 3×10^8 thymus and spleen cells/ml from animals immunized with 100 μg

GT in Maalox or Maalox alone 3 days previously. The extracts were injected intravenously on the day when the animals were immunized with GT-MBSA. The data show that cyclophosphamide treatment inhibits GT-specific suppression normally caused by GT preimmunization but not by the injection of GT-specific suppressor extracts. The suppressive activity observed in the normal animals treated with suppressor extract was greater than that seen in animals treated with cyclophosphamide. However, the difference between Groups IV and VI was not statistically significant.

The results of these experiments imply that the helper and suppressor activity for distinct thymus-dependent antigens is under a delicate balance controlled by genes within the *I* region of the *H-2* complex. The critical problem remaining to be resolved is the mechanism whereby specificity for the antigens is manifested by the processes controlled by individual Ir and Is genes, which have been shown to regulate immune responses to thymus-dependent antigens.

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