The Journal of Immunology
RESEARCH ARTICLE | JUNE 01 1980

Regulatory role of T suppressor cells on natural and induced humoral anti-tumor reactivity of C57BL/6J mice. FREE

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J Immunol (1980) 124 (6): 2530–2535.
https://doi.org/10.4049/jimmunol.124.6.2530

0022-1767/80/1246-2530$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 124, No. 6, June 1980
Printed in U.S.A.

REGULATORY ROLE OF T SUPPRESSOR CELLS ON NATURAL AND INDUCED HUMORAL ANTI-TUMOR REACTIVITY OF C57BL/6J MICE[1]

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C57BL/6J (B6) mice showed a natural humoral complement- (C) dependent cytotoxic anti-tumor reactivity starting at 6 months of age. A reverse relationship was found between spleen stimulation by PHA and level of natural anti-tumor antibody production. T-deprivation of the unresponsive 3-month-old mice induced the production of natural antibody in 40% of the mice tested. Stimulation with tumor cells of 3-month-old mice did not increase the level of the natural response, whereas it was effective in 12-month-old mice. When the 3-month-old unresponsive mice were irradiated and then transferred with spleen cells from either responsive or unresponsive old mice, the former became responsive, whereas the latter remained unresponsive. The inoculum of the unresponsive spleen cells together with the responsive ones abrogated the natural antibody production by the responsive spleen cells. This inhibition was abolished when the unresponsive spleen cells were treated with an anti-Thy-1 antiserum plus C. *In vitro* experiments confirmed the *in vivo* results, in that spleen cells from young unresponsive mice inhibited the *in vitro* antibody production by spleen cells from old mice, and the inhibition was abrogated or lowered by a treatment of the inhibiting cells with either an anti-Thy-1 or an anti-Ly-2.2 antiserum and C. When colchicine or cyclophosphamide, known to interfere with the activity of T-suppressor cells, was administered to 3-month-old unresponsive mice on the same day in which they were stimulated with blocked tumor cells, 53 to 60% of the mice became responsive, whereas an overall 6% responsive mice were found in control groups. The results suggest that T-suppressor cells, which exert a negative control on the production of natural antibodies, are present in the spleen of unresponsive mice.

Various murine strains have been shown to "spontaneously" react against tumor cells through cytotoxic lymphoid cells (1) or humoral antibodies (2–5). We have previously reported that C3Hf mice produced natural anti-tumor cytotoxic antibodies starting at 2 to 3 months of age, whereas other strains of mice, such as BALB/c and C57BL/6J (B6)[2] mice, showed sera naturally cytotoxic for tumor cells later in life, i.e., only when they were more than 6 months old (6–8). In a comparative study of the natural cytotoxic reactivity of the various strains, the early and the late responder mice have been shown to recognize different antigenic determinants, which however, all seemed to be virus related (8). On the other hand, the natural anti-tumor cytotoxic antibodies seem to be different from the natural virus-precipitating antibody found in various murine strains and directed against structural proteins of MuLV (9), since no correlation was found when individual sera were tested for both natural precipitating capacity of an ecotropic, N-tropic virus and natural anti-tumor cytotoxicity (10). The increased production of natural anti-tumor cytotoxic antibodies obtained following T deprivation in BALB/c mice and the inverse relationship found between T cell status of the animals and natural anti-tumor antibody level (7, 11) suggested that the natural reactivity for tumor cells in the late responder mice might be under the control of T cell subpopulations whose functions became impaired with age (12–14), thus allowing the spontaneous appearance of the natural response. The T cell subpopulations could interfere at either the level of the natural antibody synthesis or the level of production of the endogenous antigen(s) recognized by the natural response.

The purpose of this work was to further investigate the humoral natural response in B6 mice, another late responsive strain, and to try to elucidate the mechanisms of the negative T cell control, looking for a possible involvement of a population of T-suppressor cells, which regulate antibody production.

MATERIALS AND METHODS

Animals. C57BL/6J (B6) inbred mice of both sexes, maintained in this laboratory by brother × sister mating, were used.

Tumor. The chemically induced C57BL EL4 leukemia (15) routinely transplanted every 11 days by i.p. inoculation of 15 × 10^6 viable cells in B6 mice was used throughout the experiment as reference cell.

Sera. The sera were collected from the retro-orbital sinus of normal or experimental B6 mice and stored at −30°C. An anti-Thy-1.2 antiserum, obtained from AKR mice by hyperimmunization with C3Hf thymus cells, an anti-Ly-1.2 and an anti-Ly-2.2 antisera, obtained from the NIH serum bank, were employed to deprivate, by a complement- (C) dependent cytotoxicity assay, spleen cell suspensions of T cell subpopulations.

T deprivation and immunodepression. Two-month-old B6 mice were thymectomized by a median sternotomy under an-

Received for publication August 7, 1979.
Accepted for publication February 20, 1980.
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[1] This work was supported by a grant from Consiglio Nazionale delle Ricerche, Rome, Italy (Progetto Finalizzato Controllo della Crescita Neoplastica, Contract No. 78.02820.96).

[2] Abbreviations used in this paper: B6, C57BL/6J; HBSS, Hanks' balanced salt solution; CC, colchicine; CY, cyclophosphamide.

esthesia. The following day the thymectomized mice were whole-body irradiated (450 rads of gamma rays from a Siemens ^{60}Co Gammatron 3). The animals were used 1 month after the T deprivation. The immunodepression was obtained by total body sublethal irradiation (450 rads) of mice.

T cell separation. The anti-immunoglobulin- (IgG) coated glass bead columns were obtained by overnight incubation at 4°C of 50-ml glass beads (Potters Ballottini GmbH, Kirchheimbolanden, West Germany) with 4 ml mouse globulins obtained by saturated ammonium sulphate precipitation of normal serum. The beads were subsequently poured into a glass column (1.5 x 30 cm, Pharmacia, Uppsala, Sweden) and washed with 50 ml of phosphate-buffered saline (PBS). The columns were then filled with rabbit anti-mouse IgG serum with an excess of antibodies, left for 45 min at room temperature, and washed with 50 ml of PBS. A cell suspension in HBSS containing from 50 to 100 x 10^6 spleen cells, free of cell debris, was passed through the column using a flow rate of approximately 2 ml/min.

Spleen cell cultures. Lymphocyte cultures were prepared with 5 to 10 x 10^6 spleen cells in 1 ml of RPMI 1640 medium (Microbiological Associates, Bethesda, Md.) supplemented with 10% fetal calf serum (GIBCO, Bio-Cult, Glasgow, Scotland), 5 x 10^{-5} M 2-mercaptoethanol, and antibiotics in 24-well tissue culture plates (No. 3524, Costar, Mass.). For the mitogen stimulation the cells were cultured for 72 hr in the presence of phytohemagglutinin M (PHA), 0.1 ml, 1:10 dilution (GIBCO, Grand Island, N. Y.) at 37°C in a 5% CO_2 atmosphere. The cultures received 1 μCi of ^{3}H thymidine about 16 hr before harvesting, then the incorporation was assessed by use of a liquid scintillation counter. To verify the *in vitro* production of natural antibodies, the spleen cells were cultured for 4 days, then the supernatants were harvested and tested in the ^{51}Cr cytotoxicity assay on the EL4 target cells.

Tumor stimulation. Mice were injected i.p. once with 10^7 EL4 tumor cells. The cells were treated with mitomycin C (Kyowa Hakko Kogyo Co., Tokyo, Japan) 150 μg, for 10^7 tumor cells in 1 ml Hanks' balanced salt solution (HBSS), incubated for 3 hr at 37°C, and then repeatedly washed before being injected. Sera were harvested 7 days after the tumor inoculum.

Mice reconstitution. Three-month-old B6 mice were reconstituted the day after a sublethal irradiation (450 rads) with cells from subcutaneous lymph nodes, mesenteric lymph nodes, Peyer's patches, thymus, or spleen. The tissues were gently minced in HBSS and repeatedly washed before being injected *in vivo*. Sera were collected 8 days after irradiation and were tested for C-dependent cytotoxicity on EL4 lymphoma cells.

Antimitotic treatment. Colchicine (CC) (Sigma, St. Louis, Mo.) was freshly dissolved in physiologic saline and administered i.p. to animals at a dose of 1 mg/kg body weight. Cyclophosphamide (CY) (Endoxan Asta, Asta Werke, Germany) was also administered i.p., at the dose of 10 mg/kg body weight. The drug treatments were done the same day as the inoculum of the sensitizing tumor cells.

Cytotoxicity test. The test was performed in microplates (Sterilin, Teddington, Middlesex, U. K.); 5 x 10^4 ^{51}Cr-labeled target cells in 0.025 ml HBSS were seeded in each well with 0.025 ml of the test serum and incubated for 30 min at 37°C. Then 0.1 ml HBSS was added to each well, the microplate was centrifuged for 5 min at 2000 rpm, and the medium was removed by aspiration. After shaking the plate on a vortex, 0.025 ml of rabbit C, selected for absence of cytotoxicity on the EL4 target cells and diluted 1:10, was added, and the plate was incubated for an additional 45 min at 37°C. Each well was then refilled with 0.1 ml HBSS, the plate was centrifuged, and 0.05 ml medium was harvested and its radioactivity was measured in a gamma counter. The percentage of specific ^{51}Cr release was calculated as follows:

$$\frac{\text{Experimental release} - \text{Control release}}{\text{Maximum release} - \text{Control release}}$$

where the experimental release was the mean radioactivity released from the three replicates incubated with serum plus C, the control release was the mean radioactivity from the three replicates incubated with HBSS plus C, and the maximum release was the mean radioactivity from three replicates incubated with distilled water and frozen and thawed. Tests with a specific ^{51}Cr release >20% were considered positive.

Statistical analysis. To test significance of differences between control and experimental groups the χ^2 analysis with continuity correction was used.

RESULTS

Age dependence of the humoral natural reactivity. Individual sera from 10 B6 mice per group, having 1.5, 3, 6, 12, and 18

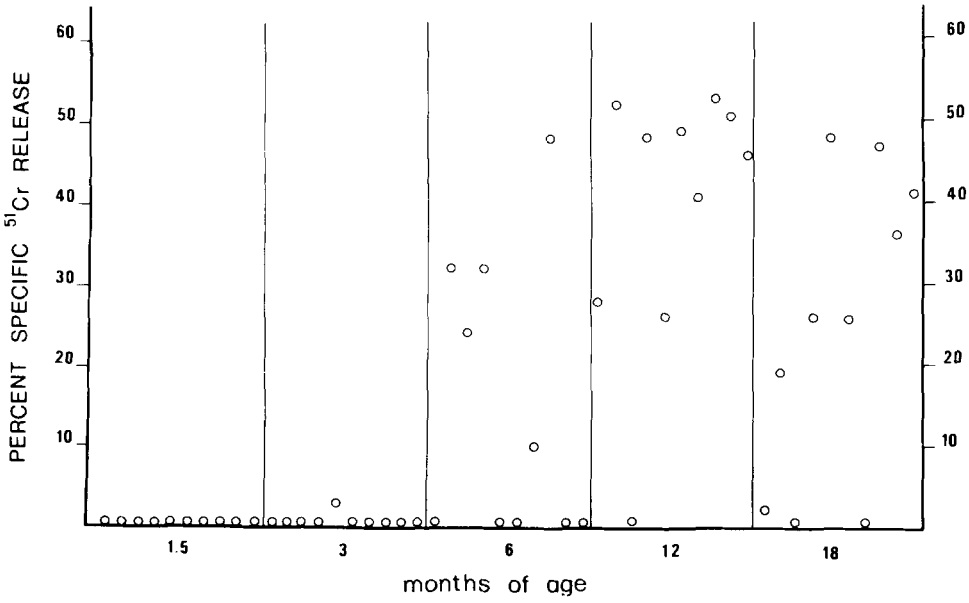

Figure 1. Age dependence of the natural anti-tumor reactivity of B6 mice. Ten individual sera per group from 50 mice aged 1.5, 3, 6, 12, and 18 months were tested in a complement-dependent cytotoxicity assay on the EL4 lymphoma target cells.

months of age, were tested for the spontaneous anti-tumor reactivity in the C-dependent cytotoxicity assay on the reference EL4 lymphoma cells. As shown in Figure 1, the B6 mice were found to acquire the natural reactivity starting only at 6 months of age, similar to BALB/c mice and at variance with C3Hf mice, which already at 2 to 3 months of age have shown a good anti-tumor reactivity (6). The effect of stimulation with tumor cells on the natural reactivity of young and old B6 mice was also investigated. One group of 10 3-month-old and one group of nine 12-month-old B6 mice were stimulated with one i.p. injection of 10^6 mitomycin- C-blocked EL4 cells, a treatment that was previously found to mount the reactivity in those 3-month-old C3Hf mice still unresponsive, but to be ineffective in B6 mice of the same age (16). As reported in Figure 2, the tumor injection was confirmed to be ineffective in young B6 mice, except in one mouse whose serum exceeded 20% cytotoxicity on the EL4 target cells, whereas the same inoculum in the older B6 mice increased the level of reactivity in the five mice already responsive and induced the response in two of the four negative mice.

Interference of T cells on anti-tumor natural reactivity. Since we have previously shown an interference of T cells on the natural anti-tumor immune response of BALB/c mice (11), we attempted to verify whether in individual B6 mice a correlation existed between level of natural reactivity and level of response to the T cell mitogen PHA. For this study, six 8-month-old B6 mice were chosen: three positive and three negative for the natural anti-tumor cytotoxic reactivity. The three positive mice, whose sera revealed 40 to 48% spontaneous cytotoxicity on EL4 lymphoma cells, had a very low stimulation index (<2) with PHA. On the contrary, the three mice negative for the natural reactivity showed stimulation indexes of 3.7, 4.2, and 15.4, respectively. This result, in agreement with our previous one, suggests a T cell involvement in the regulation of the natural response.

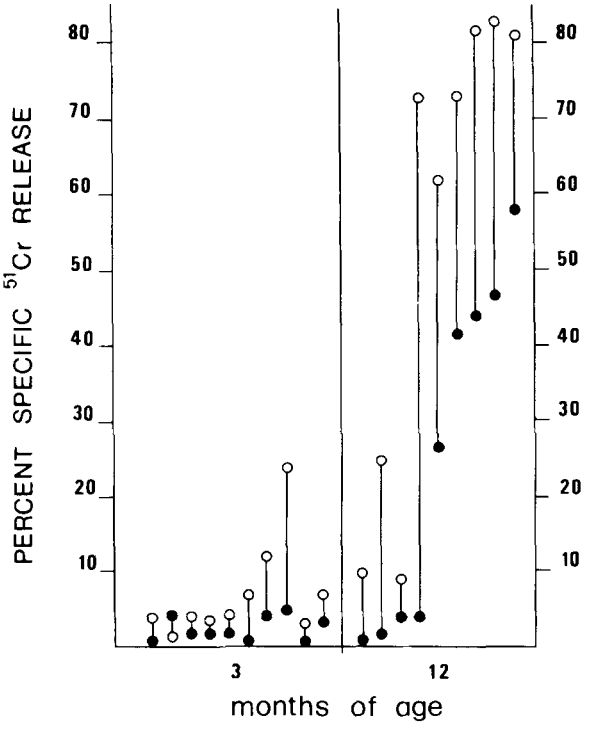

Figure 2. Effect of stimulation with mitomycin C-blocked EL4 cells on the humoral reactivity of 10 3-month-old and 9 12-month-old B6 mice. Complement-dependent cytotoxicity assay on the EL4 lymphoma target cells of the individual sera before (●) and 7 days after (○) the tumor injection.

To further demonstrate a role of T cells in the production of the natural antibodies, 2-month-old unresponsive B6 mice were T deprived by thymectomy and sublethal irradiation (450 rads), and the spontaneous cytotoxic serum reactivity was analyzed 1 month later on the EL4 lymphoma cells. As shown in Table I, the 15 untreated 3-month-old control mice were all found negative, whereas T cell deprivation induced the production of natural antibodies in six of the 15 T-deprived animals, with a mean specific cytotoxicity of 45%. The two groups differ significantly ($p < 0.05$).

Effect of reconstitution with cells from various lymphoid organs on the natural reactivity. Six groups of unresponsive 3-month-old B6 mice were immunodepressed by sublethal irradiation (450 rads) and one group, the control, was then left untreated, whereas the day after the other groups were reconstituted with 10 x 10^6 cells from various lymphoid organs of 12- to 14-month-old syngeneic mice, which were positive for the natural anti-tumor serum reactivity. Sera were collected 7 days after cell transfer and were tested for spontaneous cytotoxicity on EL4 lymphoma cells. As reported in Table II, 62% of mice reconstituted with spleen cells produced natural anti-tumor antibodies, whereas only 7% of mice reconstituted with cells from subcutaneous lymph nodes, mesenteric lymph nodes, Peyer's patches, or thymus were found responsive.

In a second experiment, reported in Table III, the mice were

TABLE I

Effect of T-deprivation of 2-month-old B6 mice on the production of natural anti-tumor antibodies

	No. Responsive Mice/Total Tested Mice	% Responsive Mice	Mean Specific Serum Cytotoxicity on EL4 Cells ± S.E.[a]	
			Responsive mice	Unresponsive mice
Untreated control mice	0/15[b]	0		5 ± 1
Sublethally irradiated thymectomized mice	6/15[b]	40	45 ± 10	4 ± 1

[a] Sera tested 1 month after T-deprivation.
[b] The two groups differ significantly ($\chi^2 = 5.21$, $p < 0.05$).

TABLE II

Individual serum reactivity on EL4 lymphoma cells of six groups of 3-month-old B6 mice sublethally irradiated and reconstituted with 10 × 10^6 cells from various lymphoid organs of 12- to 14-month-old responsive syngeneic mice

Reconstitution with	No. Responsive Mice/Total Tested Mice (%)		Mean Specific Cytotoxicity (±S.E.)	
			Responsive mice	Unresponsive mice
	0/10	(0)		0.5 ± 0.3
Subcutaneous lymph nodes	0/8	(0)		3.7 ± 2.1
Peyer's patches	1/8	(12)	30	0.3 ± 0.3
Mesenteric lymph nodes	1/8	(12)	24	2.8 ± 1.1
Thymus[a]	0/8	(0)		4.6 ± 2.0
Spleen	5/8[b]	(62)	36.6 ± 4.5	15.6 ± 2.8

[a] Including parathymic lymph nodes.
[b] The group differs significantly from the unreconstituted control mice ($\chi^2 = 5.82$, $p < 0.02$).

TABLE III

Individual serum reactivity on EL4 lymphoma cells of six groups of 3-month-old B6 mice, sublethally irradiated, and reconstituted with responsive and unresponsive spleen cells from 12- to 14-month-old syngeneic mice

Reconstitution with 10×10^6			No. Responsive Mice/ Total Tested Mice	(%)	Mean Specific Cytotoxicity (±S.E.)	
Responsive spleen cells	Unresponsive spleen cells	Unresponsive spleen cells α-Thy-1-treated			Responsive mice	Unresponsive mice
No	No	No	2/17	(12)	29.0 ± 6.0	3.1 ± 0.8
Yes	No	No	11/17[a]	(65)	33.7 ± 3.8	10.0 ± 2.9
No	Yes	No	0/18	(0)		5.4 ± 1.2
Yes	Yes	No	0/17[b]	(0)		9.4 ± 1.8
No	No	Yes	2/18	(11)	25.0 ± 1.0	5.7 ± 1.1
Yes	No	Yes	12/20[c]	(60)	35.8 ± 4.3	10.5 ± 1.6

[a, c] The groups differ significantly from the unreconstituted control mice ($\chi^2 = 7.97$, $P < 0.01$, and $\chi^2 = 7.15$, $P < 0.01$, respectively).

[b] The group differ significantly from the group *a* and *c* ($\chi^2 = 13.44$, $p < 0.01$, and $\chi^2 = 12.48$, $p < 0.01$, respectively).

similarly irradiated, reconstituted, and tested for natural serum cytotoxicity, but spleen cells only were used, selected from 12- to 14-month-old anti-tumor responsive or unresponsive mice. In the unreconstituted controls, only two mice out of 17 (12%) were found positive for natural antibodies. In the group injected with 10 x 10^6 spleen cells from responsive mice, 65% of animals became responsive, whereas all the 18 mice of the group injected with 10 x 10^6 spleen cells from unresponsive animals remained negative. When 10 x 10^6 spleen cells from responsive mice and 10 x 10^6 spleen cells from unresponsive mice were injected simultaneously, the responsivity was abrogated, and all the 17 mice remained negative; however, when the spleen cells of unresponsive mice were treated with anti-Thy-1 antiserum plus C to eliminate T cells before inoculation with the spleen cells of responsive mice, 12 of the 20 tested sera (60%) were found positive. The same anti-Thy-1 antiserum treatment of spleen cells from unresponsive mice before being transferred alone led to serum cytotoxicity in two of 18 tested mice; that is the same percentage of positivity of the control group.

Inhibition of in vitro production of natural antibodies. The inhibiting capacity of the unresponsive spleen cells from young animals on the natural antibody production by spleen cells from old mice was verified *in vitro* also. Spleen cells either from 18- or 3-month-old B6 mice, or a mixture of the two cell types, were cultured *in vitro* for 4 days, then the supernatants of the cultures were tested for their C-dependent cytotoxic reactivity on the EL4 target cells. As reported in Table IV, in two different experiments the spleen cells from 18-month-old mice produced cytotoxic antibodies *in vitro*, with a specific cytotoxicity varying between 26 and 66%, depending on the experiment and on the number of cultured cells. On the contrary, the supernatants of the cultures of spleen cells from 3-month-old mice were unreactive in both the experiments. The *in vivo*-observed inhibiting capacity of the spleen cells from the young unresponsive mice on antibody production was confirmed *in vitro* as well as was the abrogation of the inhibition by anti-Thy-1 serum treatment of the unresponsive spleen cells. On the contrary, as shown in the experiment 1 of Table IV, the inhibiting capacity was unaffected by the passage of the unresponsive spleen cells on an anti-IgG column, which by eliminating B cells and adherent cells led to an enrichment in T cells (90% of the recovered cells were Thy-1+). In addition, the data of experiment 2 show that

TABLE IV

Inhibition by spleen cells from 3-month-old B6 mice of the in vitro production of natural anti-tumor antibodies by spleen cells from 18-month-old B6 mice

No. of Inhibiting Spleen Cells ($\times 10^6$)	No. of Producing Spleen Cells ($\times 10^6$)	% Specific Cytotoxicity Exerted on EL4 Cells by Culture Supernatants	% Inhibition
Expt. 1			
0	5	26.0	
0	10	30.0	
5 Untreated	0	1.0	
5 Untreated	5	8.5	74
5 Anti-Thy-1-treated	0	0.0	
5 Anti-Thy-1-treated	5	24.0	15
5 Anti-IgG column-passed	0	5.0	
5 Anti-IgG column-passed	5	4.5	84
Expt. 2			
0	5	56.0	
0	10	66.0	
5 Untreated	0	0.0	
5 Untreated	5	5.0	91
5 Anti-Thy-1-treated	0	7.0	
5 Anti-Thy-1-treated	5	53.0	5
5 Anti-Ly-1.2-treated	0	0.0	
5 Anti-Ly-1.2-treated	5	11.0	80
5 Anti-Ly-2.2-treated	0	0.0	
5 Anti-Ly-2.2-treated	5	35.0	37

the treatment of the inhibiting spleen cells with an anti-Ly-1.2 antiserum, which recognizes Ly-1+ helper cells, did not affect the inhibitory activity, whereas the treatment with an anti-Ly-2.2 antiserum, which recognizes Ly-2+ suppressor cells, lowered the inhibition from 91% to 37%.

Interference of T-suppressor cells on natural reactivity. The above-reported results suggested that the unresponsivity of young B6 mice, either untreated or after tumor stimulation, could be due to the activity of T-suppressor cells. We have assumed that old mice become responsive when the involution of the immune system due to aging induces a decrease in precursor T-suppressor cells (17, 18), which can no longer interfere with the spontaneous raise in the anti-tumor response or with its stimulation by an inoculum of tumor cells. To test this hypothesis we treated young animals unresponsive at the time of the antigenic stimulation with x-rays, CC, or CY, which are agents known to inhibit the induction of T-suppressor cells (19–21). As reported in Table V, when 3-month-old B6 mice were treated with x-rays and the day after were inoculated with the antigen consisting of tumor cells blocked by mitomycin, or were injected with either of the two chemicals together with the antigen, 53 to 60% of the mice became responsive, whereas the inoculum of tumor cells alone induced a reactivity in only 6 to 20% of the mice, and the CY alone or the x-rays alone were uneffective. In the group that received irradiation and the day after CC and tumor cells, all animals remained negative.

DISCUSSION

T-suppressor cells have been widely demonstrated to play an important role in the regulation of the antibody response after an induced antigenic stimulation (22–25). The studies reported here, in agreement with our previous reports (11), indicate that not only the induced, but also the so-called spontaneous production of IgM cytotoxins directed against lymphoma cells in

TABLE V

Effect of irradiation, colchicine, or cyclophosphamide on the immune response of 3-month-old B6 mice injected with blocked EL4 lymphoma cells

Treatment	No. Positive Sera/ Total Tested Sera	(%)	Mean Specific Cytotoxicity on EL4 Cells ± S.E.[a]	
			Positive sera	Negative sera
Expt. 1				
	0/10	(0)		6 ± 3
CY	0/10	(0)		5 ± 2
EL4	2/10	(20)	26 ± 2	10 ± 3
EL4 + CY	6/10[b]	(60)	30 ± 2	7 ± 3
Expt. 2				
	1/17	(6)	24	2 ± 2
450 R	0/3	(0)		13 ± 3
EL4	1/17	(6)	26	8 ± 3
EL4 + CC	9/17[c]	(53)	32 ± 6	5 ± 3
450 R + EL4	4/7[d]	(57)	38 ± 6	5 ± 3
450 R + EL4 + CC	0/7	(0)		7 ± 3

[a] Sera collected 7 days after treatment.

[b,c,d] The three groups differ significantly from the respective untreated control group ($\chi^2 = 5.95$, $p < 0.02$; $\chi^2 = 6.94$, $p < 0.01$; $\chi^2 = 5.10$, $p < 0.05$, respectively).

the mouse (5, 6, 26) is regulated by a T cell subpopulation with suppressor activity.

In a previous study, we found that BALB/c mice late in life possessed natural anti-tumor antibodies, and, in fact, a good C-dependent humoral cytotoxicity for tumor cells was demonstrable when they were more than 6 months old (7, 8). The T deprivation of mice increased the spontaneous reactivity, which was found to be indirectly correlated with the T cell content, high in mice with low anti-tumor reactivity, and vice versa (7, 11).

In the present study, we found that B6 mice had a similar behavior, in that the natural anti-tumor response appeared late in life and there was a complete inverse relationship between PHA spleen stimulability and level of natural anti-tumor antibody production. The data in toto are therefore consistent with the hypothesis of a regulatory role of T cells on the spontaneous anti-tumor reactivity, possibly through a subpopulation of suppressor cells known to spontaneously decrease with age (17, 18), in agreement with the known impaired T cell function in aging. However, an increase with age of the negative control exerted by suppressor cells is also reported after a stimulation with synthetic polypeptides (27).

The other results of the present work are also in keeping with the hypothesis of a negative control by a cell subpopulation with suppressor activity. T-deprivation of unresponsive 3-month-old mice induced antibody production, although not in all the tested mice. This only partial effectiveness of the treatment could be explained by absence or inefficency of antigen stimulation in those mice that remained unresponsive and suggests that there are two conditions necessary for the presence of the natural anti-tumor cytotoxic antibodies: the decrease of T-regulator cells and the presence of a proper amount of the involved antigen, perhaps a virus-directed autogenous one, which spontaneously increases with age (9). In young mice the virus may be present in a tolerogenic amount, which establishes a tolerance status mediated by T-suppressor cells and wanes with age. In agreement with this assumption, the stimulation with tumor cells was not sufficient to increase the level of humoral anti-tumor cytotoxicity in 3-month-old tolerant mice, which have an efficient T-suppressor activity, whereas the same inoculum was effective in 12-month-old mice. At this age all mice already responsive, i.e., mice in which we can assume to have already occurred both the spontaneous decrease of T-regulator activity due to aging and the encounter with the antigen in the proper dose, had an increased level of cytotoxicity when stimulated, and in addition, some of the unresponsive animals became responsive, i.e., those that we can assume to have had a low suppressor activity but a still inadequate amount of the autogenous stimulus. For the mice that remained negative, we are inclined to think that they had a still high suppressor activity.

A further confirmation of the involvement of T-suppressor cells in the regulation of the natural humoral anti-tumor response comes from the experiments of reconstitution with various lymphoid organs of young mice given sublethal irradiation, which is known to have an enhancing effect on antibody production (28, 29), due to radiosensitivity of the suppressor effect (19). Among all the lymphoid tissues used, only the spleen was able to transfer the anti-tumor natural reactivity. The controls, irradiated only, remained almost all unresponsive, as expected, demonstrating that at 3 months of age the proper amount of the endogenous antigen essential for stimulating antibody production is not present in the majority of mice, whereas about 60% of mice irradiated and transferred with spleen cells from old responsive mice showed positive cytotoxic sera after a few days. However, in mice injected on the same day with spleen cells from both responsive and unresponsive animals, the response was abrogated. The results suggest that in the unresponsive spleens a suppressor subpopulation is present consisting of T cells, as demonstrated by the abrogation of the suppressor activity by a treatment with an anti-Thy-1 antiserum. On the contrary, the T deprivation of unresponsive spleen cells by anti-Thy-1 antiserum and their transfer without other cells in young irradiated mice did not increase the percent of positive sera in comparison to the control group, in keeping with the hypothesis of a low amount of the antigenic stimuli in the 3-month-old recipients. The *in vitro* results supported the *in vivo* findings by showing that the Thy-1+ and Ly-2+ subpopulations of young unresponsive spleen cells were able to abolish or lower the antibody production by spleen cells from old mice. On the contrary, the Ly-1+ 2- cells as well as the cells that were retained on an anti-IgG column did not result in being involved in the suppressor activity.

Drugs such as CC and CY recently have been demonstrated to regulate the antibody response by interfering with the activity of T-suppressor cells (20, 21), whose generation requires mitosis (30, 31) and therefore strongly depends on the absence of any interference with their proliferation. The suppressor regulatory cells have been found to divide soon after their encounter with the antigen (30, 31). The drugs, when administered simultaneously to the antigen and in the anti-mitotic dose range, have been shown to exert their mitosis blocking effect on the early dividing suppressor population and consequently to enhance the antibody formation (32). In this light, we presumed that irradiation, which also acts on rapidly dividing cells (33), or anti-mitotic drugs administered at the proper time, could enhance the production of the natural anti-tumor antibodies in a system such as in young B6 mice, where T cell regulation is present. The results supported this hypothesis, since in 3-month-old mice irradiation 1 day before the antigen, or CC and CY injected on the same day of the stimulating tumor cells, enhanced the production of natural anti-tumor antibodies in comparison with mice untreated or given only the antigen or only the anti-mitotic treatment. In fact, in the control groups all together only 6% mice were responsive, whereas in the

groups given the antigen plus either of the anti-mitotic treatments, 55% responder mice were found. When 3-month-old mice were given two anti-mitotic treatments at 1-day intervals, i.e., when they were irradiated for abrogation of the negative regulation, and then the day after were stimulated with tumor cells and treated with CC, no humoral response was obtained in any mouse, suggesting that in the absence of suppressor regulation, anti-mitotic drugs are unable to enhance the antibody production. The explanation for the complete unresponsiveness, in spite of abrogation of the precursors of suppressor cells and of antigen administration, might be that the drug, which could no longer act on the already abrogated suppressors, interferes only with other cell types, i.e., T-helper or B cells, thus preventing any antibody production.

Acknowledgments. We are grateful to Mrs. Piera Aiello, Mr. Mario Azzini, and Mr. Umberto Rubertelli for their technical help.

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