SUPPRESSION OF IgE ANTIBODY PRODUCTION IN SJL MICE

III. Characterization of a Suppressor Substance Extracted from Normal SJL Spleen Cells*

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Among regulators of immune responses are subpopulations of T cells (1), i.e. antigen-specific (2–4) and antigen-nonspecific suppressor cells (5, 6). Suppressor factors from these cells have been extracted and characterized (7–10). Antigen-specific suppressor cells have also been demonstrated in the case of IgE antibody responses (11, 12).

High and persistent anti-hapten IgE antibody response was induced in many strains of mice by appropriate immunization and a nemotode parasite (*Nippostrongylus brasiliensis*) infection (13, 14). However, the IgE antibody response was rapidly terminated in SJL mice. The transient IgE antibody response in SJL mice is inherited as a recessive trait controlled by a single autosomal gene and is not linked to the *H-2* complex (14). When immunized and infected SJL mice were irradiated (540 R), high titers of anti-hapten IgE antibody persisted for several weeks. Suppression of IgE response was induced in immunized and irradiated SJL mice by transfer of normal SJL spleen cells. These suppressor T cells had no specificity for the antigen (14). Moreover, when normal SJL spleen cells were treated with anti-Ly 1.2 and complement before the transfer, no suppression was observed (15).

The present report shows that the suppression of the IgE response is induced by an extract from normal SJL spleen cells. The suppressor substance is a heat-labile high molecular weight protein.

Materials and Methods

Antigen. Dinitrophenylated keyhole limpet hemocyanin ($DNP_{13}KLH$),[1] dinitrophenylated *N. brasiliensis* extract ($DNP_{17}Nb$), and Nb were used for immunization. Dinitrophenylated bovine serum albumin ($DNP_{37}BSA$), and dinitrophenylated ovalbumin ($DNP_{14}Ov$) were used as challenging antigens for passive cutaneous anaphylaxis (PCA) reactions. The subscripts refer to average number of groups per molecule of protein except for Nb and KLH where they refer to 10^5 daltons.

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; HBSS, Hanks' balanced salt solution; KLH, keyhole limpet hemocyanin; Nb, *Nippostronglyus brasiliensis* extract; Ov, ovalbumin; PCA, passive cutaneous anaphylaxis.

Preparation of these antigens has been previously described (13). The subscripts are omitted in the text.

Immunological Reagents. Rabbit globulins were precipitated by $(NH_4)_2SO_4$ (33% final concentration). Anti-mouse immunoglobulin (Ig) was prepared in rabbits. The rabbits were injected with 2 mg mouse Ig in complete Freund's adjuvant in the foot pads and boosted intravenously (i.v.) with 1 mg mouse Ig in 0.15 M NaCl on three consecutive days monthly. The rabbits were bled 4 mo after the primary immunization. The globulin fraction of the rabbit antisera was separated by $(NH_4)_2 SO_4$ precipitation (as above) and gave strong lines in double diffusion in agar (Ouchterlony) when assayed against mouse Ig preparations.

Animals. 8- to 12-wk old female SJL mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. Male Sprague-Dawley rats, weighing 250–300 g were obtained from the Blue Spruce Farms, Altamont, N. Y.

Immunization. Immunization schedules, infections, and boosters were done as published (14). Briefly, five animals in each group were immunized intraperitoneally with 1 μg DNP-KLH mixed with 1 mg $Al(OH)_3$ on day 0. On day 21 the mice were infected subcutaneously by 750 third stage *N. brasiliensis* larvae. Mice were reinjected (challenged) intraperitoneally with 1 μg DNP-Nb mixed with 1 mg $Al(OH)_3$ on day 35. Mice were bled weekly beginning 7 days after injection of DNP-Nb from the retro-orbital sinus. 0.2 ml of blood was added to 0.9 ml heparinized saline (10 U/ml), and then centrifuged for 10 min at 1,000 *g*. The supernate was considered to be a $^1/_{10}$ dilution.

In one experiment to compare a possible carrier specificity, the mice were immunized intraperitoneally with 10 μg Nb mixed with complete Freund's adjuvant or with 1 mg $Al(OH)_3$ and boosted with 10 μg Nb and the same respective adjuvant 3 wk after immunization. The spleen cells from these mice were harvested 7 days after booster. Spleen cells from mice infected with 750 larvae of *N. brasiliensis* 3 wk previously were also used as source of suppressor cells.

Irradiation. Immunized, infected, and challenged mice received 540 R of X-ray irradiation from a Gammator M (Radiation Machinery Corp., Parsippany, N. J.) on day 36 (1 day after challenge).

Cell Transfer. Spleen cells from either noninfected or infected SJL mice were prepared by gentle teasing of spleens in cold, sterile Hanks' balanced salt solution (HBSS) (Microbiological Associates, Bethesda, Md.). The cells were washed three times in HBSS and their viability was estimated by the trypan blue exclusion test. Mice were i.v. injected with 3×10^7 or 5×10^7 viable spleen cells. Thymocytes and mesenteric lymph node cells were prepared in a similar fashion.

Preparation of Spleen Cell Extract. Spleen cells from five or six normal SJL mice were washed and suspended in 3 ml HBSS (5×10^8 cells/ml). The cells were frozen in dry ice-alcohol and thawed in a 37°C water bath. This procedure was repeated five times. The tubes were then centrifuged at 4,000 *g* at 4°C for 30 min. After centrifugation the volume of the supernate was 2 ml (equivalent to 7.5×10^8 cells/ml). Extract equivalent to 1.5 or 2×10^8 normal SJL spleen cells was injected i.v. per mouse 5 h after irradiation.

Absorption with Anti-Mouse Immunoglobulin. Globulin fraction of rabbit anti-mouse Ig and normal rabbit serum were coupled to Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) according to the method of Axen et al. (16). The absorbing activity of these preparations was tested by Ouchterlony test using mouse globulin. The spleen cell extract was added to the same volume of packed, coupled Sepharose 4B and rotated at room temperature for 2 h. The extract was separated from the absorbent after centrifugation at 40 *g* at 4°C 5 min.

Heat Treatment. The extract was heated in a 56°C water bath for 2 h, then centrifuged at 1,000 *g* for 10 min at 4°C, and the supernate was injected into the mice.

Protease Treatment. Protease (Subtilopeptidase-A; Sigma Chemical Co., St. Louis, Mo.) was used. The extract from 12×10^8 normal SJL spleen cells was digested with 70 μg of protease at room temperature for 2 h. The extract equivalent to 1.5×10^8 spleen cells per mouse was injected i.v. immediately after digestion.

Gel Filtration. The extract from 10×10^8 normal SJL spleen cells in 1 ml was applied on Sephadex G-100 or G-200 columns (Pharmacia Fine Chemicals, Inc.) of 2.5×90 cm and eluted with 0.01 borate-buffered 0.15 M NaCl (pH 8) at 4°C. 2-ml fractions were collected in individual tubes with a flow rate of 10 ml/h. The optical density at 280 μm of each fraction was monitored by an LKB Uvicord Absorbtiometer (LKB Instruments, Inc., Rockville, Md.). The pooled fractions were concentrated by positive pressure dialysis and injected i.v. into the mice (see Results).

TABLE I
Suppressive Effect by Spleen Cells from Immunized SJL Donors

Group*	Source of transferred spleen cells (5×10^7)	Anti-DNP PCA titers‡			
		On day 49§		On day 63§	
		IgE	IgG1	IgE	IgG1
I	No cells transferred (control)	400	100	400	10
II	Mice immunized with 10 μg Nb with 1 mg $Al(OH)_3$	100	100	0‖	50
III	Mice immunized with 10 μg Nb in complete Freund's adjuvant	100	100	0	50
IV	Mice infected with 750 larvae of *N. brasilensis* 3 wk previously	200	100	200	50
V	Normal SJL mice	200	100	50	50

* The recipient mice were immunized as described in the Materials and Methods and irradiated (540 R) on day 36. Cell transfer was done on day 37.

‡ Sera from five mice of each group were pooled and the reciprocal of the highest dilution giving PCA reaction was taken as titer.

§ Days after immunization with DNP-KLH.

‖ 0, no PCA reactions with sera diluted $^1/_{10}$.

Ultracentrifugation. The extract was centrifuged at 100,000 *g* for 90 min. The supernate was injected i.v. into the mice.

Titration of Antibody. Titers of IgG_1 and IgE antibody of pooled sera from each group were determined by PCA reactions (17). Female SJL mice were used for IgG_1 antibody titration using a 1.5 h sensitization period (18). Mice were challenged with 500 μg $DNP_{14}Ov$ for anti-hapten antibody. Male Sprague-Dawley rats were used for IgE antibody titration (19) using a 2 h sensitization period (18). 1 mg of $DNP_{37}BSA$ was used as challenging antigen.

Results

Suppressive Effect by Spleen Cells from Immunized Mice. Table I shows the anti-DNP IgE and IgG_1 antibody titers on days 49 and 63, 2 and 4 wk after challenge. Irradiated control mice, to which no cells were transferred, showed high titers of anti-DNP IgE antibody (group I). Anti-DNP IgE responses in the recipients receiving normal SJL spleen cells were suppressed, especially on day 63 (group V).

Somewhat stronger suppression was observed in the mice of group II and group III [cells from mice immunized with Nb in $Al(OH)_3$ or in complete Freund's adjuvant, respectively]. The anti-DNP IgE titer in the mice of group IV (cells from mice infected with *N. brasiliensis*) was only slightly lower than that of group I (controls). Anti-DNP IgG_1 titers in these irradiated mice were low, probably an effect of the irradiation as already discussed (14, 15).

Organ Distribution of Suppressor Cells. The action of cells from normal SJL spleen, mesenteric lymph node, and thymus was compared. 3×10^7 cells were injected into immunized and irradiated recipients on day 37. The anti-DNP IgE titers are shown in Table II.

There was no great difference between controls (group I) and the experimental groups on day 42. However, on day 63, the suppressive effect was evident and equally strong in groups II and IV (spleen and mesenteric lymph node cells, respectively) and evident but less marked in group III (thymocytes).

TABLE II
Organ Distribution of Suppressor Cells

Group*	Transferred cells from (3×10^7):	Anti-DNP IgE PCA titer‡	
		Day 49§	Day 63§
I	No cells transferred (control)	800	800
II	Spleen	800	50
III	Thymus	800	200
IV	Mesentric lymph node	400	50

* The recipient mice were immunized as described in the Materials and Methods and irradiated (540 R) on day 36. Cell transfer was done on day 37.
‡ Sera from five mice of each group were pooled and the reciprocal of the highest dilution giving PCA reaction was taken as titer.
§ Days after immunization with DNP-KLH.

Suppression of IgE Response by the Spleen Cell Extract. Extract corresponding to 1.5 or 2×10^8 spleen cells was injected i.v. into the recipients (5 h after irradiation). As shown in Fig. 1 A, nonirradiated mice showed only a transient anti-DNP IgE antibody response. When the immunized mice were irradiated persistent high titers of IgE antibody were obtained.

The spleen cell extract was as effective in IgE antibody suppression as the viable cells. Higher doses produced greater suppression. IgG_1 antibody response persisted in all the groups, even in the group which was irradiated (Fig. 1 B). There was no great difference between the mice receiving and not receiving the extract. However, as the titers were low, it is difficult to precisely evaluate the effect of the extract on IgG_1 suppression.

Characterization of the Spleen Cell Extract

ABSORPTION WITH ANTI-MOUSE Ig. The results are shown in Table III. After absorption each recipient received the extract equivalent to 1.5×10^8 spleen cells. The extract absorbed with anti-mouse Ig (group III) and that absorbed with normal globulin (group IV) induced almost the same suppressive effect.

HEAT TREATMENT. The suppressive effect of the extract was destroyed by heating at 56°C for 2 h (Table IV, compare group III to group II).

PROTEASE TREATMENT. Protease treatment destroyed the suppressive activity of the extract (Table V).

GEL FILTRATION. The extract obtained from 10×10^8 spleen cells in a 3 ml volume was applied to the Sephadex G-200 column (see Materials and Methods). The elution profile showed two peaks. One peak at 160 ml corresponded to the void volume as determined by blue dextran as a marker. The second peak was at 284 ml. The contents of the tubes from 140 to 180 ml were pooled and designated as fraction I (void volume). Contents from tubes 181 to 240 ml were pooled and designated fraction II. Fraction III (the second peak) was the pool from 241 to 300 ml. Each fraction was concentrated to 1.5 ml. If all activity would have been in only one fraction then 0.25 ml of this fraction would correspond to the activity of 1.66×10^8 cells. Each fraction was injected i.v. into the mice (0.25 ml per mouse). As shown in Table VI only fraction I, corresponding to the void volume, could induce suppression (group I). Similarly, only the fraction corresponding to the

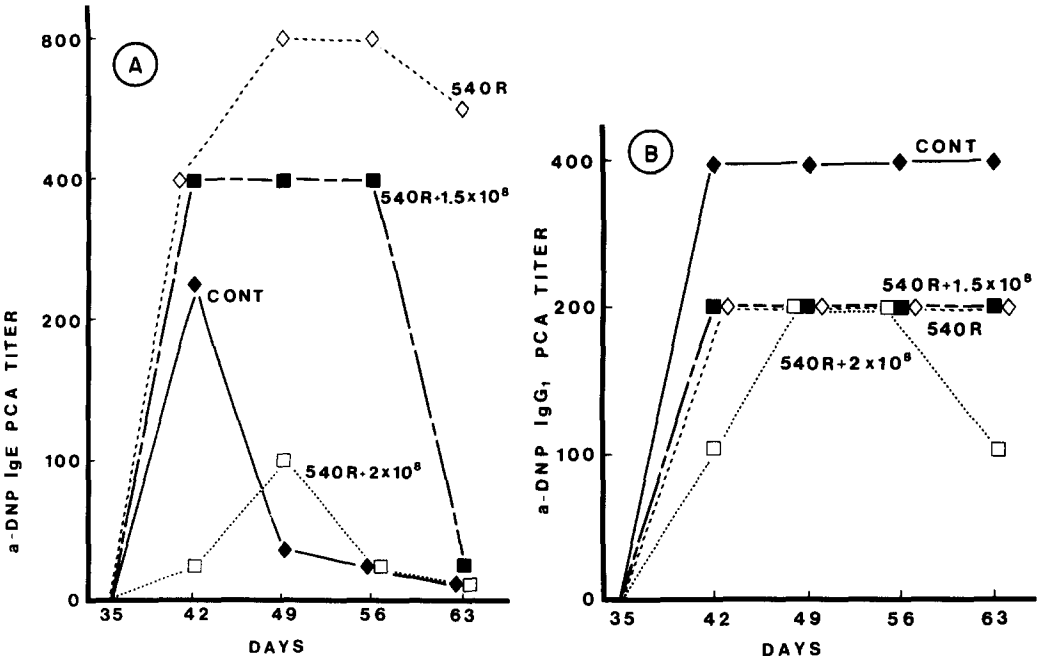

FIG. 1. Anti-DNP antibody response in SJL mice. (◆), nonirradiated mice. (◇), mice irradiated with 540 R. (■), irradiated mice (540 R) injected with extract of normal SJL spleen cells corresponding to 1.5×10^8 cells. (□), irradiated mice (540 R) injected with the same extract but corresponding to 2×10^8 cells. (A), anti-DNP IgE titers and (B), anti-DNP IgG_1 titers.

TABLE III

Suppressive Effect of Spleen Cell Extract Absorbed with Anti-Mouse Ig

Group*	Injected with:	Anti-DNP IgE titer‡ on day 63§
I	HBSS	600
II	Extract equivalent to 1.5×10^8 cells	50
III	Extract equivalent to 1.5×10^8 cells absorbed with rabbit anti-mouse Ig	200
IV	Extract equivalent to 1.5×10^8 cells absorbed with normal rabbit globulin	200

* The recipient mice were immunized as described in the Materials and Methods and irradiated (540 R) on day 36.

‡ Sera from five mice of each group were pooled and the reciprocal of the highest dilution giving PCA reaction was taken as titer.

§ Days after immunization with DNP-KLH.

void volume after passage on Sephadex G-100 column had a suppressive effect (results not shown).

ULTRACENTRIFUGATION. Ultracentrifugation of the extract was performed to examine the possibility of a virus as a suppressor substance. The suppressive effect of the extract remained in the supernate after centrifugation at 100,000 *g* for 90 min (Table VII, group I).

TABLE IV
Effect of Heat on Suppressive Activity of the Extract

Group*	Injected with:	Anti-DNP IgE titer‡ on day 63§
I	HBSS	400
II	Extract equivalent to 2×10^8 cells	50
III	Extract equivalent to 2×10^8 cells heated at 56°C for 2 h	800

* The recipient mice were immunized as described in the Materials and Methods and irradiated (540 R) on day 36.
‡ Sera from five mice of each group were pooled and the reciprocal of the highest dilution giving PCA reaction was taken as titer.
§ Days after immunization with DNP-KLH.

TABLE V
Suppressive Effect of the Spleen Cell Extract after Protease Treatment

Group*	Injected with:	Anti-DNP IgE titer‡ on day 63§
I	HBSS	400
II	Extract equivalent to 2×10^8 cells	50
III	Extract equivalent to 2×10^8 cells treated with protease	400

* The recipient mice were immunized as described in the Materials and Methods and irradiated (540 R) on day 36.
‡ Sera from five mice of each group were pooled and the reciprocal of the highest dilution giving PCA reaction was taken as titer.
§ Days after immunization with DNP-KLH.

TABLE VI
Suppressive Effect of the Fractions Obtained by Sephadex G-200 Filtration

Group*	Injected with:	Anti-DNP IgE titer‡ on day 56§
I	HBSS	800
II	Fraction I‖	0
III	Fraction II	800
IV	Fraction III	400

* The recipient mice were immunized as described in the Materials and Methods and irradiated (540 R) on day 36.
‡ Sera from five mice of each group were pooled and the reciprocal of the highest dilution giving PCA reaction was taken as titer.
§ Days after immunization with DNP-KLH.
‖ See text.

Discussion

These experiments confirmed previous results in which it has been shown that normal SJL spleen cells had a suppressing effect on anti-DNP IgE antibody production (14) (Table I; compare IgE titers on day 63 between group I and group

TABLE VII
Suppressive Effect of the Extract after Ultracentrifugation

Group*	Injected with:	Anti-DNP IgE titer‡ on day 63§
I	HBSS	400
II	Supernate of extract equivalent to 1.5×10^8 cells after centrifugation for 90 min at 100,000 *g*	50
III	Extract equivalent to 1.5×10^8 cells	50

* The recipient mice were immunized as described in the Materials and Methods and irradiated (540 R) on day 36.

‡ Sera from five mice of each group were pooled and the reciprocal of the highest dilution giving PCA reaction was taken as titer.

§ Days after immunization with DNP-KLH.

V). Somewhat greater suppression was obtained with spleen cells from mice immunized with Nb and $Al(OH)_3$ or complete Freund's adjuvant (Table I; compare group V with groups II and III). Therefore, it is possible that in addition to the antigen-nonspecific suppression by normal SJL spleen cells another suppressive mechanism is also operating, i.e., the type of suppression observed in other strains of mice on antibody production (2–4). However, our studies were directed to characterize the antigen-nonspecific suppressive effect by normal SJL spleen cells.

When spleen cells obtained from mice infected with *N. brasiliensis* larvae were used the suppression was much less evident (Table I; compare group IV with group V). It is probable that the suppressive effect in this case is masked by carrier-specific helper T cells (13).

The anti-DNP IgG_1 titers in these irradiated mice were low. As discussed previously (14) this is probably attributable to the irradiation.

Mesenteric lymph node cells and spleen cells were more effective in IgE antibody suppression than thymus cells. This fact was expected, as the antigen-nonspecific suppressor T cells from normal SJL mice are of the Ly-1 subclass (15) and it is known that the majority of thymus cells is of the Ly 1, 2, 3 subclass (20). Therefore, it was decided that spleen cells would be used for extraction of the substance responsible for suppression.

The spleen cell extract injected into anti-DNP IgE producing SJL mice was very effective in suppressing IgE production (Fig. 1 A). Extract corresponding to 1.5×10^8 cells reduced the titer considerably (from $1/400$ on day 56 to $1/20$ on day 63) when mice which did not receive the extract had still a high titer ($1/600$). Extract corresponding to 2×10^8 cells was even more effective. On anti-DNP IgG_1 production the extract seems to be much less effective. This point needs further investigation but our aims were to study the role of the extract on IgE production and not on IgG_1.

The suppressive substance in the extract was not absorbed by rabbit anti-mouse immunoglobulin (Table III; compare groups III and IV with group I). The efficacy of the extract is destroyed by exposure to heat (56°C for 2 h) (Table IV) and it was destroyed by protease (Table V).

The heat-labile protein responsible for suppression has a mol wt higher than

300,000 daltons as it is eluted in the void volume by filtration of Sephadex G-200 (Table VI) and G-100. However, it is not precipitated by ultracentrifugation at 100,000 *g* for 90 min. Therefore, it is improbable that the suppressive effect is due to a virus. It is possible but highly improbable that the suppressive substance extracted from normal SJL spleen cells could be an agglomerate of smaller molecular weight proteins. If it were an agglomerate one would expect some suppressive activity also in fractions II or III (Table VI), which was not the case.

Suppression of IgE antibody production in mice was demonstrated using urea-denatured antigen (21). This suppression is different from the one which was investigated by us previously because the former is antigen specific and the latter, observed in the SJL strain of mice, is antigen nonspecific. In another species, the rat, antigen-specific IgE suppression was also demonstrated (11).

Suppression of antibody formation using the plaque-forming cell techniques was shown in mice (2, 4). In these cases, the suppressive factor is antigen specific and its production is controlled by the *H-2* complex (4). The suppressor factor is a relatively small molecular weight protein (mol wt between 35,000 and 55,000 daltons). Again the suppressor factor reported in this paper is different from that quoted above because of the molecular weight, and the antigen specificity. Antigen-nonspecific suppressor substance obtained by stimulation with concanavalin A was reported using the plaque-forming cell assay (10). Here again the mol wt is between 48,000 and 67,000 daltons (10).

Another important point to be considered is the time lag between injection of the extract and the suppression of IgE antibody titers. The suppression occurred with an important time lag; generally about 3 wk after injection of the extract. If the extract would have direct enzymatic action on IgE and not an action on the production of IgE one would expect no time lag. We propose therefore that the extract has an action on the IgE production. Becase of the time lag it is possible that the extract has an action not directly on the anti-DNP IgE antibody-producing B cell but on some other cell which in turn influences the antibody-producing B cell.

Summary

SJL mice were immunized with 1 μg dinitrophenylated keyhole limpet hemocyanin in 1 mg $Al(OH)_3$. The mice were infected 21 days later with 750 third stage larvae of *Nippostrongylus brasiliensis*. On day 35, 14 days after infection, they were injected with 1 μg DNP-*N. brasiliensis* extract (Nb) in 1 mg $Al(OH)_3$. In order to obtain high titer and persistent anti-DNP IgE antibody the mice were irradiated (540 R) 1 day after injection of DNP-Nb. Suppression of anti-DNP IgE antibody production was induced by spleen cells from normal SJL mice. Suppression of IgE antibody response is also obtained by an extract from normal SJL spleen cells. The suppressor substance from normal SJL spleen cell extract is a heat-labile protein, and is not absorbed by anti-mouse immunoglobulin. The mol wt of this substance is larger than 300,000 daltons as determined by gel filtration on Sephadex G-200, but after ultracentifugation, the supernate still has suppressive activity on IgE antibody production.

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