# THE THYMIC SUPPRESSOR CELL

## I. Separation of Subpopulations with Suppressor Activity*

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We showed in an earlier study that thymocytes, taken from Lewis rats within a few days after a large systemic dose of bovine γ-globulin (BGG)[1] and transferred alive to normal syngeneic recipients, inhibit both antibody formation and cell-mediated immunity when the recipient is subsequently challenged with the same antigen in complete Freund's adjuvant (1). The inhibition is specific and partial rather than total, and it affects functions normally ascribed to both B and T lymphocytes. It appears to represent one of the rapidly growing groups of phenomena ascribed to "suppressor T cells" (reviewed in references 1 and 2). The present paper describes certain properties of the thymocyte subpopulation responsible for the observed inhibition.

### *Materials and Methods*

*Protocol.*—Adult, male Lewis rats (Microbiological Associates, Bethesda, Md.) (donors) received a single intraperitoneal dose of 100 mg of BGG (Cohn fraction II, Mann Research Laboratories, New York) or the control antigen OA (crystalline hen ovalbumin, Nutritional Biochemicals Corp., Cleveland, Ohio). In one group of experiments, 25 mg of hydrocortisone acetate (Nutritional Biochemicals Corp.) was injected intramuscularly at the same time.

At 2 days, thymocytes were harvested by mincing donor thymus, gently squeezing the fragments between sterile slides in ice-cold medium (M199, prepared by the Laboratory of Epidemiology and Public Health, Yale University), expelling several times through a 23-gauge needle, filtering through nylon mesh, and finally washing five times with medium. Viability was usually greater than 90%. The cells from three donors were then injected intravenously into a single normal syngeneic recipient or were separated on density gradients (see below) and the subpopulations so obtained were injected into separate recipients.

Recipients were challenged with 10 μg of BGG in complete Freund adjuvant in a footpad 24 h after cell transfer, were bled and skin tested with 30 μg of BGG and 30 μg of PPD (Parke, Davis and Co., Detroit, Mich.) 10 and 20 days after challenge, received an intraperitoneal booster dose of 1 mg of BGG at 25 days, and were subjected to a final bleeding at 32 days.

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[1] *Abbreviations used in this paper*: A, B, C, D, and P: bands (and pellet) respectively, obtained on density gradients; BGG, bovine γ-globulin; BSA, bovine serum albumin; HC, hydrocortisone acetate; OA, crystalline hen ovalbumin; and PPD, purified protein derivative of tuberculin.

Their skin reactions (Arthus at 3 h, delayed at 24 and 48 h) were scored on the basis of thickness and diameter of induration, the values ranging between 0 and a maximum of 7 (1). Antibody was quantitated by radial immunodiffusion (see below).

Each experiment was replicated several times, and each replication included all the experimental groups to be compared. Thus animals within that replicate group were challenged and tested simultaneously and with the same materials. Enough replicates were performed to provide groups of at least six to eight rats for comparison. (For further details see reference 1.)

*Density Gradient Separation.*—BSA (bovine serum albumin, 30% solution, Lot 126, Miles Laboratories, Inc., Elkhart, Ind.) was used to prepare discontinuous gradients in 35-ml cellulose tubes with successive layers containing 5 ml of 10% solution, 7 ml each of 20, 24, and 27% solution, and 5.5 ml of 30% solution in which the thymocytes were suspended (3, 4). Thymocytes were washed three times and counted before separation. An average number of $7.2 \times 10^8$ cells was used per tube. Centrifugation was at 4°C for 30 min at 12,000 rpm in a Spinco SW 25.1 rotor (Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.). This produces a maximum force of 20,000 *g* at the tip of the tube. The discrete bands which formed at the density interfaces were designated A, B, C, and D in order of increasing density and the pellet P. These were collected with a Pasteur pipette, washed twice, and counted. Usually cells from the corresponding bands of three tubes were pooled for a single experiment. It was our regular practice also to pool A with B, D with P, and, in some earlier experiments, A + B with C.

*Measurement of Precipitating Antibody* (5, 6).—A 2% solution of "Ionagar" no. 2 (Consolidated Laboratories, Inc., Chicago Heights, Ill.) in distilled water was mixed at 50°C with an equal volume of a suitably diluted antiserum in a diluent buffer containing 1.0 M glycine, 0.05 M sodium barbital, 0.0125% Cd $Cl_2$ (agent-intensifying precipitate (7)), and 0.02% Merthiolate, adjusted to pH 7.7–7.8. 2 ml of the mixture was pipetted into 35-mm cups in six-cup disposable plastic trays (Linbro Chemical Co., Inc., New Haven, Conn.) and allowed to solidify 3–4 h at 4°C. Each cup was tested with antigen at a concentration of 1 mg/ml, 5 μl, placed in five to ten replicate 3-mm wells, using Eppendorf micropipettes with disposable tips. The plates were incubated in a humidified chamber at 4°C. The diameters of the precipitin rings, after they reached constant size (normally 8 days), were determined with a measuring magnifier equipped with an eyepiece scale (Edmund Scientific Co., Barrington, N.J.).

A linear plot was obtained between the square of the measured ring diameters and the reciprocal of the dilution of the single reference serum employed throughout. A regression line was then fitted by the least square method. By use of its intercept and slope the ring diameters obtained with each "unknown" replicate could then be utilized to solve for the equivalent dilution of the reference serum which gave an equal response. The antibody levels (titers) for all sera are presented in relative terms, as percentages of that of the reference serum. The mean and standard error of the mean for each subgroup were estimated from the family of data values of that subgroup as just outlined.

Tests for significance of differences were made, between each subgroup and the OA control in that particular treatment group, by the usual *t*-test. Changes were regarded as significant when $P < 0.05$.

## RESULTS

*Hydrocortisone Resistance of "Suppressor" Cells.*—2 days after intramuscular injection of 25 mg of hydrocortisone acetate, the yield of thymocytes had fallen to less than 10% of control values (Table I). Thymocytes from either three or six BGG donors and from six OA donors treated with HC were transferred

in the usual way. In order to provide controls bracketing the actual cell numbers transferred from the treated donors, we arbitrarily chose to transfer thymocytes from BGG donors not given HC in numbers of $10 \times 10^8$ and $1 \times 10^8$, corresponding to approximately 1.5 and 0.15 donors, respectively.

Thymocytes from HC-treated BGG donors demonstrated substantial suppressor activity (Table II). The greatest suppression of skin reactions was seen with treated cells from six donors. However, on a per donor basis, one did not see a significant increase in suppressor activity of the HC-resistant thymocytes

TABLE I

*Effect of Hydrocortisone on Yields of Donor Thymocytes*

| Experiment | Average yield of thymocytes $\times 10^8$ from donors treated with | | |
|---|---|---|---|
| | BGG | BGG + HC | OA + HC |
| 1 | 8.2 | 0.80 | 0.23 |
| 2 | 8.4 | 0.60 | 0.70 |
| 3 | 8.0 | 0.63 | 0.70 |
| 4 | 8.9 | 0.78 | 0.80 |
| 5 | 7.2 | 0.50 | 1.00 |
| Average ± SE | 8.14 ± 0.25 | 0.66 ± 0.05 | 0.69 ± 0.11 |
| BGG cells, % | 100 | 8.1 | 8.5 |

TABLE II

*Immunological Responses* in Recipients of Thymocytes from Hydrocortisone-Treated Donors*

| Treatment of donors | | | | | |
|---|---|---|---|---|---|
| Antigen | BGG | BGG | BGG | BGG | OA |
| Hydrocortisone | 0 | 0 | + | + | + |
| Cells transferred | | | | | |
| Average no. $\times 10^8$ | 10.0 | 1.0 | 4.0‡ | 1.8§ | 4.8‡ |
| Skin reaction scores at 10 days | | | | | |
| Arthus (3 h) | 1.3 ± 0.4 | 3.3 ± 0.5 | 0.9 ± 0.3 | 2.5 ± 0.4 | 4.3 ± 0.3 |
| Delayed (24 h) | 3.6 ± 0.5 | 5.1 ± 0.5 | 4.4 ± 0.4 | 5.2 ± 0.2 | 6.8 ± 0.1 |
| (48 h) | 1.3 ± 0.3 | 3.0 ± 0.5 | 0.6 ± 0.4 | 2.9 ± 0.6 | 4.2 ± 0.3 |
| PPD (24 h) | 3.1 ± 0.3 | 2.8 ± 0.3 | 3.0 ± 0.3 | 3.2 ± 0.1 | 2.6 ± 0.2 |
| Skin reaction scores at 20 days | | | | | |
| Arthus (3 h) | 2.1 ± 0.4 | 3.2 ± 0.5 | 2.0 ± 0.4 | 3.3 ± 0.4 | 4.5 ± 0.4 |
| Delayed (24 h) | 2.2 ± 0.4 | 4.1 ± 0.5 | 2.6 ± 0.4 | 3.3 ± 0.4 | 4.7 ± 0.4 |
| (48 h) | 0.7 ± 0.2 | 2.6 ± 0.5 | 0.5 ± 0.4 | 2.1 ± 0.4 | 3.3 ± 0.3 |
| PPD (24 h) | 3.0 ± 0.2 | 3.2 ± 0.2 | 3.6 ± 0.4 | 3.2 ± 0.1 | 2.9 ± 0.2 |
| Precipitin titers at 20 days | 20.2 ± 4.3 | 28.5 ± 3.5 | 29.7 ± 8.2 | 21.6 ± 5.5 | 36.7 ± 7.1 |
| Precipitin titers at 32 days | 31.1 ± 8.3 | 53.5 ± 10.7 | 58.5 ± 16.5 | 52.5 ± 21.2 | 63.5 ± 5.1 |

* All values are averages of 8–10 tests ± SE.

‡ Cells transferred from six donors.

§ Cells from three donors.

as compared with thymocytes not exposed to HC. In every instance, HC-resistant thymocytes from three donors suppressed less efficiently than $10 \times 10^8$ cells from 1.5 donors not given HC. Precipitin levels were significantly lowered (at both 20 and 32 days) in recipients of $10 \times 10^8$-untreated BGG donor cells. It seems clear that some suppressor activity was lost with the hydrocortisone-sensitive population.

*Separation of Active Thymocytes on Density Gradients.*—When thymocytes were separated on BSA gradients (Table III), the yields in the different bands and the pellet agreed reasonably well with those obtained earlier by the same method (4), and corresponded in an approximate way with the yields obtained in similar density separations of mouse and human thymocytes (8–11). With great uniformity, 90% of the cells were found in band D and the pellet P, fractions which have proven largely inert by all criteria (see references 4, 8–11).

In order to facilitate comparison between responses in recipients of different numbers of different kinds of cells, the following calculation was employed to generate the values shown in Figs. 1–5. In each replication of the experiment, the difference between an individual result (skin reaction score, antibody titer) in a rat given cells from BGG-injected donors and the average value obtained from OA-injected donors (in that same replication of the experiment) was divided by the number of cells transferred to that particular recipient. We are omitting Arthus scores obtained 10 days after challenge, which were generally

TABLE III

*Cell Yields and Cells Transferred after Density Gradient Separation of Thymocytes*

| Experiment | Yield of thymocytes $\times 10^8$ (three donors) | Thymocytes* in bands | | | | Total recovered | No. of cells $\times 10^7$ transferred | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | A + B | C | D | P | | A + B + C | A + B | C | D + P |
| | | % | % | % | % | % | | | | |
| 1 | 22.0 | 1.2 | 2.2 | 14.0 | 32.7 | 50.1 | 7.6 | | | 100.3 |
| 2 | 27.0 | 0.6 | 6.7 | 9.6 | 51.9 | 68.8 | 20.0 | | | 166.0 |
| 3 | 23.4 | 1.0 | 1.1 | 17.5 | 36.0 | 55.1 | 27.0 | | | 125.0 |
| 4 | 30.0 | 1.1 | 3.3 | 6.7 | 42.7 | 53.8 | 13.3 | | | 148.0 |
| 5 | 27.6 | 1.2 | 6.9 | 10.0 | 43.4 | 61.5 | 22.7 | | | 147.6 |
| 6 | 15.6 | 0.7 | 6.6 | 14.2 | 48.0 | 69.5 | | | 10.4 | 97.0 |
| 7 | 19.8 | 0.8 | 3.6 | 18.1 | 52.5 | 75.0 | | | 7.2 | 140.0 |
| 8 | 16.8 | 1.9 | 6.0 | 14.3 | 50.0 | 72.2 | | 3.2 | 10.0 | 100.8 |
| 9 | 19.0 | 1.3 | 5.1 | 12.6 | 42.2 | 61.2 | | 2.4 | 9.6 | 104.0 |
| 10 | 20.0 | 0.7 | 5.0 | 12.8 | 54.0 | 72.5 | | 1.4 | 10.0 | 134.0 |
| 11 | 18.0 | 0.8 | 5.0 | 17.8 | 51.1 | 74.7 | | 1.4 | 9.0 | 124.0 |
| Average | 21.7 | 1.0 | 4.7 | 13.4 | 45.8 | 64.9 | 18.0 | 2.1 | 9.4 | 126.0 |
| SE | 1.4 | 0.1 | 0.6 | 1.0 | 2.0 | 2.6 | 3.0 | 0.4 | 0.4 | 6.7 |

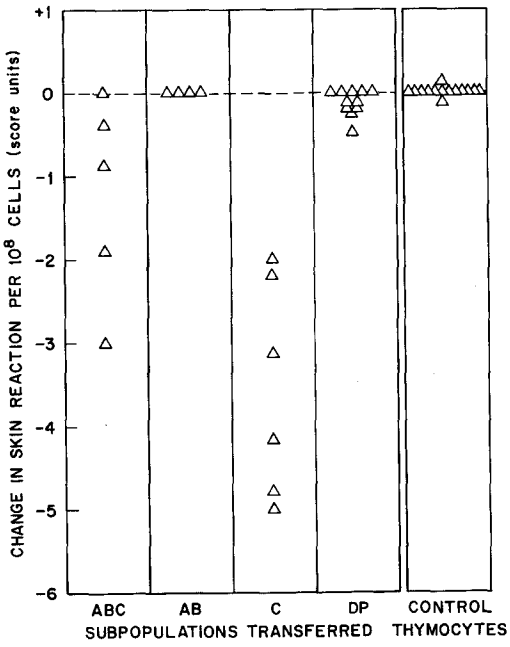
* Counted before washing. Note that cells were transferred after washing, which resulted in nonuniform cell loss in the different bands.

very low, and delayed reaction scores read at 48 h, since these mimicked the 24-h readings.

The cells of band C reproducibly and significantly inhibited the specific immunologic response, whether injected alone or in combination with bands A and B (Figs. 1–5). A and B also demonstrated some activity. As in other parts of the present investigation, Arthus and delayed reactivity at 10 and 20 days were substantially reduced, while antibody formation was low at 20 days but approached normal by 32 days.

## DISCUSSION

These results confirm and extend our earlier finding (1) that thymocytes from rats given a large dose of BGG, when transferred to syngeneic recipients, inhibit responses to BGG in the latter. The specificity of the inhibition is established both by the use as controls of thymocytes from donors treated with OA and by the demonstration that delayed reactions to PPD are un-




FIGS. 1–5. Suppression of immune reactivity in recipients of thymocyte subpopulations separated on BSA density gradients. Separation was done with cells of donors injected with BGG. Control thymocytes were from OA-injected donors. Results are plotted as difference between individual test and control animals per $10^8$ cells transferred to the test animal (calculated separately for each replication of the experiment).

FIG. 1. Arthus reactions (3-h readings) to skin test with BGG 20 days after challenge.

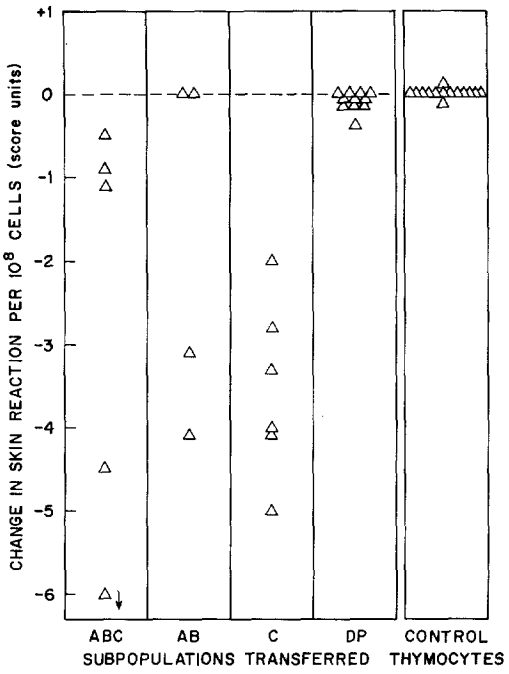



FIG. 2

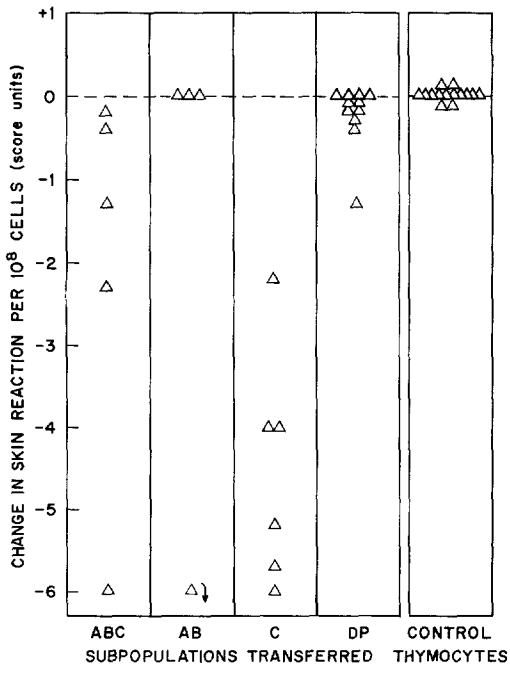



FIG. 3

FIGS. 2 and 3. Delayed reactions (24-h readings) to skin test with BGG 10 and 20 days after challenge.

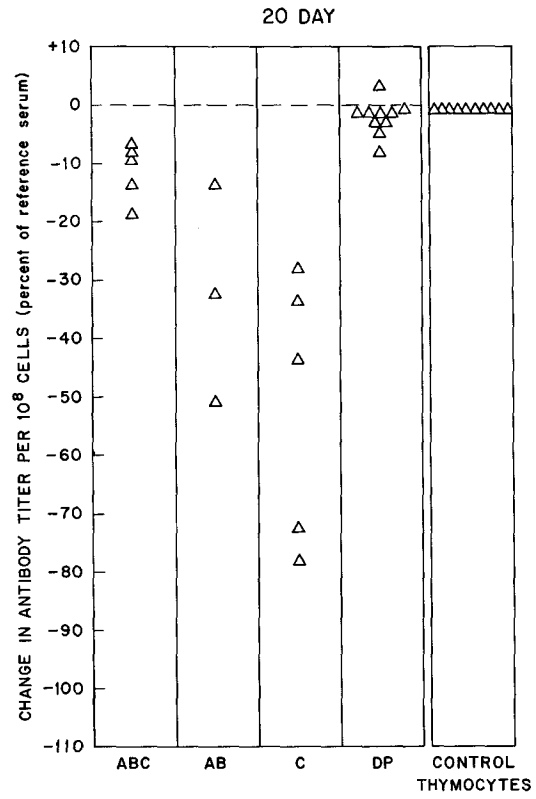



FIG. 4

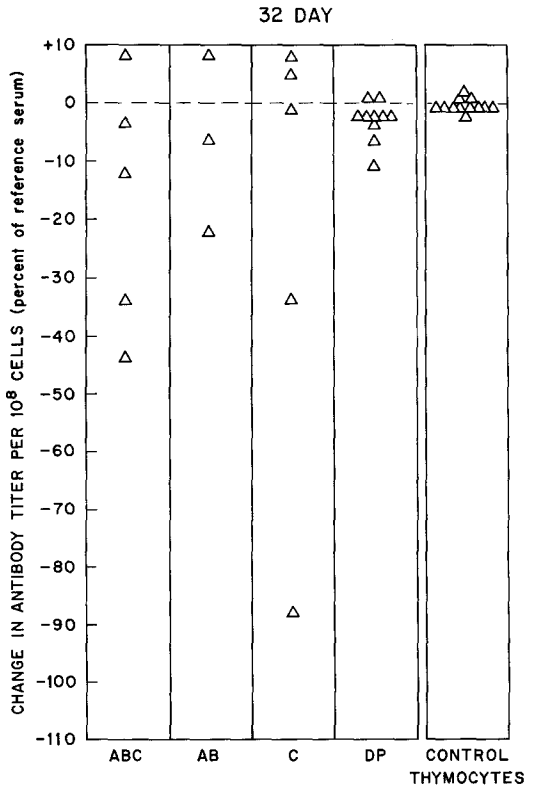



FIG. 5

FIGS. 4 and 5. Antibody titers, expressed as per cent of reference serum titer, 20 and 32 days after challenge.

affected. In a separate paper,[2] we show that inhibition is not produced by cells treated with antimycin A before transfer. This confirms the requirement for transfer of living, fully functional cells to produce inhibition in the recipient, and tends to rule out carry-over of antigen as a significant problem in the experiment.

As before, the observed inhibition affected both Arthus and delayed skin reactivity to BGG and the formation of humoral antibody. The use in the present study of a quantitative technique for measurement of precipitating antibody permitted a more precise definition of the degree of inhibition of the latter. As with other parameters of the immunological response, the inhibition was partial and, in some instances, expressed as a simple delay in antibody formation. It thus resembles competition or "preemption" more than tolerance. An attempt to define the molecular class(es) of antibody affected is in progress. However, the Arthus reactions measured in the present study may be presumed to represent slow IgG, probably IgGa (12, 13), the formation of which was obviously suppressed in appropriately treated animals.

The transferred "suppressor" cells responsible for inhibition were found, after separation on density gradients, largely in band C, but some activity was also observed in the pooled bands A + B. They appeared to represent less than 10% of the total thymocyte population. This distribution corresponds to that in which we previously found the subpopulation of thymocytes possessing activities normally ascribed to mature T cells: homing to lymph nodes and spleen; responsiveness to phytohemagglutinin and to allogeneic cells; and graft-vs.-host competence (4). Other laboratories, working with murine or human cells (8–11, 14), have confirmed this distribution and identified a number of additional properties, notably "helper cell" activity, in the same subpopulation. It should be remarked that cytotoxic or "killer cell" activity is developed in the same cells only after several days sensitization in vivo (9, 10) or in vitro (15–17).

Many of the active cells appear to be relatively resistant to hydrocortisone. Hydrocortisone resistance is a property of the thymocyte subpopulation with peripheral T cell activity (11, 18–24). Both helper activity (11, 18, 20) and the capacity to become immunized for "killer cell" activity (19, 21, 23, 24) reside in this fraction which, in several studies, was found to correspond to cells of the thymic medulla. However our quantitative data, crude though they admittedly are, imply that considerable suppressor activity resides as well in the hydrocortisone-sensitive fraction. In Gershon's studies (see reference 2 and footnote 3), part of the regulatory activity of thymocytes was found in a hydro-

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[2] Ha, T.-Y., B. H. Waksman, and H. P. Treffers. The Thymic Suppressor cell. II. Metabolic requirements of suppressor activity. Submitted for publication.

[3] Cohen, P., R. Hencin, and R. K. Gershon. 1973. The role of cortisone sensitive thymocytes in DNA synthetic responses to antigen. Submitted for publication.

cortisone-sensitive subpopulation. One must remain open to the possibility that the suppressor cells do not correspond perfectly to the thymocyte subpopulations with conventional peripheral T lymphocyte activity. Having presumably been activated by their first exposure to antigen 48 h before sacrifice of the donor, they may have undergone slight or even considerable change in size, density, resistance to such agents as X ray and corticosteroids, or other properties (25). We did, as noted above, find some activity in the largest (least dense) fractions A + B. Alternatively, there may really be distinct subpopulations within the thymus, representing either successive stages in maturation of a single cell lineage or active stages of cell development in two (or more than two) distinct cell lines (see references 10 and 26–28). Tigelaar and Asofsky's recent study of graft-vs.-host activity in thymocytes before and after cortisone treatment shows clearly the presence of active but qualitatively different cells in the sensitive and resistant subpopulations (28).

## SUMMARY

This study confirms the finding that washed thymocytes from rats given 100 mg of BGG 48 h earlier, when transferred to syngeneic recipients, exert a specific suppressor effect on immunological responses to BGG in the latter. The active cells are found in a subpopulation of low density, making up less than 10% of the total thymocytes, and are partially resistant to hydrocortisone.

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