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Stimulation of Antibody Synthesizing Lymphocytes in the Rat by Antigen Reactive Cells Sensitized *In Vitro*[1] (39303)

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(Introduced by Benito Lombardi)

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The production of serum antibody to thymic dependent antigens requires the interaction of T and B lymphocytes (1, 2). The class of antibody produced is determined by bone marrow derived lymphocytes (3) and not the thymic dependent cell. In mice, single thymic dependent lymphocytes can interact with both 7 and 19 S antibody synthesizing cells (4).

It has been previously reported that rat lymphocytes can be stimulated *in vitro* by sheep erythrocytes (5). The lymphocytes synthesize DNA but antibody formation does not occur. Such antigen reactive cells can be washed free of antigen and their interaction with antibody synthesizing cells studied *in vivo*. The study to be reported found that lymphocytes which produce either 19 or 7 S anti-sheep erythrocyte antibody are derived independently of each other and that antigen reactive cells have a limited reactivity with lymphocytes which produce antibody.

Materials and methods. Animals. Inbred male Lewis rats were purchased from Microbiological Associates. They were maintained on Purina rat food and water *ad libitum*.

Antigen. Sheep erythrocytes were collected each week from the same animal. This animal was selected because it was found to stimulate the production of a high number of plaque-forming cells (PFC) when injected into rats.

Culture conditions. A single cell suspension of spleen lymphocytes was prepared by forcing the spleen through a fine metal screen. The cells were washed by centrifugation in Hanks' balanced salt solution (BSS) and counted. One milliliter containing 15×10^6 normal Lewis rat spleen cells was cultured with 5×10^6 washed sheep erythrocytes in 35×10 mm plastic petri dishes (Falcon Plastic). Cell viability, as determined by trypan blue staining, was always greater than 90%. RPMI-1640 tissue culture medium supplemented with 15% fetal calf serum in a gas atmosphere of 83% N_2, 7% O_2, and 10% CO_2 with continuous rocking of the culture dishes was used. Cell viability was maintained better in fetal calf serum than in rat serum.

Tritiated thymidine uptake. Cultures were prepared as described. Four hours before the termination of the culture 2 μCi of T_3H (6.7 Ci/mmole) was added to each culture. Control cultures consisting of lymphocytes without sheep erythrocytes were also included. The cells were collected and the radioactivity incorporated into the DNA was counted.

Assay for cell stimulation. The lymphocyte/erythrocyte mixture was maintained in the incubator for 24 hr. The cell suspension was then washed from the plate (without scraping) and the erythrocytes were lysed with distilled water. The cell suspension was Wright stained and no macrophages were seen. The lymphocytes were washed five times in BSS and various numbers of lymphocytes were injected intravenously into normal recipient Lewis rats. Four days later the animals were sacrificed and the number of direct (19 S) and indirect (7 S) plaque-forming cells was determined by a localized hemolysis in gel assay (6). The test used was slightly modified from that described by Jerne, Nordin, and Henry (12) in that agarose (Bausch and Lomb, Rochester, N.Y.) was used. Depending on the test design some plates were flooded with rabbit antiserum to rat 7 S globulin (anti-7 S) to detect

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cells producing 7 S antibodies. Duplicate plates were always counted for each determination and random plaques were examined under high power of a light microscope to be certain of the presence of a nucleated cell in the center. The average number of plaques on plates developed without the addition of anti-7 S was subtracted from the average number of plaques on plates that had anti-7 S added to give the number of cells producing 7 S immunoglobulin.

The assumption was made that all plaques detected without the use of anti-7 S globulin were produced by cells synthesizing 19 S immunoglobulin. Sucrose density separation of rat anti-sheep erythrocyte serum showed that the 7 S antibody produced hemolysis only in the presence of anti-7 S globulin and that the 19 S antibody titer was not increased with the addition of the rabbit anti-rat 7 S globulin antiserum.

The spleen was cut into eight sections after being removed from the recipient animal and the number of PFC determined for each section. As a control, prior to injecting sensitized lymphocytes into recipient animals, the lymphocytes were given 800 R of irradiation.

Results. Stimulation of lymphocytes in vitro. Normal rat spleen lymphocytes were maintained *in vitro* with sheep erythrocytes. Tritiated thymidine was added on successive days and the amount of thymidine uptake determined (Table I). As early as 24 hr after initiation of the cultures increased amounts of thymidine uptake were found in lymphocytes cultured with erythrocytes in comparison to cultures without added erythrocytes. PFC assays were performed with the stimulated lymphocytes and no PFC's were detected on any day of culture.

PFC in recipients spleen sections. After *in vitro* stimulation of the lymphocytes, the erythrocytes were removed by lysis and washing. The sensitized lymphocytes were then injected into normal recipients. In all studies to be reported the lymphocytes were sensitized for 24 hr *in vitro* and the recipient animal spleens were assayed 96 hr after cell transfer, when the maximum number of PFC was found to occur.

In Table II the results of two representative recipient animals are shown. At the lowest number of sensitized lymphocytes injected (0.25×10^6) some spleen segments had only 19 or 7 S PFC. At higher cell numbers all cell sections had both 19 and 7 S PFC. However, either 19 or 7 S PFC predominated in individual spleen sections.

TABLE I. *IN VITRO* T_3H UPTAKE BY RAT SPLEEN CELLS CULTURED WITH S-RBC.

Day	Control	Stimulated
1	3,217 ± 187[a]	24,636 ± 1,112
2	5,592 ± 406	40,817 ± 2,642
3	7,584 ± 612	54,268 ± 2,176
4	14,769 ± 982	69,387 ± 3,837

[a] Counts per minute ± SD.

Relationship of PFC to lymphocyte number transferred. Two groups of animals, designated A and B, were injected with sensitized lymphocytes. Each group contained five animals. The number of lymphocytes injected was adjusted so that each of the five animals which comprised the B group received 1/5 of the cell number injected into each rat in the A group. Therefore, the total number of cells injected into the entire B group equaled that given one group A rat. PFC were assayed on Day 4 after transfer.

If each stimulated lymphocyte can only react with a predetermined number of antibody synthesizing cells, the expected ratio of the mean PFC in group A to the total PFC in group B would be 1.00. The results shown in Table III indicate that the mean for nine experiments closely approximated this value.

Controls. To control for the transfer of antigen with the lymphocytes 800 R of irradiation was given to sensitized lymphocytes just prior to transfer. No recipient of irradiated lymphocytes had detectable PFC.

A further control was done to rule out an effect of radiation on the antigenicity of lysed erythrocytes. Irradiation was given to lysed erythrocytes and these were then injected in normal rats. There was no difference in the number of PFC produced when either irradiated or nonirradiated lysed erythrocytes were given, indicating that the irradiation was effective only on the lymphocytes.

As a control for the possibility of the sensitized lymphocytes becoming antibody producing cells *in vivo*, the sensitized lymphocytes (50×10^6/animal) were injected into irradiated recipients. No antibody for-

TABLE II. NUMBER OF 19 S AND 7 S PFC IN SPLEEN SECTIONS OF RATS INJECTED WITH LYMPHOCYTES SENSITIZED TO SHEEP ERYTHROCYTES *IN VITRO*

	Section number							
	1	2	3	4	5	6	7	8
	Recipient injected with 0.25 × 10^6 lymphocytes							
19 S	63	0	362	50	75	88	38	50
7 S	0	150	0	88	13	13	89	0
	Recipient injected with 50 × 10^6 lymphocytes							
19 S	12,840	2,940	2,820	19,080	12,120	33,360	15,720	18,960
7 S	9,900	6,180	1,080	8,160	21,180	4,560	11,880	14,627

TABLE III. TOTAL PFC (19 S PLUS 7 S) RESULTING FROM INJECTING DIFFERENT CONCENTRATIONS OF SENSITIZED LYMPHOCYTES INTO RECIPIENTS

Cell Injection/Rat × 10^6		PFC/Spleen		
A	B	A[a]	B[b]	Ratio, A/B
1.25	0.25	13,100 ± 1,164	10,794	1.22
2.5	0.5	7,350 ± 876	7,050	1.04
5.0	1.0	20,426 ± 1,873	25,978	0.79
25.0	5.0	46,198 ± 3,412	49,171	0.94
25.0	5.0	104,883 ± 8,436	161,206	0.65
50.0	10.0	120,129 ± 7,621	88,291	1.37
50.0	10.0	299,802 ± 13,466	273,016	0.86
50.0	10.0	136,770 ± 9,927	118,874	1.15
55.0	11.0	231,454 ± 11,164	217,315	1.08
Mean				1.01 ± 0.22

[a] Mean ± SD of five rats.
[b] Total of five rats.

mation occurred in these recipients.

Discussion. Study of the *in vivo* response of lymphoid cells to antigen is difficult as several different events may be occurring simultaneously. These include antigen/lymphocyte, lymphocyte/lymphocyte, and macrophage/lymphocyte interaction. *In vitro* studies, particularly with mouse lymphocytes (7, 8), have shown that *in vitro* techniques can be used to study the mechanisms leading to antibody formation.

Culture conditions for lymphocytes were set up as described. Using this procedure, normal mouse lymphocytes were stimulated to produce antibody. However, under identical culture conditions rat lymphocytes, although stimulated to synthesize DNA, did not produce antibody. Such lymphocytes were designated antigen-reactive cells and their interaction with antibody synthesizing cells was determined *in vivo*.

The controls used confirmed the absence of immunogenic erythrocyte antigen in the washed lymphocyte preparation and that the sensitized lymphocytes could not mature into antibody synthesizing cells *in vivo*. Thus, any PFC detected in the recipients was due to the sensitized lymphocytes interacting with antibody synthesizing lymphocytes in the recipients.

The number of PFC found in the recipient spleen reached a maximum on Day 4. This coincides with the maximum number of PFC detected when sheep erythrocytes are injected intravenously into rats (6). This suggests that following the intravenous injection of sheep erythrocytes into rats the peaking of the PFC response at Day 4 is due to the time required for antibody production to occur and not the time required for the antigen reactive cells to become active. The latter event occurs within 24 hr *in vitro* (Table I).

In animals given low numbers of sensitized lymphocytes, some sections of the spleen had only 19 or 7 S PFC. A similar lack of uniformity in the distribution of antibody forming cells in the mouse spleen has

been reported (9). Other sections in these and animals given larger numbers of lymphocytes also showed a disassociation in the production of 19 and 7 S PFC. These observations suggest a different derivation for 19 and 7 S PFC and argue against the formation of all 7 S cells from cells already synthesizing 19 S antibody (10, 11).

Comparing the number of PFC resulting from the injection of a large number of lymphocytes or 1/5 that number (Table II) reveals that regardless of the number of sensitized lymphocytes injected only a given number of PFC are produced. If two sensitized lymphocytes were needed to stimulate another cell to antibody formation it would have been expected that the animals in Group A which received a higher cell number would, because of the increased probability for cellular interaction, have had more PFC than the Group B animals. Alternatively, if sensitized lymphocytes are capable of sequentially stimulating other cells to antibody formation, saturation of the spleen at high cell concentrations would be expected to occur and the total number of PFC in Group B would be expected to be greater than in Group A. The results obtained, therefore, suggest a limited reactivity of sensitized lymphocytes in inducing other lymphoid cells to antibody formation. These findings differ slightly from those reported in the mouse (4). This may be explained by species differences or the continual presence of antigen in the mouse system.

Summary. Lymphocytes obtained from rat spleens were sensitized *in vitro* to sheep erythrocytes. The sensitized lymphocytes did not synthesize antibody but when injected into unimmunized rats did stimulate antibody production to sheep erythrocytes. Analysis of the location of 7 and 19 S plaque-forming cells in the recipient rats indicated that these two antibody synthesizing cell populations arise from different lymphocytes. It was also found that the sensitized lymphocytes could react only with a limited number of antibody synthesizing cells.

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