Effect of Leucogenenol on Formation of 19S and 7S Hemolysin in Normal and Splenectomized Rats[1] (36482)

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Rats do not form appreciable hemolysin in response to the injection of sheep erythrocytes for approximately 1 month after splenectomy (1). However, rats form normal titers of hemolysin as early as 3 days after splenectomy when, in addition to sheep erythrocytes, they are injected with leucogenenol (2), 2-(1,2-dihydroxy-3-methyl-5-oxocyclohexyl)-3,11-dihydroxy-11-(hydroxymethyl)-9-methyl-1-oxa-5-azaspiro[5.5]undeca-2,4-dien-7-one (3), a compound that occurs as a metabolic product of *Penicillium gilmanii* (4) and is also present in bovine and human liver (5). Since it has been reported that splenectomy abolishes the early 19S response but not the 7S response (6) it was of interest to investigate the effect of leucogenenol on the formation of 19S and 7S hemolysin in normal and splenectomized rats.

Materials and Methods. Twelve of 24 rats (Wistar, approx 250 g in wt) were splenectomized. Three days later each of the 24 rats were injected intraperitoneally with 2×10^8 sheep erythrocytes suspended in 0.5 ml of sterile pyrogen-free isotonic saline. At the time of injection with sheep erythrocytes and at 24 hr intervals thereafter for 6 consecutive days, 6 of the normal and 6 of the splenectomized rats were injected intravenously with 1 μg of the calcium salt of leucogenenol dissolved in 0.2 ml of sterile pyrogen-free isotonic saline. Rats that were not injected with leucogenenol received in its place 0.2 ml of sterile pyrogen-free isotonic saline. Concurrently normal and splenectomized rats in groups of 6 were injected with either leucogenenol or pyrogen-free isotonic saline as above but not with sheep erythrocytes. On the seventh day following injection with sheep erythrocytes each rat was exsanguinated by cardiac puncture under ether anesthesia and the blood obtained (approx 5 ml/rat) was allowed to clot overnight at 5°. The serum was then separated, centrifuged (Sorvall, RC-2, sn24 rotor) at 10° and 8000 rpm for 10 min, heated at 56° for 30 min, and chromatographed on Sephadex (G-200-fine, obtained from AB Pharmacia, Uppsala, Sweden).

The Sephadex, suspended in 0.15 *M* unbuffered saline, was boiled for 2 to 3 min (7) to drive off gases and allow the gel to swell, then poured into a 19 × 60 mm Sephadex laboratory column (30 ml bed volume) and allowed to equilibrate for 2 hr with 0.15 *M* unbuffered saline. A portion of the serum (0.3 ml) from each rat was applied to the surface of a column and eluted from the column with 0.15 *M* unbuffered saline. Fractions (0.7 ml) were collected at 5° on a LKB Ultrorac (type 7000) fraction collector at a flow rate of approximately 2 ml/hr. The concentration of protein in each fraction was determined by its absorbance at 280 nm in a Gilford 240 spectrophotometer and expressed in absorbance units. Hemolysin titers of the chromatographically separated fractions were determined in the usual manner (2, 8) on each serum and on each chromatographic fraction. The sum of the hemolysin titers of the chromatographically separated fractions accounted for at least 97% of the hemolysin activity of the unfractionated serum. Antibody activity of the chromatographic fractions of the rat serum was also determined by the indirect hemolytic method (8, 9). A

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sheep erythrocycyte suspension (0.4 ml, containing 2 × 10^8 erythrocytes/ml) in Veronal buffer was mixed with equal volumes of the dilutions of each of the chromatographic fractions of rat serum. The mixtures were incubated for 20 min at 37° then washed with and resuspended in Veronal buffer (0.4 ml). Rabbit antirat serum antiserum (0.1 ml of a 1:10 dilution) was added to each tube and the mixture incubated for 20 min at 37°. The erythrocytes were separated by centrifugation, washed with and resuspended in Veronal buffer (8, 9). Guinea pig complement (0.4 ml, diluted 1:20) was then added to each tube. After incubation at 37° for 15 min, the amount of hemolysis was measured and the dilution of rat serum yielding 50% hemolysis was determined (2, 8, 9). Before use the rabbit antirat serum antiserum (kindly supplied by Dr. E. J. Leonard, NCI, NIH) was heated to 56° and treated for 20 min in the usual manner with packed sheep erythrocytes.

Ultracentrifugation was performed on 0.25 ml of serum in sucrose density gradients (10–28%) prepared in Veronal buffered saline (pH, 7.3; μ, 0.15, with Ca^{2+} and Mg^{2+}) as described by Samuels (10) using a Beckman L2-65 ultracentrifuge with sw39L rotor. Samples were centrifuged at 10° for 16 hr at 32,000 rpm. A 1% mixture of bovine thyroglobulin (19S) and human IgG (7S) was concurrently centrifuged with two sera. After centrifugation fractions were collected dropwise from the tops of the tubes. The distribution of protein was determined in the tube containing the bovine thyroglobulin and human IgG by measuring the absorbance of fractions at 280 nm with a Gilford 240 spectrophotometer. Corresponding fractions of serum were titrated for hemolysin activity. To verify that the sucrose gradients were linear, the percentage of sucrose in each fraction was determined in a Bausch and Lomb refractometer.

Results and Discussion. Figure 1 shows the effect of leucogenenol on the concentration, molecular weight distribution, and hemolysin activity of proteins in the serum of normal and splenectomized rats that were injected

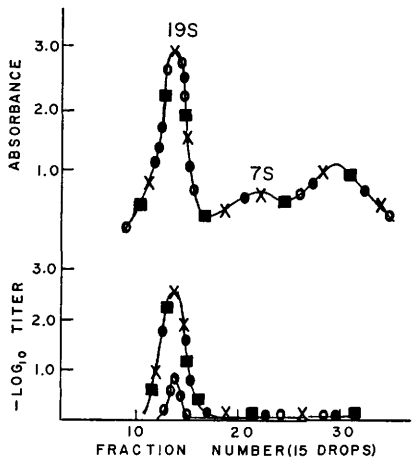

FIG. 1. Effect of leucogenenol on the concentration, molecular weight distribution, and hemolytic activity of the serum proteins from normal and splenectomized rats following their intraperitoneal injection with 2 × 10^8 sheep erythrocytes. (X) normal rat: (●) normal rat injected with leucogenenol; (○) splenectomized rat; (■) splenectomized rat injected with leucogenenol. Rats were injected intravenously with leucogenenol at the time they received the sheep erythrocytes and at 24 hr intervals thereafter for 6 days. Titers were determined on day 7. For details see text.

with sheep erythrocytes. As shown, leucogenenol does not affect the concentration and distribution of either 19S or 7S proteins. In addition, the hemolysin activity that is associated with 19S protein is not changed in normal rats by the injection of leucogenenol. As expected (1, 2) the sera of splenectomized rats that were injected with sheep erythrocytes but not with leucogenenol show insignificant quantities of hemolysin. However, splenectomized rats that received leucogenenol in addition to sheep erythrocytes show normal quantities of hemolysin, and the hemolysin is associated with the 19S proteins. Ultracentrifugation in a sucrose gradient (Fig. 2) confirms the results of the chromatography on Sephadex-200, *viz.*, that leucogenenol stimulates the 19S response in splenectomized rats.

Table I shows that the total quantity of 19S hemolysin is not increased in normal rats by the injection of leucogenenol, and that the hemolysin formed in splenectomized

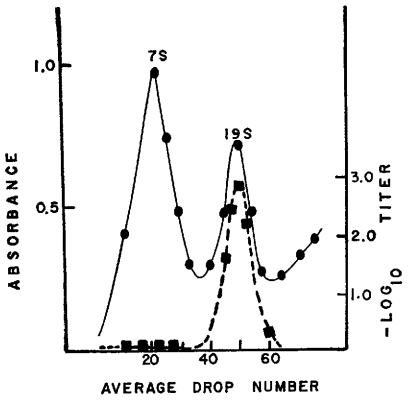

FIG. 2. The distribution by molecular weight as determined by ultracentrifugation in a sucrose gradient of the hemolysin activity in the serum of a splenectomized rat that was injected with leucogenenol and sheep erythrocytes. (●) absorbance of fractions of a 1% mixture of human IgG (7S) and bovine thyroglobulin (19S); (■) $-\log_{10}$ titers of hemolysin in the serum. Drops were obtained in order from the top of the gradient. For details see text.

rats in response to the injection of leucogenenol as well as sheep erythrocytes is associated with the 19S protein. Since rats injected with leucogenenol show by direct titration small but measurable quantities of 7S hemolysin, and rats that are not injected with leucogenenol show no measurable quantities of 7S hemolysin (Table I), the possibility existed that leucogenenol stimulates to some extent the 7S response. To ascertain whether or not the difference in 7S response was significant each of the chromatographically separated 19S and 7S fractions were titrated by the indirect hemolytic method (8, 9) using rabbit antirat serum antiserum. The results obtained by the direct titration of 19S hemolysin were confirmed. It was found, however, that the 7S hemolysin was not increased by the injection of leucogenenol.

Only normal hemolytic activity was found in the sera of rats that had not received sheep erythrocytes, and this activity was not increased by the injection of leucogenenol.

Since only a fraction of the cells that are morphologically recognizable as lymphocytes are competent to form antibodies (11), and it has been demonstrated that leucogenenol increases the rate at which the precursors of lymphocytic (12) as well as granulocytic (12, 13) cells transform into lymphocytes and granulocytes, it is reasonable to consider that our findings result from the ability of leucogenenol to increase the rate of transformation of certain immunoincompetent cells, possibly a type of lymphocyte, into antigen-reactive lymphocytes. The injection of leuco-

TABLE I. Effect of Leucogenenol on the Formation of IgM (19S) and IgG (7S) Antibody[a] by Normal and Splenectomized Rats in Response to Their Injection with Sheep Erythrocytes.[b]

	Mean hemolysin $-\log_{10}$ titers ± standard deviation[c]			
	Not injected with leucogenenol[d]		Injected with leucogenenol[e]	
	IgM	IgG	IgM	IgG
Normal	− 1.88 ± 0.12	0.0	− 2.31 ± 0.24	− 0.4 ± 0.4
Splenectomized	− 0.87 ± 0.14	0.0	− 2.27 ± 0.14	− 0.3 ± 0.3

[a] The antibody titers given are the sum of the activities found in the fractions separated on Sephadex-G-200. For details see text. Rats that received leucogenenol or saline but not sheep erythrocytes showed no measurable antibody titers. Titers were determined on day 7 after injection with sheep erythrocytes.

[b] Each rat was injected intraperitoneally with 2×10^8 sheep erythrocytes suspended in 0.5 ml of sterile pyrogen-free isotonic saline. Splenectomized rats were injected 3 days after splenectomy.

[c] Standard deviations were calculated from results on 6 animals.

[d] Animals were injected intravenously with 0.2 ml of sterile pyrogen-free isotonic saline at the time of injection with sheep erythrocytes and at 24 hr intervals thereafter for 6 days.

[e] Rats were injected intravenously with 1 μg of the calcium salt of leucogenenol dissolved in 0.2 ml of sterile pyrogen-free isotonic saline at the time of injection with sheep erythrocytes and at 24 hr intervals thereafter for 6 days.

genenol would therefore cause splenectomized animals to replace the antigen-reactive cells removed at splenectomy at a faster than normal rate and thus permit the animals to form at an earlier period the quantities of antibodies found in normal animals.

Summary. Injection of leucogenenol into splenectomized rats together with sheep erythrocytes, results in the formation of normal titers of 19S hemolysin. Injection of leucogenenol, together with sheep erythrocytes into normal rats does not result in increased hemolysin titers. The distribution and concentration of 19S and 7S proteins in the serum is not affected by leucogenenol. It is suggested that leucogenenol acts by increasing the rate of transformation of immunoincompetent cells, possibly a type of lymphocyte, into antigen-reactive lymphocytes.

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