Effect of Leucogenenol on Origin of Hemolysin in Normal and Splenectomized Rats[1] (36746)

F. A. H. Rice, Noveen D. Das, and Myoung-Jin Koo
Department of Chemistry, American University, Washington, D. C. 20016

In a previous communication it was reported that repeated injections of leucogenenol (1–3) decrease the time required for sublethally X-radiated mice to regain competency to form hemolysin in response to injection with sheep erythrocytes (4). It was also reported (5) that as early as 3 days after splenectomy, injection of leucogenenol causes splenectomized rats to form normal titers of 19S hemolysin (6) in response to the injection of sheep erythrocytes. Since it had been found that leucogenenol increases the rate of maturation or transformation of cells of the granulocytic and erythrocytic series (7–9), it was suggested (5, 6) that leucogenenol induces splenectomized rats to form normal titers of hemolysin in response to the injection of sheep erythrocytes by increasing the rate of transformation of precursors into antibody-reactive cells. An increase in the concentration of antibody-reactive cells would not be expected to affect the peak titer, presumably regulated by a feedback mechanism that is dependent on the concentration of hemolysin in the serum (10). Indeed leucogenenol does not affect the peak titer of normal animals and only induces the formation of normal peak titers in splenectomized rats that are injected once with sheep erythrocytes and daily with leucogenenol.

It was considered that if leucogeneol stimulates the formation of antibody-reactive cells it could be predicted that leucogenenol, by increasing the concentration of antibody-reactive cells, would cause an animal to reach a peak titer at a period earlier than normal and cause both normal and splenectomized animals to show an increase in the concentration of antibody-forming cells in their lymphatic tissues and possibly in their bone marrow.

Materials and Methods. Sixty-four intact and 64 splenectomized rats (Wistar, 250 g wt) were injected intraperitoneally 3 days after splenectomy with 2.5×10^8 sheep erythrocytes suspended in 0.25 ml of pyrogen-free isotonic saline (McGaw Laboratories, Glendale, CA). At approximately the same time and at 24 hr intervals thereafter, 32 of the intact and 32 of the splenectomized rats were injected intravenously with 1 μg of the calcium salt of leucogenenol (1, 2) dissolved in 0.2 ml of pyrogen-free sterile 8.4% aqueous sodium bicarbonate (Abbott Laboratories, Chicago, IL). The remaining intact and splenectomized rats were injected intravenously with 0.2 ml of sodium bicarbonate solution to which no leucogenenol had been added. From each of the 4 groups of rats so obtained; *viz* (a) intact rats injected with sheep erythrocytes and leucogenenol, (b) intact rats injected with only sheep erythrocytes, (c) splenectomized rats injected with sheep erythrocytes and leucogenenol, (d) splenectomized rats injected with only sheep erythrocytes; 4 rats were selected at random at the time of injection with sheep erythrocytes and at 3, 4, 5, 7, 14, 29–32, and 45–46 days thereafter and exsanguinated under ether anesthesia by cardiac puncture. The blood of each rat was allowed to coagulate overnight at 4°. The serum was then removed, frozen and stored at −20°. When all the rats had been sacrificed, the sera were thawed at room temperature and aliquots (0.2 ml) of the sera from each of the 4 rats that had been treated in the same manner were combined, inactivated at 56°, and titrated for hemolysin (5, 6, 11) in the usual

[1] Supported in part by Office of Naval Research, contract No. N00014-67-0275, and NIH Grant CA12736-01.

TABLE I. Titers of Hemolysin[a] in the Serum of Intact and Splenectomized Rats at Intervals Following One Injection with Sheep Erythrocytes[b] and Daily Injections of Leucogenenol.[c,d]

	Time after injection with sheep erythrocytes (days)						
	0	3	4	5	7	14	29–32
Intact rats	1/50	1/180 (1/32)	1/400 (1/80)	1/850 (1/300)	1/1200 (1/1100)	1/500 (1/200)	1/360 (1/75)
Splenectomized rats	1/50	1/130 (1/40)	1/350 (1/50)	1/650	1/1200 (1/50)	1/500 (1/50)	1/300 (1/50)

[a] Titers of hemolysin were determined on mixtures of equal volumes of serum from 4 animals that were treated in the same manner and are given to two significant figures.

[b] Injected intraperitoneally with 2.5×10^8 sheep erythrocytes suspended in 0.25 ml of sterile pyrogen-free isotonic saline.

[c] Injected intravenously at 24 hr intervals with 1 μg of the calcium salt of leucogenenol dissolved in 0.2 ml of sterile pyrogen-free 8.4% aqueous sodium bicarbonate.

[d] Titers following injection of only sheep erythrocytes are given in parentheses.

manner.

Antibody-forming cells in a number of tissues were determined by the method of Jerne and Nordin (12), using in detail the procedure described by Merchant and Hraba (13). At the time of exsanguination the liver, spleen, 4 to 10 lymph nodes from the neck region, the mesentary, and lymphatic tissue of the gut (Peyer's patches) were removed from each rat and cut into small pieces with scissors. The cells were teased out from each tissue into ice-cold Eagle's medium (2 to 4 ml) buffered with 0.1 *M* 2-amino-2-(hydroxymethyl)-1,3-propandiol (Tris). Femurs were split longitudinally and the bone marrow was suspended in Eagle's medium buffered with Tris. Pieces of tissue were removed from each of the cell suspensions by filtering them through nylon mesh (36 threads/cm) that had been previously washed 6 times and boiled 5 min in distilled water. The cells obtained from each tissue were separated from suspension by centrifugation in 4 ml test tubes at 1200 rpm for 20 min at 4° in a Sorvall RC2-B refrigerated centrifuge equipped with a SM-24 rotor. The supernatent fluid was removed with a Pasteur pipet. The cells were washed 3 times by resuspending them in 1 ml of the Tris buffered Eagle's medium and recovering them as above by centrifugation. At the final wash the concentration of cells was determined with a hemocytometer, and after centrifugation the cells were resuspended in a calculated volume of the Tris buffered Eagle's medium to yield a concentration of approximately 10^6 cells/0.1 ml. Dilutions yielding approximately 10^5 cells were also made. The actual number of cells suspended in 0.1 ml was then determined with a hemocytometer.

On hundred milliliters of 1.8% agar (Difco) in distilled water was sterilized in the autoclave for 20 min at 20 psi and added to 100 ml of 2× Eagle's Tris buffered (0.1 *M*) medium. To this solution, maintained fluid at 56°, was added 20 ml of an isotonic saline solution of DEAE-dextran (10 mg/ml, Pharmacia, Uppsala, Sweden) and 10 ml of an isotonic saline solution of fraction V bovine serum albumen (5 mg/ml, Armour Pharmaceutical Co., Chicago, IL). The washed cell suspensions (0.1 ml, containing a known number of approx 10^6 or 10^5 cells) and 0.2 ml of a 50% suspension of sheep erythrocytes (Flow Laboratories, Rockville, MD), previously washed with pyrogen-free sterile isotonic saline, were added to 2 ml of the fluid gel described above contained in 4 ml test tubes. The cells were mixed with the gel and then poured in an even layer into 100 mm diameter disposable polystyrene petri dishes. After standing 1 hr at 37°, complement (3 ml of rat serum diluted 1:10) was added. After 1 hr at 37° the complement was poured off and the number of hemolytic plaques in each petri dish was counted under a zoom micro-

TABLE II. Concentration of Antibody-forming Cells (AFC/10^6 cells) in Several Tissues of Intact and Splenectomized Rats at Intervals Following One Injection with Sheep Erythrocytes[a] and Daily Injections of Leucogenenol.[b,c]

Origin of cells	Time after injection with sheep erythrocytes (days)						
	0	3	4	5	7	14	29–32
Intact rats							
Cervical lymph nodes	10 ± 5	157 ± 50 (100 ± 50)	88 ± 10 (13 ± 5)	170 ± 70 (51 ± 15)	20 ± 5 (30 ± 10)	188 ± 60 (12 ± 5)	45 ± 10 (25 ± 5)
Mesenteric lymph nodes	3 ± 1	60 ± 14 (10 ± 5)	15 ± 6 (30 ± 15)	43 ± 12 (27 ± 10)	20 ± 10 (2 ± 1)	20 ± 15 (2 ± 2)	54 ± 5 (3 ± 2)
Lymphatic tissue of intestine	20 ± 5	467 ± 200 (400 ± 200)	55 ± 10 (98 ± 20)	1200 ± 200 (462 ± 100)	1100 ± 100 (117 ± 50)	1200 ± 500 (70 ± 30)	166 ± 60 (70 ± 20)
Liver	1 ± 1	24 ± 8 (2 ± 1)	21 ± 1 (25 ± 4)	7 ± 4 (2 ± 2)	2 ± 1 (1 ± 1)	2 ± 1 (1 ± 1)	3 ± 1 (3 ± 1)
Spleen	1 ± 1	140 ± 40 (40 ± 15)	110 ± 20 (75 ± 10)	380 ± 52 (25 ± 10)	500 ± 100 (550 ± 100)	31 ± 4 (3 ± 1)	18 ± 10 (4 ± 2)
Bone marrow	2 ± 1	180 ± 79 (2 ± 1)	80 ± 5 (45 ± 5)	98 ± 40 (15 ± 10)	55 ± 20 (60 ± 40)	115 ± 40 (16 ± 6)	18 ± 6 (16 ± 6)
Splenectomized rats							
Cervical lymph nodes	10 ± 5	256 ± 25 (12 ± 5)	97 ± 7 (20 ± 10)	42 ± 10	69 ± 15 (35 ± 10)	25 ± 5 (14 ± 5)	111 ± 50 (25 ± 10)
Mesenteric lymph nodes	3 ± 1	90 ± 10 (50 ± 10)	43 ± 6 (12 ± 4)	12 ± 5	35 ± 15 (5 ± 2)	24 ± 2 (5 ± 2)	99 ± 10 (5 ± 4)
Lymphatic tissue of intestine	20 ± 5	1600 ± 200 (100 ± 50)	350 ± 50 (80 ± 20)	580 ± 100	995 ± 30 (30 ± 20)	700 ± 200 (50 ± 10)	2600 ± 1000 (20 ± 5)
Liver	1 ± 1	4 ± 2 (1 ± 1)	24 ± 6 (2 ± 1)	20 ± 12 (1 ± 1)	2 ± 1 (1 ± 1)	5 ± 1 (1 ± 1)	32 ± 10 (1 ± 1)
Bone marrow	2 ± 1	24 ± 6 (2 ± 1)	18 ± 3 (1 ± 1)	20 ± 10	60 ± 20 (3 ± 2)	16000 ± 2000 (2 ± 1)	21 ± 5 (2 ± 1)

[a] Injected intraperitoneally with 2.5×10^8 sheep erythrocytes suspended in 0.25 ml of sterile pyrogen-free isotonic saline.

[b] Injected intravenously at 24 hr intervals with 1 μg of the calcium salt of leucogenenol dissolved in 0.2 ml of sterile pyrogen-free 8.4% aqueous sodium bicarbonate.

[c] Concentrations of antibody-forming cells following injection with only sheep erythrocytes are given in parentheses.

scope (American Optical, Model 561-C-H-1). The number of antibody-forming cells per 10^6 cells were then calculated on the assumption that 1 plaque represented 1 antibody-forming cell.

Results and Discussion. Injection of leucogenenol causes intact rats to reach maximum titers 1 to 2 days earlier than normal (Table I) and to show elevated titers of hemolysin as early as 3 days after injection with sheep erythrocytes. Consistent with this finding, Table II shows that leucogenenol causes a significant increase in the concentration of antibody-forming cells in lymphatic tissues as well as in the spleen and bone marrow as early as 3 days after the injection of sheep erythrocytes. Rats that are injected with leucogenenol show essentially maximum titers (Table I) of hemolysin 5 days after injection with sheep erythrocytes, and at this time the concentration of antibody-forming cells in lymphatic tissues, as well as in the spleen and bone marrow, is somewhat higher than that of normal rats 7 days after they received only sheep erythrocytes.

Although splenectomized rats do not form significant quantities of hemolysin in response to the injection of sheep erythrocytes 3 days after splenectomy (5, 6), repeated injections of leucogenenol, in addition to one injection with sheep erythrocytes, causes splenectomized rats to reach normal maximum titers of hemolysin in a normal period (Table I). The hemolysin apparently arises from antibody-forming cells in lymphatic tissue, particularly that of the gut and the bone marrow (Table II).

The results are consistent with the suggestion that leucogenenol transforms precursors into antibody-reactive cells which then respond to the antigen, in this case sheep erythrocytes, to form antibody-forming cells. It would seem that leucogenenol affects precursors to antibody-reactive cells in lymphoid tissues and also the bone marrow. The early increase in antibody-forming cells in the liver (Table II) suggests that this organ contains precursors to antibody-reactive cells.

Fourteen and 29–32 days after injection of sheep erythrocytes both intact and splenectomized rats that are daily injected with leucogenenol show titers of hemolysin (Table I) and concentrations of antibody-forming cells that are higher than those of animals that are not injected with leucogenenol. Presumably the increased concentration of antibody-forming cells induced by the injections of leucogenenol is responsible for the increased hemolysin formation. Leucogenenol does not maintain the increased concentration of antibody-forming cells indefinitely, and 46 days after the injection of sheep erythrocytes (not shown) the concentration of antibody-forming cells and hemolysin titers of rats that received daily injections of leucogenenol were indistinguishable from those rats that had not. The slower rate of decline in the concentration of antibody-forming cells and hemolysin might be expected since it should require a longer period for a larger than normal population of antibody-forming cells to be inhibited by the same concentration of antibody in the serum.

Summary. It has been found that daily injections of leucogenenol, at the time of and following one injection of sheep erythrocytes, causes a marked increase in the concentration of antibody-forming cells in lymphatic tissues as well as in the spleen and bone marrow. In intact animals this results in earlier formation of maximum titers of hemolysin in the serum and causes splenectomized animals to respond to the injection of sheep erythrocytes with the formation of normal maximum titers.

It is suggested that leucogenenol stimulates the formation of antibody-reactive cells from precursors.

We thank Dr. B. Merchant, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, NIH, for assistance in mastering the hemolysis-in-gel technique and for many valuable discussions.

1. Rice, F. A. H., Proc. Soc. Exp. Biol. Med. **123**, 189 (1966).

2. Rice, F. A. H., and Shaikh, B., Biochem. J. **116**, 709 (1970).

3. Rice, F. A. H., J. Chem. Soc., London 2599 (1971).

4. Rice, F. A. H., Lepick, J., and Darden, J. H., Radiat. Res. **36**, 144 (1968).

5. Rice, F. A. H., and Ciavarra, R., Proc. Soc. Exp. Biol. Med. **137**, 567 (1971).

6. Rice, F. A. H., Ciavarra, R., and Borsos, T., Proc. Soc. Exp. Biol. Med. **140**, 471 (1972).

7. Rice, F. A. H., Connolly, J., Aziz, K., and McCurdy, J. D., J. Infec. Dis. **123**, 117 (1971).

8. Rice, F. A. H., McCurdy, J. D., and Aziz, K., Proc. Soc. Exp. Biol. Med. **136**, 56 (1971).

9. Fossati, G. C., Fumarola, D., Cerra, E., and Cavalieri, E., Rivista Eoter. Immunoematol. **16**, 91 (1969).

10. Moller, G., *in* "Homeostatic Regulators" (G. E. W. Wolstenholme and J. Knight, eds.), p. 197. Churchill, London (1969).

11. Kabat, E. A., and Mayer, M. M., "Experimental Immunochemistry" 2nd ed., Thomas, Springfield, IL (1961).

12. Jerne, N. K., and Nordin, A. A., Science 140, 405 (1963).

13. Merchant, B., and Hraba, T., Science **152**, 1378 (1966).

Received May 24, 1972. P.S.E.B.M., 1972, Vol. 141.