THE EFFECT OF HETEROLOGOUS ANTI-LYMPHOCYTE SERUM ON MOUSE HEMOPOIETIC STEM CELLS*

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Heterologous anti-lymphocyte serum has been reported to be the most potent immunosuppressive agent yet described (1). It has been shown that this antiserum not only prolongs the survival of skin or renal allografts in mice (2), rats (3), and dogs (4), but that it is able to suppress an allograft rejection already in progress (5). Furthermore, this remarkable immunosuppressive effect can be achieved without causing apparent toxicity in the recipients (1). These features have stimulated the use of heterologous antilymphocyte serum in human clinical trials (6).

Previous reports have been limited to studying the action of heterologous anti-lymphocyte serum upon the lymphoid system. Little investigative work has been done on the effect of the antiserum on the closely related hemopoietic system. This is quite surprising in view of the demonstrated side effects accompanying prolonged antiserum treatment. Monaco and his associates (2) reported the occurrence of a severe wasting disease leading to death in mice receiving chronic injections with rabbit anti-mouse lymphocyte serum. Iwasaki and coworkers (7) described the development of anemia in dogs treated with horse anti-dog lymphocyte serum despite the proved absence of hemagglutinins.

The present study was undertaken to determine the effects of heterologous anti-lymphocyte serum upon hemopoietic stem cells which are essential for the maintenance of normal marrow function. The morphologic identity of the hemopoietic stem cell has not been established. For this reason, assay procedures for such cells must be based on their functional characteristics. The spleen colony assay of Till and McCullock (8) provides a unique method for quan-

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titating stem cells. This technique depends upon the capacity of multipotential cells contained in normal mouse hemopoietic tissues to give rise to clonal progeny which form macroscopic nodules in the spleens of irradiated mice. Each of these colonies have been demonstrated to be formed by a single precursor cell (9). This parent cell, designated as a colony-forming unit (CFU), possesses the properties characteristic of a stem cell. They are capable of self-renewal (10), exhibit extensive clonal proliferation, and their progeny differentiate into three cell lines: erythrocytes, myelocytes, and megakaryocytes (8, 11).

In this report, the effects of rabbit anti-mouse lymphocyte serum (RAMLS) upon mouse bone marrow were investigated using conventional morphologic studies in combination with the spleen colony assay. Particular emphasis was placed on determining the effects of such treatment on hemopoietic stem cells as represented by the CFU cell population.

Materials and Methods

Animals.—New Zealand white rabbits, purchased from commercial suppliers, were used to produce the antiserum. Mice of the A/Jax and CBA/Jax strains, obtained from the Jackson Laboratories (Bar Harbor, Maine), were used in all grafting procedures and for lymphocyte donors. Inbred mice of the C_3H/He strain, maintained in this laboratory, were utilized as both donors and recipients for the spleen colony assay.

Preparation of Anti-Lymphocyte Serum.—Rabbit anti-mouse lymphocyte serum (RAMLS) was prepared according to the method of Gray and associates (12) by immunizing New Zealand white rabbits with lymphocytes obtained from A/Jax mice. Complement in the pooled serum was inactivated by heating to 56°C for 30 min. The decomplemented serum was sterilized by passage through a 0.45 μ membrane filter, frozen, and stored at −20°C until used.

Anti-Lymphocyte Serum Assay Procedures.—

Skin grafting: All RAMLS was assayed for its ability to prolong the survival of CBA/Jax skin homografts on A/Jax mice using standard full thickness skin grafting techniques. The recipient mice were treated with 0.5 ml of antiserum on day 0 and +2. Grafts on the treated mice enjoyed a 20–30 day survival whereas the untreated recipients' grafts were rejected in less than 12 days.

Erythro-agglutinin titer: Hemagglutinin titers were determined using a modification of the microtiter technique of Sever (13). A 1% red cell suspension was prepared by adding washed peripheral erythrocytes from C_3H mice to phosphate-buffered saline (pH 7.2) containing 1% isologous serum. Serial dilutions of the serum were added to equal volumes of the above red cell suspension and incubated at room temperature for 2 hr prior to reading. The end point of the titration was the last dilution showing complete agglutination.

Leuko-agglutinin titer: Leuko-agglutinin titers were determined using a modification of the Amos and Peacocke (14) method. A cell suspension was prepared from lymph nodes obtained from A/Jax mice as described by Billingham (15). The cells were washed three times in Hanks' balanced salt solution and diluted to a final concentration of 14×10^6/ml. Equal volumes of cells and serial dilutions of serum were mixed and incubated at room temperature for two hours. 1 drop of each cell suspension was transferred to a glass slide. Agglutination was scored microscopically on these wet preparations. The end point was the last dilution showing agglutination.

Absorption of Erythro- and Leuko-Agglutinin from the Crude Anti-Lymphocyte Serum.—Erythro-agglutinin activity was removed from the crude RAMLS by overnight absorption

with washed mouse erythrocytes at 4°C utilizing a ratio of 2 volumes of serum to 1 volume of cells. The absorbed serum was decanted from the cells and sterilized by passage through a 0.45 μ membrane filter. The leuko-agglutinin was deleted from the crude serum by a similar absorption with suspensions of mouse lymph node cells. Leuko-agglutinin and erythro-agglutinin titers were established on the absorbed sera prior to use by the described assay procedures.

Spleen Colony Assay.—The spleen colony assay of Till and McCullock (8) was utilized to determine the effect of RAMLS on the CFU or hemopoietic stem cell. Within 4 hr after lethal irradiation, C_3H/He mice were inoculated with the appropriate isologous bone marrow cell suspensions by tail vein injection. 9 days later colony counts were made by dissecting the spleens from sacrificed irradiated hosts and counting in the blind the number of macroscopic nodules. Scoring was done with the aid of a dissecting microscope on both fixed and fresh specimens.

X-Ray Irradiation Procedures.—Prior to bone marrow transfusion, the recipient mice were irradiated in individual compartments of a square lucite box placed 50 cm from the target of a 200 kvp Keleket X-ray generator, operated at 15 ma tube current with a 0.5 mm Al filter. The absorbed dose received by these animals was 800 rads given over a period of 6 min and 8 sec. No autochthonous spleen colonies were observed in control animals sacrificed at 9 days postirradiation.

Preparation of Bone Marrow Cell Suspensions.—Standard bone marrow cell suspensions were prepared from the femurs of donor C_3H/He mice. Nucleated cell counts were made using trypan blue exclusion as an index of viability. The final cell suspension was adjusted by dilution with Hanks' solution to the concentration of 2.5×10^4, 3.5×10^4, or 1×10^6 cells/ml, depending upon the requirements of the experiment. Bone marrow cell suspensions used for the control and experimental groups in each in vitro study were obtained from the same donor.

Incubation Procedures.—All in vitro incubations were made by suspending 1×10^6 marrow cells in the various stated dilutions of RAMLS or normal rabbit serum (NRS). These mixtures were then incubated for 30 min at 37°C in the presence of guinea pig complement. Unless otherwise stated, the cell suspension for injection was prepared by diluting the incubated mixture with Hanks' solution to a final concentration of 25,000 cells/ml. A control incubation of cells with guinea pig complement and Hanks' solution only was included in each study. In one experiment, to be identified later, complement was purposely excluded from the incubation procedure.

Histology.—All tissues for histological examination were fixed in 10% formalin, embedded in paraffin, sectioned at 5 μ, and representative sections stained with hematoxylin and eosin. Smears of bone marrow cell suspensions and marrow prints were prepared by standard methods, air dried, and stained with Wright's stain. All differential counts were based on 1000 cells.

RESULTS

In Vitro Effect of RAMLS on the CFU Cell.—The injection of 25,000 bone marrow cells after in vitro incubation with Hanks' solution produced a mean number of 7.6 colonies per spleen in the lethally irradiated recipients. Inoculation of the same number of cells exposed to NRS resulted in a mean of 7.8 colonies. However, animals receiving bone marrow cells previously incubated in vitro with RAMLS had only 0.8 colonies per spleen (Table I). Histological examination showed no difference in the cellular composition of spleen colonies

originating from marrow suspensions variously treated with RAMLS, NRS, or Hanks' solution.

Characterization of the Bone Marrow Cell Population Surviving Treatment with RAMLS.—In the previous study cell counts were made prior to incubation, and the final number of cells inoculated was achieved by dilution based on the initial count. Subsequent observations indicated that approximately 50% of the marrow cells were lysed during the in vitro incubation with RAMLS. In the present experiment an effort was made to establish whether the decrease in CFU cells was due to a random lysis of all marrow cell types or a selective action on the stem cell population. Thus, the answers to the following questions were sought: (*a*) what portion of the original one million bone marrow cells survived incubation with RAMLS; (*b*) what was their viability based on trypan blue exclusion; (*c*) what was the CFU content of exactly 25,000 of these cells; (*d*) what morphological cell types made up this population?

TABLE I

In Vitro Effect of RAMLS on CFU Cells

Incubation			No. of cells injected per host based on dilution	No. of spleen colonies per host	Mean no. of colonies
No. of isogeneic bone marrow cells	Serum	Dilution			
1×10^6	Hanks'	—	25,000	8, 7, 7, 6, 10, 8	7.6
1×10^6	NRS	1:50	25,000	8, 7, 10, 7, 8, 7	7.8
1×10^6	RAMLS	1:50	25,000	1, 0, 0, 0, 1, 0, 0, 7, 0, 0	0.8

One million bone marrow cells were incubated in either RAMLS, NRS, or Hanks' solution for 30 min. The number of viable cells were counted after incubation. As can be seen from Table II approximately one-half of the original one million bone marrow cells survived exposure to RAMLS. Those cells lost during incubation were apparently lysed, since there were few trypan blue stained cells present in the counting chamber and virtually no cell agglutination occurred. Cells incubated in NRS and Hanks' solutions remained viable and no lysis was observed.

The cells surviving separate incubations with RAMLS, NRS, or Hanks' solution were adjusted to a concentration so that exactly 25,000 of these viable cells were injected into each irradiated recipient. The results in Table II show that those animals receiving cells incubated with NRS or Hanks' solution formed a mean of 8.6 colonies per spleen, while irradiated mice inoculated with cells incubated with RAMLS possessed a mean of 2.2 colonies per spleen. This could be interpreted as a selective destruction of CFU cells in the marrow popu-

lation incubated with RAMLS, since all irradiated recipients received an identical number of viable cells.

Table III shows the cell differential counts done on a bone marrow cell population incubated with either RAMLS, NRS, or Hanks' solution. As can be seen, incubation with RAMLS, compared to NRS or Hanks' solution, caused a 73% decrease in the number of undifferentiated blast cells and a 94 and 98% decrease in lymphocytes and nucleated erythrocytes respectively.

TABLE II

CFU Cell Content of Marrow Populations Surviving In Vitro Exposure to RAMLS

Incubation			No. of viable cells after incubation	No. of cells injected per host based on actual count	No. of spleen colonies per host	Mean No. of colonies
No. of isogeneic bone marrow cells	Serum	Dilution				
1×10^6	Hanks'	—	1×10^6	25,000	9, 9, 9, 10, 9, 8, 6	8.6
1×10^6	NRS	1:100	1×10^6	25,000	7, 12, 12, 7, 7, 7, 10, 8, 8, 8, 9, 12, 6	8.6
1×10^6	RAMLS	1:100	570,000	25,000	0, 1, 1, 7, 0, 9, 0, 0	2.2

TABLE III

Differential Cell Counts of Marrow Populations Surviving In Vitro Exposure to RAMLS*

Serum incubation	Undifferentiated blast cells	Myelocytes	Lymphocytes	Nucleated erythrocytes
Hanks' solution	50	807	90	53
NRS	52	751	101	96
RAMLS	14	980	4	2

* Based on 1000 cells counted.

In Vitro Effect of Erythrocyte-Absorbed RAMLS on the CFU Cell.—Studies were carried out to establish whether the suppression of CFU cells by RAMLS could be related to its erythro-agglutinating activity. Bone marrow cell suspensions were incubated separately with crude RAMLS (erythro-agglutinin titer 1:1024), erythrocyte absorbed RAMLS (erythro-agglutinin titer 1:4), NRS, and Hanks' solution; and their resultant CFU cell content assayed. Table IV shows that prior incubation of marrow cells in crude or erythrocyte-absorbed serum resulted in a similar number of 0.7 and 0.9 mean colonies per spleen respectively. Cells exposed to NRS gave a mean of 7.8 colonies which compared favorably with the mean of 8.0 colonies per spleen from cells incu-

bated in Hanks' solution. Thus, the effect of RAMLS of the CFU cell appeared independent of the erythro-agglutinin titer of the antiserum.

In Vitro Effect of Leukocyte-Absorbed RAMLS on the CFU Cell.—Experiments were done to determine whether the leuko-agglutinin activity of RAMLS

TABLE IV

In Vitro Effect of Erythrocyte-Absorbed RAMLS on CFU Cells

Incubation			Erythro-agglutinin titer of undiluted serum	No. of cells injected per host based on dilution	No. of spleen colonies per host	Mean No. of colonies
No. of isogeneic bone marrow cells	Serum	Dilution				
1×10^6	Hanks'	—	—	25,000	7, 12, 9, 6, 9, 7, 7, 8, 8, 7, 8	8.0
1×10^6	NRS	1:50	0	25,000	8, 6, 13, 8, 6, 8, 7, 6	7.8
1×10^6	Crude RAMLS	1:50	1:1024	25,000	1, 0, 4, 1, 0, 1, 0, 0, 0, 0	0.7
1×10^6	Absorbed RAMLS	1:50	1:4	25,000	4, 1, 0, 0, 1, 0, 1, 0, 0, 1, 2	0.9

TABLE V

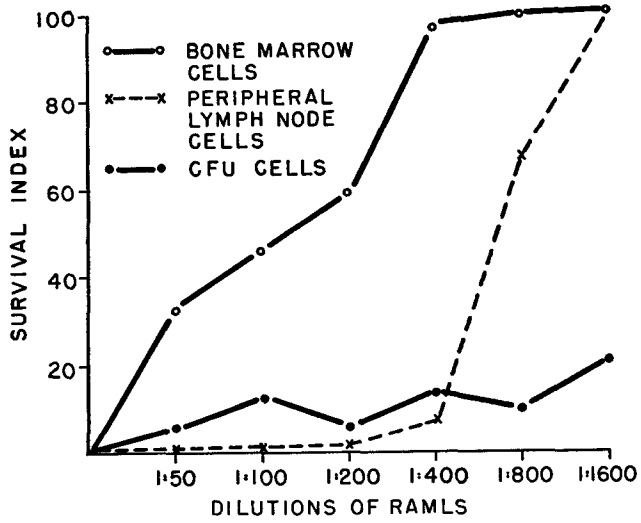
In Vitro Effect of Leukocyte-Absorbed RAMLS on CFU Cells

Incubation			Leuko-agglutinin titer of the undiluted serum	No. of cells injected per host based on dilution	No. of spleen colonies per host	Mean No. of colonies
No. of isogeneic bone marrow cells	Serum	Dilution				
1×10^6	Hanks'	—	—	25,000	9, 9, 9, 10, 9, 8, 6	8.6
1×10^6	NRS	1:800	0	25,000	7, 7, 10, 8, 8, 8, 9	8.1
1×10^6	Crude RAMLS	1:800	1:400	25,000	0, 2, 1, 1, 0, 0, 2, 1	0.9
1×10^6	Absorbed RAMLS	1:800	<1:5	25,000	1, 1, 1, 1, 3, 2, 3, 2, 1, 5, 3	2.1

was responsible for its suppressive effect on the CFU cell. Crude RAMLS (leuko-agglutinin titer 1:400), leukocyte-absorbed RAMLS (leuko-agglutinin titer < 1:5), NRS, and Hanks' solution were incubated individually with marrow cells and their effect on the CFU cells compared. Since the leuko-agglutinin assay was relatively insensitive, all incubations were done in a 1:800 dilution of serum so as to further dilute any leuko-agglutinin activity remaining

in the absorbed antiserum. Cells incubated in crude RAMLS, leukocyte absorbed RAMLS, NRS, or Hanks' solution resulted in a mean of 0.9, 2.1, 8.1, and 8.6 colonies per spleen respectively (Table V). Comparison of these results suggested that the action of RAMLS on the CFU cell was not correlated with leuko-agglutinating activity of the antiserum.

Comparison of the In Vitro Cytotoxicity of RAMLS with its Suppressive Effect upon CFU Cells.—This study was undertaken to compare the cytotoxic effect of various dilutions of RAMLS on bone marrow cells and peripheral lympho-



TEXT-FIG. 1. Comparison of the cytotoxic effect on bone marrow cells and peripheral lymph node cells with the suppression of CFU cells produced by various dilutions of RAMLS. Survival index is the per cent of cells surviving incubation in antiserum compared to the controls exposed to NRS at the same dilutions.

cytes to the suppressive action of the same dilutions of antiserum on the CFU cell content of these bone marrow preparations. Suspensions on one million bone marrow cells or lymphocytes were incubated separately at various dilutions of RAMLS or NRS. The cytotoxic effect on the incubated cells was determined by cell counts using trypan blue exclusion. The suppressive action on the CFU cells present in the same bone marrow suspensions was assayed using the spleen colony technique. The results are displayed graphically in Text-fig. 1 by plotting the survival index of CFU cells, marrow cells, and the peripheral lymphocytes against the various dilutions of RAMLS. The survival index is the percentage of cells surviving incubation with RAMLS compared to controls incubated in NRS at the same serum dilutions. Note that only 20% of the CFU cells in the marrow population survived incubation with 1:1600 dilution of RAMLS com-

pared to a 100% survival of marrow cells and peripheral lymphocytes incubated at the same concentration. This indicated that a significant suppressive effect of RAMLS on the CFU cells occurred in vitro at a dilution which showed no detectable cytotoxic effects on the marrow cell population or peripheral lymphocytes, and that stem cell proliferation was prevented by very small concentrations of the antiserum.

In Vitro Effect of RAMLS on the CFU Cell When Incubated without Complement.—In all previous studies, incubations were done in the presence of guinea pig complement and, as stated, a significant degree of cell lysis occurred. Similar studies, using mouse serum as the source of complement, showed that no cytolysis or demonstrable cell injury occurred when bone marrow cells were incubated with RAMLS. This observation raised the question whether the detrimental effect of RAMLS on CFU cells was dependent upon a cytotoxic action produced only in the presence of guinea pig complement. Standard bone

TABLE VI

The Effect of RAMLS on CFU Cells When Incubated without Complement Added In Vitro

Incubation (without complement)			No. of cells injected per host based on dilution	No. of spleen colonies per host	Mean No. of colonies
No. of isogeneic bone marrow cells	Serum	Dilution			
1×10^6	Hanks'	—	25,000	5, 5, 5, 6, 1	4.4
1×10^6	NRS	1:2	25,000	6, 6, 5, 5, 4	5.2
1×10^6	RAMLS	1:2	25,000	0, 0, 0, 1, 2, 0, 0	0.4

marrow suspensions were incubated with either RAMLS, NRS, or Hanks' solution in the absence of complement. After incubation, cell counts demonstrated that virtually all cells survived without injury as manifested by trypan blue exclusion. These cells were then injected into irradiated isogeneic hosts to determine the number of CFU cells capable of colony formation. The results are shown in Table VI and indicated that the suppression of CFU cells by RAMLS was not dependent upon in vitro exposure to guinea pig complement.

In Vivo Effect of Erythrocyte-Absorbed RAMLS on CFU Cells Transplanted into a Lethally Irradiated Recipient.—The previous experiments have demonstrated that RAMLS, when incubated with a bone marrow cell suspension in vitro, reduced the number of CFU cells present in the treated population. Studies were then initiated to determine whether a similar effect could be demonstrated in vivo. Suspensions containing 35,000 normal bone marrow cells were injected intravenously into lethally irradiated isogeneic mice. Immediately afterwards, these animals received an intravenous injection of 0.1 ml of RAMLS or Hanks' solution. For control purposes a second separate suspension contain-

ing 25,000 isogeneic bone marrow cells per inoculum was similarly injected and chased with 0.1 ml of NRS or Hanks' solution. Table VII shows the results of these experiments. A mean of 4.8 colonies were formed when the marrow inoculum was followed by a RAMLS injection compared to a mean of 9.9 resulting from similar treatment with Hanks' solution. The intravenous injection of NRS or Hanks' solution after inoculation of the marrow suspension yielded the same mean number of spleen colonies. Thus, the administration of RAMLS in vivo caused a 50% reduction in the number of spleen colonies formed by the transplanted marrow cells. It was observed that this activity of RAMLS was dependent on the time interval between the injection of the marrow cell suspension and the antiserum. The inoculation of 0.1–0.2 ml of RAMLS into irradiated hosts 1 or more hr prior to infusion of the marrow cells failed to reduce the

TABLE VII

In Vivo Effect of RAMLS on CFU Cells Transplanted into Irradiated Mice

Treatment ofIrradiated Host			No. of spleen colonies per host	Mean No. of colonies	SD
No. of isogeneic bone marrow cells	Serum	Dose injected*			
35,000	Hanks'	0.1 cc i.v.	10, 10, 9, 8, 9, 13, 10	9.9	1.2
35,000	RAMLS erythrocyte-absorbed	0.1 cc i.v.	5, 2, 4, 7, 3, 6, 6, 3, 4, 8	4.8	1.8
25,000	Hanks'	0.1 cc i.v.	7, 4, 9, 9, 7, 5, 9, 9	7.3	—
25,000	NRS	0.1 cc i.v.	5, 8, 6, 9, 7, 6, 8, 7 8, 9, 10, 8	7.5	—

* All injections were made immediately after marrow infusion.

number of spleen colonies formed, suggesting that the heterologous antibodies were rapidly removed from the recipient's peripheral blood.

The In Vivo Effect of Erythrocyte-Absorbed RAMLS on Normal Bone Marrow.—Having established that RAMLS could act in the irradiated animal to reduce the number of spleen colonies produced by transplanted marrow cells, studies were undertaken to determine the possible effect of this antiserum upon hemopoietic stem cells situated in the marrow tissues of normal mice. Three groups of eight mice received a single 1 ml intravenous injection of RAMLS, NRS, or Hanks' solution respectively. 24 hr later the mice were sacrificed. Pooled bone marrow cell suspensions were made from the animals in each group and their CFU cell content assayed. Mice acutely treated with RAMLS had a mean of 5.1 colonies per spleen. Animals similarly treated with NRS or Hanks' solution showed means of 10.3 and 9.7 colonies per spleen (Table VIII). No

significant difference was observed in the total cell content of femoral marrows obtained from animals treated with RAMLS, NRS, or Hanks' solution. Thus, a single injection with RAMLS caused, within 24 hr, a 50% decrease in the number of CFU cells contained in the bone marrow of treated mice.

An additional effort was made to ascertain the effect of chronic RAMLS treatment on the bone marrow of individual mice. Three groups of mice were treated daily with 0.25 cc subcutaneous injection of either RAMLS, NRS, or Hanks' solution. Members of each group were sacrificed after 1 and 3 wk of treatment. A cell suspension was prepared from the bone marrow of each animal and analyzed for the CFU cell content. Table IX shows that after 1 wk, animals treated with RAMLS had a group mean of 3.5 colonies per spleen. Injection of the same number of bone marrow cells from mice similarly treated

TABLE VIII

CFU Cell Content of Mouse Bone Marrow 24 Hr after Injection of RAMLS In Vivo

Treatment of marrow donors			No. of cells injected per host from pooled marrow of treated donors	No. of spleen colonies per host	Mean No. of colonies	SD
No. of mice treated	Serum	Dose per mouse				
8	Hanks'	1 cc i.v.	25,000	9, 10, 10, 7, 15, 7, 10	9.7	2.5
8	NRS	1 cc i.v.	25,000	12, 10, 12, 16, 10, 9, 6, 17	10.3	3.6
8	RAMLS erythrocyte-absorbed	1 cc i.v.	25,000	9, 8, 0, 6, 5, 2, 5, 5, 6	5.1	2.6

with NRS and Hanks' solution formed a group mean of 8.0 and 7.9 spleen colonies. A similar suppression of CFU cell content was found in the marrows of animals treated with RAMLS for 3 wk.

Microscopic studies of the marrow imprints from mice treated with RAMLS for 3 wk showed marked depletion of lymphocytes, erythroid precursors, and a decrease in the number of undifferentiated blast cells. The majority of the marrow population was composed of myelocytes in various stages of maturation (Fig. 1 *a* and 1 *b*). Table X shows differential cell counts made on individual marrow suspensions prepared from mice treated for 3 wk with RAMLS. These marrow specimens showed a 50% decrease in undifferentiated blast cells and greater than an 85% reduction in the number of lymphocytes and nucleated erythrocytes when compared to marrow specimens from control animals. These in vivo findings were similar to the results observed in marrow suspensions incubated in vitro with antiserum (Table III). Thus, chronic treatment with

TABLE IX

CFU Cell Content of Mouse Bone Marrow after Prolonged In Vivo Treatment with RAMLS

Donor treatment			No. of cells injected per host	Donor No.	No. of spleen colonies per host	Mean No. of colonies per donor	Mean No. of colonies per group	SD
Duration of treatment	Serum	Dose per day						
wk								
1	Hanks'	0.25 cc s.c.	25,000	1	7, 12, 9, 6, 9, 7, 7, 8, 8, 7, 8	8.0	7.9	1.4
				2	8, 7, 7, 6, 10, 8	7.6		
	NRS	0.25 cc s.c.	25,000	1	6, 5, 7, 10, 9, 5, 8, 8, 7, 9, 9, 8	7.6	8.0	1.7
				2	11, 7, 11, 8, 9	9.2		
	RAMLS erythrocyte-absorbed	0.25 cc s.c.	25,000	1	2, 1, 3, 1, 2, 1, 1	1.6	3.5	2.2
				2	4, 1, 6, 4, 5, 9, 4, 7, 3, 6, 4, 4	4.7		
3	Hanks'	0.25 cc s.c.	25,000	1	9, 9, 9, 10, 9, 8,6	8.6	7.2	2.1
				2	4, 8, 6, 8, 4, 6, 4, 8, 3, 8, 9, 9	6.4		
	NRS	0.25 cc s.c.	25,000	1	9, 10, 5, 14, 8, 6, 9, 9, 6, 12, 17	9.5	9.0	2.6
				2	9, 10, 11, 9, 7, 7, 6, 9, 8, 8, 10, 10	8.5		
	RAMLS erythrocyte-absorbed	0.25 cc s.c.	25,000	1	1, 1, 2, 3, 2, 2, 4, 1, 5	2.3	3.0	2.1
				2	5, 8, 1, 5, 1, 2, 1, 0, 3, 0, 5	3.0		
				3	9, 5, 4, 2, 5, 2, 4	4.5		
				4	4, 5, 2, 2, 4, 4, 1, 2	2.9		
				5	3, 7, 6, 3, 2, 1, 2, 1, 2	3.0		

RAMLS altered the cellular composition and decreased the number of functional stem cells in the bone marrow.

The Effect of Erythrocyte-Absorbed RAMLS Treatment on the Survival of Mice Exposed to a Sublethal Dose of X-Ray Irradiation.—The previous study illustrated that the treatment of normal mice with RAMLS reduced the number of CFU cells in their bone marrow. The following experiment was carried out to determine if this process could summate with the bone marrow insult produced by sublethal irradiation and cause increased mortality in the treated animals. Three groups of mice were irradiated with 600 R and then injected subcutaneously with 0.25 cc. of either RAMLS, NRS, or Hanks' solution daily. By 30 days after irradiation, 100% (15/15) of mice receiving RAMLS in combination with irradiation had died with 80% of the deaths occurring between

TABLE X

Differential Cell Counts of Marrow Populations from Mice Treated Daily with RAMLS, NRS, or Hanks' Solution for 3 Wk*

Serum treatment	Undifferentiated blast cells	Myelocytes	Lymphocytes	Nucleated erythrocytes
Hanks' solution				
Donor No. 1	48	704	112	136
Donor No. 2	44	654	66	236
NRS				
Donor No. 1	34	708	108	150
Donor No. 2	23	683	85	209
RAMLS erythocyte-absorbed				
Donor No. 1	17	976	7	0
Donor No. 2	11	966	18	5
Donor No. 3	12	970	14	4

* Based on 1000 cells counted.

the 8th and 19th postirradiation day. No mortality was observed in a comparable panel of normal mice treated with RAMLS only, or irradiated mice treated with NRS or Hanks' solution respectively. These findings indicated that RAMLS given concomitantly with sublethal irradiation caused an increased mortality in the recipients.

DISCUSSION

These experiments have demonstrated that RAMLS has a deleterious effect on mouse bone marrow cells in vitro and in vivo. Antiserum treatment caused a marked loss of functional hemopoietic stem cells from the treated marrow population, and this was accompanied by a depletion of marrow lymphocytes, erythroid precursors, and undifferentiated blast cells. The concept that a humoral antibody can destroy bone marrow is not new. There have been several experiments indicating that heterologous antiserum evoked against bone marrow cells or peripheral leukocytes can disrupt hemopoiesis and cause marrow

aplasia when injected into normal animals of the original donor species (16, 17, 18). Although hypoplastic marrows were not produced by the dosage of RAMLS used in the present experiments, the results suggested that heterologous antilymphocyte serum had a similar effect.

The attempt was made to establish the mechanism by which RAMLS suppressed stem cell function. Since the crude antiserum possessed significant erythro- and leuko-agglutinating activity it was postulated that such antibodies might cause marrow cell agglutination. The resulting cell aggregates could have lodged in the pulmonary capillaries of the irradiated recipient after intravenous injection and thus be removed from the circulation. Such a mechanism would have decreased the number of spleen colonies formed on a purely mechanical basis. This explanation seemed unlikely since stem cell suppression was produced by dilutions of RAMLS which did not cause cell agglutination detectable by direct microscopic observation. Furthermore, the treatment of marrow suspensions with RAMLS from which the erythro- and leuko-agglutinins had been removed by absorption caused a decrease in spleen colony formation equal to that produced by the crude antiserum, indicating that the loss of CFU cells could not be correlated with agglutinating activity.

The cytotoxic action of RAMLS on lymphocyte has been amply demonstrated by other investigators (12, 19). The present study has shown that RAMLS has a similar cytotoxic effect upon mouse bone marrow cells in vitro. Since this was accompanied by a loss of functional CFU cells, these results suggested that a cytotoxic mechanism might explain the effect of RAMLS upon stem cells. As CFU cells cannot be identified morphologically, the direct demonstration of their injury by RAMLS was not possible. Because of this limitation, studies were undertaken to determine whether the in vitro cytotoxic action of RAMLS on a bone marrow cell population correlated with its suppression of the CFU cell population. If the effect on the stem cells paralled the degree of marrow cell destruction, it would be reasonable to assume that the decrease in CFU cell function was due to a random lysis of all marrow cell types by RAMLS. However, these experiments established that marrow cell populations which survived exposure to RAMLS without injury detectable by vital staining techniques contained significantly fewer CFU cells than did a comparable number of normal cells. This suggested that antiserum treatment caused an apparent selective loss of stem cells from the marrow preparations. Similarly, serial dilution experiments indicated that profound CFU cell loss followed exposure of bone marrow preparations to antiserum concentrations which had no discernible cytotoxic effects on the total marrow population. As standard cytotoxicity studies cannot detect minor variations in cell survival (20), and since CFU cells account for only 0.2% of the marrow population, it is possible that all the stem cells in the treated preparation could have been selectively lysed at high dilutions of RAMLS and their destruction would have been missed by the assay procedure.

This apparent selective destruction of CFU cells could have resulted from RAMLS containing high titers of cytotoxic antibody directed against specific antigenic determinants of stem cells. This hypothesis would be particularly attractive if the morphological small lymphocytes of mouse bone marrow were the multipotential hemopoietic stem cells, as has been postulated (21). However, it seems unlikely that RAMLS—produced by using lymphocytes as the antigenic source—would contain a tissue-specific antibody which reacted only with the lymphoid-like stem cells and did not effect the remaining bone marrow lymphocytes. Since the lymphoid cells account for nearly 10% of the marrow population, it seems doubtful that significant destruction of these cells would have been overlooked by the test procedures.

The probable explanation for the apparent selective action of RAMLS upon CFU cells resides in the sensitivity of such cells to injury and not in their specific lysis. The cytotoxicity studies previously described have demonstrated that high concentrations of RAMLS can cause lysis or death of all marrow cell types. It seems likely that the same reaction between antibody and marrow cells could occur at lower antiserum concentrations. The resulting cell injury may not have produced lysis or death demonstrable by trypan blue staining, but could have been sufficient to prevent sophisticated biological functions such as proliferation and differentiation (20). If this explanation were correct, then the apparent selective loss of CFU cells would result from the different methods used to assay injury to stem cells and the general marrow population.

Previous reports have established that the cytotoxic action of RAMLS upon lymphocytes in vitro was complement dependent (12, 19). In these studies a similar complement requirement has been found necessary to produce the cytotoxic action of RAMLS on bone marrow cells. Marrow preparation exposed to RAMLS in vitro in the absence of complement formed fewer spleen colonies than did control suspensions, indicating that in vitro cytotoxicity mediated by heterologous guinea pig complement was not a prerequisite for causing CFU cell loss. However, these findings do not exclude the possibility that the irradiated host could provide complement in vivo to complete the cytotoxic action on antibody-coated cells. Failure to demonstrate an in vitro complement requirement in this process raised the possibility that RAMLS prevents stem cell function merely by coating these cells. This could interfere with their response to proliferative stimulae in the manner analogous to the "blindfolding" hypothesis proposed by Levey and Medawar (5) to explain the immunosuppressive action of the antiserum. Such a mechanism cannot be excluded by the present studies, but it fails to account for the demonstrated cytotoxic properties of the RAMLS.

It could be argued that the marrow changes produced by RAMLS resulted from antiserum-stimulated transformation of stem cells into differentiated cell lines or nonfunctional blast cells (1). However, in its simplest form, this hypothesis would not explain the selective loss of lymphocytes, erythroid pre-

cursors, and undifferentiated blast cells from the marrow population, and would ignore the demonstrated lysis of these cells following in vitro exposure to RAMLS. Finally, if such stimulation occurred, the injection of antiserum into irradiated hosts transfused with normal marrow should have increased the number of spleen colonies formed, while in fact, such treatment decreased splenic colonization.

Regardless of the precise mechanism involved in RAMLS action, these in vitro studies have established that the antiserum can produce marrow alterations mediated by a cytotoxic action directed against diverse marrow cell types. It seems plausible that RAMLS has a similar action against stem cells and their apparent preferential destruction results from the ease with which their proliferative capacity is impaired after injury by the antiserum.

Since the maintenance of normal marrow function is dependent upon the continued replacement of cellular elements by stem cell proliferation and differentiation, injury to these cells would alter hemopoiesis. It seems surprising, therefore, that short term treatment of animals with conventional doses of anti-lymphocyte serum does not cause pancytopenia. However, the destruction of stem cells by small doses of RAMLS could be compensated by increased proliferation and self-renewal of the unaffected cells resulting in a more rapid turnover of the stem cell pool. The net result of this process would be a diminished hemopoietic reserve which would not be reflected by a change in the peripheral hemogram. Higher doses or more prolonged administration of RAMLS could conceivably overwhelm this compensatory mechanism and result in obvious clinical abnormalities. Indeed this could help explain the varied hematological finding seen in normal animals chronically treated with anti-lymphocyte serum such as the thrombocytopenia described in primates (22) and the unexplained anemia noted in dogs (7). Alternatively, animals having decreased marrow reserve because of existing disease or concomitant immunosuppressive treatment might be unable to compensate for the marrow insult produced by standard doses of anti-lymphocyte serum. There is evidence for this hypothesis in the present study, as RAMLS was found to cause a marked mortality in mice receiving prior sublethal irradiation. Further, preliminary histological studies in these animals have indicated that hemopoietic tissue regeneration was severely impaired. If these results could be generalized to humans, such a mechanism could help explain the complication of leukopenia and thrombocytopenia seen in patients receiving anti-lymphocyte serum in combination with azathioprine after renal transplantation (23).

The mechanism of the immunosuppressive action of heterologous anti-lymphocyte serum remains unknown. The experiments reported here have illustrated that minute concentrations of RAMLS can injure hemopoietic stem cells and prevent their proliferating to form a clonal progeny. If this action could be generalized to other stem cells, it would be reasonable to suggest that RAMLS-coated lymphocytes undergoing proliferation in response to antigenic

stimulation might be equally susceptible to injury by the antiserum. Such a selective interference with these responding lymphocytes would prevent their yielding a progeny of committed effector cells, and thus inhibit the immune response without causing a detectable depletion of the general lymphoid population as noted by Anderson and associates (24). If the analogy between the effects of RAMLS upon hemopoietic stem cells and antigen-induced proliferation of lymphocytes were valid, this stem cell injury could be achieved in vivo at doses which cause minimal peripheral cytotoxic effects (Text fig. 1).

Previous experiments provide precedence for such a hypothesis. Mice treated with anti-lymphocyte serum have exhibited initial destruction of the lymph node germinal centers (12) suggesting active lymphocyte proliferation is affected by doses of RAMLS that do not apparently affect the remaining lymphoid population. Other investigators have demonstrated that the delay of antiserum treatment for 5 or more days after skin grafting resulted in only modest prolongation of graft survival (2, 5). Such a delay would provide adequate time for proliferation of specific effector cells (25), and even if some of these cells were destroyed by the cytotoxic action of RAMLS administered in high doses, sufficient numbers could remain to carry out homograft rejection in an attenuated form.

SUMMARY

The present study has demonstrated that rabbit anti-mouse lymphocyte serum (RAMLS) has the capability of destroying bone marrow cells and suppressing hemopoietic stem cell function. The in vitro incubation of bone marrow suspensions with RAMLS caused extensive cell lysis with an apparent preferential destruction of lymphoid, erythroid, and blastoid elements. Using the spleen colony assay, the number of functional hemopoietic stem cells was found to be markedly reduced in bone marrow populations exposed to RAMLS in vitro. Further, this loss of stem cell function could be produced by exposing marrow suspensions to small concentrations of antiserum which did not produce detectable cytotoxic effects on the general marrow population.

A similar effect of RAMLS upon hemopoietic stem cells was found in vivo. The intravenous injection of RAMLS into lethally irradiated mice immediately after the infusion of isogeneic marrow cells reduced the number of spleen colonies formed, indicating that the antiserum could exhibit a deleterious effect upon stem cells in the bloodstream of the intact animal. Normal animals treated with daily subcutaneous injections of RAMLS for 3 wk had a significantly reduced marrow content of functional hemopoietic stem cells, suggesting that RAMLS can affect stem cells located in situ in the bone marrow. The experiments indicate that RAMLS possesses potential marrow toxicity.

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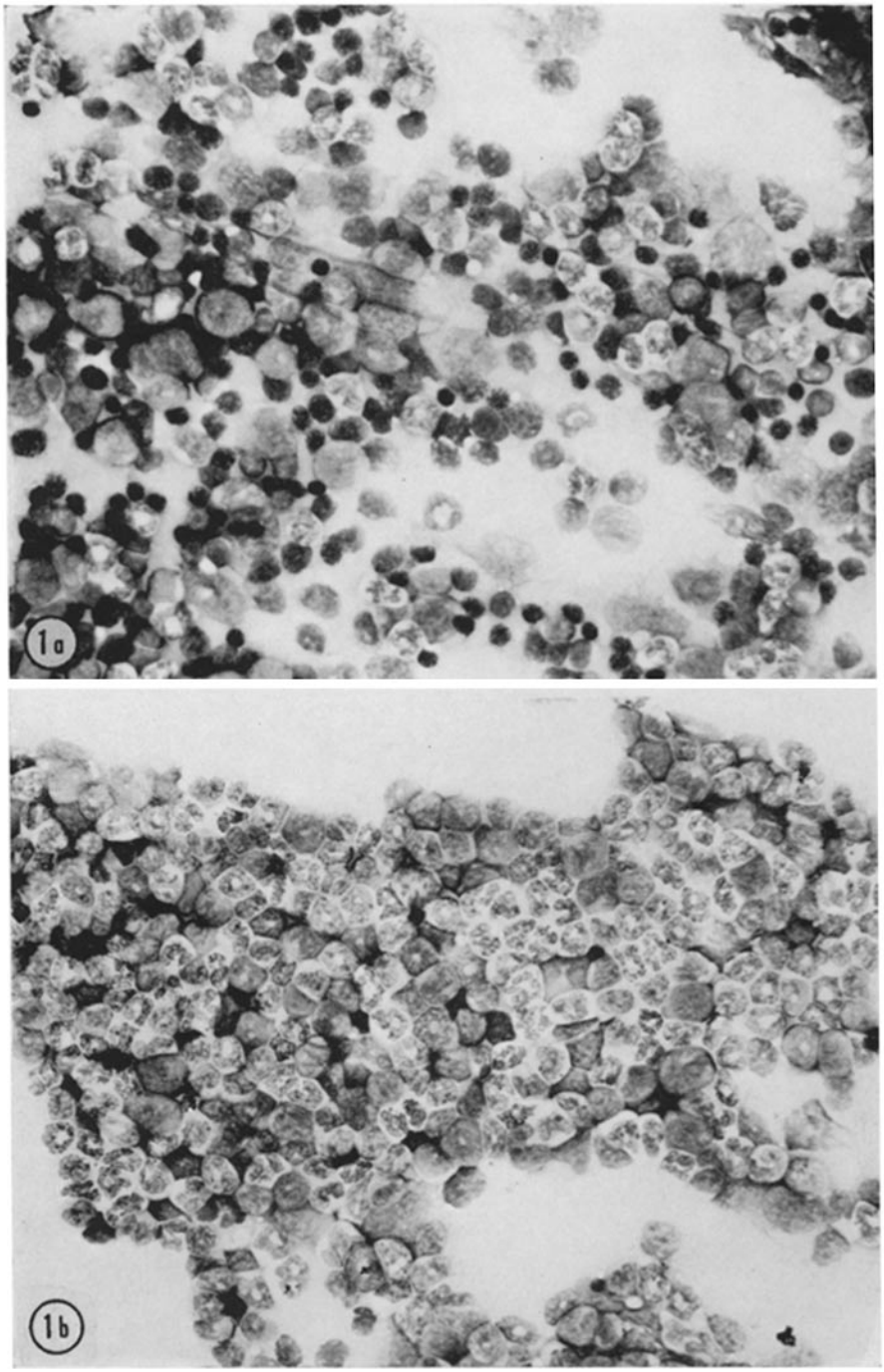
EXPLANATION OF PLATE 81

FIG. 1*a*. Imprint of normal C_3H mouse bone marrow showing numerous erythroid and lymphoid elements. × 400.

FIG. 1*b*. Imprint of C_3H mouse bone marrow after 3 wk of daily treatment with 0.25 ml s.c. of RAMLS showing a marrow population composed almost entirely of myeloid cells. × 400.



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