THE EFFECT OF HETEROLOGOUS ANTI-LYMPHO CYTE SERUM ON LYMPHOCyTES OF THYMUS AND MARROW ORIGIN*

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Heterologous anti-lymphocyte serum (ALS) is a potent and effective immunosuppressive agent. Administration of ALS decreases the ability of treated animals to reject allografts (1–4) and to produce humoral antibody when immunized with certain antigens such as sheep red blood cells (SRBC) (2, 3, 5, 6). The production of antibody against SRBC requires a synergistic interaction between cells of thymus and marrow origin (7, 8) and Martin and Miller (9) have shown that thymus-derived spleen cells are inactivated when ALS treatment is used to depress the ability of a mouse to produce anti-SRBC antibody.

We have recently reported (10) that cells of marrow origin appear to be likewise affected. These preliminary results have been extended and the findings obtained from the subject of this communication.

**Materials and Methods**

*Animals Used.*—A local farm horse was used for production of ALS, and male CBA mice (18–22 g body weight) served as skin graft donors. All other animals used in these experiments were male C57BL/6J mice (20–25 g body weight). The mice were obtained from the Jackson Laboratory, Bar Harbor, Maine.

*Preparation of ALS.*—A single pool of horse anti-mouse lymphocyte serum was used throughout these experiments. For the preparation of ALS, a horse was repeatedly injected with a mixture of thymus and lymph node cells obtained from male C57BL/6J mice. Each inoculum contained $10^9$ cells and injections were given subcutaneously on days 0, 3, 6, and 9 and intravenously on days 10, 14, and 21. The horse was bled on day 29 and the serum obtained was stored in portions of 2 ml at $-20^\circ$C. Before use, all ALS was decomplemented (heating at $56^\circ$C for 30 min) and absorbed with an equal volume of packed SRBC to remove natural anti-SRBC antibodies.

*Skin Grafting of Mice.*—The method employed and the criteria used for assessing graft rejection have been previously described (11).

*Immunization with SRBC and Determination of Hemagglutinin Titters.*—SRBC were obtained from freshly bled sheep and stored at 4°C in Alsever's solution. The cells were always

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†Abbreviations used in this paper: ALS, anti-lymphocyte serum; CY, cyclophosphamide; PFC, plaque-forming cells; SRBC, sheep red blood cells.
used within a week after collection. For purposes of immunization, the cells were washed three times in 0.9% NaCl, suspended in TC 199 (Difco Laboratories, Inc., Detroit, Mich.), and intravenously injected into mice. Each mouse received 0.5 ml of a 2% suspension of SRBC. Hemagglutinin titers were determined 7 days after immunization using a technique which we previously described (12). The titer recorded was the dilution in the last well in which the cell pattern differed from the buttons of cells present in the control wells.

Preparation of Cell Suspensions.—Thymus and spleen cell suspensions were prepared by a previously described method (11). For preparation of marrow cell suspensions, the long bones were removed from mice and after their ends had been cut off, the marrow cavities were flushed out with TC 199. Before use, the resulting cell suspension was passed through a stainless steel mesh to remove debris.

Statistical Analyses.—To determine the ranges of the 95% confidence limits of the means, the t value for the appropriate degrees of freedom was multiplied by the standard error of the mean (13).

Plan of Experiments.—The basic experimental model used has been previously described (14) and derives from the work of Santos and Owens (15). C57BL/6J mice were killed 24 hr after the last of four successive daily subcutaneous injections of 0.5 ml ALS. A suspension of cells from the spleens of these animals was mixed with SRBC and intravenously injected into syngeneic mice rendered immunologically anergic by the intraperitoneal injection of cyclophosphamide (250 mg/kg body weight) 6-8 hr earlier. Serum hemagglutinin titers were determined 7 days later.

Titors produced by cyclophosphamide (CY)-treated mice injected with SRBC and 2 X 10^7 normal spleen cells were much higher than those found when the CY-treated mice received SRBC and 2 X 10^7 spleen cells from mice previously given ALS. In subsequent experiments attempts were made to see if the depressed titers in this latter group could be elevated to normal levels by intravenously injecting the CY-treated mice with normal syngeneic thymus and/or marrow cells, in addition to SRBC and spleen cells from ALS-treated donors. The thymus and marrow cells were injected into a different vein immediately after the injection of spleen cells.

SRBC and spleen cells were always injected 6-8 hr after the mice had received CY. Because of the danger of acute death from pulmonary emboli, not more than a total of 10^8 nucleated thymus and marrow cells were intravenously injected on any one occasion. If larger numbers of thymus and marrow cells had to be injected, they were given in divided doses of 0.8-1 X 10^8 cells each, at 8-12 hr intervals. The first injection of 10^8 cells was given at the same time as the SRBC and spleen cells, and all subsequent injections were completed within the next 18 hr.

In a final series of experiments, SRBC and mixtures of thymus and marrow cells from both untreated and ALS-treated donors were adoptively transferred to mice previously given CY. These experiments were done to determine whether, and to what degree, ALS treatment altered the immunological competence of cells located within the thymus and marrow.

RESULTS

Suppression of Immune Responses by ALS.—The ALS used in these experiments was a moderately good immunosuppressive agent. The median survival time of CBA grafts on male C57BL/6J mice given 0.5 ml of ALS subcutaneously on days +1, +4, +7, and +10 after grafting was 17 days, with a range of 14-23 days. All grafts on untreated or normal horse serum-treated recipients were rejected in 11 days or less.
Table I shows that the ALS used was also capable of decreasing the immune response to SRBC. Administration of ALS for 3 consecutive days before immunization with SRBC significantly depressed the serum hemagglutinin response of treated animals. A still greater depression of serum hemagglutinin levels was found when mice received 4 days of pretreatment with ALS. This latter dose of ALS reduced the 19S plaque-forming cell (PFC) responses in the spleens of treated animals to background levels.

**Antibody Production by CY-Treated Mice Given SRBC and Normal Syngeneic Lymphoid Cells.**—Table II shows that hemagglutinin titers produced by CY-treated mice given SRBC were significantly lower than those produced by mice given SRBC but not CY. Titers could be raised to the levels found in the latter group if the CY-treated mice received a mixture of SRBC and $2 \times 10^7$ normal syngeneic spleen cells. Similar serum hemagglutinin levels resulted when the CY-treated mice were given SRBC and a mixture containing $1.2 \times 10^8$ normal thymus and $1.2 \times 10^8$ normal marrow cells. Mixtures containing fewer thymus and marrow cells produced correspondingly smaller amounts of antibody. Titers were very low when large numbers of either thymus ($1.2 \times 10^9$) or marrow cells ($1.2 \times 10^9$) alone were given to the CY-treated animals.

A synergistic interaction between normal thymus and marrow cells was demonstrable when a mixture containing SRBC and these two cell types was given to CY-treated mice. Serum hemagglutinin titers produced by animals given the mixture were invariably higher than the sum of the titers produced by mice given SRBC and thymus or marrow cells alone in that experiment. (Throughout this paper hemagglutinin titers have been expressed in terms of

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### TABLE I

**Hemagglutinin Titers Produced by Mice Given SRBC after Injections of ALS**

| Mice received | Serum given on days | No. of mice producing hemagglutinin titers of |
|---------------|--------------------|-------------------------------------------|
| Nil           | --                 | 2  20                                     |
| Normal horse  | $-4, -3, -2, -1$   | 10                                        |
| ALS           | $-2, -1$           | 16                                        |
| ALS           | $-3, -2, -1$       | 1  4  3  4  4  4                         |
| ALS           | $-4, -3, -2, -1$   | 4  3  2  3                               |

Male C57BL/6J mice intravenously injected with 0.5 ml of a 2% suspension of SRBC 24 hr after the last injection of serum. Hemagglutinin titers determined 7 days later.

* Serum given as subcutaneous injections of 0.5 ml each time. SRBC were injected on day 0.

† Titers expressed in terms of $\log_2$ of their reciprocals.
log, of their reciprocals. Hence, summation is not a simple arithmetical process. For example, \(4 + 4 = 5\).

*Antibody Production by CY-Treated Mice Given SRBC and Spleen Cells*

**TABLE II**

Hemagglutinin Titers Produced by Cyclophosphamide (CY)-Treated C57BL/6J Mice Given SRBC and Normal Syngeneic Spleen, Thymus, and Marrow Cells

| Experiment No. | CY-treated mice given | No. of mice | Hemagglutinin titers* |
|----------------|-----------------------|-------------|-----------------------|
|                |                       |             | Mean                  | 95% confidence limits of means |
| 1              | SRBC (no CY)          | 14          | 9.6                   | 8.3-10.9                      |
|                | SRBC                  | 12          | 0.4                   | 0.2-0.6                       |
|                | SRBC + 0.5 \(\times 10^7\) spleen cells | 8          | 0.4                   | 0-0.9                         |
|                | SRBC + 1 \(\times 10^7\) spleen cells | 8          | 5.0                   | 3.7-6.3                       |
|                | SRBC + 2 \(\times 10^7\) spleen cells | 12         | 9.1                   | 8.3-9.9                       |
|                | SRBC + 3.5 \(\times 10^7\) spleen cells | 8          | 9.4                   | 8.8-10.0                      |
|                | SRBC + 2 \(\times 10^7\) inactivated spleen cells | 12         | 0.5                   | 0-1.5                         |
| 2              | SRBC + 2 \(\times 10^7\) spleen cells | 7          | 11.1                  | 10.8-11.4                     |
|                | SRBC + 2 \(\times 10^7\) thymus and 2 \(\times 10^7\) marrow cells | 8          | 5.3                   | 4.0-6.6                       |
|                | SRBC + 4 \(\times 10^7\) thymus and 4 \(\times 10^7\) marrow cells | 8          | 6.0                   | 4.5-7.5                       |
| 3              | SRBC + 2 \(\times 10^7\) spleen cells | 8          | 11.4                  | 10.4-12.4                     |
|                | SRBC + 8 \(\times 10^7\) thymus cells | 8          | 2.6                   | 1.6-3.6                       |
|                | SRBC + 8 \(\times 10^7\) marrow cells | 7          | 3.5                   | 2.4-4.6                       |
|                | SRBC + 8 \(\times 10^7\) thymus and 8 \(\times 10^7\) marrow cells | 8          | 7.9                   | 5.5-10.3                      |
| 4              | SRBC + 2 \(\times 10^7\) spleen cells | 8          | 9.5                   | 8.9-10.1                      |
|                | SRBC + 1.2 \(\times 10^6\) thymus cells | 8          | 3.6                   | 3.0-4.2                       |
|                | SRBC + 1.2 \(\times 10^6\) marrow cells | 7          | 3.6                   | 2.5-4.7                       |
|                | SRBC + 1.2 \(\times 10^6\) thymus and 1.2 \(\times 10^6\) marrow cells | 7          | 9.2                   | 8.4-10.0                      |

Mice were given CY (250 mg/kg body weight) intraperitoneally 6-8 hr prior to intravenous injection of SRBC and syngeneic lymphoid cells. Serum hemagglutinin titers were determined 7 days later.

*Titers expressed in terms of logs of their reciprocals. All mice received 0.5 ml of a 2% suspension of SRBC.

*from ALS-Treated Donors.*—Table III shows that spleen cells from ALS-treated animals are deficient in their ability to produce anti-SRBC antibody. These immunological deficits were only demonstrable after 3 days of pretreatment with ALS (Table III), and Table I shows that this was the minimum amount of ALS required to significantly depress the serum hemagglutinin response of mice immunized with SRBC.
Antibody Production by CY-Treated Mice Given SRBC, Spleen Cells from ALS-Treated Animals, and Normal Thymus and/or Marrow Cells.—The next series of experiments (Table IV) were performed in order to determine whether the immunological deficits which appeared in the spleens of animals given ALS might have been due to the inactivation of cells which were functionally similar to thymus and/or marrow cells. The additional injection of a mixture containing normal thymus and marrow cells significantly raised the serum hemagglutinin levels found when CY-treated mice were given SRBC and \(2 \times 10^7\) spleen cells from ALS-treated donors. If the mixture contained \(8 \times 10^7\) thymus cells and \(8 \times 10^7\) marrow cells, hemagglutinin titers produced by the immuno-

| TABLE III |

Hemagglutinin Titers Produced by Cyclophosphamide (CY)-Treated C57BL/6J Mice Given SRBC and Syngeneic Spleen Cells from ALS-Treated Donors

| Experiment no. | Spleen cell donors received | Serum given on days* | No. of CY-treated recipients | Hemagglutinin titers† |
|----------------|----------------------------|----------------------|-----------------------------|----------------------|
|                |                            |                      | Mean | 95% confidence limits of means |
| 1              | Nil                        | —                    | 8    | 12.2 11.6-12.8 |
|                | Normal horse serum         | —4, —3, —2, —1       | 8    | 11.8 11.1-12.5 |
|                | ALS                        | —1                   | 8    | 11.4 10.7-12.1 |
| 2              | Nil                        | —                    | 8    | 11.1 10.6-11.6 |
|                | ALS                        | —2, —1               | 8    | 10.7 10.1-11.3 |
|                | ALS                        | —3, —2, —1           | 8    | 4.1 3.7-4.5 |

Mice were intravenously injected with 0.5 ml of a 2% suspension of SRBC containing \(2 \times 10^7\) syngeneic spleen cells 6-8 hr after intraperitoneal injection of CY (250 mg/kg body weight). Hemagglutinin titers were determined 7 days later.

* Serum given as subcutaneous injections of 0.5 ml each time. Cells transferred 24 hr after the last injection.

† Titers expressed in terms of logs of their reciprocals.

logically anergic recipients were similar to those found after their injection with SRBC and \(2 \times 10^7\) normal spleen cells. Mixtures containing fewer thymus and marrow cells were correspondingly less effective. Marrow cells (\(1.2 \times 10^6\)) alone were completely ineffective and only a partial elevation of hemagglutinin titers to normal levels was found when the inoculum contained thymus cells (\(1.2 \times 10^6\)) alone. Titers produced by CY-treated mice given SRBC, spleen cells from ALS-treated donors, and normal thymus and marrow cells were always higher than the sum of the titers separately produced by the groups given SRBC and either spleen cells from ALS-treated animals or normal thymus and marrow cells. This suggests that there was a synergistic interaction between spleen cells from ALS-treated donors on the one hand and normal thymus and marrow cells on the other. A synergy was also demonstrable when
the CY-treated recipients of SRBC and spleen cells from ALS-treated donors were given $1.2 \times 10^8$ normal thymus cells alone (Table IV, experiment 1).

**Antibody Production by CY-Treated Mice Given SRBC and Mixtures of Thymus and Marrow Cells from ALS-Treated and Untreated Donors.**—The next two experiments were performed in order to see what effect, if any, ALS had on the immunological competence of cells located within the thymus and marrow. CY-treated mice were given SRBC and mixtures of syngeneic thymus and

| Exper- | CY-treated hosts received SRBC and* | No. of | Hemagglutinin titers† |
|--------|-----------------------------------|--------|-----------------------|
| ment no. |                                    | recipients | Mean | 95% confidence limits of means |
| 1      | Normal spleen cells ($2 \times 10^7$) | 8       | 10.1 | 9.3–10.9 |
|        | Normal thymus cells ($1.2 \times 10^8$) | 8       | 1.4  | 0.4–2.4 |
|        | ALS spleen cells ($2 \times 10^7$) | 7       | 2.4  | 1.7–3.1 |
|        | ALS spleen cells ($2 \times 10^7$) + normal thymus cells ($2 \times 10^8$) | 8       | 3.4  | 2.1–4.7 |
|        | ALS spleen cells ($2 \times 10^7$) + normal thymus cells ($1.2 \times 10^8$) | 8       | 5.5  | 4.9–6.1 |
| 2      | Normal spleen cells ($2 \times 10^7$) | 8       | 11.3 | 8.9–13.7 |
|        | Normal marrow cells ($1.2 \times 10^8$) | 8       | 3.9  | 2.8–5.0 |
|        | ALS spleen cells ($2 \times 10^7$) | 7       | 2.0  | 1.0–3.0 |
|        | ALS spleen cells ($2 \times 10^7$) + normal marrow cells ($1.2 \times 10^8$) | 8       | 3.9  | 2.2–5.6 |
| 3      | Normal spleen cells ($2 \times 10^7$) | 8       | 13.6 | 12.2–15.0 |
|        | Normal thymus ($4 \times 10^7$) + normal marrow cells ($4 \times 10^8$) | 8       | 6.1  | 5.4–6.8 |
|        | ALS spleen cells ($2 \times 10^7$) | 8       | 3.1  | 2.2–4.0 |
|        | ALS spleen cells ($2 \times 10^7$) + normal thymus ($4 \times 10^7$) and normal marrow cells ($4 \times 10^7$) | 8       | 9.4  | 8.3–10.5 |
| 4      | Normal spleen cells ($2 \times 10^7$) | 8       | 11.5 | 11.0–12.0 |
|        | Normal thymus ($8 \times 10^7$) + normal marrow cells ($8 \times 10^7$) | 8       | 6.1  | 5.2–7.0 |
|        | ALS spleen cells ($2 \times 10^7$) | 8       | 4.0  | 2.9–5.1 |
|        | ALS spleen cells ($2 \times 10^7$) + normal thymus ($8 \times 10^7$) + normal marrow cells ($8 \times 10^7$) | 7       | 10.3 | 9.1–11.5 |

* Normal cells were obtained from untreated mice. ALS spleen cells were obtained from mice 24 hr after the last of four successive daily subcutaneous injections of ALS (0.5 ml each time). The figures in parentheses indicate the number of cells injected on each occasion. SRBC were given as 0.5 ml of a 2% suspension.

† Titers expressed in terms of logs of their reciprocals.
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Table V shows that ALS treatment had no effect on the immunological competence of cells located within the thymus, and that marrow cells were only slightly affected. Moreover, the second experiment in Table V shows that the depressed hemagglutinin titers produced by CY-treated mice given SRBC and spleen cells from ALS-treated donors could be elevated to normal levels by injections of thymus and marrow cells from the same ALS-treated animals.

### TABLE V

| Experiment no. | CY treated hosts received SRBC and* | No. of recipients | Hemagglutinin titer† mean ± 95% confidence limits of means |
|---------------|-----------------------------------|------------------|-------------------------------------------------------------|
| 1 Normal thymus cells (8 X 10⁷) + normal marrow cells (8 X 10⁷) | 8 | 7.8 | 6.9-8.7 |
| ALS thymus cells (8 X 10⁷) + normal marrow cells (8 X 10⁷) | 8 | 7.3 | 6.8-7.8 |
| Normal thymus cells (8 X 10⁷) + ALS marrow cells (8 X 10⁷) | 8 | 6.4 | 5.7-7.1 |
| 2 Normal spleen cells (2 X 10⁷) | 8 | 9.0 | 8.3-9.7 |
| ALS spleen cells (2 X 10⁷) | 8 | 3.8 | 2.9-4.7 |
| ALS thymus cells (8 X 10⁷) + ALS marrow cells (8 X 10⁷) | 7 | 5.5 | 4.7-6.3 |
| ALS spleen cells (2 X 10⁷) + ALS thymus (8 X 10⁷) and ALS marrow cells (8 X 10⁷) | 8 | 8.6 | 7.9-9.3 |

* Normal cells were obtained from untreated mice and ALS cells from mice 24 hr after the last of four successive daily subcutaneous injections of ALS (0.5 ml each time). In Experiment 2, the ALS spleen, thymus, and marrow cells were obtained from the same ALS-treated animals. SRBC given as 0.5 ml of a 2% suspension.

† Titers expressed in terms of log₂ of their reciprocals.

### DISCUSSION

Hemagglutinin titers produced by CY-treated mice given SRBC and a mixture of normal thymus and marrow cells were always higher than the sum of the titers which were produced by the groups given SRBC and normal thymus cells or marrow cells alone in that same experiment. These findings are in agreement with observations made by Claman et al. (7) and others (8) which have shown that the production of antibody to SRBC requires a synergistic interaction between cells of thymus and marrow origin. In addition, the present experiments show that this synergism can be demonstrated when the recipients of SRBC and normal syngeneic thymus and marrow cells have
been rendered immunologically anergic by prior treatment with CY instead of the X-irradiation used by previous workers (7, 8).

It was necessary to inject the CY-treated mice with SRBC and a mixture containing $1.2 \times 10^6$ normal thymus cells and $1.2 \times 10^6$ normal marrow cells to obtain serum hemagglutinin levels similar to those which were found when the mice received SRBC and $2 \times 10^7$ normal spleen cells. It is difficult to explain satisfactorily why such large numbers of thymus and marrow cells were needed. Perhaps cells which are involved in antibody production form a smaller proportion of the thymus and marrow cell populations than they do of the spleen cell population. Another possibility is that cells which are located within the thymus and marrow might require a period of residence maturation and, possibly, differentiation in another environment before achieving their full potential as participants in the immune response.

The present experiments confirm previously reported observations (2, 3, 5, 6, 9) which have shown that administration of ALS prior to injection of SRBC significantly depresses the ability of treated animals to produce anti-SRBC antibody. The ALS used was absorbed with SRBC prior to use. In addition, normal horse serum was completely ineffective in suppressing the immune response to SRBC and this serum was the preimmunization serum of the horse used for production of ALS. Hence, it is unlikely that the depression of the immune response to SRBC by this ALS could have been due to the passive transfer of anti-SRBC antibodies, as suggested by Guttman et al. (16).

It has been shown that administration of ALS decreases the ability of cells in the lymph nodes and spleen to participate in immune responses (9, 17, 18). Similar results have been obtained in this present series of experiments. Administration of ALS significantly decreased the ability of spleen cells from treated animals to produce anti-SRBC antibody when they were adoptively transferred with SRBC to mice given CY. Tables I and III show that this decrease in the immunological competence of spleen cells was only demonstrable when the amount of ALS given was sufficient to depress the animal's immune response to SRBC.

Hemagglutinin titers produced by CY-treated mice given SRBC and $2 \times 10^7$ normal spleen cells were always much higher than those which were found when the spleen cells were obtained from ALS-treated mice. Titers could be raised to the levels found in the former group if CY-treated recipients of SRBC and spleen cells from ALS-treated donors were also injected with mixtures containing normal thymus and marrow cells. Inocula which contained only marrow cells were completely ineffective while thymus cells alone were much less effective than mixtures containing both thymus and marrow cells. The ability of a mixture of thymus and marrow cells to restore titers to normal levels could not have been due to a simple additive effect, as a synergistic interaction was regularly demonstrable between spleen cells from ALS-treated
donors on the one hand, and normal thymus and marrow cells on the other. The work of Micklem et al. (19) and of Globerson and Auerbach (20) suggests that the spleen contains a mixture of cells which have originated in the thymus and marrow. If this is so, our results would suggest that immunosuppression by ALS is associated with the inactivation of both thymus- and marrow-derived lymphocytes.

Inactivation of thymus-derived lymphocytes by ALS would explain why recovery from the effects of ALS is thymus-dependent (2, 12, 21) and why treatment with ALS depletes the thymus-dependent areas of the lymph nodes and spleen (22-24). The possibility that cells, other than those of thymus origin, might also be inactivated as the result of treatment with ALS is one interpretation of findings reported by Mitchison (25). This possibility is also suggested by the failure of Levey and Medawar (4) to demonstrate any curtailment of allograft survival when mice were given \(2 \times 10^8\) thymus cells, 25 days after skin grafting. On the other hand, Gray et al. (3) were able to show a significant reduction in allograft survival times when ALS-treated mice were given \(10^8\) syngeneic spleen cells. It has been recently shown by Lubaroff (26) that cells of marrow origin play an important role in allograft rejection. These observations, together with those of Gray et al. (3), suggest that allograft survival times were not decreased when ALS-treated animals received thymus cells alone (4) because the animals might also have been deficient in marrow-derived lymphocytes.

Inactivation of marrow-derived spleen cells by ALS was not demonstrable in experiments reported by Martin and Miller (9). They showed that thymus cells alone could raise the peak PFC response of irradiated recipients of SRBC and spleen cells from ALS-treated donors to levels found when the animals were given SRBC and normal spleen cells. There were differences between their experiments and those reported in this paper, and any one or more of these differences might have been responsible for their failure to show that ALS inactivates cells of marrow origin. The use of irradiation rather than CY to depress the immune responses of the recipients of adoptively transferred spleen cells was probably not a factor of much importance. However, in order to quantitate anti-SRBC antibody production by the transferred cells they measured the PFC responses in the spleens of the recipient animals rather than their serum hemagglutinin levels. The former measures early 19S antibody production while the latter is predominantly a 7S antibody response to immunization with SRBC. Perhaps more important was that the amount of ALG given to their donor mice, though immunosuppressive, still allowed considerable numbers of PFC to be produced when given to other mice which were subsequently immunized with SRBC. In our experiments, the amount of ALS given to the donor mice was more strongly immunosuppressive. Administration of ALS prior to injection of SRBC reduced the PFC responses in the spleens.
of treated animals to background levels. A subsequent communication will show that when mice are given ALS, the first cells to be inactivated are those of thymus origin. Deficiencies of marrow-derived spleen cells only become evident when strongly immunosuppressive amounts of ALS are administered.

The ability of ALS to inactivate marrow-derived spleen cells, as described in these present experiments, could not have been due to the fact that the horse used for preparation of ALS had been immunized with inocula containing thymus and lymph node cells and therefore, presumably, cells of both thymus and marrow origin. Inactivation of marrow-derived spleen cells was also demonstrable (our unpublished findings) when mice received ALS which had been prepared by immunizing horses with mouse thymus cells alone.

Studies with radioactively labeled ALS IGG (27) have shown that anti-lymphocyte antibody fails to penetrate lymphoid tissue to any significant degree. The present functional studies are in agreement with these observations. They show that administration of ALS does not alter the immunological competence of cells located within the thymus and that cells in the bone marrow are only affected to a slight degree. In contrast, administration of immunosuppressive doses of ALS markedly decreases antibody production by spleen cells. It has been shown by Cantor et al. (28) that the majority of immunologically competent cells in the spleen are derived from the peripheral recirculating pool of lymphocytes. Hence our findings are consistent with the generalization (17, 27, 29) that the principal site of action of ALS is the peripheral recirculating lymphocyte.

It is difficult to explain satisfactorily why cells in the thymus and bone marrow are relatively immune to the effects of ALS, since in vitro incubation with ALS can be shown to decrease the ability of thymus cells to participate in immune responses. Agglutinating and cytotoxic antibody titers of ALS are approximately similar regardless of whether cells from mouse thymus, marrow, lymph node, or spleen are used as target cells and this suggests that approximately similar numbers of antibody binding sites are present on each of these different lymphoid cell types. Differences in avidity for antibody, as between central and peripheral lymphoid cells, is also unlikely to account for the relative immunity of cells in the thymus and marrow. With large doses of ALS we have been able to show (unpublished observations) that, despite the presence of free ALS in the circulation, the immunological competence of cells in the thymus and, to a lesser degree, in the marrow, remain unaffected.

Finally, the second experiment in Table V suggests that the unaffected cells within the thymus and marrow could provide a mechanism for recovery from the effects of ALS. This would explain why the immunosuppressive properties of ALS can be so markedly potentiated by prior thymectomy (2, 12, 21) or irradiation (4) of the recipient.
SUMMARY

When CY-treated mice were given sheep red blood cells the serum hemagglutinin titers produced were significantly lower than those found when mice received SRBC but not CY. Titers could be raised to the levels found in the latter group if, in addition to SRBC, the CY-treated mice received $2 \times 10^7$ normal syngeneic spleen cells or a mixture containing $1.2 \times 10^8$ normal thymus and $1.2 \times 10^8$ normal marrow cells. Inocula which contained fewer cells produced correspondingly smaller amounts of antibody. A synergistic interaction between normal thymus and marrow cells was always demonstrable in these experiments.

Hemagglutinin titers produced by CY-treated mice given SRBC and $2 \times 10^7$ normal syngeneic spleen cells were always much higher than those found when the spleen cells were obtained from animals previously given ALS. Titers could be raised to normal levels if the animals in this latter group received additional injections containing mixtures of normal syngeneic thymus and marrow cells. Marrow cells alone were completely ineffective, while inocula which only contained thymus cells were much less effective than mixtures of thymus and marrow cells. These results suggest that immunosuppression by ALS is associated with the inactivation of both thymus and marrow-derived lymphocytes.

In other experiments CY-treated mice received SRBC and mixtures of thymus and marrow cells from both untreated and ALS-treated donors. No decrease in the immunological competence of cells located within the thymus of ALS-treated donors was demonstrable in these experiments. Marrow cells were slightly affected but to a markedly lesser degree than were spleen cells of ALS-treated animals. In a final experiment, it was possible to show that the thymus and marrow cells of ALS-treated animals could repair the immunological defects which were present in their own spleen cell populations.

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