CELL TO CELL INTERACTION IN THE IMMUNE RESPONSE

IV. Site of Action of Antilymphocyte Globulin*, ‡

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A knowledge of the site of action of antilymphocyte serum is required in order to develop a meaningful in vitro assay of its immunosuppressive activity and to gain insight into its mechanism of action. Mice treated with antilymphocyte serum exhibit defects similar in nature to those characteristically found in neonatally thymectomized mice. Thus, in general, cell-mediated immune responses are depressed to a greater extent than humoral antibody responses (1, 2) and cell depletion involves particularly that class of lymphocytes found in the periarteriolar lymphocyte sheaths of the spleen and diffuse cortex of the lymph nodes (1–4), that is in those areas traversed by long-lived recirculating small lymphocytes (5). The question may thus be raised as to whether antilymphocyte serum might act selectively on a population of lymphocytes the development of which is under thymus control.

The previous papers of this series have indicated that the IgM response of mice to sheep erythrocytes requires an interaction between thymus-derived antigen-reactive cells and bone marrow–derived antibody-forming cell precursors (6, 7). Neonatally thymectomized mice challenged with sheep red blood cells fail to produce in their spleens a normal number of hemolysin-forming cells (8). This deficiency can be corrected by injecting either thoracic duct cells (6, 8) or thymus cells (6). The restorative capacity of thymus cells clearly identifies the specific cellular defect induced by thymectomy since the cell population of the thymus, unlike that of thoracic duct lymph, contains no antibody-forming cell precursors (7).

Antilymphocyte serum depresses the immune response of mice to sheep erythrocytes (9, 10). If the target cells of antilymphocyte serum were primarily thymus-derived cells, then normal reactivity should be restored by injecting thymus cells. Accordingly, experiments were set up to test this possibility. In addition, an investigation was made on the influence of thymus cells on the response of heavily irradiated mice to an injection of sheep red blood cells and spleen cells from antilymphocyte serum–treated mice.

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Materials and Methods

Mice.—2- to 3-month-old male and female mice of the highly inbred CBA strain were used. The origin and maintenance of these mice has been described previously (6).

Cell suspensions from spleen and thymus were prepared as before (6). Sheep erythrocytes were obtained from a single sheep assigned to this work as reported in a previous paper (6).

Injections were made either intraperitoneally or intravenously into the tail vein as stated in the text. In the case of thymus cells, in doses exceeding 10 million per mouse, intravenous injections were given slowly, over 1–2 min, and in a large volume (0.4–0.5 ml) as reported before (6).

Irradiation.—Mice were exposed to doses of 800 rads total-body irradiation as explained in a previous paper (7).

Thymectomy was performed by the method of Miller (11). Routine macroscopic and microscopic checks of thymus areas were made at autopsy and mice with thymus remnants were excluded from the experiments.

Immunization and Assay for Hemolysin Plaque-Forming Cells.—Mice were immunized with 0.1 ml of a 20% suspension of sheep erythrocytes (approximately 1–2 $\times 10^8$ cells) either intraperitoneally or intravenously. The number of hemolysin plaque-forming cells per spleen was determined according to the technique of Cunningham and Szenberg (12) which is a modification of that of Jerne et al. (13).

Preparation of Antilymphocyte Globulin (ALG).[1]—Two preparations of rabbit anti-mouse lymphocyte globulin were used. The first, (A), was obtained from four rabbits by giving two injections of 10^9 washed thymus cells intravenously 14 days apart, followed by two further injections of 5×10^8 thymus cells 4 and 6 wk later. The rabbits were bled 7 days after the last injection. The ability of the preparation to prolong the survival of skin grafts on mice has been described previously (14). The second, (B), was obtained from three rabbits by injecting 10^9 washed mouse thymus cells intravenously at two-weekly intervals. The rabbits were bled 1 wk after both the second and the third injections and the sera pooled. Both preparations were absorbed with washed mouse erythrocytes and the second preparation was also absorbed with sheep erythrocytes. Neither antisera contained detectable antibodies against mouse gamma globulin.[2] The gamma globulin fraction of both sera was obtained by ammonium sulfate precipitation and passage through a DEAE-cellulose column using 0.0175 M phosphate buffer pH 6.3. NRG was prepared similarly. The final products were adjusted to 10 mg/ml and their purity checked by polyacrylamide gel electrophoresis and agar gel precipitation using anti-rabbit gamma globulin antiserum. Mice received a single intraperitoneal injection of 5 mg ALG or NRG.

Labeling of Thymus Cells with ^{51}Cr.—Thymus cells were washed once and then suspended in Eisen's balanced salt solution containing 10% heat-inactivated fetal calf serum. The cell concentration was 5×10^7/ml. ^{51}Cr, as Na_2CrO_4 in isotonic saline, was obtained from the Radiochemical Centre, Amersham, England. The specific activity varied between 150 and 263 μc/μg of chromium. The concentration of radioactivity was 1000 μc/ml at the time of use. 20 μc of radioactivity was added per milliliter of the cell suspension. The cells were then incubated in a water bath for 30 min at 37°C with occasional mixing. Cold Eisen's solution plus fetal calf serum was added and the tubes centrifuged. The cells were washed three times and finally resuspended in a concentration of about 10^8 cells per milliliter. The amount of activity

[1] The following abbreviations are used: ALS, antilymphocyte serum; ALG, antilymphocyte globulin; NRG, normal rabbit globulin; SRBC, sheep erythrocytes; PFC, hemolysin plaque-forming cells; NS, not significant; and SE, standard error.

[2] Assayed by the sensitive microprecipitation technique using a range of ^{125}I-labeled mouse myeloma proteins.

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in a sample was determined and mice were then injected with about 5×10^7 cells containing 5000 counts per minute. 6 hr later the mice were bled and the spleens, livers, and lungs removed for organ counting. The amount of radioactivity present in these organs was determined using a Packard "Tricarb" scintillation counter.

Statistical Analysis.—The standard errors of the means were calculated. The P values were determined by Student's t test except where the variance ratio F test exceeded 0.05 probability. An appropriate modification of the t test was then utilized.[3]

RESULTS

Effect of Antilymphocyte Globulin on the Hemolysin Plaque-Forming Cell Response to Sheep Erythrocytes.—The immunosuppressive capacity of our ALG preparations was tested in mice challenged with SRBC. In Fig. 1 are shown the results of experiments in which the animals were injected intraperitoneally with SRBC 48 hr after administration of 5 mg ALG/A. The controls were treated in an identical way but received NRG instead of ALG, or no rabbit globulin at all. In all groups the peak number of PFC produced in the spleen occurred on day 4 but it was 1 $\log_{10}$ lower in the case of ALG-treated mice.

Another group of mice was then used to determine when, in relation to the challenge with SRBC, the ALG preparation should be given in order to get a maximum immunosuppressive effect. The time of administration of ALG and NRG was therefore varied relative to the injection of SRBC and the PFC response per spleen was determined on day 4. As can be seen from Fig. 2, ALG exerted significant immunosuppression but only when given before or simultaneously with the SRBC. By contrast NRG, whether given before, or together with SRBC, produced no depression of the PFC response.

Effects of Antilymphocyte Globulin on Hemolysin Plaque-Forming Cells In Vitro.—Since the incubation of spleen cells containing PFC with specific anti-H2 isoantisera and complement in vitro was associated with a marked reduction in the number of detectable PFC (6), it was of interest to determine whether incubation with ALG would produce a similar effect. Therefore, 10 million spleen cells from an immunized mouse were incubated with varying

[3]

$$t_M = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

where $\bar{x}_1$ and $\bar{x}_2$ are the means of the observations in two groups; S_1^2 and S_2^2 are the variances of the observations in each group; n_1 and n_2 are the number of observations in the two groups; M is the number of degrees of freedom and is given by

$$\frac{1}{M} = \frac{C^2}{n_1 - 1} + \frac{(1 - C)^2}{n_2 - 1} \quad \text{where } C = \frac{\frac{S_1^2}{n_1}}{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}$$

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concentrations of ALG in the presence of 1:10 guinea pig serum as a source of complement. For controls, cells were incubated under similar conditions but in the absence of ALG. After 30 min at 37°C, the number of PFC remaining was determined and expressed as a percentage of the number remaining in the ab-

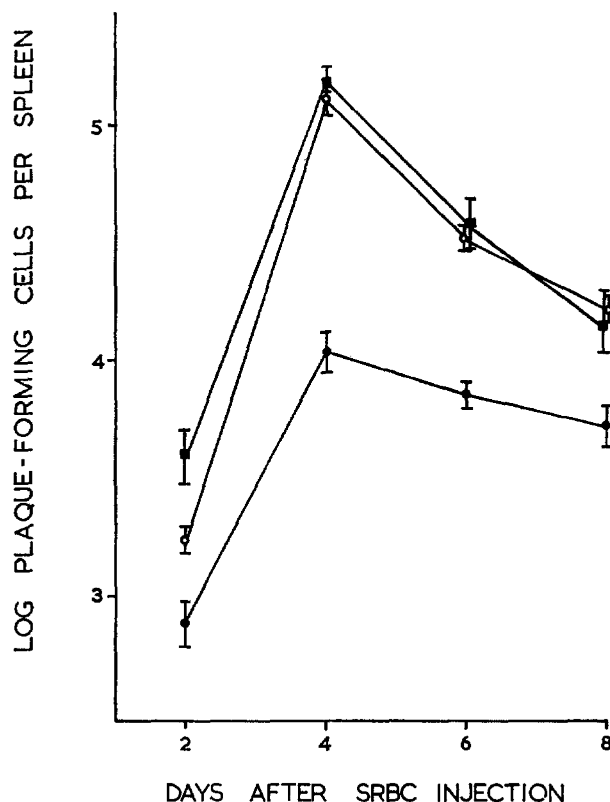

FIG. 1. PFC produced in the spleens of normal mice (O——O) and of mice given a single dose of ALG (●——●) or NRG (■——■) 48 hr prior to an intraperitoneal injection of SRBC. Each point represents the mean of determinations on four to six mice. The SE of the means are indicated by the vertical bars.

sence of ALG. At concentrations of 100 μg/ml or less, neither ALG/A nor ALG/B inhibited PFC. Even at 1 mg/ml, only ALG/B had an inhibitory effect (Fig. 3).

Effect of Injecting Thymus Cells into ALG-treated Mice.—The effect of administering thymus cells on the immune response of ALG-treated mice was examined. It was found that an intravenous injection of 100 million thymus cells had no effect on the response of ALG/A-treated mice receiving SRBC *intraperitoneally* although some increase was apparent in the case of ALG/B-

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treated recipients (Table I). When the erythrocytes were given *intravenously* together with thymus cells, a marked increase in the response occurred in both ALG/A- and ALG/B-treated mice (Table I and Fig. 4). A similar increase was achieved with 20 million normal spleen cells given intravenously (Fig. 4

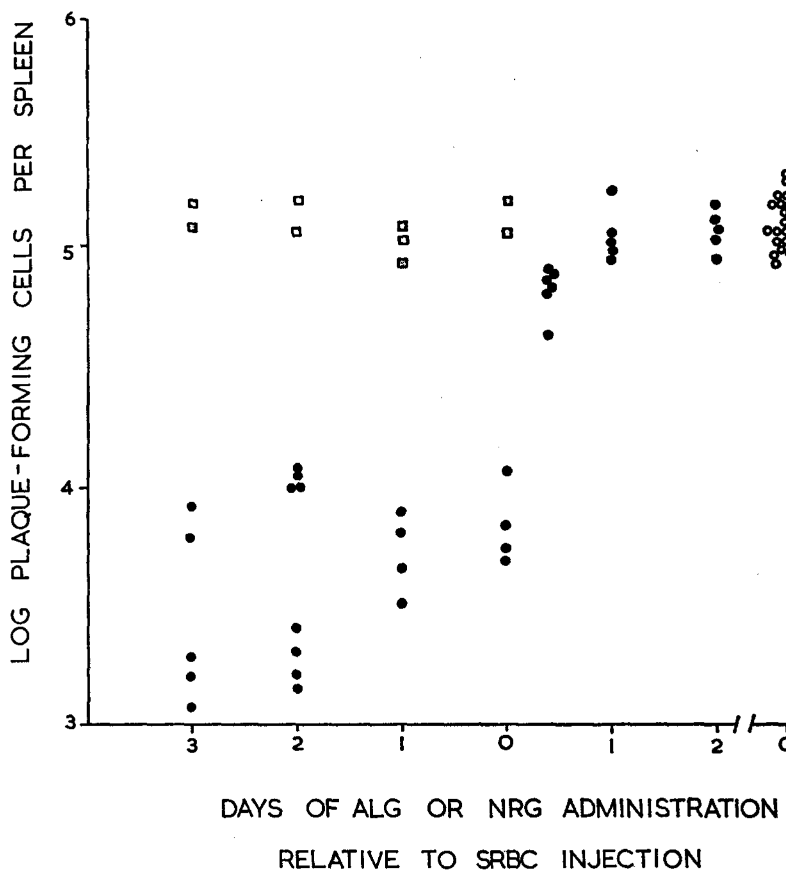

FIG. 2. PFC produced in the spleens of normal mice (C on abscissa) (○) and of mice given a single dose of ALG (●) or NRG (□) at varying times relative to an intraperitoneal injection of SRBC.

and Table II), whereas 100 million bone marrow cells had no significant effect (Table II). In addition, it can readily be seen from Table I that the response of ALG-treated mice not receiving thymus cells was greater when SRBC were given intravenously than intraperitoneally. In normal mice, however, neither thymus cells nor the route of injection of SRBC materially affected the PFC response (Table I and Fig. 5).

Localization of ^{51}*Cr-labeled Thymus Cells in ALG-treated Mice.*—It was of

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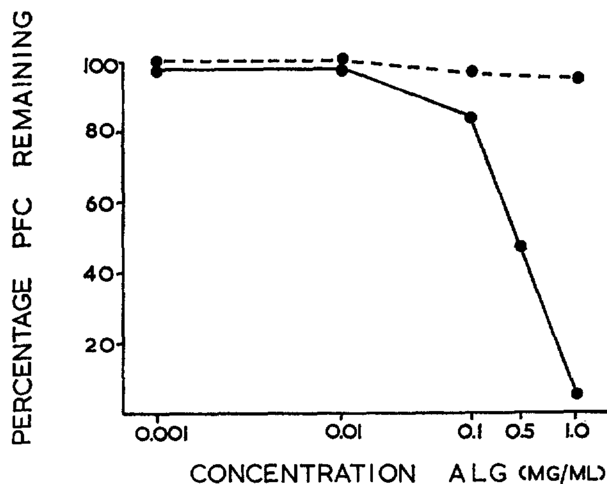

FIG. 3. Percentage of PFC remaining after in vitro incubation with different concentrations of ALG/A (●----●) and ALG/B (●——●) in the presence of complement.

TABLE I

PFC Produced in the Spleens of ALG-Treated Mice after Injection of SRBC and Thymus Cells

Pretreatment	Route of SRBC injection	No. of thymus cells injected i.v.	No. of mice in group	Peak PFC per spleen (± SE)	Significance level
ALG/A	i.p.	10^8	5	12,800 ± 4,270	NS
	i.p.	Nil	6	11,000 ± 2,288	$P < 0.01$
	i.v.	Nil	10	30,100 ± 3,684	$P < 0.01$
	i.v.	10^8	9	64,956 ± 7,893	
ALG/B	i.p.	10^8	5	16,280 ± 2,703	NS
	i.p.	Nil	6	9,533 ± 959	$P < 0.01$
	i.v.	Nil	10	18,540 ± 1,863	$P < 0.01$
	i.v.	10^8	8	65,700 ± 9,986	$P < 0.001$
NIL	i.v.	Nil	10	154,909 ± 14,690	NS
	i.v.	5×10^7	5	140,800 ± 27,733	NS
	i.p.	Nil	5	142,600 ± 14,270	

interest to determine whether treatment with ALG affected the distribution of injected thymus cells and particularly the extent of their localization in the spleen. For this purpose, 50 million ^{51}Cr-labeled thymus cells were injected into normal mice and mice pretreated with ALG/A 24 hr previously. The localization of radioactivity was determined 6 hr later. Approximately equal amounts of radioactivity were detected in the circulation in mice of both groups. In the spleens of ALG-treated mice, the amount of radioactivity was

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only half that recorded in the spleens of normal mice. Somewhat more radioactivity was present in the liver and lungs of ALG-treated mice than of normal mice (Fig. 6).

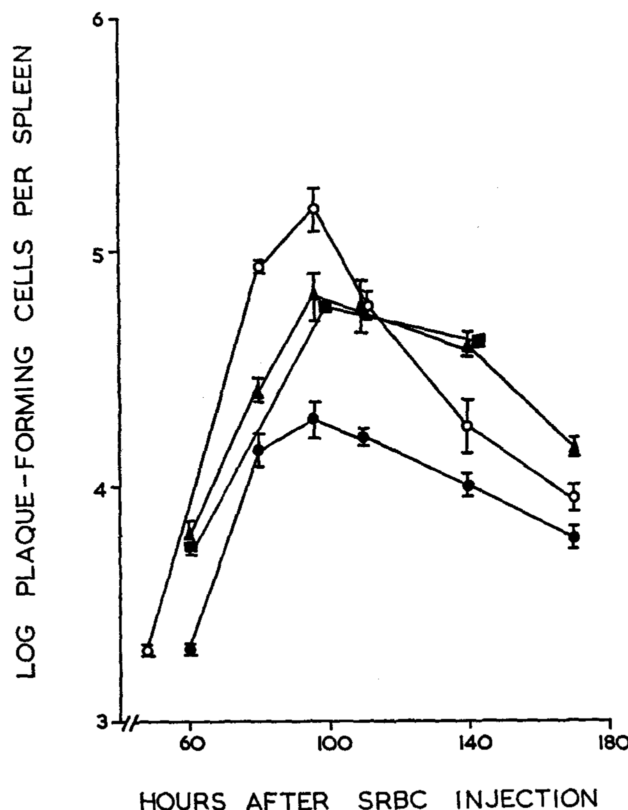

FIG. 4. PFC produced in the spleens of normal mice (O——O) and of ALG-treated mice injected intravenously with SRBC alone (●——●), or together with either 10^8 thymus cells (▲——▲) or 2×10^7 spleen cells (■——■). Each point represents the mean of determinations on 4–10 mice. The SE of the means are indicated by the vertical bars.

Hemolysin Plaque-Forming Cells Produced in Irradiated Mice Injected with Spleen Cells from ALG-Treated Mice.—The results obtained in Table I can be interpreted to mean that the spleens of ALG-treated mice were deficient in thymus-derived cells. Further support for this contention would be obtained if it could be shown that irradiated recipients of spleen cells from ALG-treated donors produced fewer PFC in response to SRBC than irradiated recipients of the same number of normal spleen cells, and that additon of thymus cells could reverse this deficiency. Accordingly, 10 million spleen cells from normal

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TABLE II

PFC Produced in the Spleens of ALG/B-Treated Mice after Intravenous Injection of SRBC and either Spleen or Bone Marrow Cells

Inoculum	No. of mice in group	Peak PFC per spleen (± SE)
SRBC	10	18,540 ± 1,863
2 × 10^7 spleen cells + SRBC	6	66,670 ± 11,850
10^8 bone marrow cells + SRBC	3	22,133 ± 2,599

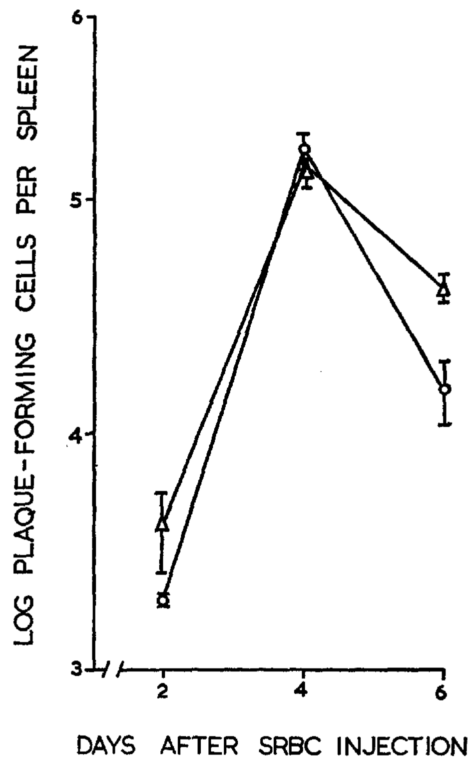

FIG. 5. PFC produced in the spleens of normal mice after an intravenous injection of SRBC alone (O——O) or together with 5 × 10^7 thymus cells (Δ——Δ). Each point represents the mean of determinations made on five mice. The SE of the means are indicated by the vertical bars.

or ALG-treated mice were injected intravenously together with SRBC into two groups of heavily irradiated syngeneic recipients. As can be seen in Fig. 7 and Table III, the response was lower in the recipients of cells from ALG-treated donors. By adding 50 million thymus cells to spleen cells from these

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donors, the response was raised to a value similar to that obtained in recipients of normal spleen cells. By contrast, 50 million bone marrow cells did not enhance the response.

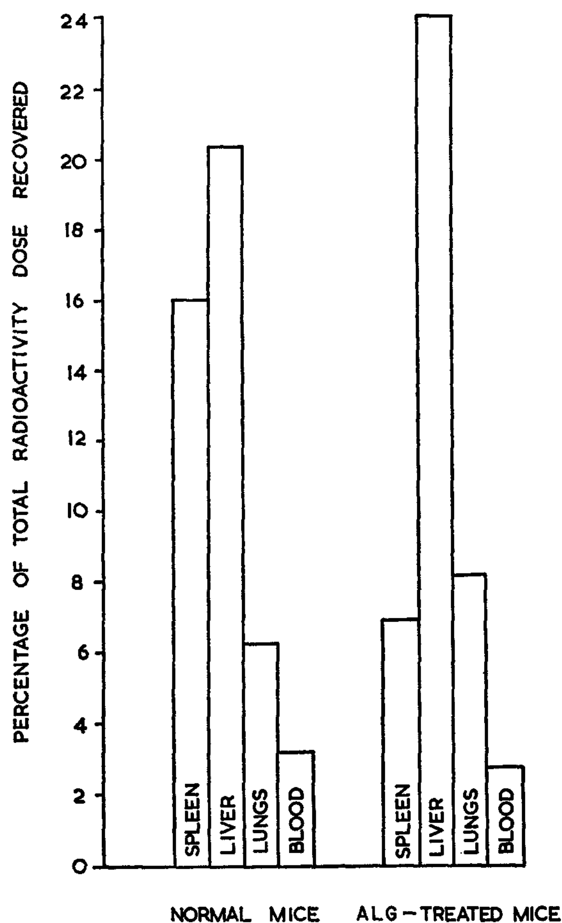

FIG. 6. Localization of radioactivity in the blood and in various organs of normal and ALG-treated mice injected with ^{51}Cr-labeled thymus cells 6 hr previously. The values represent the mean of determinations on four normal and eight ALG-treated mice.

In other experiments, 50 million thymus cells were mixed with different numbers of normal spleen cells ranging from half a million to 20 million and injected intravenously together with SRBC into irradiated syngeneic recipients. Thymus cells did not enhance the response obtained with normal spleen cells throughout this dose range (Fig. 8).

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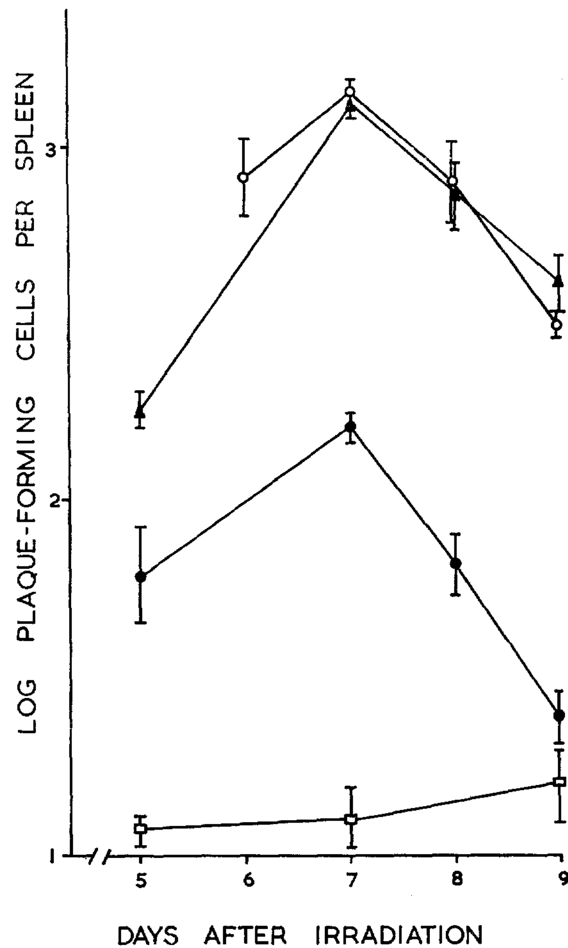

FIG. 7. PFC produced in the spleens of irradiated mice injected intravenously soon after irradiation with SRBC and either 5 × 10^7 thymus cells (□——□), 10^7 normal spleen cells (○——○), 10^7 spleen cells from ALG-treated mice (●——●), or a mixed inoculum of 10^7 spleen cells from ALG-treated mice and 5 × 10^7 thymus cells (▲——▲). Each point represents the mean of determinations on 4–20 mice. The SE of the means are indicated by the vertical bars.

It is known that nonthymectomized irradiated mice protected with hemopoietic cells, such as those in bone marrow and spleen of both normal and neonatally thymectomized donors, begin to recover their capacity to respond to SRBC after a period of 1–2 wk, and normal responsiveness is achieved after 5 wk. By contrast, only partial recovery occurs in mice thymectomized prior to irradiation and given either bone marrow from normal mice or spleen from

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neonatally thymectomized donors. Injection of normal spleen cells however, immediately confers some immunological capacity, presumably by providing immunologically competent cells already present in normal spleen (15). An experiment was thus set up to determine whether spleen cells from ALG-treated mice could in time adoptively restore the response to SRBC in thymectomized irradiated hosts to the level achieved by normal spleen cells. Both

TABLE III

PFC Produced in the Spleens of Irradiated Mice after Intravenous Injection of SRBC, Thymus, or Bone Marrow Cells, and Spleen Cells from Normal or ALG-Treated Mice

Inoculum	No. of mice in group	Peak PFC per spleen (± SE)	Significance level
10^7 normal spleen cells + SRBC	21	1500 ± 187	NS
10^7 normal spleen cells + 5 × 10^7 thymus cellt + SRBC	18	1818 ± 244	
10^7 normal spleen cells + 5 × 10^7 marrow cells + SRBC	5	1444 ± 202	NS
10^7 spleen cells from ALG/A-treated mice + SRBC	6	289 ± 88	$P < 0.001$
10^7 spleen cells from ALG/A-treated mice + 5 × 10^7 thymus cells + SRBC	8	1114 ± 112	
10^7 spleen cells from ALG/B-treated mice + SRBC	20	173 ± 23	$P < 0.001$
10^7 spleen cells from ALG/B-treated mice + 5 × 10^7 thymus cells + SRBC	16	1415 ± 198	
10^7 spleen cells from ALG/B-treated mice + 5 × 10^7 marrow cells + SRBC	4	330 ± 108	
5 × 10^7 thymus cells + SRBC	8	25 ± 9	

thymectomized and nonthymectomized irradiated mice were given 10 million normal spleen cells, 10 million spleen cells from ALG-treated mice, or 10 million normal bone marrow cells. The mice were tested for their ability to respond to SRBC either immediately, or 2–4 wk later. The peak PFC response of mice challenged immediately after irradiation occurred on day 7 (Fig. 7) while that of irradiated mice challenged 2–4 wk later occurred on day 4 (unpublished work). The results are shown in Fig. 9. In nonthymectomized mice the responses, at 2–4 wk postirradiation, were essentially similar whether the mice received normal spleen cells, spleen cells from ALG-treated mice, or normal bone marrow cells. On the other hand, the response of thymectomized irradiated mice given normal spleen cells remained significantly higher than that of mice given

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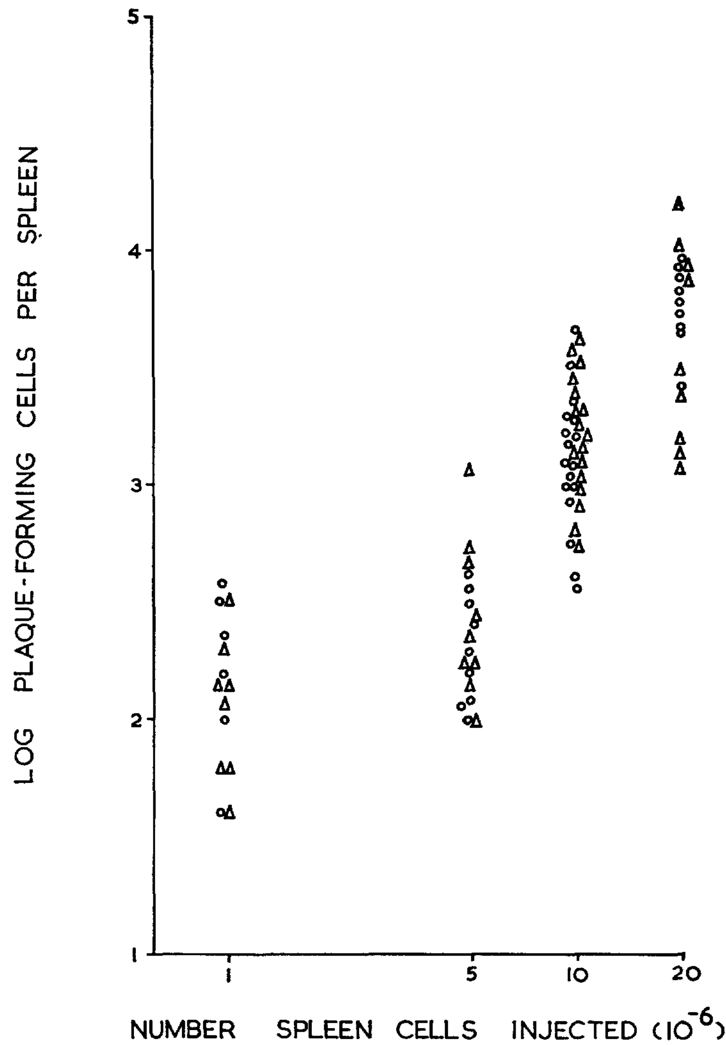

FIG. 8. PFC produced in the spleens of irradiated mice injected intravenously soon after irradiation with varying numbers of normal spleen cells with (△) or without (○) the addition of 5 × 10^7 thymus cells.

spleen cells from ALG-treated donors. The partial recovery in the latter mice was no greater than that in mice given only bone marrow cells.

Hemolysin Plaque-Forming Cells Produced in Irradiated Mice Injected with Spleen Cells Incubated with ALG In Vitro.—ALG had no apparent effect in vivo either on hemolysin-forming cells or on their precursors. Similarly ALG/A

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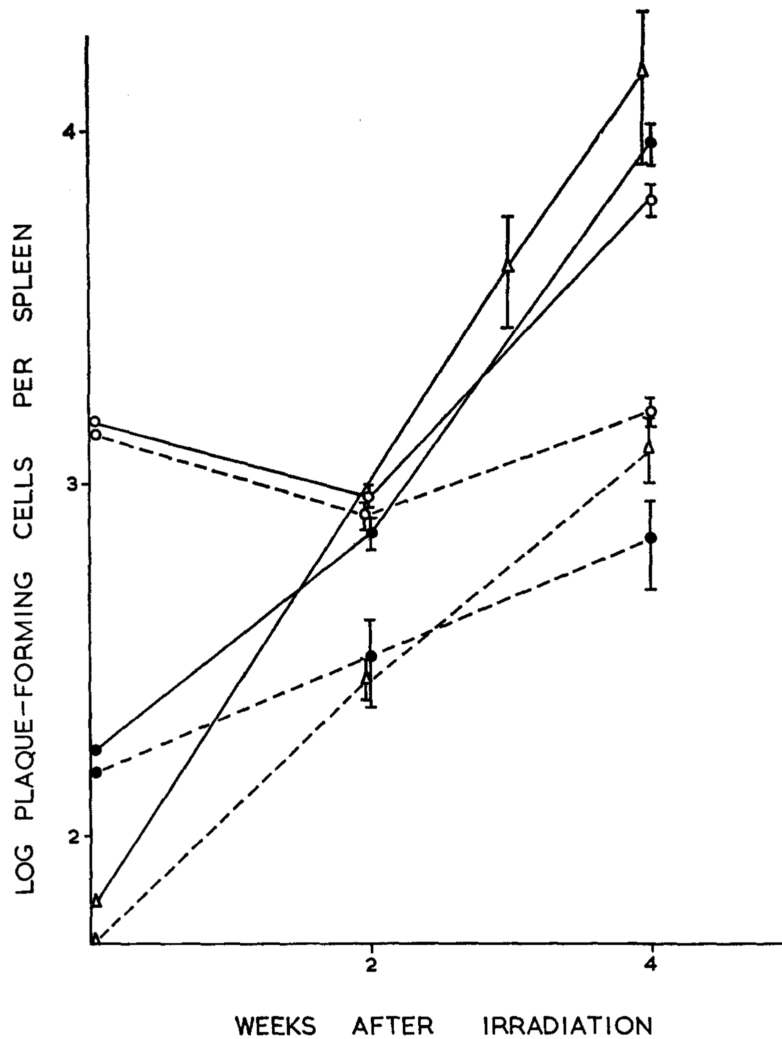

FIG. 9. PFC produced in the spleens of nonthymectomized irradiated (——) and thymectomized irradiated (----) mice given spleen cells from normal (○) or ALG-treated (●) mice, or bone marrow cells from normal mice (△). The recipients were challenged with SRBC immediately after irradiation and cell transfer, or 2–4 wk later. Each point represents the mean of determinations made on four to eight mice. The SE of the means are indicated by the vertical bars.

did not affect plaque formation by hemolysin-forming cells in vitro (Fig. 3). The following experiment was performed in order to see whether ALG/A would affect antibody-forming cell precursors in vitro. Normal spleen cells were incubated at 37°C for 15 min in the presence of ALG/A at a concentra-

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tion of 100 μg/ml. The cells were washed twice and then 10 million spleen cells were injected together with SRBC into irradiated recipients with or without 5×10^7 thymus cells. The number of PFC in the recipients' spleens was determined 7 days later and compared with that in mice given normal spleen cells incubated in the absence of ALG. Thymus cells did not significantly elevate the response of spleen cells incubated in vitro with ALG (Table IV). Thus, it appears that antibody-forming cell precursors can be eliminated from a population of spleen cells exposed to ALG in vitro. This is in contrast to the effect of ALG in vivo which is exerted selectively on thymus-derived cells.

TABLE IV

PFC Produced in the Spleens of Irradiated Mice Injected with Spleen Cells Preincubated In Vitro with ALG/A

Inoculum	No. of mice in group	Peak PFC per spleen (± SE)	Significance level
10^7 normal spleen cells + SRBC	7	1301 ± 149	NS (1301 vs. 1159)
10^7 normal spleen cells + 5×10^7 thymus cells + SRBC	4	1159 ± 166	$P < 0.001$ (1159 vs. 109)
10^7 spleen cells preincubated with ALG + SRBC	8	109 ± 27	NS (109 vs. 344)
10^7 spleen cells preincubated with ALG + 5×10^7 thymus cells + SRBC	5	344 ± 88	

DISCUSSION

The immune response of mice to SRBC requires a participation by both thymus-derived cells and bone marrow–derived antibody-forming cell precursors (6, 7). The ability of thymus cells alone to restore to near-normal the reactivity of ALG-treated mice demonstrates that these mice are deficient primarily in thymus-derived cells. The failure to obtain complete restoration of the response might indicate that ALG has also some effect in vivo, on antibody-forming cell precursors. This possibility appears unlikely for the following reasons: (*a*) bone marrow failed to increase the responsiveness to SRBC in both ALG-treated mice and in irradiated recipients of spleen cells from ALG-treated donors; (*b*) ALG did not exert any immunosuppressive effect when given to mice *after* SRBC, that is presumably after the interaction between thymus-derived cells and antibody-forming cell precursors had occurred; and (*c*) thymus cells restored the peak PFC response to SRBC in irradiated recipients of spleen cells from ALG-treated donors to a level similar to that observed in irradiated recipients of normal spleen cells. The fact that the PFC response was not completely restored to normal may therefore result not from any significant effect of ALG on PFC precursors, but from other effects on the spleen.

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Thus the diminished localization of ^{51}Cr-labeled thymus cells in the spleens of these mice suggests that some damage may have occurred in this organ. Since previous work had shown that lymphocytes, preincubated with ALG in vitro, were rapidly eliminated from the circulation (14), the present finding that labeled cells remained circulating indicates that free ALG was no longer present in effective concentrations 24 hr after injection.

Levey and Medawar (16) observed that 200 million thymus cells given to ALS-treated mice 25 days after skin grafting, did not curtail graft survival. Thymus cells injected systemically do not localize to any significant extent in the peripheral lymph nodes (17)—the predominant site where sensitization to skin grafts occurs (18, 19). The inability of thymus cells to hasten the rejection of skin grafts might simply be linked to a failure of these cells to make contact with graft antigens. Similarly, inadequate contact between thymus cells and antigen might explain why in our experiments the PFC response of ALG-treated recipients of thymus cells was increased to a greater extent when SRBC were given intravenously than intraperitoneally. Denman et al. (20) reported that a mixed inoculum of 150 million cells from spleen, lymph nodes, and bone marrow given intravenously did not enable ALG-treated mice to produce hemagglutinins in response to intraperitoneal SRBC. In our experiments 20 million spleen cells elevated the PFC response of ALG-treated mice injected with SRBC intravenously. The failure of Denman et al. (20) to show an effect with these cells is difficult to explain particularly since lymph node cells home to peripheral lymph nodes after injection (21) and are effective in shortening the survival time of skin grafts on ALS-treated mice (16, 22).

Levey and Medawar (16) observed that a single injection of ALS was almost incapable of preventing sensitization when allogeneic lymphoid cells were injected intravenously, but could prevent sensitization resulting from an allogeneic skin graft. This was taken as evidence that ALS affected circulating lymphocytes to a greater extent than lymphocytes within lymphoid organs. In these experiments, however, ALS was administered 1 day *after* the mice had received either the allogeneic cells or the skin graft. It is known from the present experiments and those of Berenbaum (10) that ALG cannot depress the response to an injection of SRBC if it is given 1 day after antigen. The situation with allogeneic lymphoid cells may well be the same. On the other hand, the ability of ALS to prevent sensitization when given after the application of a skin graft might well be because sensitization in effect does not begin until at least 48 hr postgrafting (23, 24). From our own results, it is apparent that ALG depressed the PFC response to SRBC to a greater extent when the antigen was given intraperitoneally than when given intravenously. This can be taken to indicate that ALG interferes with migration of thymus-derived lymphocytes from the "periphery" to the lymphoid organs. It might explain why ALG is particularly effective in prolonging survival of organ

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grafts where it is likely that sensitization occurs as a result of contact between circulating lymphocytes and graft antigens (25). It is also evident from our experiments that ALG does not affect solely "peripheral" lymphocytes but also alters the cell population within lymphoid organs, as shown by the poor PFC response of irradiated recipients of spleen cells from ALG-treated donors.

Levey and Medawar (16) also reported delayed recovery of reactivity against allogeneic skin grafts in irradiated recipients of a mixed inoculum of spleen and bone marrow cells obtained from mice that had received five injections of ALS. They argued from their results that ALS inactivated rather than eliminated lymphoid cells (even those cells within lymphoid organs); that the progeny of ALS-treated cells were incompetent for one or more generations; and that the inactivation eventually wore off in the irradiated recipient. Since, however, both bone marrow and spleen are a source of stem cells which can differentiate to immunologically competent cells in an irradiated, *nonthymectomized* host (15), their data do not exclude the more likely possibility that competent cells were eliminated by ALS in the donor and that reactivity in the irradiated recipient returned only after *new* immunologically competent cells had differentiated from stem cells provided in the inoculum. Our experiments employing irradiated and thymectomized irradiated recipients of spleen cells from ALG-treated donors clearly show the importance of using thymectomized recipients in this type of experiment. They show in addition that ALG did not damage the stem cells since the return of reactivity occurred at the same rate in irradiated recipients of both normal bone marrow and spleen cells from ALG-treated donors. The peak response in thymectomized irradiated recipients of normal spleen cells was not significantly different whether the mice were challenged immediately after irradiation or 2 or 4 wk later. In thymectomized irradiated recipients of spleen cells from ALG-treated donors, the response increased as the time interval between irradiation and challenge was lengthened, but was always significantly lower than that in recipients of normal spleen cells. In fact it did not increase beyond that of thymectomized irradiaated mice protected with normal bone marrow. These findings do not support the idea that ALS temporarily inactivates lymphoid cells, whether by "blindfolding" or by "sterile activation" (26). They are more consistent with the concept that the thymus-derived cells are eliminated. The data we have obtained in thymectomized irradiated mice are thus in general agreement with the results of others showing a prolongation of the immunosuppressive effect of ALS in thymectomized recipients (27, 28).

Guttmann et al. (29) reported that the suppression of the response to SRBC in the rat by ALG was due to the presence of anti-SRBC antibodies within the globulin preparation and not to antilymphocyte antibodies. In our experiments, ALG/B was completely absorbed with SRBC and yet was still strongly immunosuppressive. This difference in the results might be due to the species used. Thus the response to SRBC in the rat is not depressed by neonatal thy-

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mectomy (30, 31) possibly because SRBC are stronger antigens in the rat which lacks the Forssman antigen than in the mouse which is Forssman positive (32). If ALG produces the same effect as neonatal thymectomy, then one would not expect it to have, in the rat, a marked immunosuppressive effect with respect to the response to SRBC. Consistent with this, are our findings that the peak antibody response of mice to the flagella antigen of *Salmonella adelaide* was depressed neither by neonatal thymectomy nor by ALG/A.[4]

The findings reported here suggest the generalization that, in vivo, the specific primary target cell for the immunosuppressive activity of ALS is the thymus-derived antigen-reactive cell—the recirculating small lymphocyte which can initiate certain immune responses—and that the effect of ALG is to eliminate these cells. While the mechanism of elimination is unknown, unpublished work from our laboratory suggests that opsonization followed by phagocytosis plays a major role. We have shown that, in vitro, antibody-forming cells precursors can be affected by ALG although there was no evidence for such an effect in vivo. The selective action of ALS on thymus-derived cells in vivo may be linked (*a*) to the specificity of the antibody raised during the preparation of ALS;[5] (*b*) to the greater susceptibility of thymus-derived cells, in contrast to other cell types, to the action of this antibody; and (*c*) to the probability that recirculating cells will be exposed to ALG more extensively than noncirculating cells. From these considerations, it would seem that the thymus may provide the best source of cells when attempts are made to purify ALG by elution methods, such as the one described by Woodruff (33).

The capacity of thymus cells to reconstitute ALG-treated mice supports the concept that cooperation between bone marrow–derived and thymus-derived cells occurs in the immune response of mice to SRBC. The lack of effect of ALG on bone marrow–derived precursors might also explain why ALS does not necessarily cause lymphopenia for, as hypothesized in a preliminary communication (14), the deficiency resulting from the elimination of thymus-derived lymphocytes might be offset by an excessive production of other types of lymphoid cells. The selective action of ALS makes it a useful tool in characterizing further the functions of the thymus-derived cells.

SUMMARY

In this series of papers it has been shown that the immune response of mice to sheep erythrocytes requires the participation of two classes of lymphoid cells. Thymus-derived cells initially react with antigen and then interact with

[4] Martin, W. J., and J. F. A. P. Miller. Influence of thymectomy and antilymphocyte globulin on the antibody response to *Salmonella adelaide* flagellin in mice. Manuscript in preparation.

[5] Unpublished studies utilizing ^{125}I-labeled ALG/B have shown that thymus cells absorb three times as much radioactivity as bone marrow cells and six times as much as liver or kidney cells.

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another class of cells, the antibody-forming cell precursors, to cause their differentiation to antibody-forming cells.

Antilymphocyte globulin depressed the ability of mice to respond to sheep erythrocytes. This effect was more marked when the antigen was injected intraperitoneally than intravenously, and occurred only when the antilymphocyte globulin was given before or simultaneously with antigen. Injection of thymus cells restored to near normal the ability to respond to an intravenous injection of sheep erythrocytes. Spleen cells from antilymphocyte globulin–treated mice gave a weak adoptive immune response in irradiated recipients. The addition of thymus cells however enabled a response similar to that given by normal spleen cells.

When thymectomized irradiated recipients were used, normal spleen cells continued to give a higher response to a challenge of sheep erythrocytes at 2 and 4 wk postirradiation than did spleen cells from ALG-treated donors. This result is more consistent with the notion that thymus-derived target cells are eliminated, rather than temporarily inactivated, by antilymphocyte globulin.

These findings suggest that, in vivo, antilymphocyte globulin acts selectively on the thymus-derived antigen-reactive cells.

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