Antilymphocytic serum (ALS) is the name given to an antiserum raised in members of one species by the injection of lymphocytes or lymphoid cells taken from members of another species. It has the power not merely to prevent or delay the onset of immunological reactions leading to the rejection of homografts, but also to arrest reactions already in progress. This combination of properties is unique, and since ALS is devoid of acute toxicity, and is indeed, in transplantation systems, the most powerful immunosuppressive agent yet described, its clinical and experimental potentialities are worth close attention.

The experiments reported here are intended to throw further light on the nature and mode of action of ALS.

Materials and Methods.—Preparation of antiserum: Antisera were raised by giving New Zealand rabbits two successive intravenous injections 14 days apart of a single-celled suspension of 10⁶ living thymocytes from young female CBA mice. The rabbits were bled 7 days after the second injection, and the serum was heated to 56°C for 30 min, filtered, and stored at −20°C. This simple regimen served also for lymphocyte fractions and for cells other than lymphocytes, and the antiserum so prepared required no absorption with red cells. Longer courses of injection usually yielded less effective antisera, perhaps because of a change in the physical character of the operative antibodies, or because of interference by antibodies to minor or irrelevant constituents of the immunizing cells. Antisera were always injected by the subcutaneous (subintegumentary) route.

Assay; Skin grafting: The strength of a sample of ALS was measured in terms of its power to prolong the life of A-strain tail skin homografts on adult male CBA mice. In a typical experiment, each member of a uniform panel of mice received 0.5 ml ALS on the second and again on the fifth day after grafting (i.e., on days +2 and +5 in the now widely used notation). The results are figured as survival curves showing, day by day, the number of mice still bearing surviving grafts, and are expressed numerically either as the mean expectation of life (MEL) of a graft at grafting, or as the median survival time (MST). The error function cited is the standard deviation (sd). The MEL of A-strain tail skin homografts on adult CBA males is 11.6 ± 1.3 days sd (MST 11.5 days).

Abrogation of the Second-Set Response.—The sensitivity aroused in CBA mice by grafts of A skin, as measured by the survival time of a second graft transplanted at various intervals after the rejection of a first, is still clearly in force after 35 weeks. For the first 10 weeks after the rejection of first-set grafts, the MST of second-set grafts does not exceed 6 days. We have already shown that under the protection afforded by 4 × 0.5 ml ALS given on days −1, +1, +3, +5, the MST of second-set grafts exceeded 40 days (maximum 210 days). However, when 11 days was allowed to pass between the completion of such a course of injections and the transplantation of second-set grafts, the MST fell to between 14 and 15 days (maximum 31 days). If the delay between antiserum injections and the trans-
plantation of second-set grafts were to be delayed still further, would the recipients be found to have reverted to a sensitized state?

In the experiments illustrated by Figure 1, 44 CBA mice received first-set A-strain tail skin grafts which enjoyed an MEL of $11.5 \pm 1.3$ days (range 10–14 days). Starting 15 days after this first grafting, all mice then received 2.0 ml ALS (0.5 ml followed by $6 \times 0.25$ ml) over the next 12 days. They were then subdivided into three roughly equal groups which received second-set grafts, 7, 10, and 14 weeks, respectively, after receiving their first-set grafts. Figure 1 shows that the effect of ALS was still significantly in force after 7 weeks (MEL $16.2 \pm 3.5$, range 12–25 days), but that the 10-week and 14-week groups showed a virtually complete reversion to virgin (first-set) reactivity (MEL $12.4 \pm 1.8$ and $12.1 \pm 2.0$, respectively). There was no suggestion of a return to a state of sensitivity.

Population Turnover.—Irradiation: The experiments illustrated by Figures 2 and 3 were an attempt to assess the influence of an enforced turnover of the lymphoid population on the duration of the effect of ALS. We reasoned that if ALS was injected into mice containing a predominantly newly formed and regenerating population of lymphocytes, its effect should be specially long-lasting; whereas if the population were caused to turn over after the injection of ALS, its effect should die away abnormally soon.

An exposure of mice to 450 r whole-body irradiation from a Co$^{60}$ source confirmed these expectations (Fig. 2). Two sets of 12 CBA mice received 0.5 ml ALS on days +2 and +5, and the mice of one set were exposed to 450 r on day +15. The grafts on the irradiated mice broke down more quickly than their controls. The experiment illustrated by Figure 3 was of essentially the same design except that the mice were irradiated 2 days before grafting (−2) and 1.25 ml ALS was injected over the period of lymphoid regeneration (0.5 ml on +2, 0.25 ml on +5, +8, +11). The MST of grafts on mice that received ALS alone was 46$1/2$ days and on the preirradiated mice 57$1/2$ days; no graft in the latter group broke down before the 40th day. This experiment is still in progress.

Adrenalectomy and the effect of steroids: Figure 3 also illustrates an experiment sharing the same controls and designed to compare the effects of adrenalectomy and of the administration of extra hydrocortisone$^{4}$ on the efficacy of ALS. Two sets of mice received the regimen of ALS injections described in

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**Fig. 1.**—Survival on CBA mice of second-set A-strain skin grafts transplanted at various intervals after the abolition, by ALS, of the sensitivity provoked by the transplantation of first-set grafts. The mice revert slowly to a state of "virgin" (i.e., first-set) reactivity. For details, see text.

**Fig. 2.**—Showing that the survival of A skin on CBA mice under the protection of ALS ($2 \times 0.5$ ml) is curtailed by exposure to 450 r whole-body irradiation 15 days after grafting (contrast Fig. 3). MEL: irradiated mice $28.3 \pm 8.7$, controls $32.5 \pm 6.8$. 
the preceding paragraph and were then grafted. Thirteen days later, all mice were given 0.25 mg hydrocortisone acetate, and on the 14th day the members of one group were adrenalectomized by the dorsolateral approach. The adrenalectomized mice were protected by a single subcutaneous injection of 2.5 mg deoxycorticosterone trimethylacetate; the others received weekly subcutaneous injections of 0.5 mg hydrocortisone acetate. Figure 3 shows that adrenalectomy hastened (MST 36\(\frac{1}{2}\) days) and hydrocortisone profoundly retarded (MST 90 days) the rejection of homografts (control MST 46\(\frac{1}{2}\) days as before).

Effect of Thymectomy.—If ALS acted mainly as a lymphocytolytic agent, it would be rational to abet its action by auxiliary treatments having the same effect, such as chronic drainage of lymphocytes from the thoracic duct\(^1\) or thymectomy.\(^2,\)\(^6\) Figure 4 shows that in our experience, thymectomy did not potentiate the effect of ALS. Life tables for three groups of grafts are illustrated: (1) thymectomy on day -4, with no further treatment; (2) ALS alone (7 × 0.25 ml at 3-day intervals from day +2); and (3) ALS combined with thymectomy. That the two longest-lived grafts (>100 days) belonged to (3) may have been due to luck in sampling. Another possibility is that as much as thymectomy delays recruitment of immunologically competent cells in adults,\(^7\) its effect is of the same general kind as that attributed to hydrocortisone or to preirradiation. It should be added that the injection or implantation of normal thymocytes or thymus grafts does not restore immunological competence to mice rendered unreactive by injections of ALS.

Does Treatment with ALS Induce Immunological Tolerance?—A serious formal objection to classifying ALS-induced nonreactivity as tolerance is that it is neither brought into being nor maintained by antigenic stimulation; unlike tolerance it is, in fact, very insensitive to antigenic differences. Figure 5 illustrates life tables of A,
C57, and C3H grafts on CBA mice under the protection given by 2 × 0.5 ml ALS injected on days +2 and +5. The "difficult" combinations A → CBA and C57 → CBA are made too easy and the "easy" combination C3H → CBA not easy enough to fit an interpretation based on the idea of tolerance. The same applies a fortiori to the induced acceptance of heterografts: the regimen of ALS injections that allows homografts to survive 6 weeks will allow guinea pig or human skin to survive 2 weeks, and longer survivals could doubtless be achieved if they were felt to serve a useful purpose.

Specificity of Antilymphocytic Antiserum.—The term "antilymphocytic serum" can be justified on two counts: the injection of lymphoid cells is a very good way to produce ALS (no better way is yet known), and lymphoid cells are almost certainly the target on which it acts. The balance of evidence turns against the idea that ALS acts through the thymus. Nevertheless, cells other than lymphocytes can raise antisera having the properties of ALS.

Sera were raised against L cells by injecting them intravenously as if they were lymphocytes (2 × 500,000,000 cells on days 0 and 14 with first withdrawal of serum on day 21; a third injection of 500,000,000 cells on day 28 with a second withdrawal of serum on day 35). Sera against epidermal cells were produced by giving intravenous injections of 20,000,000–50,000,000 pure basal layer cells on three, four, or five occasions to yield first, second, and third serum samples, respectively. The epidermal cells were prepared by scraping the basal layer off the epidermis of CBA tail skin disengaged in one sheet from the dermis by the action of trypsin.

Table 1 records the results of routine assays (2 × 0.5 ml on days +2 and +5) of these various antisera in terms of their power to extend the life of A-strain skin on CBA mice. The best serum was as powerful as some of our earlier antilymphocytic sera.

Antigenic Activity of Crude Thymocyte Fractions.—Well-washed single-celled suspensions of thymocytes in 0.15 M NaCl (not in media containing Ca++ or Mg++ ions) were made the starting point of a crude separation into three fractions: (a) insoluble matter including membranes; (b) soluble matter; (c) nucleoprotein, prepared by two different methods.

(1) In the "low-salt" preparation the washed packed cells were homogenized in 10 vol water, using first an Ultraturrax blender for 20–25 min at moderate speeds and then an MSE-Mullard ultrasonic generator (20 kc/sec ~ 50 w) for 90–120 sec. One volume of 0.6 M NaCl was added to 3 vol aqueous homogenate to restore the salt concentration to 0.15 M. The precipitate so formed was washed two or three times by centrifugations in phosphate-buffered (pH 7.0) saline, redissolved in water, spun lightly, reprecipitated, and washed two or three times again as before. The final preparation is referred to as "crude nucleoprotein"; though not wholly de-

| TABLE 1 |
| Mean and Maximum Survival Times of A-Strain Homografts on CBA Mice Treated with Antiepidermal and Anti-L-Cell Sera* |

<table>
<thead>
<tr>
<th></th>
<th>First Serum Mean</th>
<th>Max</th>
<th>Second Serum Mean</th>
<th>Max</th>
<th>Third Serum Mean</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiepidermal</td>
<td>13.1 ± 3.5</td>
<td>16</td>
<td>23.7 ± 4.5</td>
<td>29</td>
<td>17.6 ± 2.7</td>
<td>21</td>
</tr>
<tr>
<td>Anti-L cell</td>
<td>15.3 ± 3.7</td>
<td>17</td>
<td>15.0 ± 1.3</td>
<td>17</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Survival times in days. Control value, 11.6 ± 1.3.
polymerized, it was sufficiently nonfibrous when prepared in this way to make it possible to inject it intravenously in suspension.

The densely opalescent supernatant fluid left after the removal of matter insoluble in 0.15 M NaCl was spun for 60 min at 25,000 rpm in a Spinco SW-25 rotor.\textsuperscript{13} The sediment, which contains most of the cells' transplantation antigens,\textsuperscript{14} is referred to as the “crude insoluble” and the supernatant fluid as the “crude soluble” fraction. Both were injected intravenously.

(2) In the alternative nucleoprotein preparation (“high-salt” method), the washed packed sediment of thymocytes was suspended in 5–10 vol 0.15 M NaCl and then blended to achieve full dispersion in an equal volume of 3 M NaCl. The immensely viscous solution was spun for 60 min at 25,000 rpm in the SW-25 rotor as above. The nucleoprotein present in the supernatant fluid was partially purified by three times successively reprecipitating by the addition of 10 vol water, winding out the flossy fibrous precipitate on a glass rod, and redissolving in 1.5 M NaCl. One additional ultracentrifugation removed the remaining insoluble matter. After its final precipitation, the highly fibrous nucleoprotein was broken down into shorter fibers in the Ultraturrax blender and injected intramuscularly and intraperitoneally.

All the operations described above were done in the cold. Up to 100 IU heparin per rabbit was added to all preparations injected intravenously.

To raise antisera, these various preparations were injected as if they were suspensions of living thymocytes: injections were made on days 0 and 14 with withdrawal of blood for a first serum sample on day 21, sometimes followed by a third injection on day 28 and a second withdrawal of blood for serum on day 35. Assay was carried out by the usual method (0.5 ml antiserum per mouse on days +2 and +5).

The results are shown in Table 2. The crude soluble cell fraction had little activity, but the first antiserum raised against the insoluble fraction, including the membranous matter, was the most potent we have tested. The rapid decline in potency of the second serum, which required absorption with red cells, is not yet understood (see above), though on a purely empirical level it serves as a warning against the dangers of hyperimmunization. Both nucleoprotein preparations yielded moderately active antisera.

These findings make a prima-facie case for thinking that “ALS” may be a heterogeneous assemblage of antibodies directed against several different antigenic specificities. The alternative is to suppose that single operative antigen abundant in the insoluble fraction is present as a contaminant in the nucleoprotein preparations. The hypothesis of “sterile activation” (see below) will fit with either interpretation.

\textbf{How Does Antilymphocytic Serum Work?—}ALS prevents not only the primary immune response to homografts but also the expression of a preexisting state of

\begin{table}
\centering
\caption{Assays of Antisera Raised by Various Thymocyte Fractions}
\begin{tabular}{|l|l|l|l|l|l|l|}
\hline
Fraction & 1st injection & 2nd injection & 3rd injection & MEL Corresponding to: & 1st serum & 2nd serum \\
\hline
Crude soluble & 1.3 & 5.0 & 5.0 & & 12.3 ± 1.6 & 17.4 ± 4.2 \\
Crude insoluble & 2.5 & 2.5 & 2.1 & & 44.2 ± 8.4 & 14.5 ± 2.0 \\
Nucleoprotein (low salt) & 1.3 & 5.0 & 5.0 & & 25.3 ± 3.7 & 21.5 ± 4.2 \\
Nucleoprotein (low salt) & 4.0 & 3.0 & --- & & 22.0 ± 2.8 & --- \\
Nucleoprotein (high salt) & 4.2 & 3.4 & 5.2 & & 25.8 ± 5.3 & 17.4 ± 6.7 \\
\hline
\end{tabular}
\end{table}
sensitivity. The presumption is therefore that, unlike all other immunosuppressive agents that have been studied from this standpoint,15 ALS prevents the recognition of antigen. This interpretation is supported by the fact3 that ALS can suppress the first inflammatory episode of the normal and immune transfer reactions in guinea pigs,16 a property that will be the subject of a separate communication.

The hypothesis we originally thought most plausible was that ALS coated ("blindfolded") lymphocytes and thus prevented any recognition of antigen, whether in the afferent or the efferent sectors of the immune response. This hypothesis would be greatly weakened if it could be shown that the descendants of affected lymphoid cells remained unreactive for one or more cellular generations. The following experiments show that this is probably the case.

Two sets of 15 CBA mice were exposed to 950 r in a Co60 source and then given intravenous injections of 2,000,000 marrow cells and 90,000,000 splenic cells from either (a) normal CBA mice or (b) CBA mice which had received four injections of 0.25 ml ALS at 2-day intervals before transfer. Nine days later all mice were given A-strain tail skin grafts. The grafts on the mice reequipped with normal lymphoid cells enjoyed an MEL of 17.9 ± 2.7 days; on mice reequipped with cells from ALS-treated donors the corresponding figure was 30.1 ± 8.1 days. Under the conditions of this experiment, the cells used to recolonize the heavily irradiated mice must have gone through several divisions without regaining immunological competence. "Blindfolding" cannot therefore be the whole story. Full details of these and similar experiments will be published elsewhere.

The chronic treatment of mice with ALS, as in the experiment just described, led to a certain degree of lymphoid hypertrophy accompanied by the formation of blast cells. This pointed to some form of activation, and revived our interest in the possibility that antilymphocytic rabbit immunoglobulin acts as an obligative antigen in mice,3 preoccupying all potentially reactive cells. This hypothesis can hardly be correct, for the immune response of ALS-treated mice to rabbit immunoglobulin is feeble or absent.2 If ALS is not antigenic, it cannot be acting as a competitive antigen in the conventional sense.

It is noteworthy that mice strongly sensitized against rabbit IgG with the help of pertussis vaccine and Freund's complete adjuvant are still capable of giving effect to ALS: in one experiment skin grafts on presensitized mice under ALS treatment survived 19.4 ± 3.8 days where in normal controls they survived 21.8 ± 3.1 days. Long-term survivors, however, seem not to occur in mice immunized against IgG. The fact that ALS seems to abolish or prevent the inception of a reaction against itself3 is of some clinical importance.

There remains the possibility of a generalized sterile activation of lymphoid cells—perhaps analogous to that produced by phytohemagglutinin—which forestalls or supplants all other immunological commitments. In this hypothetical process, blast formation and cell division are not accompanied by a specific immunological performance. (It is already known that heterologous antisera can activate lymphocytes mitotically16 and in terms of lysosomal activity.17) Though it may not stand up to closer scrutiny, this hypothesis accounts adequately for the properties of ALS described so far. If it is true, it follows that the most appropriate in vitro test for the potency of a sample of ALS would be one based on its power to induce blast formation. There seems little room for a theory that attributes the effect of ALS
to lymphocytolysis or lymphocytic depletion. Indeed, if we are right in thinking that agents which stimulate the turnover of lymphocyte populations curtail the action of ALS, lymphocytic depletion may turn out to be an undesirable side effect of the action of ALS.

Summary.—Antiserum (ALS) raised in rabbits against CBA lymphoid cells was assayed with respect to its power to prolong the life of A-strain skin homografts on CBA mice.

Mice in which a preexisting sensitivity to homografts had been abolished by a short course of injections of ALS slowly reverted to a state of "virgin" reactivity, not to a sensitive state. Agents that delay the turnover of lymphoid populations prolong the effectiveness of ALS, and conversely: the median survival time of homografts on mice that received 1.25 ml ALS followed by weekly injections of 0.5 mg hydrocortisone was about 90 days. Thymectomy did not enhance the effect of ALS; adrenalectomy reduced it. Sublethal irradiation before ALS treatment enhanced and after ALS treatment reduced its effectiveness. ALS was effective in mice strongly sensitized beforehand against rabbit immunoglobulin.

Antisera formed after the injection of pure epidermal cells and of L cells had the properties of ALS, which is thus not specific in any histological sense. More than one crude anatomical fraction of thymocytes—notably a fraction containing membranous and other insoluble matter, but also nucleoprotein fractions prepared in two different ways—could excite the formation of effective antisera, so raising the possibility that ALS is a heterogeneous mixture of antibodies directed against several antigenic species.

The state of nonreactivity produced by ALS is not antigen-dependent, and reasons are given for not classifying it as immunological tolerance. There is some indication that, for a few generations, the descendants of cells rendered incompetent by ALS are themselves incompetent. ALS may act by bringing about a generalized "sterile activation" of lymphoid cells which forestalls or supplants all other immunological commitments.

ALS appears to be devoid of intrinsic or acute toxicity, and its clinical possibilities therefore deserve close attention.

We gratefully acknowledge the invaluable help of Miss S. Jooste, Miss S. Reeves, and Miss R. Sharman.

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In the combination C3H → CBA, graft survival times exceeding a year can be achieved merely by injections of cell-free antigen, by irradiation, or, most effectively, by both combined. A state of true tolerance is secured. See Medawar, P. B., Transplant., 1, 21 (1963).


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