SUPPRESSION OF ADJUVANT DISEASE IN THE RAT BY HETEROLOGOUS ANTILYMPHOCYTE GLOBULIN*

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Interest in heterologous antilymphocyte serum (ALS) as an immunosuppressive agent dates from the studies of Wilhelm et al. (1), Interbitzin (2), and Waksman et al. (3). These authors showed that an antiserum raised in rabbits against guinea pig lymph node cells, when injected into the latter animal, depleted lymphoid structures, reduced the numbers of circulating lymphocytes, and depressed immune responses. Similar results were subsequently obtained in mice (4) and in rats (5). A new impetus was given to the subject by the report of Woodruff and Anderson (6) that administration of ALS could prolong the survival of experimental homografts in rats. This has since been confirmed in mice (4), dogs (7), and man (8). Particular interest is attached to reports (9, 10) that suppression of graft rejection could be achieved by doses of ALS insufficient to lower significantly the numbers of circulating lymphocytes, suggesting that ALS may act by a nondestructive interference with the participation of lymphocytes in immunological reactions.

These reports raised the possibility that ALS may offer a new approach in the treatment of those human diseases in which immune responses play an essential pathogenic role. It was therefore decided to study the influence of ALS on adjuvant disease in the rat (11). In this experimental model, a single intracutaneous injection of Freund's complete adjuvant results, 9-14 days later, in the appearance of a marked and prolonged polyarthritis, as well as other lesions. The arthritis is generally regarded as a delayed type of hypersensitivity response to disseminated mycobacterial antigen (12), and thus appeared to be an appropriate model in which to study the action of ALS.

This report describes the influence of rabbit anti-rat lymphocyte globulin (ALG) on adjuvant arthritis and other immune responses in the rat, and it compares the efficacy of antisera raised separately against thymus and lymph....
node cells. Rats receiving ALG developed antibodies against rabbit globulin, and the influence of such circulating antibodies on the activity of ALG was also studied. Two preliminary reports on this work have appeared (13, 14).

**Materials and Methods**

**Preparation of Antilymphocyte Serum.**—Peritoneal lymph nodes, spleens, and thymuses, excised from various strains of rats, were crushed with forceps in Hanks' solution, then filtered through glass-wool columns (15). The resulting cell suspension, of which over 95% resembled small lymphocytes, was centrifuged once, then resuspended in Hanks' solution, and injected intraperitoneally into white New Zealand rabbits weighing approximately 3 kg. Each rabbit received between 200 X 10⁶ and 1200 X 10⁶ cells on each occasion. Three injections were given during the 1st wk, and one at the end of the 2nd, 3rd, and 7th wk. The rabbits were bled 7 days after the last injection, and subsequently 7 days after each booster injection. In all, they were bled nine times. Sera were inactivated by heating to 56°C for 30 min, then stored at −20°C.

**Processing of Serum.**—Initially, individual sera were tested by injecting 2 ml of heat-inactivated, but otherwise untreated, serum intraperitoneally into rats and by comparing the absolute lymphocyte count before and 4 hr after injection. A majority of sera so tested reduced the count from an initial value of about 12,000/mm³ to less than 15% of this value after 4 hr. However, many of the injected rats suffered a severe reaction with collapse, and subsequent blood smears and hematocrit examinations showed evidence of a severe hemolytic anemia. This method of selecting sera was therefore given up in favor of the lymphocyte agglutination test described below. Using this procedure, sera were included only if they agglutinated rat lymphocytes in a dilution greater than 1/64.

The pooled sera were absorbed twice with 1/4 volume of packed rat erythrocytes from which the "buffy coat" had been removed during three washings with normal saline solution. Each absorption involved incubation of the cells and serum at 37°C with intermittent agitation for 1 hr. The red cells were removed by centrifugation either immediately or after standing at 4°C overnight.

A crude globulin preparation was obtained by precipitation with 50% saturated ammonium sulphate, followed by washing with a 40% saturated ammonium sulphate solution. The precipitate was redissolved in normal saline, then dialyzed for 4 days against normal saline. After dialysis, the volume was made up to that of the original serum, and the concentration of globulin determined from the optical density at 280 nm. At this stage, control normal rabbit globulin, prepared by an identical process from normal rabbit serum (Microbiological Associates, Bethesda, Md.), was adjusted in volume to give a similar final globulin concentration (this varied from 6.3 to 9.2 mg/ml in the various batches). Finally, both preparations were sterilized by passage through 0.45 μ Millipore filters and stored sterile at −20°C in 12 ml aliquots.

**Adjuvant Injection.**—Adjuvant was prepared by homogenizing dried, heat-killed *Mycobacterium tuberculosis* (H37 Ra, Difco Laboratories, Inc., Detroit, Mich.) in heavy mineral oil (E. R. Squibb & Sons, New York, N.Y.) to give a suspension of 6 mg *M. tuberculosis* per milliliter. The suspension was autoclaved, and rats under light ether anesthesia received 0.1 ml by intracutaneous injection into the base of the tail.

**Rats.**—Adult female rats weighing between 165 g and 210 g were housed in individual sheet metal cages and received a diet of commercial food pellets and water ad lib. Both Lewis rats (Microbiological Associates) and Sprague-Dawley rats (Simonsen Laboratories, Gilroy, Calif.) were found to develop arthritis consistently following adjuvant injection and, except as specified below, one or the other of these strains was used for each experiment. Samples of
blood were obtained under ether anesthesia from the orbital sinuses or (for blood counts) from the tail. All globulin preparations were administered intraperitoneally.

Quantitation of Arthritis.---From the 9th day after adjuvant injection, all rats were examined regularly for arthritis. At each examination, the wrists, ankles, tarsi, and tail were graded 0-4 depending on the degree of swelling and redness, and each of the smaller joints (metacarpophalangeal, metatarsophalangeal, and interphalangeal) 0-2. The total score for all these joints was used as an index of the severity of polyarthritis on a particular occasion.

Lymphocyte Agglutination.---Lymph nodes or thymuses from rats killed by exsanguination were crushed in Hank's solution, then filtered through glass-wool columns. Next the cells were washed three times in a buffer solution containing 1.96 g Na₂HPO₄, 0.65 g NaH₂PO₄, 3.0 g Na₂EDTA, and 3.5 g NaCl per liter. Before use, the cells were resuspended in sufficient volume of this solution to give a concentration of 50 × 10⁶ cells per milliliter, and 1% decomplemented normal rabbit serum was added. Cells stored overnight at 4°C proved satisfactory for use. During preparation, some cells formed clumps which could not be resuspended, hence the cell concentration had to be adjusted immediately before use. An occasional batch of

<table>
<thead>
<tr>
<th>Antilymphocyte preparation</th>
<th>Agglutination titer</th>
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<tbody>
<tr>
<td>Unabsorbed serum</td>
<td>1/256</td>
<td>1/512</td>
</tr>
<tr>
<td>Ammonium sulphate precipitated globulins after absorption with rat erythrocytes</td>
<td>1/256</td>
<td>1/8</td>
</tr>
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TABLE I
Agglutinating Properties of Crude Antilymphocyte Serum and Absorbed Antilymphocyte Globulin

Hemagglutination Tests.---All hemagglutination tests were performed using Microtiter equipment with disposable U plates. 0.05 ml of a 1% suspension of the appropriate washed erythrocytes (rat or sheep) was added to 0.05 ml of serum in serial dilutions. The results were read after 4 hr incubation at room temperature.

Indirect hemagglutination tests for measuring rat anti-rabbit globulin and rabbit anti-rat globulin antibodies were performed by a modification of the above procedure. Serum from a rat or a rabbit hyperimmunized to sheep erythrocytes was used to sensitize sheep erythrocytes by incubating the cells at 37°C for 30 min with the appropriate serum diluted to 1/4 the basic agglutinating titer, after which the erythrocytes were washed three times. The sheep erythrocytes, thus coated with a subagglutinating dose of rabbit or rat immune globulin, were then used in hemagglutination tests as above. These sera were tested also against unsensitized sheep cells, with negative results, indicating the absence of "heterophile" antibody. Agglutination of sensitized cells was interpreted as indicating the presence of antibody to the immune globulin (rat or rabbit) coating the erythrocytes. In some instances, rat sera were tested against rat erythrocytes sensitized with a subagglutinating dose of an appropriate rabbit antiserum.
Corticosterone Determinations.—These were performed by the fluorometric technique of Guillemin et al. (16) using 0.2 ml of serum. Blood samples were drawn between 10 a.m. and 12 noon.

Urinalysis.—Rats receiving intraperitoneal injections almost invariably passed at least a few drops of urine at the time of injection. Advantage was taken of this to test the urine with Labstix (Ames Co., Inc., Elkhart, Ind.) as a simple screening test for abnormal quantities of protein, glucose, and blood.

Immunofluorescence.—Goat anti-rabbit globulin was labeled with fluorescein isothiocyanate by the method of Spendlove (17).

RESULTS

In Vitro Properties of ALG.—Table I shows the results obtained with a typical batch of rabbit antilymphocyte serum. The pooled serum, before processing, agglutinated rat lymphocytes in a titer of 1/256 and rat erythrocytes in a titer of 1/512. Absorption twice with 1/4 volume of packed rat erythrocytes and ammonium sulphate fractionation reduced the titer of hemagglutinins to 1/8 without affecting the antilymphocyte titer. The titers of individual animals remained fairly constant through successive bleedings.

An indirect hemagglutination technique utilizing sheep cells sensitized with rat antibody was used to test for the presence of antibodies to rat globulin. Sera from early bleedings gave low titers (0-1/16), while in later bleedings the titers rose to between 1/64 and 1/128. However, processing of the serum, as described, appeared to remove these antibodies, for all batches of ALG gave negative results. ALG also gave negative results in the standard sensitized sheep cell agglutination test and latex fixation test (RA, Hyland Laboratories, Los Angeles, Calif.) for rheumatoid factors.

Response to Injection of ALG.—Table II shows the changes in the absolute lymphocyte count following a single 2 ml injection of ALG, compared with a similar injection of normal rabbit globulin (NRG). 2 hr after injection of ALG,
the lymphocyte count was falling, and some effect was still apparent at 24 hr. By contrast, NRG produced only a modest depression at 4 hr. Daily injections of 1 ml of ALG for 3 wk maintained the lymphocyte count at approximately 3,500/mm³. Rats so treated showed no evidence of hemolytic anemia or other toxic effect, and maintained normal hematocrit values.

Effect of ALG on Adjuvant Arthritis.—Fig. 1 shows the influence of a 21 day course of ALG on the progress of adjuvant arthritis in the rat. Treated animals received 2 ml of ALG on the day before adjuvant administration, followed by 1 ml daily thereafter. Each point indicates the mean joint score in a group of 10 rats on a particular day. All animals in the control groups, which received either no treatment or NRG, developed either moderate or severe polyarthritis beginning on the 9th to 12th day, increasing rapidly in severity up to the 15th day. Gross changes were still present when the experiment ended at 8 wk. By contrast, rats treated with ALG received considerable protection, showing either mild arthritis or none at all. After stopping treatment, there was no evidence of the "escape" which has been described after corticosteroid suppression (18).

Clinically, the animals that received ALG appeared healthier and were more active. Fig. 2 shows that they were spared the weight loss (which averaged 17 g) and the fall in hematocrit (which averaged 8%) experienced by the other two groups. The absolute lymphocyte counts are also shown. Compared with the lymphocytosis seen in control animals, the ALG-treated animals receiving adjuvant developed a modest lymphopenia. There was, however, some escape
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from this lymphopenia in comparison with animals given the same course of ALG without the adjuvant injection.

*Rat Anti-Rabbit Globulin Antibodies.*—Sera obtained from five rats in each

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**Fig. 2.** Mean values of body weight, hematocrit, and peripheral lymphocyte count in three groups of rats before and after adjuvant injection. 10 rats in each group. (Same experiment as Fig. 1). Note that ALG-treated rats were spared the weight loss and anemia seen in controls.

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group on day 27, 1 wk after stopping globulin treatment, were examined for the presence of antibodies to rabbit globulin. The results of an indirect hemagglutination test using sheep erythrocytes sensitized with a subagglutinating titer of rabbit amboceptor are shown in Table III. Both the ALG- and NRG-
treated animals had high titers of anti-globulin antibodies, the titers of the ALG-treated group being, if anything, slightly greater. Titers of earlier sera obtained on day 14 were about three dilutions lower in these two groups.

**Rat Serum Corticosterone Levels.**—It was felt necessary to exclude the possibility that ALG suppressed adjuvant arthritis through an adrenocortical mechanism. Serum corticosterone levels were therefore determined (16) on serum obtained from five rats in each group on day 14, at a time when the arthritis was increasing in the control groups and the globulin preparations were still being administered. Table IV shows the results. The distribution of serum levels indicates no adrenocortical overactivity in the group of animals treated with ALG.

**Table III**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>No. of rats showing titers of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt;1/4</td>
</tr>
<tr>
<td>None</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>NRG</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>ALG</td>
<td>5</td>
<td>3</td>
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</tbody>
</table>

**Table IV**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Mean (and range) serum corticosterone* μg/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5</td>
<td>15.2 (10-27)</td>
</tr>
<tr>
<td>NRG</td>
<td>5</td>
<td>8.6 (5-16)</td>
</tr>
<tr>
<td>ALG</td>
<td>5</td>
<td>9.6 (5-22)</td>
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</table>

* Normal values under completely basal conditions are from 6 to 15 μg/100 ml. (16). Stress involved in the ether anesthesia used to bleed these rats might account for the somewhat higher values in some animals.

Effect of Varying the Timing and Dosage of ALG.—Fig. 3 shows the influence of various ALG treatment regimens on the course of adjuvant arthritis. A single injection of 2 ml, given on the day before adjuvant injection, had little if any effect, while three daily injections of 2 ml, bracketing the time of adjuvant injection, produced almost as efficient suppression as the full 21 day course. Later administration of ALG for periods of 1 wk produced progressively less...
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However, there appeared to be some depression in the curve of the mean joint score, even in the groups started on ALG either late in the incubation period or actually after the onset of arthritis. The shape of these curves suggests that ALG given late produced mild suppression limited strictly to the time of administration—a pattern suggestive of antiinflammatory activity rather than immune suppression.

Effect of ALG on the Primary Antibody Response to Sheep Red Blood Cells.—To assess immunological competence in the animals used in these experiments, five rats in each group were injected intraperitoneally with 1 ml of a 10% sus-

![Graph showing the influence of timing and dosage of ALG on the suppression of adjuvant arthritis.](image-url)
pension of washed sheep erythrocytes on day 15. They were bled 1 wk later (day 22), and the distribution of hemagglutination titers among the various groups is given in Fig. 3. The brisk antibody response seen in the two control groups was completely prevented by ALG treatment for 3 wk. Shorter courses produced partial suppression. The numbers concerned are too few to draw firm conclusions, although the pattern suggests that ALG suppressed this primary antibody response most efficiently when given shortly before antigen administration.

Comparison of Antisera to Lymph Node and Thymus Cells.—Two groups of rabbits were used to produce separate antisera against lymphoid cells from rat lymph nodes and rat thymuses. The immunizing program and method of processing the globulin were identical with that used in the preparation of ALG, except that the different groups of rabbits were injected only with the appro-

| TABLE V |
| Agglutinating Properties of “Antilymphocyte” and “Antithymocyte” Globulin Preparations |
| Antiserum preparation | Agglutination titer for cells obtained from* |
| | Lymph nodes | Thymus |
| Antilymphocyte globulin | 1/64 | 1/128 |
| Antithymocyte globulin | 1/64 | 1/128 |

* Repeated testing of different batches of cells from these sources revealed no consistent difference in sensitivity to agglutination. The one-tube difference shown here is not significant.
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appropriate type of cell. Table V shows the result of an agglutination test comparing the properties of these two globulin preparations. The two were equally potent, and showed no differences in specificity for the two types of cells used in immunization. Differences in specificity were also looked for by immunofluorescent methods. Sections of rat lymph node and thymus, after incubation with one or the other globulin preparation, were exposed to fluoresceinated goat anti-rabbit globulin antibody. There appeared to be equal affinity of the two preparations for each type of tissue. These results, therefore, indicated no gross differences in specificity between the two antisera with regard to their affinity for cells obtained from the thymus or from lymph nodes. (They were also taken to indicate that either type of cell was suitable for use in lymphocyte agglutination tests. In practice, the larger number of cells obtainable from the thymus, with less erythrocyte contamination, made this the more convenient source).

Fig. 5. Comparison of in vivo activity of antisera prepared against thymus cells and lymph node cells (results of experiment illustrated in Fig. 4). Lymphocyte counts were determined 3 hr after the fourth injection of globulin. Joint scores represent the maximum score achieved by each rat. The antibody response to a challenge with sheep erythrocytes and the result of a skin test using PPD are also shown. Note that there is no difference in activity between antisera to thymus and lymph node cells, and that the PPD skin reaction was almost unaffected despite marked suppression of arthritis. Control rats, not given adjuvant, had completely negative skin tests to PPD.

<table>
<thead>
<tr>
<th>Absolute Lymphocyte Count ($\times 10^3/\text{mm}^3$)</th>
<th>Joint Score (max)</th>
<th>Hemagglutinin Titer (log$_2$ recip titer)</th>
<th>PPD Skin Reaction at 24 hr (mm diam induration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L: Anti-lymph node cell globulin T: Anti-thymus cell globulin N: Normal rabbit globulin</td>
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</tbody>
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The biological activity of the two globulin preparations in vivo was compared in the experiment shown in Fig. 4. Three groups of 10 rats were each given 2 ml of a globulin preparation (antilymphocyte, antithymocyte, or NRG) daily for 5 days. 3 hr after the fourth injection (day 0), absolute lymphocyte counts were performed, and each animal was injected with adjuvant. 1 ml of a 10% suspension of sheep red blood cells was injected on day 9, and blood was taken for a hemagglutination test on day 16, antigen challenge being intentionally delayed to avoid complete suppression of the response. All animals were skin-tested, with 0.1 ml (5 μg) of "second strength" purified protein derivative (PPD, Merck Sharp & Dohme, Rahway, N.J.) on day 14.

Fig. 6. Design of experiment to determine the influence on ALG activity of prior hyperimmunization with rabbit globulin. All rats given alum-precipitated rabbit globulin had high titers of antibodies to rabbit globulin on day −3.

Fig. 5 summarizes the results. Compared to NRG, both the active preparations reduced the peripheral lymphocyte count and suppressed both adjuvant arthritis and the primary antibody response, and to an approximately equal extent. Both may have produced a slight reduction in the intensity of the skin reaction to PPD, but it is of interest that the reaction was still clearly positive compared to control rats not injected with adjuvant, even in those animals completely protected from arthritis.

The Influence of Circulating Anti-Rabbit Globulin Antibodies on the Activity of ALG.—The experiments described above had shown that all rats receiving ALG developed precipitating antibodies to rabbit globulin in their serum. This raised the question of whether the presence of such antibodies modified the
activity of ALG, or made its administration hazardous to the recipient. The experiment illustrated in Fig. 6 was designed to investigate these questions. The parameters used in the previous experiment were again examined in comparing the activity of ALG in a group of five rats hyperimmunized to normal rabbit globulin with its activity in animals hyperimmunized to an unrelated antigen, bovine serum albumin (BSA). Immunization was achieved by two intraperitoneal injections of 2.8 mg of the appropriate alum-precipitated antigen (19). An indirect hemagglutination test on serum obtained on day -3, before giving the first ALG injection, showed all rats pretreated with alum-precipitated rabbit globulin to have antiglobulin titers of between 1/256 and 1/1024. Untreated rats gave negative results. As illustrated, one group of controls received alum-precipitated globulin followed by NRG, another received no globulin treatment.

Fig. 7 shows the results. In both groups receiving ALG (groups A and B), there was a reduction in the lymphocyte count and suppression of arthritis.
There was also a tendency for the primary antibody response to sheep erythrocytes and the PPD skin reaction to be reduced. These effects were at least as great in the group hyperimmunized to globulin compared to the group pretreated with BSA. The results thus indicate no inhibition of ALG activity by the presence of circulating antiglobulin antibodies, and they do not exclude the possibility of slight potentiation. It will be noted that there is a tendency for normal rabbit globulin given to hyperimmune rats (group C) to produce somewhat similar, although less marked, effects. This appears to be an exaggeration of a tendency, noted throughout these studies, for NRG, when given in larger doses, to interfere with immune responses and lower the absolute lymphocyte count.

Urine Examination and Renal Histology.—During the course of the experiment illustrated in Figs. 6 and 7, the urine of rats receiving globulin preparations was, with few exceptions, tested on each occasion that globulin was administered. By the simple screening test used, none of the specimens contained abnormal concentrations of protein, blood, or glucose. Histological sections were obtained on day 25 from animals in group A (those immunized to rabbit globulin, then given ALG). Sections were also obtained from four rats immunized to rabbit globulin then given NRG for 5 days (like group C) and killed 6 hr after the last injection. All sections were stained with hematoxylin and eosin and with periodic acid–Schiff stain. None showed any abnormal changes in the basement membrane or elsewhere.

Discussion

In the preparation of ALG, Freund's complete adjuvant was omitted from the immunizing program used, lest its inclusion might confuse the interpretation of the influence of ALG upon adjuvant arthritis. This probably resulted in a less potent preparation than might otherwise have been obtained. Also, the number of cells used for immunization was relatively small compared to the massive doses which enabled Iwasaki et al. (20) to produce in horses an antiserum of very much greater agglutinating potency. The results obtained in these experiments, therefore, probably reflect those to be expected from a relatively mild antilymphocyte treatment regimen, although the criteria for evaluation of the potency of antilymphocyte preparations are still uncertain. This decreased potency was apparent in the relatively modest lymphopenia resulting from single or repeated injections of ALG. However, some reduction in the numbers of circulating lymphocytes did occur in all animals that showed depressed immune responses.

Adjuvant disease in the rat probably represents an immune response to a disseminated antigen present in \textit{M. tuberculosis} (12). It provides a convenient experimental model for evaluating antiinflammatory and/or immunosuppressive drugs and, in general, the results of such experiments have correlated well with the activity of the
same drugs in conditions such as rheumatoid arthritis (18, 21-24). Measured by this yardstick, ALG was observed to be a potent suppressive agent in doses which were not toxic to the rats. The efficient suppression obtained by a short course of ALG covering the time of adjuvant injection, and the absence of "escape" after stopping treatment, suggest that ALG interfered with the initiation of an immune reaction. However, when given later in the course of the disease, ALG produced slight suppression, apparent only during the period of administration, suggesting an antiinflammatory effect in this instance. Such combined immunosuppressive and antiinflammatory effects are known to occur with the use of antimetabolites such as 6-mercaptopurine (21). Another similarity between ALG and drugs like 6-mercaptopurine is their ability to suppress adjuvant arthritis without influencing the skin reaction to PPD (Fig. 5) suggesting that tuberculoprotein is not the responsible antigen in adjuvant arthritis (21). Evidence for an antiinflammatory action of ALG was in fact obtained by Morris et al. (25), who showed that an antilymphocyte preparation suppressed the inflammatory response to bacterial infection in guinea pigs. From the viewpoint of treating disease in man, ALG thus emerges as an alternative to the antimetabolite group of drugs for the treatment of the so-called autoimmune diseases. The advantage of ALG lies in its ability to produce profound immune suppression without critical reduction of formed elements in the circulation or elsewhere. Against this, of course, the hazards of repeated injections of foreign protein must be weighed.

Antilymphocyte preparations have been shown to suppress a variety of other experimentally induced disorders, including allergic encephalomyelitis in the guinea pig (3), Coombs-positive hemolytic anemia in NZB mice (26), lymphocyte choriomeningitis in the mouse (27), and graft-versus-host disease (28). However, ALG did not prevent the appearance of renal disease in (NZB x NZW) F1 mice (29).

Gray et al. (30) explored the possibility that the immunosuppressive and lymphocytopenic effects of ALG might be mediated through adrenocortical stimulation. Although they considered this unlikely, the results of their experiments in mice were not conclusive. Attempts were made in the present experiments to exclude this possibility. Efforts to repeat these experiments in appropriately supplemented, adrenalectomized animals were unsuccessful both because of poor survival of adrenalectomized rats with adjuvant disease, and also because of undue susceptibility of these animals to the stress of ALG injections. However, the relatively normal serum corticosterone levels measured in rats receiving ALG (Table IV) are interpreted as strong evidence against any significant contribution to the results by increased adrenal steroid secretion.

ALG is produced against crude whole lymphocytes and would be expected to contain antibodies to a wide range of cellular antigens. Some of these antigens would be shared by many cells in the donor animals, while others might be specific for narrower cell lines. Iwasaki et al. (20) found that absorption of ALS with either liver or kidney cells reduced the lymphocyte agglutinating titer by 90% although, like ourselves, they found no loss during absorption with red blood cells. In addition, Levey and Medawar (31) have produced antisera both to epidermal and to L cells which can prolong the survival of mouse homografts. These results suggest a much broader specificity for these preparations than is implied in the term "antilymphocyte." However, Nagaya and Sieker (5) and Kubista et al. (32) found differences in the biological activity of an antiserum directed against lymphocytes compared with one directed against thymus
cells, suggesting that the latter might be more potent. In addition, Potworowski and Nairn (33) have detected an antigen present in subcellular fractions of thymocytes not shared by lymphocytes.

It seemed important, therefore, to determine whether there were any significant differences between antisera raised separately against lymph node cells and against thymus cells. In vivo biological differences between such antisera might represent either differences in the relative strength of the preparations, i.e., the concentration of antibody, or differences in the pattern of specificity, i.e., varying activity against different target cells. We were careful for this reason to use identical immunizing programs in producing antisera against cells from these two sources. The globulin preparations obtained from pools of such antisera had identical agglutinating titers for both lymph node and thymus cells (Table V). This we have taken to indicate that the two preparations were equipotent in a general sense, and that neither showed any greater affinity for one or the other cell type. The latter observation was confirmed by immunofluorescence testing. In vivo, both preparations reduced peripheral lymphocyte counts, suppressed adjuvant arthritis, and inhibited primary antibody responses, all to an equal extent. Thus we have been able to detect no quantitative or qualitative differences between these preparations.

Levey and Medawar (31) found that, in rabbits immunized against mouse lymphocytes, the initial bleeding produced the most potent antilymphocyte serum, and that longer courses of injections yielded less effective sera. In the experiments described here, the rabbits were boosted and rebled a total of nine times. Comparison of early and late bleedings showed no significant differences in lymphocyte agglutination titer, and immunosuppression was achieved with all batches of ALG, although the relative efficiency of early and late sera in this regard was not specifically compared. Successive bleedings were found to have increasing titers of antibodies against rat immunoglobulin. Thus, the specificity of ALG probably broadens with continued immunization without increase in the specific antilymphocyte activity.

Gray et al. (30) found that rabbit anti-mouse lymphocyte serum inhibited the appearance of antibodies to rabbit immune globulin in recipient mice, and suggested that this might explain the long-term effectiveness of their preparation. Our experience in rats differs in that all animals given ALG developed precipitating antibodies to rabbit immunoglobulin, and the resulting titers were at least as high in rats given ALG as in animals given a similar course of NRG. These antibodies might be expected to modify the biological activity of ALG, either by accelerating immune elimination, or by forming immune complexes with ALG which left the lymphocyte-binding sites intact. The former would be expected to decrease, and the latter, perhaps, to leave unchanged or even to increase "antilymphocyte" activity. In the present experiments, ALG, given to rats having high titers of circulating antibodies against rabbit immunoglobulin, produced at least as much immunosuppression and lymphopenia as in animals not so pre-immunized. To what extent this represents a balance of effects is not clear.

Circulating antibodies to globulin also represent a potential hazard to the recipient, if ALG is used therapeutically. A situation would arise in which there would be a risk, at least in theory, of anaphylactic shock, serum sickness, and renal damage. The extent to which information about this can be obtained from the results of experiments in rats is clearly limited. However, it is encouraging that none of the rats used in these
experiments showed any evidence of toxicity to ALG. There was no sign of collapse or other reactions following injections, and treated rats did not become anemic. Repeated tests for proteinuria and hematuria were always negative and, even in the provocative situation where ALG was given to rats already hyperimmunized to rabbit globulin, no abnormality of renal histology was detected by light microscopy, although the tissues were not examined with the electron microscope. During the course of these experiments, a few rats developed chronic respiratory infection, but this complication was no more frequent among the ALG-treated animals than among controls.

The slight reduction in peripheral lymphocyte counts and the slight suppression of immune responses noted when larger doses of NRG were given to control animals remain unexplained. Both might represent a stress reaction. Alternatively, the immune suppression might be due to antigen competition, or to the presence of the $\alpha_2$-glycoprotein fraction of serum globulin which Mowbray (34) has shown to interfere with allograft rejection. In the case of adjuvant arthritis, Pearson and Wood have shown that incorporation of an irrelevant protein such as ovalbumin into the adjuvant reduces the severity of the subsequent arthritis. Whatever the explanation, the implications about the necessity for adequate controls in experiments with antilymphocyte preparations are obvious.

The group of human diseases sometimes referred to as autoimmune includes a variety of conditions of unknown etiology in which immunological overactivity is prominent. Current concepts assign an essentially pathogenic role to such immune processes, and this provides the rationale for treatment with immunosuppressive drugs such as azathioprine and cyclophosphamide. The usefulness of immunosuppressive agents in these conditions is not yet established, but across a wide therapeutic front the reports are at least encouraging (35). The studies reported here have shown that the known efficacy of ALG in suppressing allograft rejection also extends to suppression of an immunologically induced disease in the rat by a relatively mild dosage regimen, and encourages the hope that it may prove an effective form of therapy in human disease. Only clinical experience will show whether the lack of toxicity seen in the rat extends to man.

SUMMARY

The effect of antilymphocyte globulin (ALG) on adjuvant arthritis, an immunologically induced disease in the rat, was studied. ALG was prepared from the serum of rabbits immunized against rat lymphocytes.

Adjuvant arthritis was induced in rats by a single intracutaneous injection of Freund's complete adjuvant; after 9 to 12 days, all control rats developed polyarthritis. Administration of antilymphocyte globulin about the time of adjuvant injection produced marked inhibition of clinical disease. Some suppression was apparent even when ALG was started after the onset of arthritis. Rats receiving ALG remained conspicuously healthy compared to controls. Urinary findings and renal histology showed no evidence of nephritis. The results of serum corticosterone determinations made it unlikely that adrenal stimulation contributed to the actions of ALG.

1 Pearson, C. M., and F. D. Wood. Personal communication.
Antilymphocyte preparations lowered peripheral lymphocyte counts and suppressed primary antibody responses to sheep erythrocytes, but had little effect on the skin reaction to PPD, even in rats protected from arthritis.

All rats given ALG developed antibodies to rabbit globulin; there was no evidence that ALG inhibited the appearance of antibodies to itself, and prior hyperimmunization of rats with rabbit globulin did not interfere with the biological activity of ALG subsequently injected.

Antisera produced separately against lymph node and thymus cells had identical properties with regard to agglutination of lymphocytes and thymus cells. Administered to rats, these preparations were equally potent in lowering lymphocyte counts and suppressing both adjuvant arthritis and the primary antibody response to sheep erythrocytes.

It is concluded that ALG, as used in these experiments, is a potent immunosuppressive agent without obvious toxic effects.

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