pinealectomy as a second order effect, no different, except in the manner by which it is achieved, from the effect of LL itself. This possibility cannot be excluded on the basis of our data, but it appears unlikely in view of the fact that pinealectomized birds can be entrained by light cycles.

4) The pineal could be a self-sustained oscillator, normally driving a damped oscillator which in turn directly controls locomotor activity. In this case, the pattern of "decay" into arrhythmicity after entrainment would reflect the damping of the remaining oscillator. On the basis of this interpretation, the entrainment response of pinealectomized birds would result from direct driving of the damped oscillator by the light cycle.

5) The pineal could be a coupling device within a multi-oscillator clock system. Two or more oscillating components of the timing machinery might be coupled, and fixed phase relationships might be maintained among them by some action of the intact pineal organ. Arrhythmicity in DD could result from their uncoupling by removal of the pineal organ and their subsequent drifting out of phase with each other. Entrainment in the absence of the pineal organ would indicate that the light cycle alone is sufficient to maintain fixed phase relationships among the component oscillators.

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5. Activity recordings were obtained under conditions previously described; M. Menaker, in *Circadian Clocks*, J. Aschoff, Ed. (North-Holland, Amsterdam, 1965), p. 386. The birds were fed about every 14 days without interruption of constant darkness.
6. Pinealectomy was accomplished as follows: After the bird was anesthetized with Equithesin, the skin on the dorsal surface of the skull was slit and pulled laterally while the head was steadied by a stereotactic instrument. A piece of skull directly over the pineal body was drilled out with a dental burr and removed. The pineal organ was excised along with the meninges to which the dorsal part of the gland adheres. The removed skull piece was then replaced and sealed with Replica plastic filling material, and the skin sutured. Large venous sinuses which surround the pineal gland and receive blood from it, as well as from other parts of the central nervous system, were necessarily cut in removing the pineal body. Sham operated birds underwent an identical surgical procedure, including the cutting into these sinuses; however, the organ was not removed. During all surgical procedures the birds were exposed to light for 1 to 2 hours, for most of which they were under anesthesia.
7. "Arrhythmicity" is provisionally used in this context, as quantitative analysis of the activity data is not yet available. The day on which onset and offset become indistinguishable is arbitrarily designated as the beginning of arrhythmicity.
8. Although published documentation is rare, it is a common observation that most circadian rhythms are not expressed in constant bright light. As the basis of this response to constant light is not understood for normal birds, the meaning of the similar arrhythmic behavior produced by pinealectomy and that produced by LL remains obscure.
9. Light was obtained from a fluorescent bulb (Ken Rad, 4 watt, cool white F4T5/cw) providing 200 to 900 lux depending on the bird's position in the cage.
10. Birds were injected with Bouin's solution and decapitated. Skin and eyes were removed from the skull, and the dorsal part of the skull was drilled and carefully removed (including Replica cap) under a dissecting microscope. The brain with remaining skull was placed in Bouin's solution for 3 to 14 days, after which the skull was dissected from the hardened brain tissue which was then left in running tap water for 12 to 24 hours. Brains were then dehydrated in an ethanol series, cleared in xylene, and embedded in Paraplast. They were sagittally sectioned at 15 to 20 $\mu$ and stained in Mayer's hemalum and eosin.
11. Supported in part by NSF grant GB-3806; by NIH training grant 5-TI-CM-836-03; and by AFOSR grant 637-67.

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## Antiserum to Lymphocytes: Interactions with Chemical Immunosuppressants

Abstract. *An injection of rabbit antiserum to mouse lymphocytes causes temporary lymphopenia and prolonged survival of A-strain skin grafts on CBA mice. Cortisone or amethopterin without further antilymphocytic serum prolongs lymphopenia and immunosupression. When cortisone or amethopterin precedes the administration of the antiserum, the immunosuppressant action of the combination is less than that of the antiserum given alone. Whether the serum or the drug is given first does not affect the induction of lymphopenia by the serum. Thus, immunosuppressant action of antilymphocytic serum can be distinguished from its ability to induce lymphopenia. The results suggest that this serum may act as a mitogenic agent redirecting the proliferation of lymphocytes into immunologically incompetent pathways.*

The immunosuppressant effects of heterologous antiserums against lymphocytes in prolonging skin and kidney allograft survival have been well documented (*1–3*); however, the many theoretical and practical hazards associated with the prolonged administration of this material or of any heterologous serum product to organ transplantation need to be considered. In addition to the obvious dangers of serum sickness and anaphylaxis (*3*), the administration of antilymphocyte serum (ALS) has been associated with severe systemic reactions (*2*), marked local reaction at the site of injection, anemia, fever (*3*, *4*), generalized wasting syndromes (*5*), the development of antibodies to the injected proteins (*6*), and deposition of proteinaceous material along the basement membranes of renal glomeruli (*7*). Some of these reactions may be avoided by suitable purification of antigen (*8*), by fractionation of the serum, or by absorption (*3*, *4*). We have been interested in determining whether the immunosuppressant effect induced by ALS could be prolonged by other immunosuppressant agents (*9–11*), in order to try to shorten the period of ALS administration and to reduce the required dosage of other toxic, immunosuppressant materials. It is possible to maintain the immunosuppressant action induced by ALS with a combination of purine and pyrimidine antagonists in mice (*9*) or with azathioprine in dogs (*10*). Our experiments are designed to find out whether the additive or synergistic interaction of ALS with chemical immunosuppressant agents is dependent on their sequence of administration.

Rabbit antiserum to mouse lymphocytes (ALS) was prepared according to the technique of Gray *et al.* (*11*), with some modifications (*12*). Suspensions of washed A/Jax mouse lymph node cells (90 percent small lymphocytes) in Hanks's solution were mixed with equal volumes of complete Freund's adjuvant (Difco) and injected into each of the four footpads of an adult New Zealand white rabbit; the total dose was 400 $\times 10^6$ cells in 1.0 ml. Three weeks later, booster injections of the same cells ($100 \times 10^6$ cells in 0.5 ml) were given

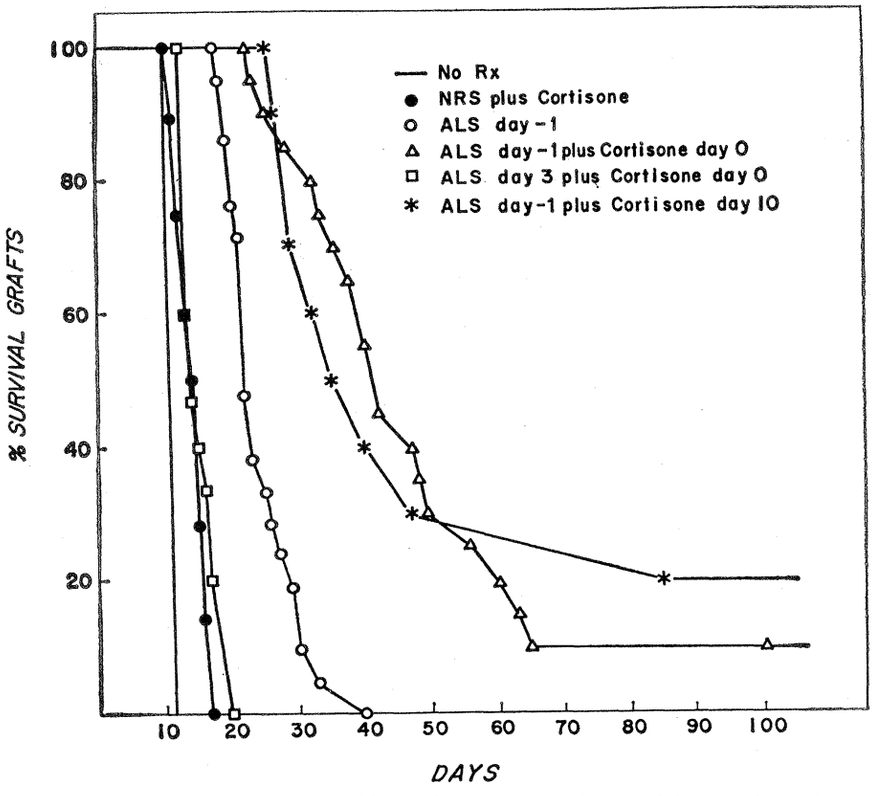


Fig. 1. Cumulative survival of A skin grafts on CBA mice receiving cortisone in addition to rabbit antiserum to mouse lymphocytes (ALS) or normal rabbit serum (NRS). Each line represents 16 to 35 recipient mice.

intravenously on 4 successive days, and the rabbits were bled from an ear artery 4 and 5 days later. The serum was separated and pooled; the complement was removed by heating at 56°C for 30 minutes; the serum was frozen at −20°C. Leukoagglutination titers against A/Jax lymph node cells were determined by a modification of the method of Amos and Peacocke (*13*) described by Gray *et al.* (*11*). The leukoagglutination titer of the several pooled batches of ALS used was in the range 1:256 to 1:512. A single intraperitoneal injection of 0.5 ml of serum was administered to each CBA/J female recipient mouse (20 to 25 g) at various intervals before or after it had received a tail-skin graft from A/Jax donor mice. Grafting was performed as described by Billingham and Medawar (*14*). Dressings were removed on the 6th day after grafting, and grafts were examined daily for total epithelial destruction. Skin-graft survival is recorded according to the percentage of the number of original grafted animals bearing viable skin grafts at various intervals after grafting.

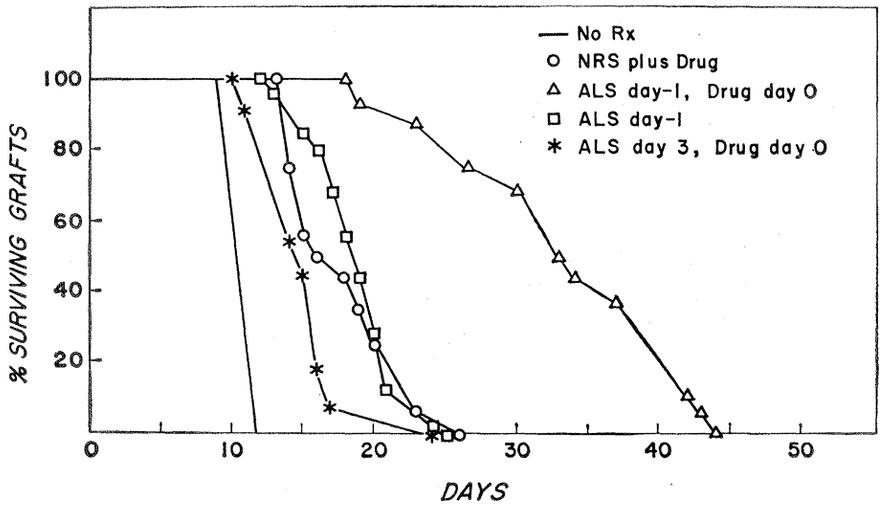


Fig. 2. Cumulative survival of A skin grafts on CBA mice receiving amethopterin in addition to rabbit antiserum to mouse lymphocytes (ALS) or normal rabbit serum (NRS).

Cortisone acetate in a concentration of 400 mg per 100 ml of sterile saline was administered subcutaneously in a dosage of 16 mg per kilogram of body weight three times weekly. Each day methotrexate was dissolved in sterile saline in a concentration of 60 mg per 100 ml of sterile saline, and three times weekly it was administered intraperitoneally to recipient mice in a dosage of 3.0 mg per kilogram of body weight. Normal rabbit serum (NRS) from which complement was removed was used for control recipient mice. Control female mice received a 0.5-ml injection of NRS 24 hours before the application of A/Jax tail-skin grafts. The median survival time (MST) in days was 10.4 ± 0.4 (S.E.) (Fig. 1). This is identical to the response in the untreated host. When experimental mice received a single injection of 0.5 ml of ALS, A-strain skin-graft survival varied depending on the batch of ALS used, but generally ranged from 19 to 23 days (Figs. 1 and 2). There was no difference in skin-graft survival when ALS was administered 1 day before the application of the skin grafts (day −1), or 4 days later (day 3).

The same batch of ALS was used to compare skin-graft survival by various schedules of adjuvant chemotherapy (Fig. 1). Cortisone administered to NRS recipients beginning on the day of grafting (day 0) caused slightly more graft prolongation (MST = 14.1 ± 0.3) than that seen in control mice. In another experiment, mice were treated with 0.5 ml of ALS, and on the next day skin grafts were done; at the same time a regimen of cortisone (3 times weekly) was begun. In these animals the graft survival was prolonged. Some grafts even survived as long as cortisone was administered. When cortisone was finally stopped, after 100 days, the grafts persisted for 50 to 75 days more. Cortisone administration that was started up to 10 days after grafting yielded similar

results (Fig. 1). Cortisone administration started on the day of skin grafting (day 0) and ALS administered on day 3 gave the same results as cortisone given alone (MST = 15.5 ± 0.7). The effect of ALS could thus no longer be detected.

Similar results were obtained when methotrexate was used instead of cortisone (Fig. 2). Methotrexate therapy started on the day of grafting in mice that had been given a single injection of ALS 24 hours previously caused a significant further prolongation of graft survival (MST = 33.8 ± 1.9) compared to that achieved with either ALS alone (MST = 18.9 ± 0.6) or with methotrexate plus NRS (MST = 16.3 ± 0.6). However, methotrexate started on the day of grafting and ALS started 3 days later prolonged graft survival less (MST = 15.5 ± 0.9) than did either ALS or methotrexate alone.

In order to determine whether prior administration of either cortisone or methotrexate had interfered with the lymphopenic action of ALS, peripheral blood lymphocyte counts were carried out in drug- and serum-treated CBA female mice that had not received skin grafts (Fig. 3). A single injection of ALS usually produced a moderate degree of lymphopenia that persisted for 4 to 7 days and then returned toward normal in about 17 to 21 days (*12*). The addition of cortisone or methotrexate after administration of ALS did not significantly alter the initial response, although the return of the peripheral lymphocytes to normal levels was markedly delayed, and, depending on dosage, this return did not occur until drug administration was stopped. When drug administration preceded the injection of ALS, neither the initial lymphopenic response to ALS or the persistence of the lymphopenia was greatly diminished.

Our results coincide with mounting evidence that the immunosuppressant action of ALS is not wholly dependent upon the accompanying lymphopenia (*15*, *16*). Lymphoid depletion may indeed be one major mode of ALS action, because graft rejection occurs at the approximate time of peripheral lymphocyte repopulation, and the prolonged administration of ALS results in profound lymphoid destruction (*1*, *6*, *17*). Prolongation of ALS activity can be obtained with thoracic duct drainage (*17*) or thymectomy (*13*). However, Levey and Medawar obtained similar degrees of immunosuppression with serums

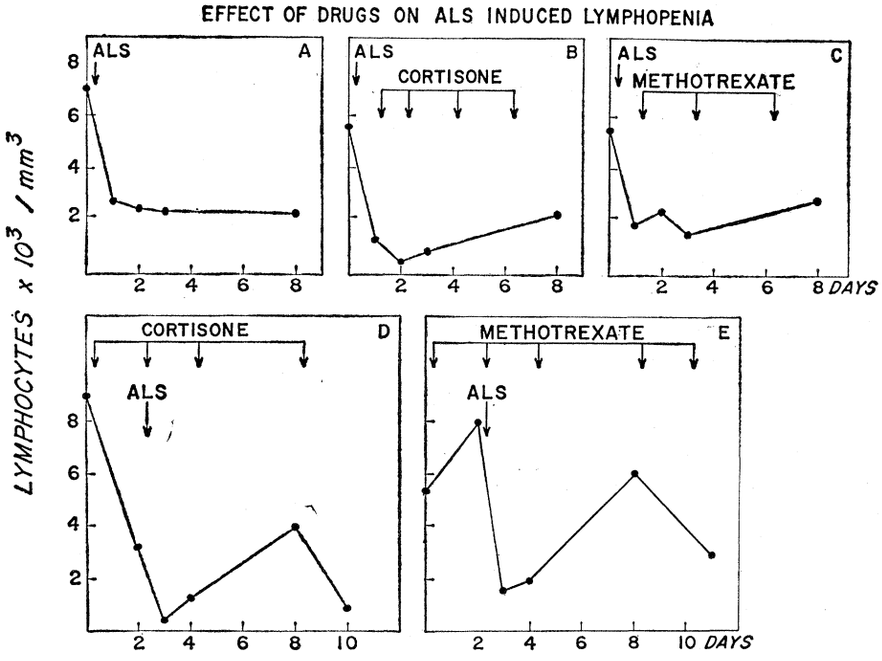


Fig. 3. Mean total peripheral blood lymphocyte counts in CBA mice treated with a single dose of rabbit antiserum to mouse lymphocytes plus cortisone or amethopterin. (A) ALS alone. (B) ALS followed by cortisone. (C) ALS followed by amethopterin. (D) ALS given after cortisone course begun. (E) ALS given after amethopterin course begun.

which did not induce much lymphopenia (*15*, *16*). More recent studies have shown that the lymphocytes which persist after ALS treatment may themselves be immunologically incompetent (*16*, *19*, *20*). Our studies demonstrate that ALS may not be effective even when a significant degree of peripheral lymphopenia is achieved. This strongly suggests that its mechanism of action may lie in other modes of lymphocyte inactivation that are independent of lymphocyte death.

Investigators have suggested that ALS may act as a lymphocyte mitogen (*16*) inasmuch as the addition of ALS to lymphocyte cultures induced lymphocyte transformation (*21*). In this respect ALS resembles phytohemagglutinin and other plant mitogens (*23*). Since such compounds are weakly immunosuppressive (*22*, *23*), and since DNA synthesis by lymphocytes has been correlated with an inability to produce antibodies (*24*), it has been hypothesized that compounds like ALS act by inducing mitosis along lines of differentiation that produce immunologically incompetent lymphocytes (sterile activation theory) (*16*). This hypothesis would be consistent with the observations that the lymphoid cells of recipients of ALS are less capable of initiating a graft-versus-host reaction (*19*, *20*), and that increasing the lymphoid population turnover by sublethal irradiation interferes with the prolonged action of previously injected ALS (*16*); however, this hypothesis disregards the comparatively weak and transient immunosuppressive action of the best mitogens. In fact, under some circumstances, mitogens may act as adjuvant agents (*25*).

In our experiments we used agents which interfere with lymphocyte transformation in vitro and whose mode of immunosuppressive action depends to some extent on their antimitotic action. Both these agents interfere with the immunosuppressive action of ALS when they precede the administration of ALS. These results lend support to the idea that the immunosuppressant action of ALS may be a function of its mitogenic characteristics. The finding that acceleration of lymphocyte repopulation interferes with the action of previously administered ALS (*16*) makes it not surprising that the use of drugs which suppress lymphoid mitosis also prolong its immunosuppressant action under similar circumstances.

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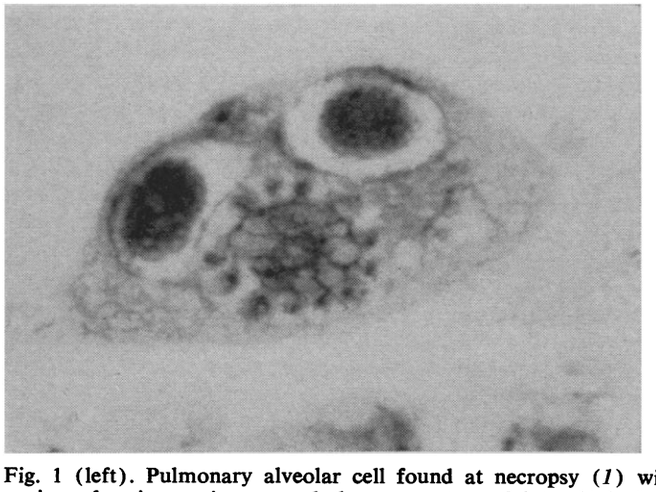
## Toxoplasma gondii and Cytomegalovirus: Mixed Infection by a Parasite and a Virus


Abstract. *Human fibroblasts infected in vitro with cytomegalovirus are relatively resistant to infection by* Toxoplasma gondii *during the first 4 days of virus infection. After 5 days, however, the cytomegalovirus-infected cells become susceptible to the parasites. The toxoplasmas replicate in paracentral rosettes surrounded by host cell mitochondria. This growth configuration differs from that seen in human fibroblasts infected in vitro with toxoplasma only but resembles the pattern seen in doubly infected cells found in human necropsy tissue.*


Simultaneous infections by the obligate intracellular parasite *Toxoplasma gondii* and cytomegalovirus (CMV) occur with an unexpected frequency in patients with disseminated neoplastic disease (*1*). In necropsy tissues from several such cases, we have observed toxoplasma organisms in the same cells with CMV nuclear inclusions (*1*). The growth pattern of the parasite in some of these doubly infected cells is unusual in that the toxoplasma rosettes form a ring around the cytoplasmic body (*2*) or cytocentrum of the host cell (Fig. 1). The double infection has now been reproduced for investigation in vitro. Experiments demonstrate that virus-infected host cells, after an early period of host cell resistance to parasite invasion and suppression of toxoplasma replication, eventually become receptive to double infection. The receptive period occurs after 72 hours when CMV accumulates in the host cell cytoplasm (*2*, *3*). The conspicuous circular configuration of toxoplasma rosettes in doubly infected cells is apparently related to a rearrangement of the host cells organelles during late virus infection (*2*, *3*).

The time course of toxoplasma infection in tissue culture is relatively rapid and the host cells in culture are destroyed within 3 days by any inoculum of toxoplasma providing a multiplicity of at least one organism per cell. In comparison, a 24- to 48-hour eclipse period precedes detectable CMV replication. The in vitro studies therefore were limited to the effects of initial CMV infection on the growth characteristics of toxoplasma.

In all experiments, fibroblast cell cultures (WI-38) were infected with cytomegalovirus (Davis strain) at least 2 days before toxoplasma organisms (RH strain) were introduced. In most of the experiments the fibroblasts were grown on cover slips in Leighton tubes and infected with 7800 PFU (plaque-forming units) of cytomegalovirus per tube. At specified times, the cover slips were fixed in a mixture of acetate-buffered mercuric chloride and formalin (*4*) and stained with hematoxylin and eosin (*4*). These preparations permitted observa-



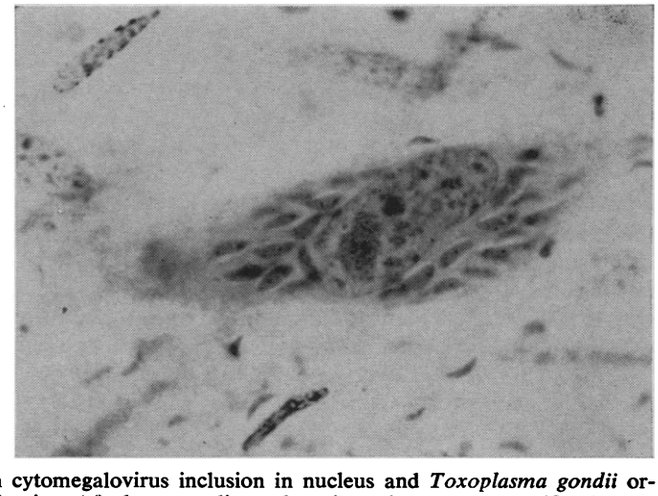


Fig. 1 (left). Pulmonary alveolar cell found at necropsy (*1*) with cytomegalovirus inclusion in nucleus and *Toxoplasma gondii* organisms forming a ring around the cytocentrum [glutaraldehyde fixation (*4*); hematoxylin and eosin; microscope magnification × 1000]. Fig. 2 (right). Nonreplicating toxoplasma in a cell infected with cytomegalovirus. The toxoplasma was inoculated 2 days after virus infection and allowed to grow for 48 hours. B5 fixation; hematoxylin and eosin stain; microscope magnification, × 1000.