HISTOPATHOLOGICAL EFFECTS IN MICE OF HETEROLOGOUS ANTILYMPHOCYTE SERUM

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Antiserum raised in one species against lymphoid cells of another (heterologous antilymphocyte serum, ALS) exerts powerful immunosuppressive effects when injected into members of the lymphoid donor species (8, 20, 34). Despite intensive investigation there is yet no clear agreement concerning the mechanism of this immunosuppression. Various possibilities have been summarized by Levey and Medawar (20).

All workers are agreed that the administration of ALS causes widespread changes in lymphoid tissues; however, the type and magnitude of the changes reported have been variable and often conflicting. Some studies have emphasized generalized lymphoid depletion following ALS treatment (8, 25) while others have noted discrete lymphoid tissue lesions (27, 31) or lymphoid hyperplasia (2, 12, 20). Similarly, the correlation between the degree of immunosuppression and lymphopenia obtained by some workers (8) was not noted by others (14). The determination of the histologic features that are invariably linked with immunosuppression by ALS would be helpful in clarifying its mode of action.

Toxic effects of ALS have been noted, particularly on red blood cells and on glomerular capillaries (10, 12, 16). It would be helpful to learn whether these effects are due to antibodies irrelevant to the immunosuppressive activity of ALS. It would also be important to characterize other possible toxic consequences of ALS and the relation of their pathogenesis to the desired immunosuppressive effects.

Materials and Methods

Mice.—CBA mice of either sex, between 2 and 4 months old, obtained from the breeding unit of the National Institute for Medical Research, were used throughout these studies.

Preparation and Assay of ALS.—Antilymphocytic serum for routine use was prepared by the method of Levey and Medawar (20). Adult New Zealand rabbits were given two intravenous injections of 10^9 CBA thymocytes a fortnight apart, and bled 1 wk after the last injection. The serum was heated to 56°C for 30 min, sterilized by Seitz filtration, and stored at −20°C until use. Normal rabbit serum (NRS) was obtained from unimmunized NZ rabbits and processed in an identical manner.

Several antisera were prepared by incorporating adjuvants into the cell suspension and immunizing the rabbits by intramuscular inoculation. Adjuvants used included aluminium phosphate, and Arquad-2-HT (dioctadecyl dimethylammonium chloride). Various other antisera were also examined with respect to the histological changes evoked in the lymph nodes of recipients. These included: (*a*) horse-anti-mouse lymphocyte globulin (HALGG)—batch 4096A supplied for these experiments by Dr. David Long of the Wellcome Foundation; (*b*) duck-anti-mouse lymphocyte serum (DAMLS)—this was antiserum prepared in ducks in a manner similar to that used in rabbits (15).

The 7S IgG fraction of ALS was isolated by ammonium sulfate precipitation, and column chromatography using either Sephadex G-150 or DEAE cellulose, by standard techniques.

Some antisera were absorbed overnight at 4°C with 4:1 (v/v) of washed packed CBA erythrocytes before use.

Immunosuppressive potency of various antisera was assayed by their ability to prolong the survival of female A strain tail skin transplanted to CBA mice. Routine antiserum (0.5 ml), given subcutaneously on day +2 and day +5 for a total dose of 1.0 ml, regularly prolonged the mean survival time (MST) from 11.3 ± 0.6 days to more than 20 days. The "adjuvant" antisera were at least as effective as routine antisera. The HALGG was somewhat less effective (MST, 18.0 days); the duck antiserum was totally ineffective.

Skin Grafts.—Grafts of tail skin from A strain mice to the right flank of CBA mice were performed according to the technique of Billingham and Medawar (3).

Administration of ALS.—The effects of antiserum administred in the following regimens were studied:

1. Routine antiserum, single dose: Parallel groups of mice were injected subcutaneously with a single 0.5 ml dose of either ALS or NRS, in the right axillary region, and sacrificed three at a time on days 1, 2, 4, 7, 10, 14, and 21 after injection.

2. Routine antiserum, short course: Groups of mice were given three doses of 0.5 ml ALS or NRS subcutaneously at 2-day intervals, and sacrificed 1, 2, 4, 7, 14, and 21 days after the last dose.

3. Routine antiserum, chronic treatment: Groups of mice were given an initial loading dose of three closely spaced injections of whole unabsorbed ALS followed by weekly maintenance doses of either 0.25 ml or 0.5 ml. Control animals received weekly injections of NRS. This regimen was continued for as long as 6 months. Mice were sacrificed in pairs from each group at various intervals, or, if death occured, were autopsied on that day.

4. Purified IgG fraction of ALS, single dose: Groups of mice were given 5 mg of the purified IgG fraction of ALS subcutaneously and sacrificed two at a time, 1, 2, 4, and 6 days after injection. Some groups of mice were given a paralyzing dose of 200 μg NRS IgG (19) 3 wk before the ALS IgG dose.

5. Adjuvant antisera: Groups of recipients were given one or two doses of 0.5 ml of whole unabsorbed antiserum 3 days apart, and sacrificed at 1, 2, and 3 wk after injection.

6. Miscellaneous antisera: Groups of recipients were given a single dose of 0.5 ml of whole unabsorbed antiserum, and sacrificed at 1, 2, 4, 6, and 10 days after injection.

7. High dose intravenous ALS: Recipients were given intravenous injections of either 0.5 ml or 1.0 ml ALS or NRS via the tail vein over a period of 3–5 min. Animals were sacrificed 1, 2, and 4 days after injection.

Hematologic and Morphologic Studies.—Mice were bled from the tail artery or by cardiac puncture under ether anesthesia; the capillary microhematocrit, total leukocyte count, and Wright-stained differential leukocyte count were performed by routine methods. Blood smears were scanned visually for gross changes in platelet numbers or morphology.

Histology.—Mice were sacrificed by cervical dislocation and the peripheral lymph nodes (brachial, axillary, inguinal), mesenteric nodes, Peyer's patch, spleen, liver, kidney, adrenal, thymus, sternal bone marrow, lung, heart, skeletal muscle, brain, and grafted skin were fixed in 10% neutral formalin for histological processing. Sections were stained with either

hematoxylin and eosin or methyl green–pyronine. Smears of lymph nodes and femoral bone marrow were also taken and stained with Wright's stain.

OBSERVATIONS

Effects of a Single Subcutaneous Dose of ALS or NRS.—

Hematologic changes: Anemia was a constant feature in animals given a single dose of unabsorbed ALS; a fall in hematocrit of 10% or more was observed within 48 hr after injection and persisted for 1 wk or longer. Prior absorption of the antiserum with mouse erythrocytes ameliorated or abolished the anemia.

A marked drop in both circulating granulocytes and lymphocytes was observed within 6 hr of injection. Absolute lymphocyte levels remained profoundly depressed to 10–20% of normal for 48 hr, and continued to remain somewhat below normal levels for 2 wk or longer (Fig. 1 *a*). Antiserum absorbed with mouse red cells retained the ability to induce lymphopenia.

Anemia or lymphopenia were not seen 2 days after a single dose of normal rabbit serum; rather, a distinct lymphocytosis was seen on the 6th day postinjection.

Abundant platelets were present in peripheral blood smears taken from both ALS and NRS-treated animals. An abnormal bleeding tendency was never observed in this group.

Lymph nodes: Within the first 48 hr after ALS injection, focal accumulations of cellular debris and pyknotic nuclei could be detected in the small vessels of the loose cortex or paracortical regions of lymph nodes draining the injection site (right axillary), as well as more distant lymph nodes (Fig. 7).

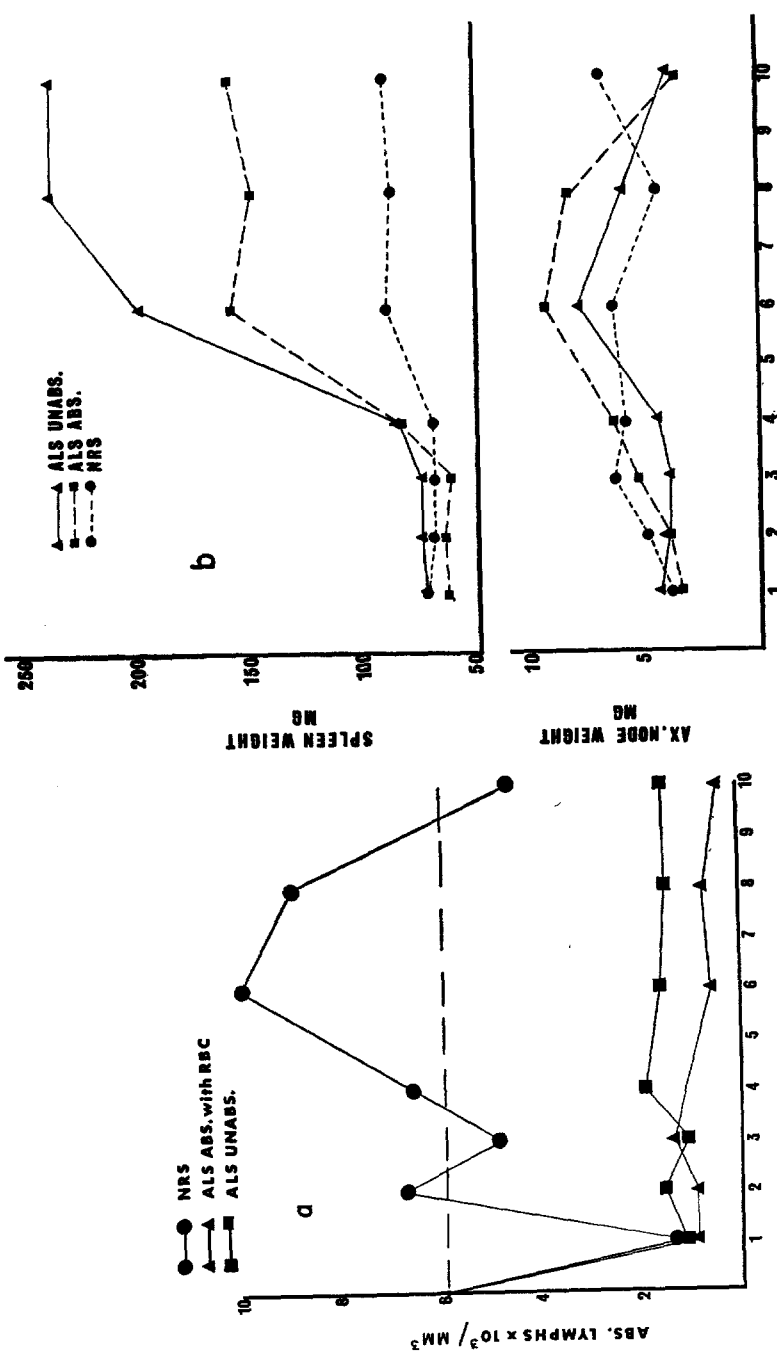
By 48 hr a striking depletion of small lymphocytes was noted in both ipsi- and contralateral axillary, brachial, and inguinal nodes, and to a lesser extent in mesenteric nodes; this depletion was sharply confined to the paracortical region. A few small lymphocytes remained, closely associated with postcapillary venules, but the contents of this region consisted for the most part of reticulum cells, large lymphocytes, and immunoblasts (Fig. 3).

By 96 hr, lymphocytes began to reappear in this area, but significant depletion remained for 1 wk or longer after some antisera. By this time, also, increased numbers of immunoblasts and reticulum cells could be seen in the cortex and paracortical area. Moreover, a pronounced medullary hyperplasia with the production of plasma cell precursors was in evidence.

Between 4 and 10 days after injection, the medullary hyperplasia increased, and plasma cells appeared in large numbers within medullary sinusoids. Germinal follicles appeared in some axillary nodes (Fig. 5).

Animals treated with a single dose of normal rabbit serum showed no lymphocyte depletion. Between 4 and 10 days after injection, medullary hypertrophy, germinal center formation, and plasmacytosis were noted. These changes were slightly less intense than in ALS-treated animals (Figs. 4 and 6).

In both ALS- and NRS-treated animals, the weight of peripheral (axillary)



FIGS. 1*a–b*. Changes in peripheral lymphocyte counts and sizes of lymphoid organs after a single 0.5 ml dose of either unabsorbed ALS, ALS absorbed with mouse erythrocytes, or normal rabbit serum. Fig. 1*a*. Peripheral lymphocyte levels at various times after a single dose of antiserum. Each point represents the mean value of determinations from three animals. Fig. 1*b*. Wet weights of spleen and axillary lymph nodes from the same groups of mice. Each point in the upper curve represents the spleen weight from three animals; each point in the lower curve represents the average weight of a single axillary node (average of 2 axillary nodes from each of three mice).

lymph nodes increased for 6 days after injection, and approached normal by 10 days (Fig. 1*b*).

Spleen: By 48 hr after a single dose of ALS, there was usually a slight increase in spleen weight due to hyperplasia of the red pulp with increased myeloid and erythroid precursor cells. This hyperplasia could be lessened but was not abolished by prior absorption of the antiserum with mouse red cells.

By 96 hr after injection, occasional follicles showed mild depletion of small lymphocytes from periarteriolar areas; this was neither striking nor uniform.

NRS-treated animals showed neither increased splenic erythropoiesis nor lymphoid depletion.

In both ALS- and NRS-treated animals, a moderate follicular hyperplasia of immunoblasts and plasma cells occurred between the 4th and 10th postinjection day, accompanied by a gradual increase in spleen weight (Fig. 1*b*). This had begun to recede at 2 wk postinjection.

Peyer's patch: No lymphoid depletion was seen after ALS. However, many necrotic lymphocytes could be seen within small vessels at 24 and 48 hr after a single dose of ALS (Fig. 8).

Bone marrow: Neither ALS- nor NRS-treated animals showed any consistent change from the usual predominance of myeloid cells. No definite decrease in marrow small lymphocytes was detected in either group.

Thymus: A drop of 10–15% in thymus weight was occasionally noted 4 days or more after a single dose of ALS; no other abnormalities in either thymic architecture or cellularity were noted after a single dose of ALS or NRS. In the ALS-treated animals, the thymus appeared normal despite striking small lymphocyte depletion in other organs (Fig. 9).

Liver, lungs, adrenals, heart, kidneys, and brain were judged grossly and microscopically normal after either ALS or NRS.

Effects of a Short Course of ALS or NRS.—

Hematologic changes: Anemia usually had corrected itself by one or 2 wk after the last dose of a course of antiserum, whether the course consisted of 1, 2, or 3 doses of 0.5 ml. Likewise, lymphocyte counts had reached normal levels by 4 wk after the last injection, whether one, two or three doses of ALS were given initially (Fig. 2). Occasionally, large transitional lymphocytes with basophilic cytoplasm could be seen in peripheral blood smears from either ALS- or NRS-treated animals. No platelet abnormalities were seen in either group.

Lymph nodes: Paracortical depletion, noted after a single dose of ALS, was well established 24 hr after the last of three doses of ALS; it tended to persist for longer periods, being noticeable 2 wk or more after the last dose.

Lymph nodes were grossly enlarged after a short course of either ALS or NRS, because of intense medullary hyperplasia and germinal center formation. The greatly expanded medulla tended to encroach upon and obscure the

boundaries of the paracortical region, so that ALS-induced lymphoid depletion could often not be detected in routine hematoxylin and eosin-stained material. Lymphoid depletion could be easily discerned, however, in sections stained with methyl green-pyronine.

Spleen: After three doses of ALS, the spleen showed striking depletion of small lymphocytes from the periarteriolar regions of all lymphoid follicles; this persisted longer than 2 wk (Fig. 10). In this markedly depleted area, a rim of

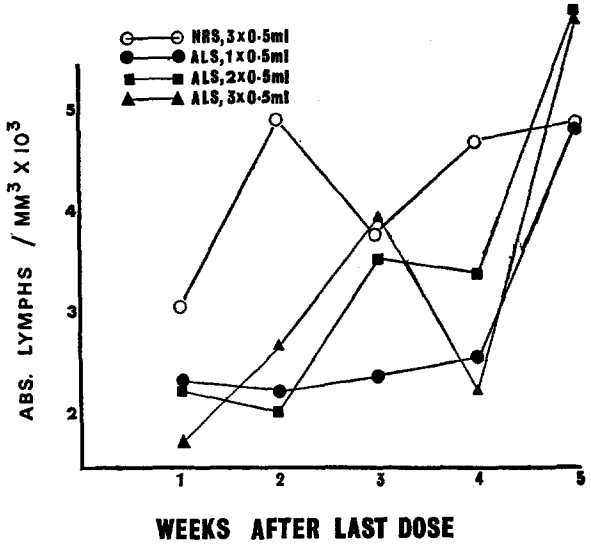


FIG. 2. Recovery of lymphocyte levels after one, two, or three doses of 0.5 ml ALS given at 2-day intervals. Each point represents the mean of determinations of lymphocyte count of 3 or 4 animals. Recovery to initial lymphocyte levels was not complete until 4–5 wk after injection, whether one, two, or three doses had been given.

immunoblasts could be made out surrounding the central arteriole (Fig. 11). No such lesions were observed in NRS-treated animals.

The spleens from both ALS- and NRS-treated animals were enlarged, with generalized follicular hyperplasia with immunoblasts and plasma cells within and at the edge of follicles. Moderate erythroid hyperplasia of the red pulp was present, more intense in the ALS-treated animals.

Bone marrow: After three doses of ALS, moderate erythroid hyperplasia was usually seen in marrow sections and smears. Small lymphocytes were present, although their numbers were somewhat reduced. NRS-treated animals showed no changes in the bone marrow.

The thymus, adrenals, kidneys, liver, lungs, and heart were all morphologically

within normal limits. The absence of either thymic or adrenal atrophy in these animals seems noteworthy.

Skin grafts: The evolution of the homograft reaction was studied in animals that received 0.5 ml of ALS 2 and 5 days after being grafted with A strain tail skin.

In both normal and ALS-treated animals, infiltration of the graft bed with lymphocytes and plasma cells was noticeable by the 5th day after grafting. In untreated animals, this increased until by day +10 the graft was edematous and heavily infiltrated, and showed degeneration of dermal appendage structures; epidermal necrosis invariably occurred by day +12 (Fig. 12).

In the ALS-treated animals, the infiltrate was very sparse, and failed to evolve with the characteristic intensity or perifollicular distribution seen in the usual homograft response. By day 10, only a few lymphocytes and plasma cells were present throughout the basal layers of the dermis, with little tendency to aggregate around hair follicles. Grafts examined at the 14th day (3 days after the median rejection time in untreated recipients) appeared grossly normal, with intact epidermis and no induration. Microscopically, the infiltrate had receded (Fig. 13). Grafts which rejected after 20 to 25 days showed a rapid and heavy accumulation of mononuclear cells in all layers of the dermis and surrounding hair follicles.

Effects of Chronic Treatment with ALS or NRS.—Mice treated for 3–6 months with weekly maintenance doses of ALS or NRS maintained a weight of 26–30 g, and showed no evidence of wasting, overt infections, or tumor formation. This regimen of ALS treatment prolonged (180 days or longer) the survival of skin homografts as long as it was continued (16). Deaths in the chronic treatment group sometimes occurred within a few hours after a serum injection, presumably due to anaphylaxis. After 3 months of treatment, additional mortality was noted and attributed to renal disease (see below under Kidney) (Table I).

Hematologic effects: Anemia was present in all animals treated with ALS throughout the period of treatment; hematocrits generally remained stable at 25–30%. Lymphopenia was a constant feature with lymphocyte values stabilizing at about one-half the normal absolute value; recovery time of the normal lymphocyte number was delayed for at least 1 month after ALS was discontinued. Platelets were present in all blood smears examined; no bleeding tendencies were noted.

Lymph nodes: Striking paracortical depletion was uniformly present in ALS-treated animals after discontinuing ALS; significant depletion was present as long as 1 month later. The cortex appeared intact; as a rule, well-developed germinal centers were noted. Some lymph nodes showed foci of cortical fibrosis after prolonged treatment.

In animals treated chronically with either ALS or NRS, there was striking

medullary hyperplasia with sheets of plasma cells and many eosinophils overrunning the medullary sinusoids.

The *bone marrow* showed marked to moderate erythroid hyperplasia throughout the period of chronic ALS treatment.

The *thymus* was histologically normal after 6 months of treatment with either ALS or NRS. Thymic cortical depletion was noted in several animals who

TABLE I

Some Effects of Prolonged Chronic Administration of 0.5 ml Weekly of Whole Rabbit Anti-Mouse Lymphocyte Serum (ALS) or Normal Rabbit Serum (NRS)

Treatment	Duration	Animal weight	Spleen weight	Absolute lymphocytes	Cause of death
	days	*g*	*mg*	*per mm³*	
ALS, 0.5 ml per wk	75	28	168	—	Sacrificed
subcutaneously	75	25	76	—	"
	92	33.5	210	3500	"
	110	27	150	3500	"
	110	31	219	3300	"
	110	30	153	1900	"
	129	31	148	4400	"
	129	20	110	1400	"
	132	18	110		Renal disease
	140	20	55		"
	140	29	72		"
	142	24	70		"
	156	31	200		"
	160	19	24		"
NRS, 0.5 ml per wk	161	20	160	12,000	Renal disease
subcutaneously	180	23	114	4,800	"
	180	38	105	6,000	"

ALS-treated animals maintained moderate lymphopenia and splenomegaly relative to NRS-treated animals. Both groups eventually developed a wasting syndrome with renal lesions (see text).

developed severe renal disease as a result of treatment with either ALS or NRS.

The *liver, lungs, heart, adrenals, and brain* were all within normal histological limits in both ALS- and NRS-treated groups.

Skin grafts were tolerated for prolonged periods in the ALS-treated group. These showed findings similar to those described after a short course of ALS. With continued treatment, most grafts showed no microscopic evidence of infiltration at all after 3 months of treatment. The lymph node draining a viable graft at the end of 8 wk of chronic ALS treatment showed a combination of marked germinal center formation and moderate paracortical depletion; in

some cases the demarcation of the paracortical areas was obscured by hyperplasia of the adjacent medullary region (Fig. 14).

Kidneys: After 3 months of weekly treatment with 0.25 ml of either ALS or NRS, most animals developed histologic evidence of nephritis, with fibrinoid accumulations within glomeruli and thickening of Bowman's capsule, similar to lesions seen in serum sickness nephritis (Fig. 15). Immunofluorescent stains were negative for rabbit gamma globulin, but markedly positive for mouse gamma globulin, tending to confirm this impression. The nephritic changes in many animals seemed sufficiently advanced to account for the constitutional effects of wasting, ascites, pleural effusion, and death seen in a portion of animals treated with either ALS or NRS.

Effects of the Purified Gamma Globulin (IgG) Fraction of ALS or NRS.—The histological effects of a single dose of ALS IgG were entirely similar to those produced by whole antiserum. Lymphopenia was observed after 6 hr, and marked paracortical depletion after 48 hr. By 6 days, there was some depletion of the paracortical areas and vigorous medullary hyperplasia.

Animals given a single dose of NRS IgG developed neither lymphopenia nor lymphoid depletion, but did develop moderate medullary hyperplasia.

To determine if the depletion of lymphocytes observed after ALS was related to the immunogenicity of ALS IgG (19), a single dose of ALS IgG was administered to recipients 3 wk after they had received a dose of NRS IgG sufficient to induce paralysis. In these animals, lymphoid depletion proceeded as in nonparalyzed recipients. However, less medullary hyperplasia was seen in these animals than in animals given ALS IgG without prior paralysis.

Toxic Effects of Antisera Manufactured Using Adjuvants.—Some antisera were prepared by inoculating donor rabbits with suspensions of murine thymocytes incorporated into adjuvants such as aluminium phosphate or Arquad-2-HT (15). These antisera were highly toxic to recipient mice and required extensive absorption with both red cells and minced tissues before use. Mice given 0.5 ml of one such antiserum without previous absorption (serum 33) died within 10 days with wasting, hunched posture, and ruffled fur.

Numerous pathological lesions were seen in these mice which had no counterpart in those animals given ALS prepared by our routine method. These included: thymic involution with pronounced depletion of cortical thymocytes; cortical as well as paracortical depletion of lymphocytes from lymph nodes; extreme erythroid hyperplasia with four-fold splenomegaly and hepatic foci of extramedullary hematopoiesis; and numerous 0.2–0.5 mm liver microabscesses (Fig. 16).

Histologic Effects of Miscellaneous Antisera.—

Horse anti-mouse lymphocyte globulin: Lymph nodes from recipients of this immunosuppressively weak antiserum showed mild to moderate paracortical

depletion of lymphocytes 48 hr after injection, less pronounced than in animals treated with routine rabbit ALS. By the 6th day after injection, there was intense medullary hyperplasia which completely obscured the boundaries of the paracortical areas.

Duck anti-mouse lymphocyte serum: This antiserum, which was completely ineffective as an immunosuppressant, produced no lymphoid depletion at any time after a single dose. By the 6th postinjection day, some hyperplasia of the medulla was in evidence.

Toxic Effects of High Dose Intravenous ALS.—50% of recipients given 1.0 ml whole unabsorbed ALS by intravenous injection succumbed within 48 hr. At the time of death, all animals showed hemoglobinemia and hemoglobinuria; there was massive hemorrhage from the gastrointestinal tract. Obvious infarcts were present in liver, kidneys, and lungs. Lymphoid tisses showed paracortical and perifollicular depletion, although the thymus did not appear significantly affected.

DISCUSSION

In order to classify better the histological changes which develop after ALS treatment, it is convenient to regard the whole unabsorbed antiserum as a mixture of three categories of substances (*a*, *b*, *c*): (*a*) consists only of that fraction of ALS which is immunosuppressively active, contained within the small fraction (3–5%) of ALS IgG molecules directed specifically against lymphocyte antigens (17, 32, 33); (*b*) consists of other rabbit anti-mouse antibodies, either IgG or IgM, irrelevant to the immunosuppressive activity of ALS, but potentially or actively toxic to the mouse; and (*c*) is a category to which we may assign all other rabbit serum components, non–gamma globulins as well as gamma globulins, which may be immunogenic in mice.

Each category of substances gives rise to a distinctive histologic picture; under the usual conditions of ALS administration all three types of histological changes could occur simultaneously.

Lesions Due to the Immunosuppressive Fraction of ALS.—A consistent pattern of histologic changes was specifically associated with the immunosuppressive capacity of antilymphocytic antiserum. This consisted of peripheral lymphopenia occurring at 48 hr after the first injection of antiserum, selective depletion of small lymphocytes from lymph nodes, paracortical areas and splenic follicular periarteriolar regions, and relative preservation of thymic architecture. All animals bearing skin grafts under the aegis of ALS, whether for 20 or 150 days, showed signs of paracortical and periarteriolar lymphocyte depletion; antisera which were weakly immunosuppressive, such as HALGG, showed a lesser depletive effect. Duck anti-mouse antiserum, totally ineffective at prolonging skin homografts (15) seemed incapable of producing such depletion. Other histologic features of ALS administration discussed more fully below, such as

hemolytic anemia or hyperplasia of lymphoid organs, seemed irrelevant to the immunosuppressive effect and could occur in its absence.

Lymph node changes similar to those we have observed have been described previously by other workers (27, 31), and we concur with them in suggesting that this depletion is one characteristic accompaniment of immunosuppression of transplantation immunity by antilymphocyte serum. Although extensive depletion of the cortical as well as the paracortical area has been achieved with intensive treatment with ALS (8) it appears from our studies that paracortical depletion alone is sufficient to procure indefinitely prolonged immunosuppression.

It seems likely that a rapid depletion of lymphocytes is brought about by the lymphocytotoxic action of ALS; the large numbers of pyknotic nuclei which quickly accumulate in lymphoid tissue capillaries after a single dose of ALS, attest to this. Other evidence obtained from in vivo studies of Cr-51–labeled lymphocytes (30) have established that a single dose of antiserum can destroy a large proportion of such lymphocytes in the recipient.

The restriction of lymphoid tissue depletion to the paracortical and periarteriolar zones is indicative of a preferential action of ALS on the rapidly recirculating lymphocyte pool. These zones, which consist largely of long-lived recirculating cells, can be selectively depleted after maneuvers such as prolonged thoracic duct cannulation (22), extracorporeal irradiation of the blood (4), and neonatal thymectomy (26).

Levey and Medawar (21) suggested that ALS acts in the first instance on peripheral lymphocytes. Our previous observations (30) on the distribution of radioactively-labeled lymphocytes in ALS-treated recipients indicated that ALS removed lymphocytes from that portion of the rapidly recirculating pool of cells that is external to lymph nodes, and that labeled lymphocytes which had already "homed" to lymphoid organs were relatively impervious to the action of ALS. In further support of this view, we have determined by radioautographic studies of lymphoid tissues of ALS-treated mice perfused with intravenous tritiated thymidine, that ALS treatment results in a selective and extensive removal of long-lived small lymphocytes from lymph node paracortical areas (29). Moreover, it has been determined that fluorescein-labeled antilymphocyte globulin penetrated lymphoid tissues poorly (5).

The thymus appeared histologically normal in the vast majority of animals given repeated doses of routine ALS. It seems unlikely that a direct effect on either thymocytes or thymic "humoral factor" is operative in producing the specific histologic accompaniments of immunosuppression by ALS. This does not exclude that a portion of the immunosuppressive action of ALS may be mediated through the thymus, as has been suggested by some workers (27, 28). The fact that the characteristic lesions are found in those areas of lymphoid tissue designated as "thymus-dependent" (26) implies to us only that these

areas reflect the state of the total recirculating lymphocyte compartment with which they are in equilibrium.

The histological course of skin allograft rejection under ALS treatment is of interest. The tendency of lymphoid infiltration to decrease and finally disappear after chronic, prolonged treatment may herald a state resembling specific tolerance in a portion of chronically treated, skin-allografted animals. Recent observations have indicated that ALS treatment when combined with thymectomy (24) or administration of additional antigen[1] may predispose to the acquisition of specific immunological tolerance.

Lesions Due to "Irrelevant" Antibodies.—Medawar (23) has pointed out that ALS must consist of a mixture of antibodies directed against numerous cellular antigens, most of which are irrelevant to its desired immunosuppressive activity. Among the identified but irrelevant antibodies which may be generated concomitantly with ALS are included 7S and 19S hemagglutinins and hemolysins (1), 19S anti-leukocyte activity (13), and anti-mouse gamma globulins (8); none of these has the capacity to prolong skin allograft survival.

It is surprising that more in the way of side effects due to irrelevant antibodies did not occur in our experimental animals. The most prominent toxic effect produced was that of hemolytic anemia, in severe instances accompanied by hemoglobinemia and hemoglobinuria. Bone marrow and splenic erythroid hyperplasia occurred after treatment even with ALS that had been absorbed with red cells, although no anemia developed, making it likely that even absorbed ALS possesses some, albeit controllable anti-red cell activity.

Antisera manufactured with the use of aluminium phosphate or Arquar incorporated into the immunizing cell suspensions were considerably more toxic than routinely prepared sera, and it is possible that adjuvant immunization increased the amount of irrelevant antibodies present. Apart from an intense hemolytic anemia, accompanied by extreme erythroid metaplasia in the spleen, marrow, and even the liver, widespread and variegated lesions were seen in other organs:

The thymus was usually depleted of cortical lymphocytes after two injections of such antiserum. Denman and Frenkel (5) and Lance (17) have determined that ALS does not enter the thymus to any appreciable extent. It thus seems likely to us that this depletion is brought about by a generalized stress response rather than by any direct effect of ALS on thymic tissue. The generalized lymph node depletion seen in these mice also reflects a process qualitatively different from that obtaining with our usual antisera, and one that is unnecessary for allograft prolongation.

The punctate lesions seen in the liver of these animals may represent invasion of pathogens as a result of lowered host resistance, as in the HMV infections

[1] Lance, E. M., and P. B. Medawar. Data to be published.

described in thymectomized mice (6). We have not, however, excluded that they are due to a directly toxic effect of the antiserum on liver tissue.

The administration of large intravenous doses of routine ALS results in marked damage to erythrocytes and platelets, and widespread hemorrhages and infarction in many organs. It seems possible that irrelevant and potentially toxic antibodies are usually present in even our routine antiserum, but are absorbed onto tissue receptor sites when the antiserum is injected subcutaneously.

Lesions Due to Immunization by Immunogenic Components of ALS.—In animals given either ALS or NRS, the histologic features of immunization develop. These consist of hypertrophy of lymphoid tissue with pronounced medullary hyperplasia and germinal center formation in lymph nodes, and follicular hyperplasia in spleens. Blast forms and plasma cells are prominent in these regions. In some animals, eosinophilia in peripheral blood and lymph nodes may develop as in genuine serum sickness.

The medullary hyperplasia which occurs in lymph nodes during the period when cellular hypersensitivity has been virtually abolished by ALS treatment is of particular interest. It confirms the unique capacity of ALS to discriminate between humoral and cellular responses, and selectively to suppress the latter, as noted by Lance and Batchelor (18). It also provides additional evidence that the suppressive effects of ALS are exerted preferentially on that population of cells which mediates cellular immunity. Although several investigators have noted the ability of ALS to transform lymphocytes to immunoblasts in vitro (7, 9, 11, 33), there is little in our studies to suggest that lymphocyte activation by means other than antigenic stimulation takes place in vivo. Medullary proliferation with blast transformation takes place slightly more vigorously in ALS than NRS-treated animals, doubtless due to the greater immunogenicity of ALS IgG (19). Previous immunization of animals with NRS IgG can markedly augment this transformation, while prior paralysis to NRS IgG can significantly inhibit it. It thus seems that these changes are due to an orthodox immune response to rabbit serum constituents, and are not directly relevant to the immunosuppressive action of ALS.

In chronically treated animals, "complex" nephritis may develop as a result of hyperimmunization to rabbit serum components. Similar lesions have been noted by others in other species (10, 12). Lance and Dresser's finding that paralysis of ALS recipients with NRS IgG abolishes a subsequent immune response to purified ALS gamma G (19) may provide a means for circumventing this hazard. Our present findings in animals tolerant of NRS IgG would support this consideration.

SUMMARY

The effects of heterologous rabbit anti-mouse lymphocyte antiserum on the morphology of lymphoid and other tissues was investigated in CBA mice.

The lymphoid tissues exhibited characteristic changes specific for ALS treatment, which were an invariable accompaniment to its immunosuppressive effects. These consisted of peripheral lymphopenia occurring at some time during a course of ALS treatment and persistent depletion of small lymphocytes in lymph node paracortical areas and splenic follicular periarteriolar zones. The thymic histology was generally well preserved.

It is suggested that the relevant lesions reflect a rapid depletion of the pool of recirculating lymphocytes, possibly by a primary cytotoxic effect exerted on cells peripheral to lymphoid tissue.

Other histologic features attendant to the administration of ALS were accounted for as consequences of immunization of ALS recipients to rabbit serum constituents or by the deleterious effects of antibodies directed against tissues other than lymphoid cells.

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BIBLIOGRAPHY

1. Abaza, H. M., and M. F. A. Woodruff. 1966. *In vitro* assay of antilymphocytic serum. *Rev. Franc. Etudes. Clin. Biol.* **2**:821.
2. Balner, H., and H. Dersjant. 1967. Effects of antilymphocyte sera in primates. *In* Antilymphocytic Serum. *Ciba Found. Study Group, 29*. G. E. W. Wolstenholme and M. O'Connor, editors. Churchill. London.
3. Billingham, R. E., and P. B. Medawar. 1951. The technique of free skin grafting in mammals. *J. Exptl. Biol.* **28**:385.
4. Cronkite, E. P., C. R. Jansen, H. Cottier, K. Rai, and C. R. Sipe. 1964. Lymphocyte production measured by extracorporeal irradiation, cannulation, and labelling techniques. *Ann. N. Y. Acad. Sci.* **113**:566.
5. Denman, A. M., and E. P. Frenkel. 1968. Mode of action of antilymphocyte globulin. I. The distribution of rabbit antilymphocyte globulin injected into rats and mice. *Immunology.* **14**:107.
6. East, J., D. M. V. Parrott, F. C. Chesterman, and A. Pomerance. 1963. The appearance of a hepatotrophic virus in mice thymectomized at birth. *J. Expil. Med.* **118**:1069.
7. Grasbeck, R., C. M. Nordman, and A. de la Chapelle. 1963. Mitogenic action of antileukocyte immune serum on peripheral leukocytes *in vitro*. *Lancet.* **2**:385.
8. Gray, J. G., A. P. Monaco, M. L. Wood, and P. S. Russell. 1966. Studies on heterologous antilymphocyte serum in mice. I. *In vitro* and *in vivo* properties. *J. Immunol.* **96**:217.
9. Greaves, M. F., I. M. Roitt, R. Zamir, and R. B. A. Carnaghan. 1967. Effect of

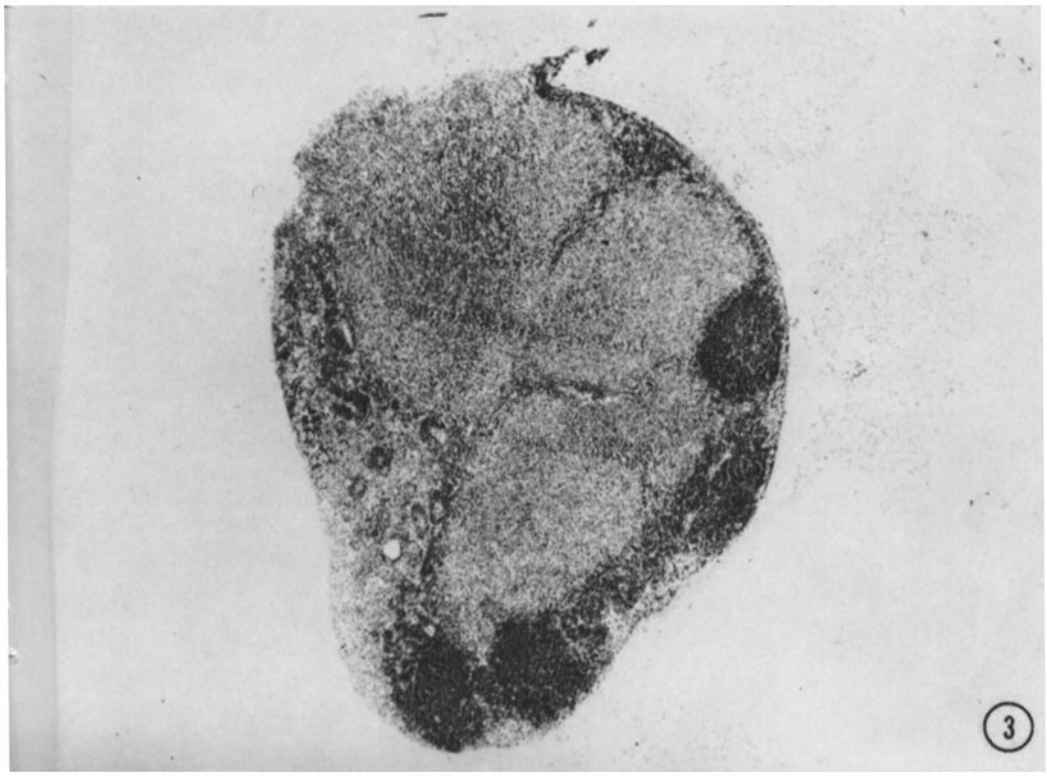
anti-lymphocyte serum on responses of human peripheral-blood lymphocytes to specific and non-specific stimulants *in vitro*. *Lancet*. **2**:7530.

10. Guttmann, R. D., C. B. Carpenter, R. R. Lindquist, and J. P. Merrill. 1967. Renal transplantation in the inbred rat. III. A study of heterologous antithymocyte sera. *J. Exptl. Med.* **126**:1099.
11. Holt, L. J., N. R. Ling, and D. R. Stanworth. 1966. The effect of heterologous antisera and rheumatoid factor on the synthesis of DNA and protein by human peripheral lymphocytes. *Immunochemistry*. **3**:359.
12. Iwasaki, Y., K. A. Porter, J. R. Amend, T. H. Marchioro, V. Zuhlke, and T. E. Starzl. 1967. The preparation and testing of horse anti-dog and anti-human antilymphoid plasma or serum and its protein fractions. *Surg. Gynecol. Obstet.* **124**:1.
13. James, K., and P. B. Medawar. 1967. Characterization of anti-lymphocyte antibody. *Nature*. **214**:1052.
14. Jeejeebhoy, H. F. 1964. Studies on the mode of action of heterologous antilymphocyte plasma. I. A comparison of the immunosuppressive properties of dog and rabbit anti-rat lymphocyte plasma. *Transplantation*. **5**:273.
15. Jooste, S. V., E. M. Lance, R. H. Levey, P. B. Medawar, M. Ruszkiewicz, R. Sharman, and R. N. Taub. 1968. Notes on the preparation and assay of antiphocytic serum for use in mice. *Immunology*. In press.
16. Lance, E. M. 1967. The effects of chronic ALS administration in mice. *In* Advance in Transplantation. J. Dausset, J. Hamburger, and G. Mathe, editors. Munksgaard, Copenhagen. 108.
17. Lance, E. M. 1968. Experimental observations bearing on the clinical use of ALS. *In* The Immune Response and its Suppression. E. Sorkin, editor. S. Karger, Basel. In press.
18. Lance, E. M., and R. Batchelor. 1968. Selective suppression of cellular immunity by antilymphocyte serum. *Transplantation*. **6**:490.
19. Lance, E. M., and D. W. Dresser. 1967. Antigenicity in mice of antilymphocyte gamma globulin. *Nature*. **215**:488.
20. Levey, R. H., and P. B. Medawar. 1966. Some experiments on the action of antilymphoid antisera. *Ann. N. Y. Acad. Sci.* **129**:164.
21. Levey, R. H., and P. B. Medawar. 1967. Further experiments on the action of antilymphocytic antiserum. *Proc. Natl. Acad. Sci.* **57**:470.
22. McGregor, D. D., and J. L. Gowans. 1963. The antibody responses of rats depleted of lymphocytes by chronic drainage from the thoracic duct. *J. Exptl. Med.* **117**: 303.
23. Medawar, P. B. 1967. Biological effects of heterologus antilymphocyte antisera. *In* Human Transplantation. F. T. Rapaport and J. Dausset, editors. Grune and Stratton, New York. 501.
24. Monaco, A. P., M. L. Wood, and P. S. Russell. 1966. Studies on heterologous antilymphocyte serum in mice. III. Immunologic tolerance and chimerism produced across the H-2 locus with adult thymectomy and antilymphocyte serum. *Ann. N. Y. Acad. Sci.* **129**:190.
25. Nagaya, H., and H. O. Sieker. 1965. Allograft survival; effect of antiserums to thymus glands and lymphocytes. *Science*. **150**:1181.

26. Parrott, D. M. V., M. A. B. De Sousa, and J. East. 1966. Thymus-dependent areas in the lymphoid organs of neonatally thymectomized mice. *J. Exptl. Med.* **123**:191.
27. Parrott, D. M. V. 1967. The response of draining lymph nodes to immunological stimulation in intact and thymectomized animals. *In* Symposium on Tissue and Organ Transplantation. *J. Clin. Pathol.* **20**:456.
28. Russe, H. P., and A. J. Crowle. 1965. A comparison of thymectomized and anti-thymocyte serum-treated mice in their development of hypersensitivity to protein antigens. *J. Immunol.* **94**:74.
29. Taub, R. N. 1968. Lymphocyte kinetics and lymphoid tissue morphology accompanying immunosuppression by antilymphocyte serum (ALS). *In* The Immune Response and its Suppression. E. Sorkin, editor. *Antibiot. Chemotherapia.* In press.
30. Taub, R. N., and E. M. Lance. 1968. Effects of heterologous antilymphocyte serum on the distribution of Cr-51 labelled lymph node cells in mice. *Immunology.* **15**:633.
31. Turk, J. L., and D. A. Willoughby. 1967. Central and peripheral effects of antilymphocyte sera. *Lancet.* **1**:249.
32. Woodruff, M. F. A. 1968. Purification of antilymphocytic antibody. *Nature.* **217**: 821.
33. Woodruff, M. F. A., B. Reid, and K. James. 1967. Effect of antilymphocytic antibody and antibody fragments on human lymphocytes *in vitro*. *Nature.* **215**:591.
34. Woodruff, M. F. A., and N. F. Anderson. 1963. Effect of lymphocyte depletion by thoracic duct fistula and administration of antilymphocytic serum on the survival of skin homografts in rats. *Nature.* **200**:702.

FIG. 3. Brachial lymph node of CBA mouse 48 hr after a single injection of 0.5 ml rabbit anit-mouse lymphocyte serum. There is striking depletion of lymphocytes from the paracortical areas, with relative sparing of the cortical and primary follicular zones. Methyl green–pyronine. × 52.

FIG. 4. Brachial lymph node 48 hr after a single dose of normal rabbit serum. The paracortical areas show no significant depletion. Methyl green–pyronine. × 52.



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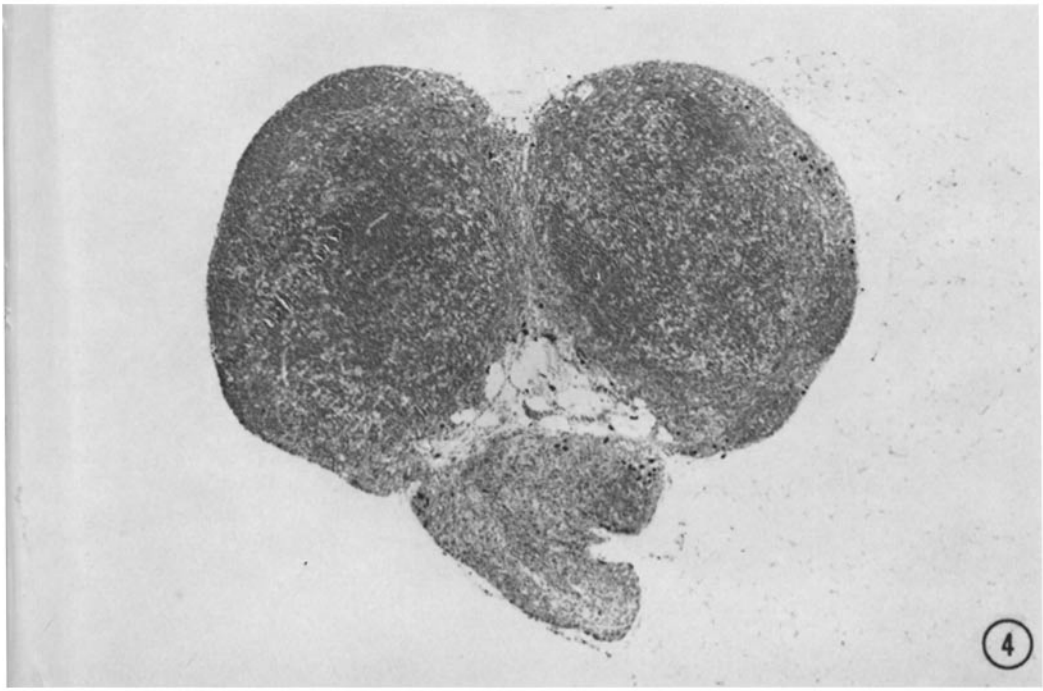

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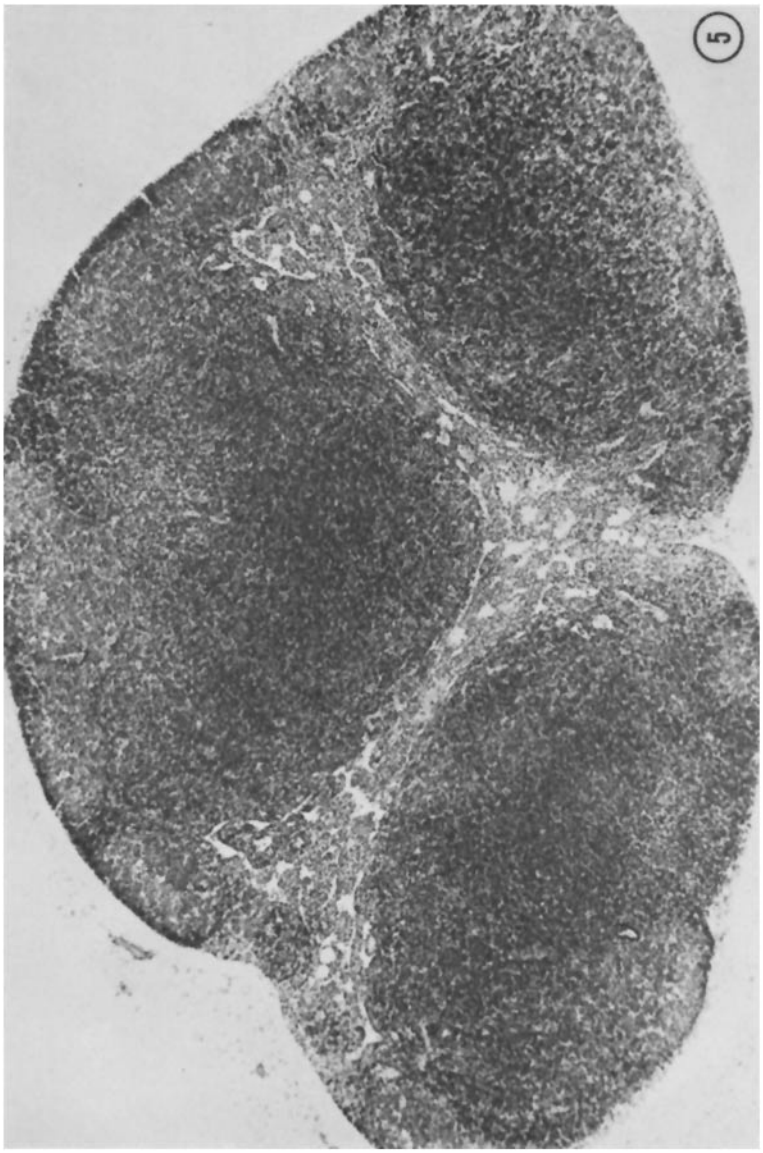
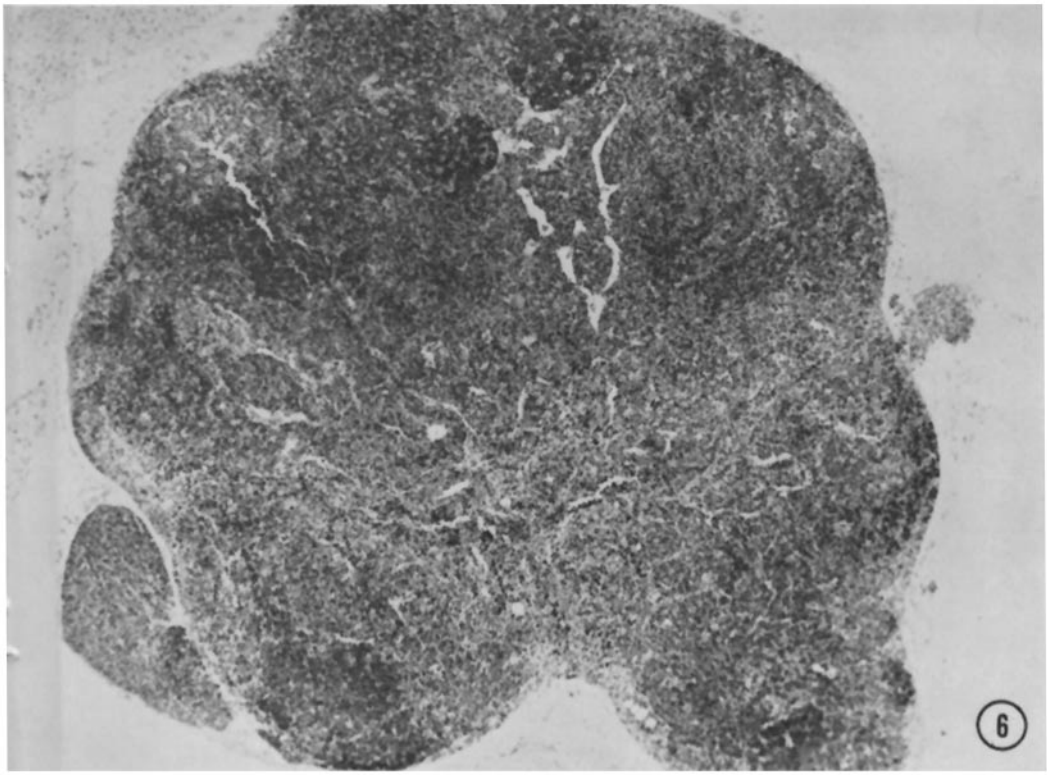


FIG. 5. Brachial lymph node 8 days after a single dose of 0.5 ml ALS. The entire node is enlarged (same magnification as Fig. 1). Paracortical depletion is still discernible; there is marked medullary hyperplasia with large numbers of plasma cells within sinusoids; some of these cells have infiltrated into the paracortical regions. Hematoxylin and eosin. × 52.

FIG. 6. Brachial lymph node 8 days after injection of normal rabbit serum. Note the enlargement and medullary hypertrophy as in Fig. 5 However, the paracortical areas show neither depletion nor infiltration by medullary cells. Hematoxylin and eosin. × 52.

FIG. 7. Axillary lymph node 24 hr after a single subcutaneous dose of ALS. Note cellular debris and pyknotic nuclei within small vessels in the cortical and paracortical areas. There is a striking reduction in numbers of small lymphocytes, the cells in the paracortical areas, leaving mostly large lymphocytes, immunoblasts, and reticulum cells. Methyl green-pyronine. × 256.



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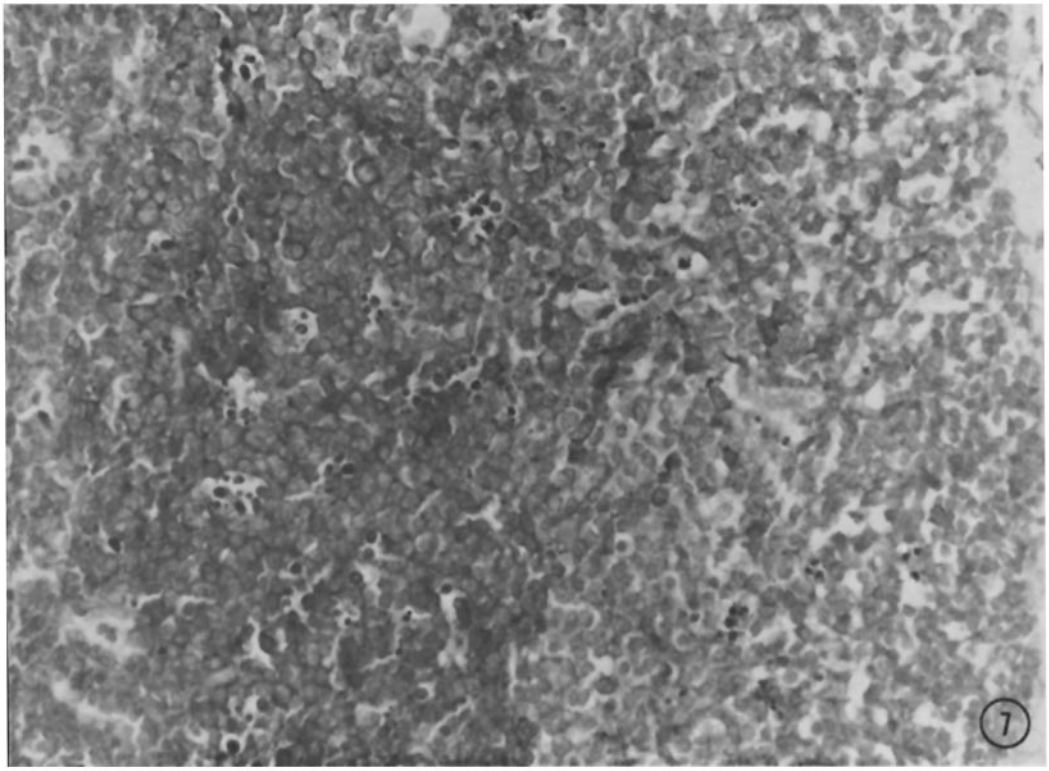

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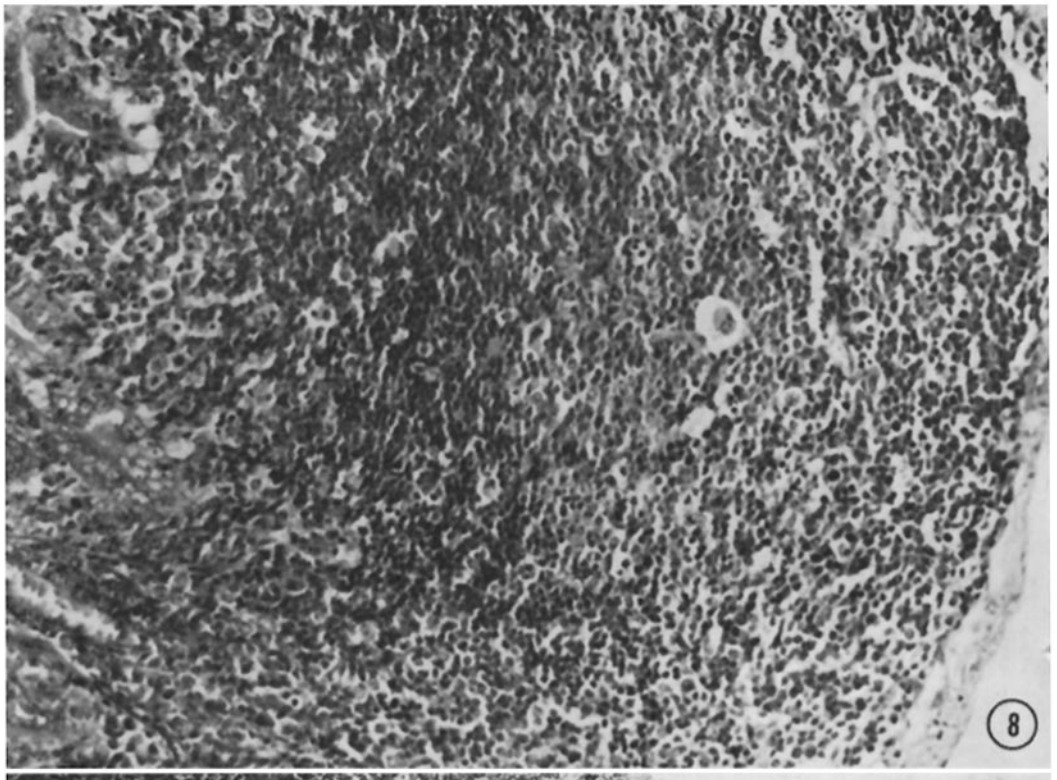
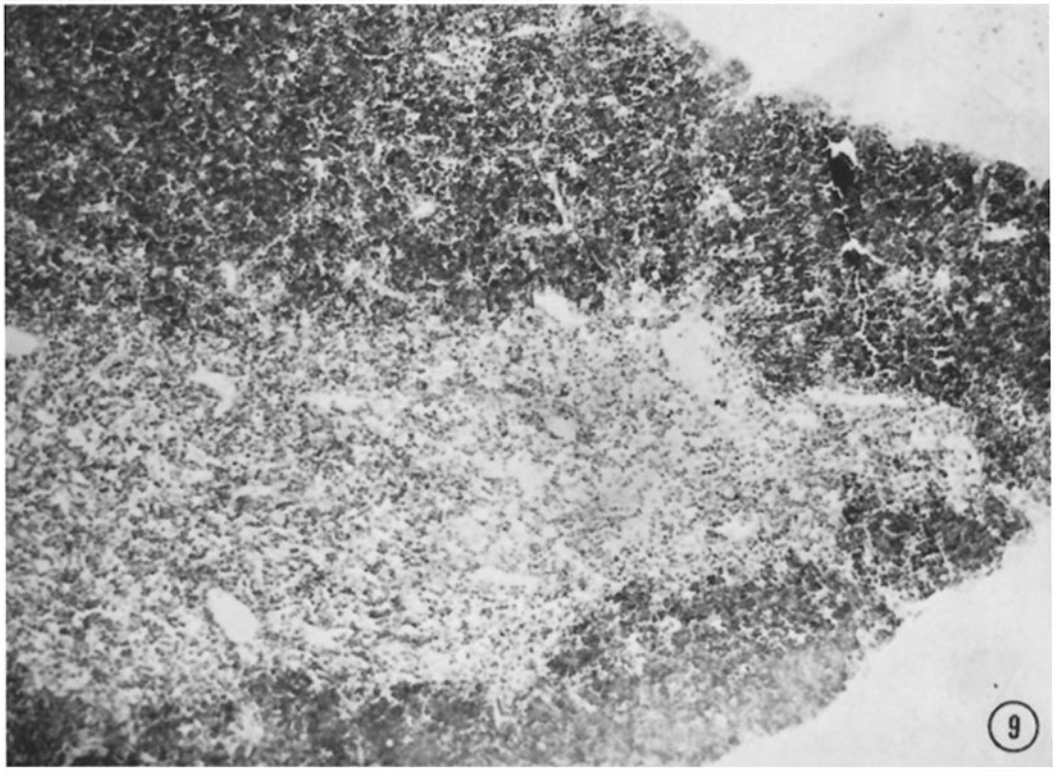




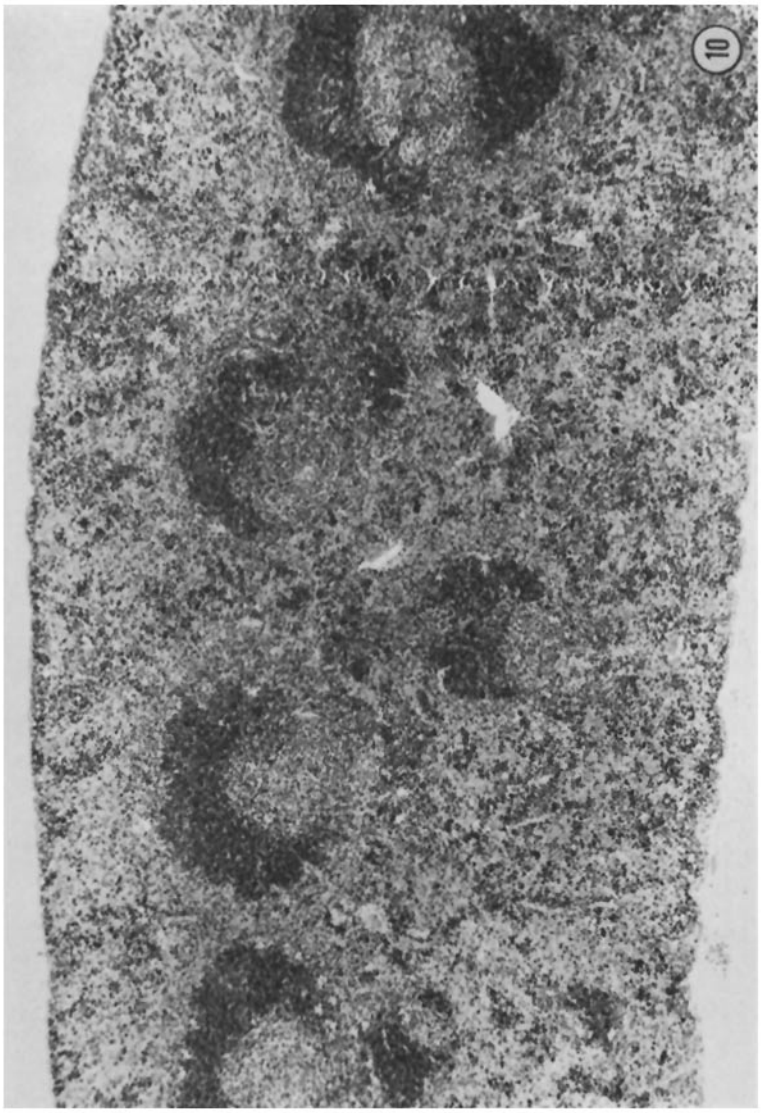


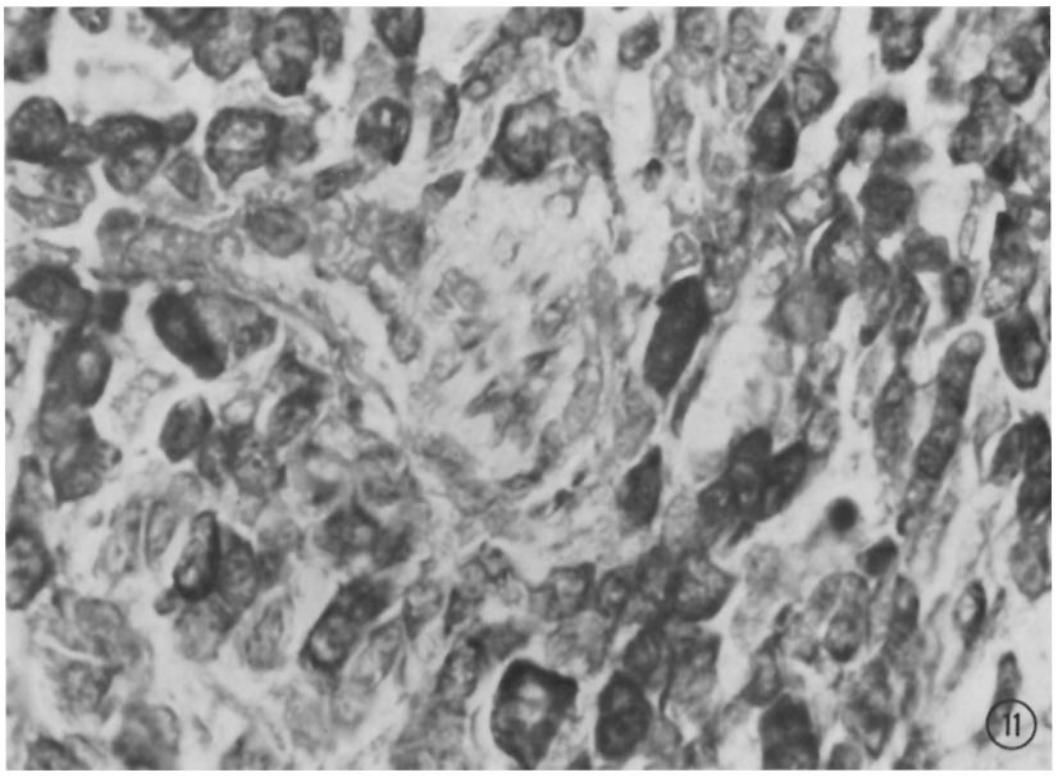
FIG. 10. CBA spleen 1 wk after the last of three subcutaneous doses of 0.5 ml RAMLS administered at 2-day intervals. Note the "punched-out" lesions, indicating depletion of small lymphocytes from peri-arteriolar regions of lymphoid follicles. Hematoxylin and eosin. × 52.

FIG. 8. Intestinal Peyer's patch 48 hr after a single dose of ALS. Only minimal lymphocyte depletion is present. However, many necrotic cells can be seen within small vessels. Methyl green–pyronine. × 132.

FIG. 9. Thymus 4 days after a single dose of ALS. No consistent abnormality in either thymic architecture or cellularity is noted. Hematoxylin and eosin. × 132.

FIG. 11. Higher power view of periarteriolar region. Note the persistence of immunoblasts surrounding the central arteriole. × 640.

FIG. 12. A strain tail skin homograft on CBA recipient, 12 days after grafting. There is edema, heavy mononuclear infiltration, and epidermal necrosis, typical of graft rejection. Hematoxylin and eosin. × 96.



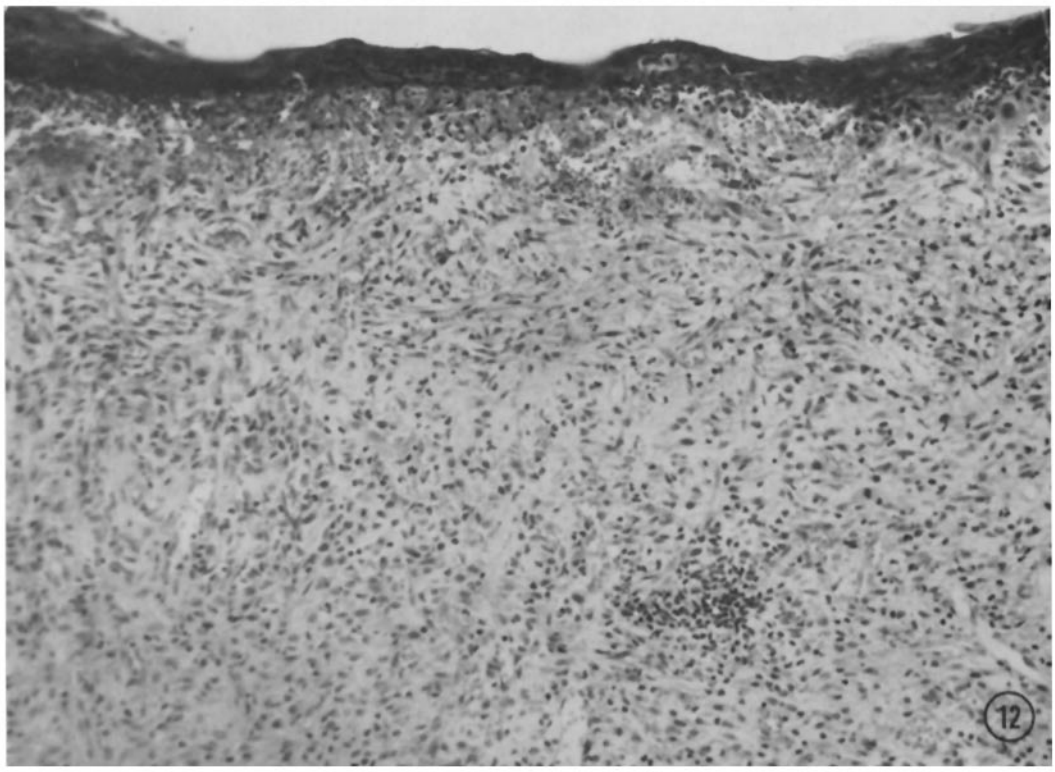
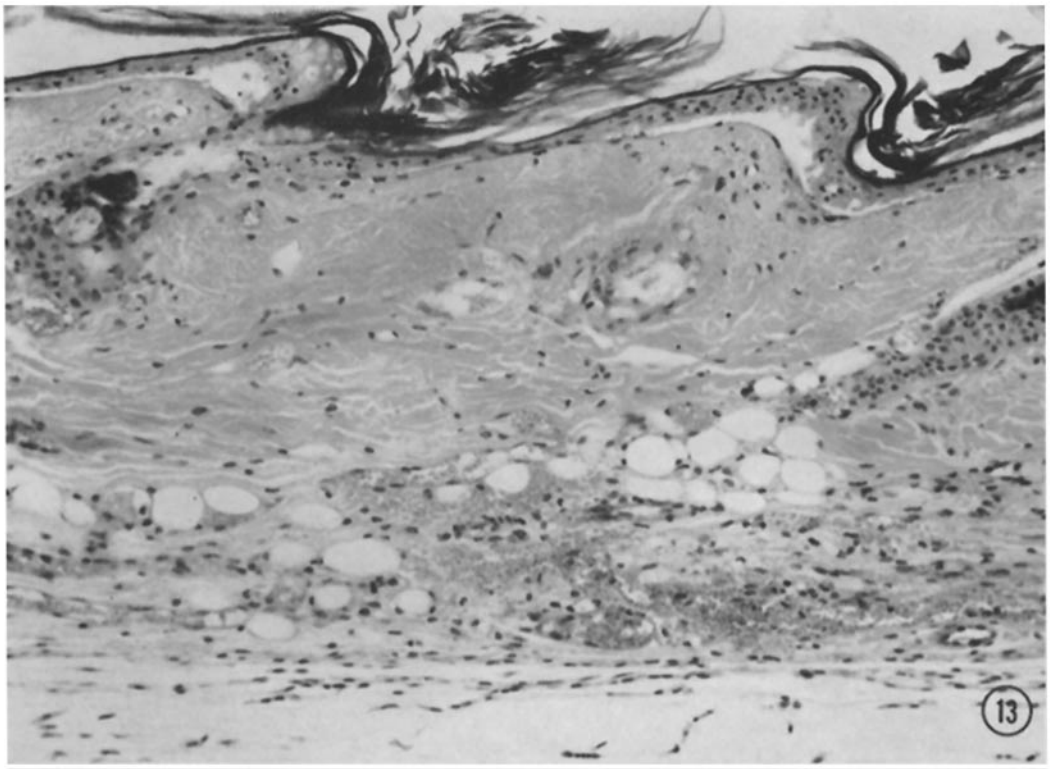


FIG. 13. A strain graft on CBA recipient that received 0.5 ml ALS on the 2nd and 5th days after grafting. 13 days after grafting, the epidermis is intact with no edema and minimal infiltrate. Hematoxylin and eosin. × 96.

FIG. 14. Axillary lymph node draining a viable A strain skin allograft after 8 wk of chronic ALS treatment. Note paracortical depletion, somewhat obscured in outline by hyperplasia of adjacent medullary tissue. Note formation of germinal centers. Hematoxylin and eosin. × 52.



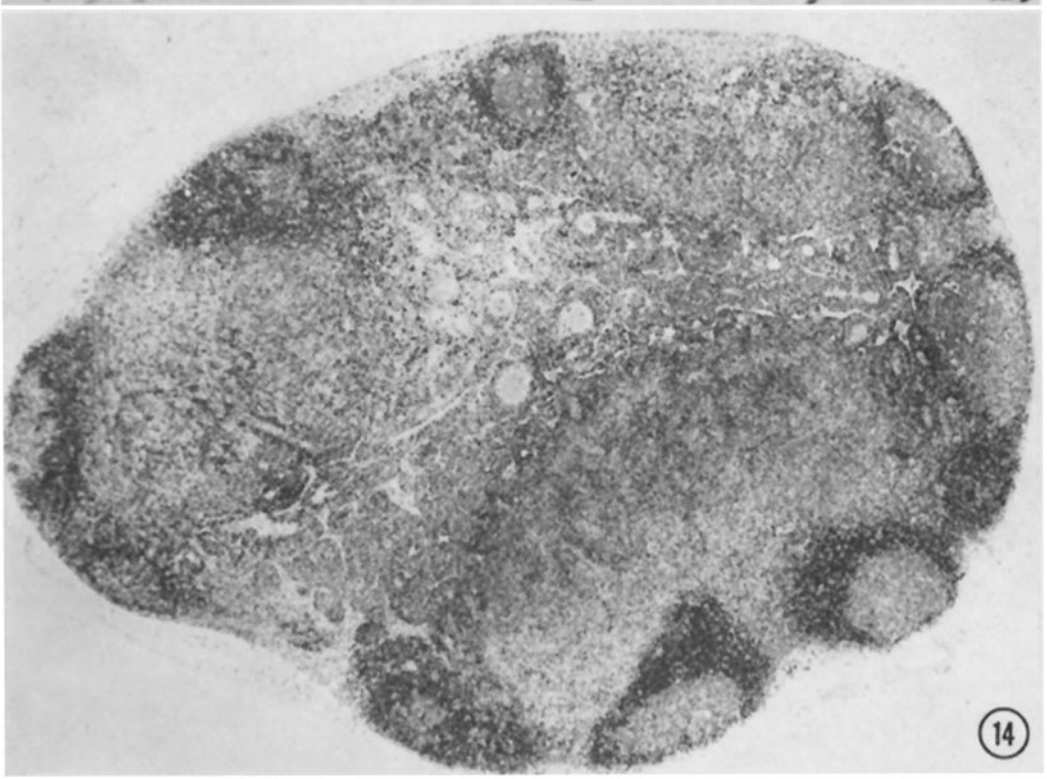


FIG. 15. Glomerular alterations seen as a result of either ALS or NRS given twice weekly for 3 months. There is a thickening of Bowman's capsule and fibrinoid deposition within glomerular capillaries. The insert shows an immunofluorescent stain demonstrating the presence of mouse gamma globulin within the fibrinoid deposits. Hematoxylin and eosin. × 291.

FIG. 16. Focal hepatic necrosis with microabscess formation produced by a single dose of adjunct antiserum. Hematoxylin and eosin. × 256.