Depression of Phagocytic Activity of the Reticuloendothelial System by Antilymphocytic Serum[1] (34250)

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The role of the phagocyte in the removal of invasive microorganisms has been termed "native resistance" (1). Concomitant with this function, the macrophage population of the reticuloendothelial system (RES), has been shown to be intimately involved in humoral immune processes, such as antibody production (2) and the development of tolerance (3).

In addition to the role of the RES in "native resistance" and humoral antibody formation, it was demonstrated that the RES plays a regulatory role in transplantation rejections. Di Luzio (4) showed that stimulation or depression of RES function in donor animals can cause a significant corresponding alteration of the development of graft-versus-host reactions. In addition, methyl palmitate-induced RES depression was shown to diminish the ability of recipient animals to reject tumor transplants (5) and bone marrow transplants (6). In contrast, glucan-induced stimulation of the RES was associated with enhanced rejection of both tumor and bone marrow transplants (5, 6).

In addition to its role in the immunology of transplantation, the RES is probably involved in such posttransplant complications as infections, which have been associated with up to 30% of renal transplantation failures (7), and tumor metastases (8). The increased susceptibility to infection, and possibly neoplasia, following transplantation may be a result of an impaired activity of the RES of the recipient induced by the employed immunosuppressive agents.

One such immunosuppressive agent that is being used with increasing frequency in transplantation is antilymphocytic serum (ALS) (8–10). While studies have attempted to elucidate the mode of action of ALS (11–13), there remains little agreement as to the mechanisms involved.

Since RES depression greatly influences the immune response (14) to particulate antigens and the vascular clearance and tissue distribution of particulate antigens, the immunosuppressive activity of ALS may be mediated, in part, by inducing altered activity of the macrophage cells. A modification of macrophage activity by ALS may influence antibody production during its initial phases, namely the macrophage recognition mechanism or the macrophage-processing of the antigen. The ALS-induced depression of the RES may be a direct action of ALS upon RE cells (15), or a depletion of plasma opsonic activity, as was shown to be the mechanism of "RES blockade" (16–18).

The present study was undertaken to define the influence of ALS upon phagocytosis, and subsequently, to determine the mechanism of ALS-induced depression of the RES. The functional impairment of the RES by ALS may well contribute to certain of the immunological effects observed with ALS administration.

Methods. In the preparation of ALS, adult rabbits received four weekly intraperitoneal injections of 2×10^8 rat (Sprague-Dawley) thymocytes suspended in Tyrode's solution. Ten days later the rabbits were bled from the marginal ear vein and the serum was heat-inactivated at 56° for 45 min. The rabbit serum was titered by adding equal volumes of sequential dilutions of rabbit serum, test thymocyte cell suspension (about 4×10^6 cells/ml), and fresh guinea pig serum to ag-

[1] Supported in part by Atomic Energy Commission, USPHS grant HE 12573-01 and the Cancer Association of Greater New Orleans, Inc. (Grant No. 125-68).

glutination tubes. The cell–serum suspension was incubated for 2 hr at 37°, and thymocyte viability was ascertained by utilizing the trypan blue exclusion test. If the resulting ALS titer (LD_{50}) was less that 1:32, the rabbits were given another injection of thymocytes, rested 4 days and bled. Average titer attained was approximately 1:40 (range, 1:32–1:128). The above technique for ALS titration was also employed for titration of the cytotoxic effects of ALS upon isolated Kupffer cells from rat liver (19).

Normal or control rabbit serum (NRS) was obtained by injecting rabbits with Tyrode's solution isovolumetrically with those rabbits used for ALS preparation. These animals never demonstrated a lymphocytotoxicity titer greater than 1:2.

In all experiments male rats, weighing between 250 and 300 g, received a single injection of either 1 ml of ALS or 1 ml of NRS prior to evaluation of RES functional activity. The test sera were injected either intravenously or intraperitoneally.

The phagocytic activity of the RES was evaluated by determining the intravascular clearance rates of the gelatinized "RE test lipid emulsion" labeled with ^{131}I-triolein prepared and utilized according to the method of Di Luzio and Riggi (20) and Saba *et al.* (21). The rats were injected intravenously with the lipid emulsion at a dosage of 50 mg/100 g of body weight and tail blood samples were obtained every 2 min for 10 min and intravascular clearance rates were determined (14, 17, 20, 21). Liver, lung, and spleen samples, obtained 10 min postinjection, and blood samples were assessed for radioactivity with a Nuclear Chicago Auto-Gamma scintillation system. Results are expressed as percentage of the injected ^{131}I-labeled gelatinized RE test emulsion per gm of organ weight (%ID/g) or percentage of injected dose per total organ (%ID/TO).

The *in vitro* techniques employed for assay of liver macrophage phagocytic activity and the evaluation of serum opsonic activity have been previously described (17, 21). Briefly, in all *in vitro* experiments 100 units of heparin, 2 mg of gelatinized RE test lipid emulsion, and liver slices weighing approximately 250 mg were added to the incubation media which consisted of either 3 ml of serum of Krebs–Ringer–phosphate buffer (pH 7.4). All flasks were gassed with 95% oxygen and 5% carbon dioxide, then incubated for 30 min at 37° on a Dubnoff metabolic shaker. The amount of lipid emulsion phagocytized by the Kupffer cells of the liver slices was determined with the Nuclear Chicago Auto-Gamma scintillation system. Results are expressed as the percentage of added ^{131}I-gelatinized RE test lipid emulsion phagocytized per 100 mg of liver tissue (%ID/100 mg).

Results. The ability of ALS to significantly alter RES functional activity was dependent upon the route of administration. Intravenous administration of ALS induced no significant effects upon either intravascular clearance rates or hepatic localization of gelatinized "RE test lipid emulsion" either 4 or 24 hr after ALS administration (Table I). However, 24 hr after ALS administration, splenic uptake was significantly depressed and the half-time for intravascular clearance manifested a tendency towards the depressed state.

The intraperitoneal injection of ALS caused a profound depression in phagocytic function (Table II). At both 4- and 24-hr post intraperitoneal-ALS administration, the vascular clearance of the lipid emulsion was significantly impaired, the degree of impairment, when compared to control values, was 63%. Hepatic localization of the lipid emulsion had decreased 44 and 33%, 4- and 24-hr post-ALS administration, respectively. No significant alteration of pulmonary or splenic phagocytic activity was noted in rats treated with either NRS or ALS.

In an attempt to elucidate the site of ALS-induced RES depression, both Kupffer cell phagocytic activity and serum opsonic activity were evaluated 4 hr after the intraperitoneal administration of ALS (Tables III and IV). In assessing serum opsonic activity, liver slices were obtained from normal (uninjected) rats and incubated with either buffer, or serum, which was obtained from either normal rats, ALS-treated rats, or rats which received normal rabbit serum. The ability of

TABLE I. Effect of Intravenously Injected Rabbit Antirat Lymphocyte Serum on Intravascular Clearance and Tissue Distribution of Gelatinized "RE Test Lipid Emulsion" in Rats.[a]

Treatment	No. of animals	Time[b] (hr)	Intravascular $t/2$ (min)	Organ uptake					
				Liver		Lung		Spleen	
				(% ID/g)	(% ID/TO)	(% ID/g)	(% ID/TO)	(% ID/g)	(% ID/TO)
None	8	—	12.1 ± 1.3	3.5 ± 0.3	42.5 ± 2.0	0.6 ± 0.02	0.8 ± 0.02	6.1 ± 0.7	4.4 ± 0.6
NRS[c]	6	4	16.6 ± 4.6	3.9 ± 0.4	49.7 ± 5.7	0.9 ± 0.04	1.4 ± 0.03	5.9 ± 0.4	4.7 ± 0.4
ALS[d]	7	4	13.9 ± 1.0	3.0 ± 0.1	32.3 ± 2.5	0.9 ± 0.03	1.4 ± 0.02	4.8 ± 0.7	4.1 ± 0.4
ALS	8	24	22.0 ± 4.1	4.4 ± 0.8	49.6 ± 7.4	0.8 ± 0.02	1.2 ± 0.02	3.2 ± 0.8[e]	2.9 ± 0.5[e]

[a] Values expressed as mean ± SEM.
[b] Hours postinjection of 1 ml of control and experimental test serum.
[c] NRS = normal rabbit serum.
[d] ALS = rabbit antirat lymphocyte serum.
[e] Significant ($p < 0.05$) as compared to NRS-injected controls.

the various incubation media to enhance phagocytic uptake of gelatinized "RE test lipid emulsion" by the liver slices was quantitated (Table III).

The incubation of normal rat liver slices in the presence of Krebs–Ringer–phosphate buffer was associated with minimal uptake of gelatinized RE test lipid emulsion. The presence of normal rat serum induced a mean 10-fold increase in Kupffer cell phagocytosis. This finding is in accord with previously published data (16, 17, 19). The employment of serum obtained from ALS-treated rats resulted in a comparable degree of phagocytosis, denoting the maintenance of opsonic activity in ALS-treated rats.

In an effort to assess the influence of ALS upon hepatic phagocytic activity, liver slices derived from either untreated, NRS-treated, or ALS-treated rats were incubated in normal rat serum (Table IV) to which the gelatinized RE test lipid emulsion was added and the degree of phagocytosis quantitated. Liver slices derived from normal or NRS-treated rats removed 21% of the added lipid emulsion per 100 mg of tissue. In contrast, liver slices from ALS-treated animals demonstrated a decrease of 43% in uptake. This finding suggests that the depressive effects of acute ALS administration upon phagocytosis is dependent upon an ALS-induced alteration of the Kupffer cell and not upon any humoral or opsonic factor involved in phagocytosis. The cytotoxicity of ALS on Kupffer cells was determined to evaluate the validity of this hypothesis (Fig. 1). Isolated rat Kupffer cells were incubated with guinea pig serum as a source of complement, and various dilutions of either NRS or ALS. The incubations were conducted for 2 hr. After incubation, the trypan blue exclusion test as described for titration of lymphocytotoxic titer of ALS was used as an indication of cellular viability. As shown in Fig. 1, in contrast to NRS, ALS had a definite killing effect upon Kupffer cells. The LD_{50} for Kupffer cells, which was 1:16, was significantly less than the LD_{50} of ALS on thymocytes, however, ALS clearly exerts a cytotoxic effect upon Kupffer cells.

Discussion. Whereas, the intraperitoneal

TABLE II. Depressant Effect of Intraperitoneally Injected Rabbit Antirat Lymphocytic Serum on the Intravascular Clearance and Tissue Distribution of Gelatinized "RE Test Lipid Emulsion."[a]

Treatment	No. of animals	Time[b] (hr)	Intravascular $t/2$ (min)	Organ uptake					
				Liver		Lung		Spleen	
				(% ID/g)	(% ID/TO)	(% ID/g)	(% ID/TO)	(% ID/g)	(% ID/TO)
NRS[c]	6	4	19.4 ± 4.6	3.6 ± 0.5	48.3 ± 5.8	0.8 ± 0.02	1.3 ± 0.04	5.3 ± 0.6	4.5 ± 0.5
ALS[d]	6	4	31.2 ± 1.4[e]	2.4 ± 0.4[e]	26.7 ± 3.0[e]	0.7 ± 0.02	1.0 ± 0.03	4.8 ± 0.4	3.6 ± 0.6
ALS	8	24	31.0 ± 2.5[e]	2.6 ± 0.2[e]	32.0 ± 2.1[e]	0.5 ± 0.01	0.8 ± 0.03	5.6 ± 0.6	4.2 ± 0.5

[a] Values expressed as mean ± SEM.
[b] Hours postinjection of 1 ml of test serum.
[c] NRS = normal rabbit serum.
[d] ALS = rabbit antirat lymphocyte serum.
[e] Significant ($p < 0.05$) as compared to NRS-injected controls.

administration of ALS has a profound depressant action upon RES function as manifested by the prolongation of the intravascular clearance of gelatinized RE test lipid emulsion, the absence of a significant effect on RES activity when ALS is administered intravenously suggests rapid inactivation or preferential binding of ALS to lymphocytes, particularly with splenic lymphoid elements, with a reduced exposure of the macrophage cells to ALS. The depressed clearance rate following intraperitoneal injection of ALS was associated with a selective impairment in hepatic localization of the emulsion; while splenic and pulmonary localization remained unaltered. The ALS-induced depression of phagocytosis was associated with a direct cellular effect upon the hepatic phagocytes as reflected by the cytotoxicity of ALS to Kupffer cells and inhibition of phagocytic activity of isolated liver slices. The quantitation of opsonic activity by the *in vitro* phagocytic assay procedure (17, 21) demonstrated that serum from ALS-treated rats, manifesting phagocytic impairment as reflected by decreased vascular clearances, revealed normal levels of opsonic activity. Thus, phagocytic impairment induced by ALS administration is not comparable to RE depression induced by administration of particulate materials which is related to depletion of opsonins (16, 17).

TABLE III. Effect of Intraperitoneally Injected Rabbit Antirat Lymphocytic Serum on Opsonic Activity as Evaluated by *in Vitro* Hepatic Phagocytosis.[a]

Incubation medium	No. of livers	No. of slices[e]	Phagocytic uptake (% ID/100 mg)[b]
Krebs-Ringer buffer	2	8	2.0 ± 0.8
Normal rat serum	4	10	21.1 ± 2.4
Rat,[c] NRS	5	10	21.3 ± 3.6
Rat,[d] ALS	5	10	20.8 ± 4.4

[a] Rats injected with 1.0 ml of test serum 4 hr before exsanguination.
[b] Values expressed as mean ± SEM.
[c] Serum obtained from rats, which were injected intraperitoneally with normal rabbit serum.
[d] Serum obtained from rats, which were injected intraperitoneally with antirat lymphocyte serum.
[e] Liver slices employed in the *in vitro* opsonin assay procedure were obtained from normal rats.

TABLE IV. Effect of Intraperitoneally Administered Rabbit Antirat Lymphocytic Serum on the *in Vitro* Phagocytic Activity of Rat Liver Slices.[a]

Liver donor	No. of livers	No. of slices	Phagocytic uptake (% ID/100 mg)[b]
Normal rat	4	8	21.3 ± 2.1
NRS, rat[c]	5	10	21.0 ± 2.9
ALS, rat[d]	9	18	12.1 ± 1.4[e]

[a] Rats injected with 1.0 ml of test serum 4 hr before sacrifice and liver extirpation.

[b] Values expressed as mean ± SEM.

[c] Rats injected with normal rabbit serum.

[d] Rats injected with rabbit antirat lymphocyte serum.

[e] Significant ($p < 0.05$) as compared to normal or NRS-treated rat controls.

The phagocytic depression induced by ALS is, in part, comparable to methyl palmitate (22) or X-irradiation-induced depression (23). Methyl palmitate, and X-irradiation, like ALS are also immunosuppressive agents. However, in contrast to the absence of an impairment in splenic phagocytosis in ALS treated animals, both methyl palmitate and X-irradiated-treated animals demonstrate an impairment in splenic phagocytosis (22, 23).

The injurious effect of ALS upon Kupffer cells is not only reflected by the impaired phagocytosis but also by the manifested cytotoxicity. While the resulting titer (1:16) was significantly less than that obtained with rat thymocytes (1:40), a cross-reaction between macrophage and thymocyte surface antigens is clearly suggested. Such a cross-reaction may result through simple compatibility of common surface antigens of the lymphocytic and Kupffer cell, due to the possible lymphocytic origin of the Kupffer cell. In this regard, it was been demonstrated that lymphocytes can transform into hepatic macrophages (24).

The mode of action of ALS-induced immunosuppression has been extensively investigated (11–13). Several possible mechanisms have been suggested *e.g.*, a nonspecific serum agent (11), interaction with recipient lymphoid cells (12) and an action against a thymus-mediated mechanism (13). Presently, there is no agreement as to which, if any, of the proposed mechanisms is correct.

The cytotoxic effects of ALS upon Kupffer cells may have relevance to the mode of immunosuppressive action of ALS due to the unique role of the macrophage in immunogenesis. Fishman (2) demonstrated that the macrophage has a definite role in the initiation of immunogenesis through its antigen processing activity, a concept which has been confirmed (3, 14, 25–27). Frei *et al.* (28) demonstrated that phagocytosis is an essential in the development of the primary immune response. Macrophages have also been shown to reverse the immunosuppressive action of X-irradiation towards bacterial antigens (29) and to be a mediator in the induction of immune tolerance (3).

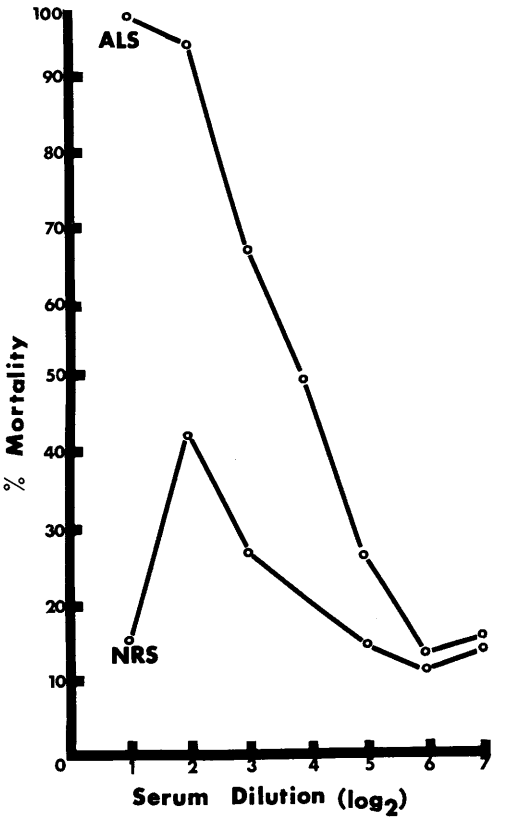

FIG. 1. The comparative cytotoxic effect of ALS and NRS on Kupffer cell viability. Kupffer cells were incubated *in vitro* with either ALS or NRS and guinea pig serum for 2 hr. The trypan blue exclusion test was used to determine viability.

In view of the primary role of the macrophage in immune phenomena, the demonstrated cytotoxic effect of ALS on Kupffer cells suggests the possibility that ALS may exert its immunosuppressive activity by its antimacrophage activity. The validity of this hypothesis is also supported by the observations that selective RES depressants and stimulants significantly alter certain immunological events (14, 30).

Methyl palmitate, an RES depressant (22) was shown to significantly reduce the immune response of mice against particulate antigen (14); whereas, glucan, an RES stimulant (31), was shown to enhance the development of immunity towards particulate antigen (14). These agents have been demonstrated to alter transplantation reactions (32). In accordance with the presently postulated concept that the detrimental effect of ALS on host defense may be related, in part, to its antimacrophage activity, Panijel and Cayeux (33) have recently reported that heterologous antiperitoneal macrophage serum, which destroys or damages macrophages *in vitro,* exerted an immunosuppressive effect.

The depression of RES functional activity by ALS may also play a major role in altering host defense against infections since the RES is responsible for the removal of invasive microorganisms. This concept is supported by the observation of enhanced mortality following the administration of yellow fever virus to mice which received antimacrophage serum (33).

The beneficial employment of ALS in modifying transplantation reactions (9, 10) may be negated, in part, by its detrimental effect on the RES. Thus, while ALS does reduce humoral (34) and cellular immunity (12, 35), the ALS induced depression of overt phagocytosis through selective phagocytic impairment of Kupffer cells and its macrophage cytotoxicity, may be the basis, not only of the possible mechanism of ALS-induced suppression but also a contributing factor in the development of infection, and neoplasia (7, 8, 36–38).

Summary. Antilymphocytic serum induced a profound decrease in phagocytic activity of the RES as reflected by the impairment in the intravascular clearance of gelatinized "RE test lipid emulsion." The phagocytic alterations were due to a selective impairment in hepatic phagocytosis, as localization of the labeled test emulsion by lung and spleen was unaltered. The depression of hepatic phagocytosis was not related to an alteration in plasma opsonic activity. A direct injurious effect of ALS on Kupffer cells was demonstrated not only by the ALS-induced impairment in Kupffer cell phagocytic activity, as assayed by *in vitro* techniques, but also by the cytotoxicity of ALS on macrophages. The immunosuppressive activity of ALS, as well as certain other detrimental consequences of ALS administration may be related, in part, to ALS-induced alteration of the macrophage host–defense system.

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Received June 11, 1969. P.S.E.B.M., 1969, Vol. 132.