THE EFFECT OF ANTI-LYMPHOCYTE GLOBULIN ON CELL-MEDIATED RESISTANCE TO INFECTION*

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Mice which survive infection with *Listeria monocytogenes* are highly resistant to reinfection and specifically hypersensitive to *Listeria* antigens (1). The resistance and the accompanying state of delayed-type hypersensitivity can both be transferred to normal recipients with immune lymphoid cells obtained from actively infected donors (2). Since neither of these immunological modalities can be transferred with immune serum (1, 2, 3), it is a useful model for studying the action of anti-lymphocyte globulin on an immune mechanism which is mediated by activated lymphoid cells to the apparent exclusion of humoral antibody.

According to current dogma, cell-mediated mechanisms of immunity are initiated by uncommitted lymphoid cells which transform, divide, and differentiate to form immunologically committed cells. In this circumstance, anti-lymphocyte globulin (ALG) would have two potential targets against which to act. The first would consist of the immunocompetent cells which become engaged early in the infection, and the second would be the immunologically committed progeny of the first. The former belong to the afferent and the latter to the efferent limb of the arc. The cells which were shown in the accompanying paper (2) to protect normal recipients against a lethal challenge infection and render them hypersensitive to *Listeria* antigens are obviously committed cells. The present paper describes the action of ALG on this cell type. The effect of ALG on the afferent arc, and its influence on delayed-type hypersensitivity, will be reported separately.

Materials and Methods

General.—The animals, organisms, method of immunization, preparation of spleen cell suspensions, and measurement of anti-*Listeria* resistance were described in the preceding paper (2).

Anti-Lymphocyte Globulin.—An anti-serum against mouse lymphoid cells was prepared in rabbits by the intravenous injection of 10^9 living thymocytes, given on two occasions at an interval of 2 wk. Beginning 1 wk later, the animals were bled twice weekly. The serum col-

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lected over a period of 4 wk was pooled and processed. Bleedings were continued for a further 4 months without further immunization. This serum was also pooled to make a second batch of antiserum. The two batches of pooled serum were brought to 37% saturation with ammonium sulfate. The precipitate was redissolved in saline and absorbed with mouse RBC to remove residual hemagglutinins. The globulins were reprecipitated and washed twice with 37% saturated ammonium sulfate. They were then dissolved in, and dialyzed against a phosphate buffer (0.01 M, pH 7.3) prior to separation on a 2.5 × 45 cm column of DEAE-cellulose which had been equilibrated with the same buffer. The proteins which were not retained on the column (ALG) were lyophilized and redissolved in saline to a concentration of 9.75 mg/ml. The final product was devoid of hemagglutinins and gave a single precipitin line which corresponded to IgG by immunoelectrophoresis. Serum from unimmunized rabbits was processed in the same way to obtain a preparation of normal rabbit globulin (NRG) containing 9.0 mg protein/ml.

Titration of Lymphagglutinating Activity.—Lymphagglutinating activity was assayed against mouse thymocytes and thoracic duct lymphocytes which had been washed and suspended in basic saline solution (BSS) at a concentration of 2×10^7 per ml. These were added to equal volumes of serial dilutions of NRG or ALG. The mixtures, in a volume of 0.4 ml in 12 × 75 mm tubes, were incubated for 30 min at 37°C in a water bath oscillating at 120 cpm. After standing for 1 hr at 4°C, they were gently resuspended and examined microscopically for evidence of agglutination.

Assay of Lymphocytolysis.—The cytotoxicity of ALG was assayed only in the presence and absence of fresh, normal mouse serum. Serial dilutions of NRG and ALG in Hanks' BSS were prepared and added to mouse thymocytes suspended in 20% normal mouse serum at a concentration of 2×10^7 per ml. The suspensions were let stand for 1 hr at 4°C and were then incubated without shaking at 37°C for 60 min. At the end of incubation, samples from each tube were diluted in trypan blue (0.05%) and examined after 5 min for evidence of nuclear staining.

RESULTS

Activity of ALG in Vitro.—The two preparations of ALG used in the present study showed comparable lymphaggulutinating activity. Except for a prozone effect, high concentration of each preparation gave massive agglutination of thymocytes and thoracic duct lymphocytes, and sharp agglutinating end points at a dilution of 1:128. When RBC were added artificially to a suspension of thymocytes in the presence of ALG, the former remained randomly distributed, while the thymocytes became aggregated into clumps devoid of RBC.

When tested for cytotoxicity against thymocytes, both NRG and ALG caused considerable cell death (approximately 70% under the conditions of test) at protein concentration of less than 0.3 mg/ml. In the presence of a constant concentration (20%) of fresh mouse serum, however, the anti-lymphocyte globulin showed no cytotoxic activity at the highest concentration (4.9 mg protein/ml). It is presumed, therefore, that the ALG used in these studies was not cytotoxic for mouse thymocytes in the presece of whatever complement is available in fresh normal mouse serum.

Effect of ALG on the Passive Transfer of Antibacterial Resistance with Immune Spleen Cells.—Filtered spleen cell suspensions were prepared from immunized

donors on the 7th day after an intravenous injection of 8.6×10^2 viable *Listeria*. Cells from unimmunized donors were prepared at the same time. After the third washing, the cells were suspended in 1% fetal calf serum (FCS) at a concentration of 4×10^8/ml. The immune cells were divided into two parts to which ALG or NRG were added in doses of 0.97 and 0.90 mg protein/100 million cells. Normal cells were treated with NRG only. The cell suspensions were injected immediately into the tail vein of normal recipients which had been challenged 30 min previously with 2.1×10^4 (4 LD_{50}) viable *Listeria*. Five injected animals which did not receive cells were killed at this time to establish the initial levels of bacterial implantation in spleen and liver. Bacterial counts were made after 24 and 48 hr on the spleens and livers of five mice from each of the three groups.

Fig. 1 illustrates the effect of ALG on the transfer of anti-*Listeria* immunity with lymphoid cells obtained from the spleens of 7-day immune donors. It shows that a small dose, equivalent to the specific antibody contained in 0.97 mg protein, was effective in abolishing the protection conferred by immune lymphoid cells which had been treated with NRG.

In the foregoing experiment, the transferred lymphoid cells were inactivated by exposure to ALG in vitro. They were equally sensitive to inactivation in vivo. When given intravenously to recipients before, or soon after cell transfer, a similar dose of ALG (0.97 mg/mouse) totally inhibited the protective activity of immune lymphoid cells. The results in Fig. 2 show that heat-inactivation of cells and an injection of ALG given 1 hr *after* cell transfer were equally effective in abolishing the immunity conferred with 7-day immune lymphoid cells (10^8 cells of 86% viability). The effect of treatment of recipients with ALG *before* cell transfer is described in later experiments.

Assay of Antilymphocyte Activity in Vivo.—Equal volumes of a serial 2-fold dilution of ALG were added to five aliquots of a pooled suspension of filtered spleen cells prepared from 7-day immune mice. The additions were made after the cells and dilutions of ALG had been chilled to 2°C. As each addition was made, it was injected ito groups of 10 mice which had received an intravenous challenge dose of *Listeria* (1.9×10^4) 15 min previously. The cells were injected in a volume of 0.5 ml containing 10^8 spleen cells (64% viability) and amounts of ALG which ranged from 2.0 to 0.1 mg protein. A group of control mice received the same number of immune spleen cells which had been treated with NRG at the highest protein concentration (2.0 mg/10^8 cells). The initial level of bacterial implantation in spleens and livers was determined in mice which did not receive cells.

The effect of varying doses of ALG on the protective activity of 10^8 *Listeria*-immune spleen cells is depicted in Fig. 3. All but the lowest dose of ALG (0.1 mg/10^8 cells) produced a total suppression of protective activity in both liver and spleen. Only the liver, which is slower to respond (2), showed clear evidence that the lowest dose of ALG was subinhibitory.

Persistence of ALG Activity in Vivo.—The neutralizing effect of ALG on passive protection against challenge with *L. monocytogenes* was used to study the persistence of anti-lymphocyte activity in vivo.

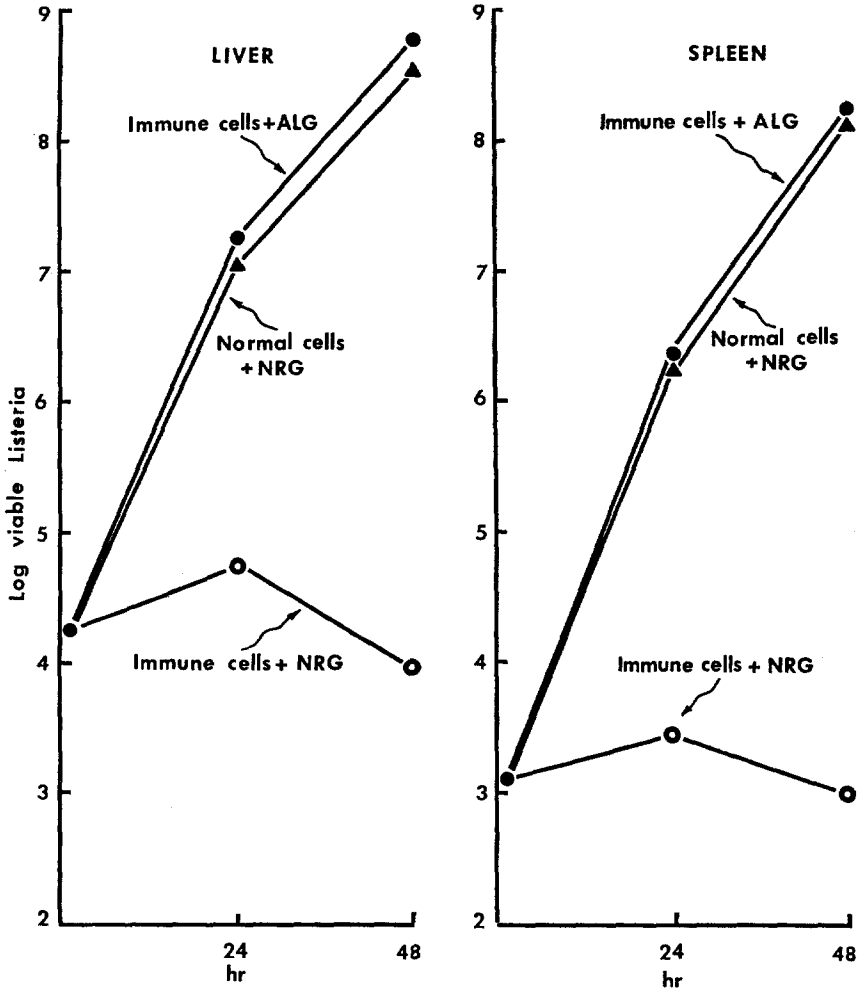

FIG. 1. Growth curves of *L. monocytogenes* in livers and spleens of mice which were protected passively with normal or *Listeria*-immune spleen cell suspensions. The cells were treated with ALG or NRG immediately prior to transfer. Means of 5.

At intervals from 0–24 hr prior to the transfer of immune spleen cells, groups of prospective recipients were injected intravenously with 0.1 ml ALG containing 0.97 mg protein. At zero time, all mice were passively immunized with a pool of filtered spleen cells prepared from 7-day immune donors. Each mouse re-

ceived the challenge inoculum of *L. monocytogenes* (8.6×10^3) admixed with 2.1×10^8 spleen cells of 78% viability. The initial implantation of *Listeria* in spleens and livers was determined in a group of 5 untreated mice which were sacrificed 30 min after injection. Spleen and liver counts were performed on mice of all groups after an interval of 48 hr.

Fig. 4 shows that anti-lymphocyte activity persisted in the blood for at least 24 hr after an intravenous injection of ALG (0.97 mg total protein). In comparison with untreated mice, those receiving ALG between 0–3 hr before cell trans-

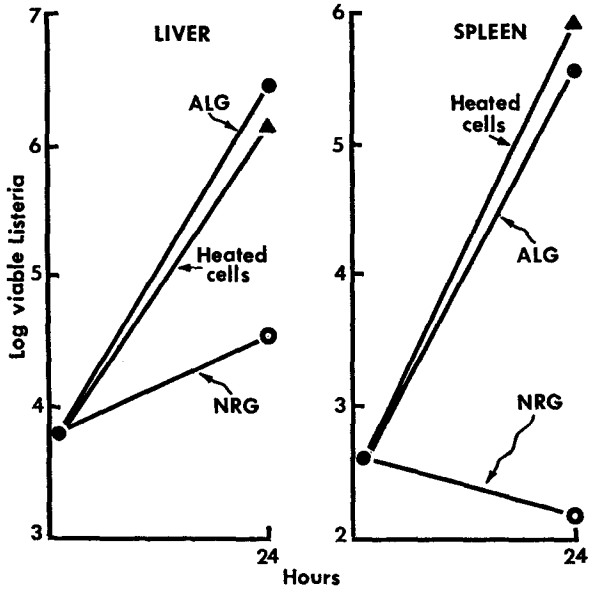

FIG. 2. Growth of *L. monocytogenes* in livers and spleens of mice which had received living or heat-killed spleen cells from *Listeria*-immune donors. One hr after transfer the recipients of living cells were injected intravenously with either ALG or NRG (approx. 1.0 mg/mouse). Means of 5.

fer showed what must be regarded as complete suppression of passively acquired resistance. The inhibitory effect was somewhat less in animals which received their injections of ALG at intervals ranging from 6–24 hr before transfer. The spleens of these animals contained 100-fold fewer organisms than were found in animals treated with ALG at the time of transfer, but 10,000-fold more than were present in untreated controls (Fig. 4, C). The partial loss of anti-lymphocyte activity in the blood of mice treated 6 or more hr prior to cell transfer may have been due to absorption of antibody by specifically reactive cells encountered in the circulation, to equilibration of antibody between intra- and extravascular fluids, or to its absorption by lymphoid cells outside the circulation.

The Accessibility of Immune Lymphoid Cells in Vivo.—Fluorescent antibody

studies (4) have suggested that immunoglobulin molecules penetrate poorly into lymphoreticular tissues, so that lymphoid cells within spleen and lymph nodes are not easily accessible to the action of ALG. In this circumstance, it might be

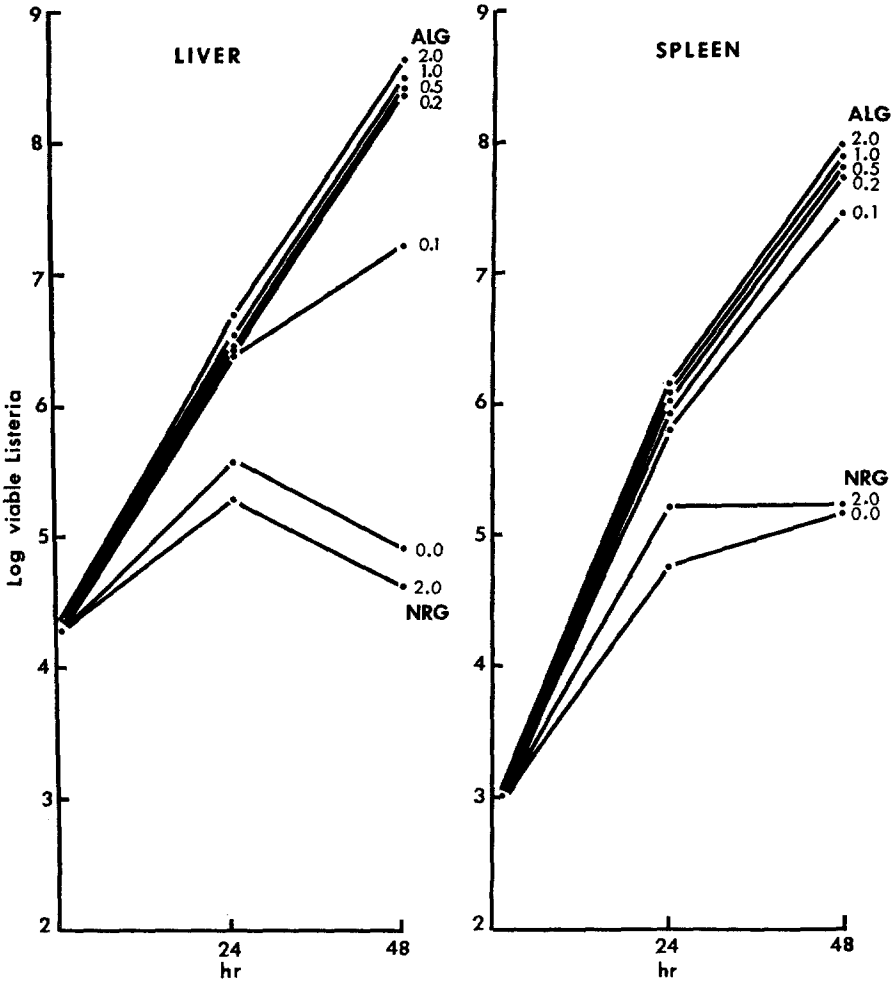

FIG. 3. Growth curves of *L. monocytogenes* in the livers and spleens of mice which were protected passively with untreated immune spleen cells (0.0) or with immune cells treated with NRG (2.0 mg.) or ALG in doses ranging from 2.0 to 0.1 mg. Each mouse received 10^8 spleen cells mixed with ALG or NRG immediately prior to transfer. Means of 5.

difficult to neutralize immune lymphoid cells within the spleens of immune donors. The fact that a substantial level of anti-lymphocyte activity persisted in the circulation for 24 hr after the injection of 1 mg ALG signifies that the total

mass of lymphoid cells in the body is insufficient to absorb this dose of ALG or that ALG does not have access to all lymphoid cells in vivo.

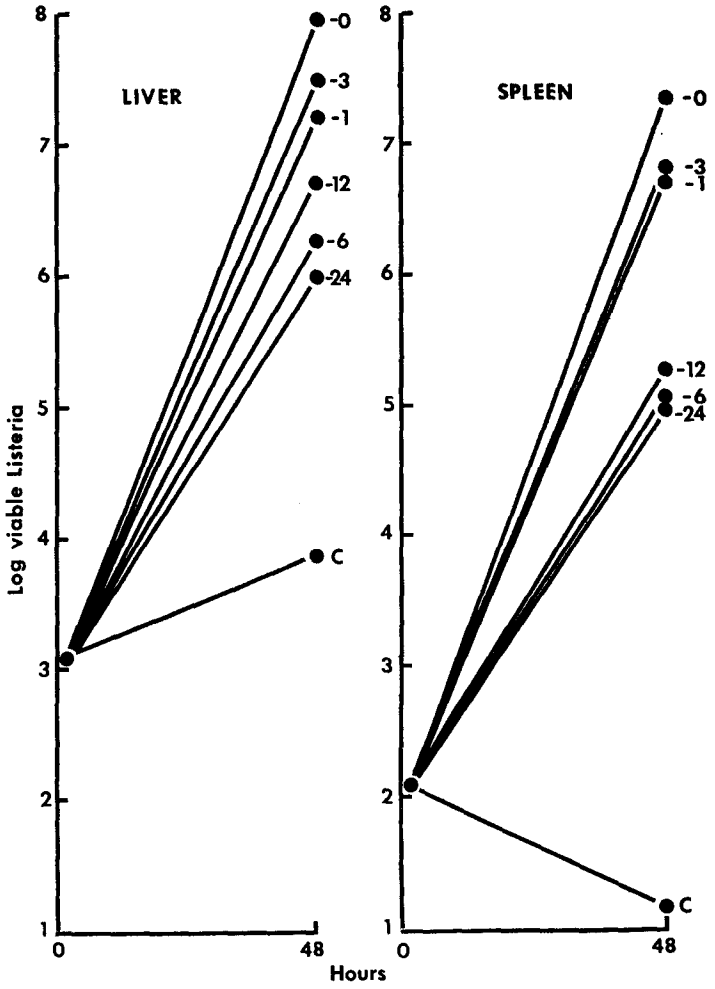

FIG. 4. Growth of *L. monocytogenes* in livers and spleens of untreated mice (C) and mice which received a single intravenous injection of ALG (1.0 mg) at varying times (0 to −24 hr) before the injection of 2.0×10^8 immune spleen cells. Means of 5.

The present model offers an opportunity to examine this question. If ALG penetrates into lymphoid tissues, the immunologically committed cells in the spleen could be inactivated *in situ*. In the first attempt to investigate this question donor mice were injected intravenously with ALG (4.9 mg total protein) or NRG (4.5 mg total protein) on the 6th day of the immunizing infection. 24 hr

later, both groups of donors were exsanguinated to remove as much of the circulating ALG as possible. Spleen cell suspensions were then prepared and transferred, together with the challenge inoculum of *Listeria*, to two groups of normal recipients. Normal cells were given to a group of controls. The recipients of cells

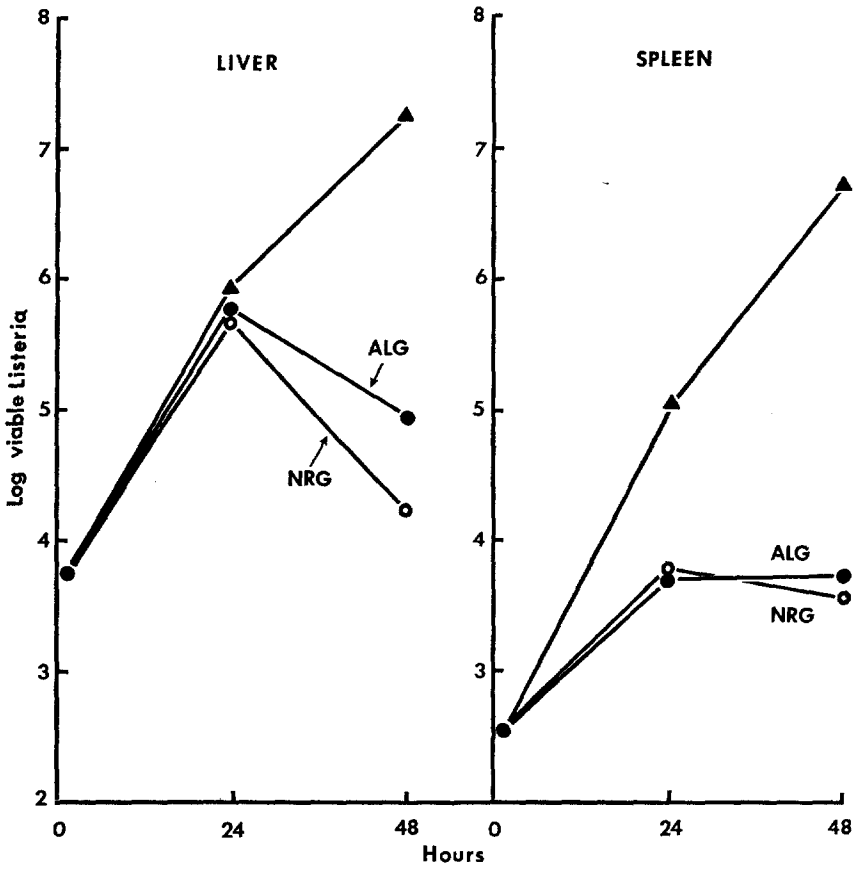

FIG. 5. Growth curves of *L. monocytogenes* in livers and spleens of mice which had received spleen cells from normal donors (▲) or immune donors which had been injected 24 hr previously with 5.0 mg of ALG (●) or NRG (O). Means of 5.

from ALG-treated donors were not protected even though they received a larger number of viable spleen cells (6.1×10^7) than did the recipients of NRG-treated donors (4.7×10^7).

Since the foregoing result could not be reconciled with subsequent observations, the experiment was repeated with one difference. After the spleens had been removed from exsanguinated donors, they were sectioned into 5 or 6 parts

and suspended in 100 ml of BSS at 4°C. After 30 min, they were again washed in BSS before being dissociated by screening through a 50-mesh grid. They were then treated in the usual way. The washing of the sliced spleens was done in the hope that unbound antibody would be leached from the tissue before it could attach to cells during dissociation. The results, recorded in Fig. 5, show that immune lymphoid cells from ALG-treated donors were still highly active when transferred to recipients. In this experiment, the recipients of cells from ALG-treated and NRG-treated donors were injected with 1.9×10^8 and 1.3×10^8 viable spleen cells, respectively. The relatively low level of passive protection obtained with this dose of cells is attributed to the small infecting dose (240 viable organisms) which was used to immunize the donors.

The Question of Antimacrophage Activity in ALG

All of the preceding observations could be taken to indicate that ALG interferes with passive immunization through its capacity to kill or neutralize the activity of immune lymphoid cells. The findings would be as easily explained, however, if ALG were active against the macrophages of the recipients rather than the lymphoid cells of the donor. Were it capable of preventing phagocytosis or interfering with the activation of host phagocytes, the ALG-treated recipient might fail to benefit from the protection normally conferred with immune lymphoid cells. To examine this possibility, a number of experiments were performed. In the first experiment, immune lymphoid cells were treated with ALG in vitro and were then washed exhaustively to remove excess ALG prior to injection into normal recipients.

Transfer with Washed Cells.—Filtered spleen cells from 8-day immune mice were washed 3 times and brought to a concentration of 4.3×10^8/ml in 1% FCS. At this stage, a dye-exclusion test showed that 78% of the cells were viable. They were divided into two parts, one of which was treated with NRG (1.8 mg protein/10^8 cells) and the other with ALG (1.9 mg/10^8 cells). After these additions, the cells were let stand for 1 hr at 4°C. The cell suspensions were then washed 3 times in 100 ml of 1% FCS in BSS. They were finally suspended at a concentration of 4×10^8/ml in a medium containing approximately 2×10^4 viable *Listeria*/ml. At this stage, the NRG and ALG treated cells were respectively 69 and 79% viable and contained 1.7×10^4 and 1.2×10^4 viable *Listeria* per ml as determined by plate count. They were injected intravenously into two groups of normal recipients in a volume of 0.5 ml. Spleen and liver counts were performed on 5 mice from each group after 15 min and again after 24 and 48 hr.

The results of the foregoing experiment showed that ALG-treated cells, though viable at the time of transfer, were devoid of protective activity in recipients (Fig. 6). It must be reasoned that extensive washing has removed all but trace amounts of free antibody from the cell. Since it is unlikely that the

macrophages of recipient mice would be damaged by antibody bound to lymphoid cells, it is concluded that ALG owes its suppressive effect to its action on donor cells.

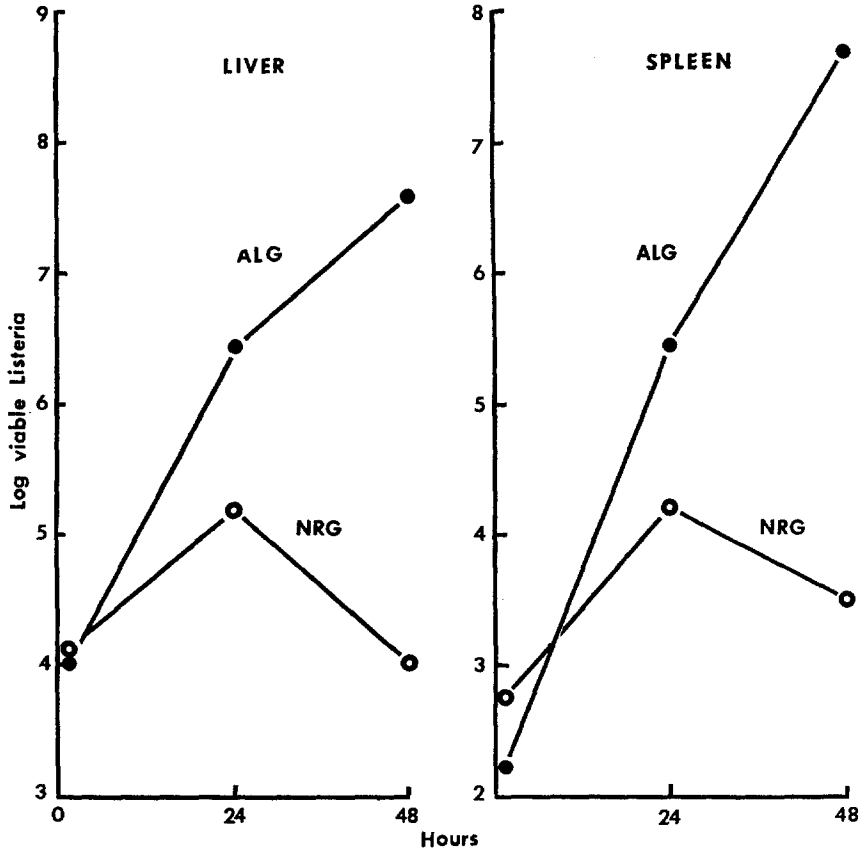

FIG. 6. Growth curves of *L. monocytogenes* in livers and spleens of mice injected with immune spleen cells which had been washed repeatedly after treatment with ALG or NRG. Means of 5.

Macrophage Agglutinating Activity of ALG.—As a further test for anti-macrophage activity in the two ALG preparations used in these experiments, they were assayed for agglutinating activity against normal peritoneal macrophages. The test posed a problem, for mouse macrophages agglutinate spontaneously under the conditions usually employed for assaying leukagglutinating activity. This difficulty was largely overcome by performing the tests at 2°C.

Cells were harvested from the peritoneal cavities of 10 normal mice. They

were washed twice and suspended in BSS at a concentration of 2×10^7/ml. Thymus glands from the same animals were used to prepare a washed suspension of thymocytes at the same density. The preparations of ALG (9.7 mg/ml) and NRG (9.0 mg/ml) were serially diluted and added to equal volumes (0.2 ml) of the two cells suspensions in 12 × 75 mm tubes. The mixtures were shaken at 120 cpm in an ice water bath at 2°C for 60 min. Samples from each tube were then examined in hemocytometer chambers for evidence of agglutination.

Table I shows a titration of the thymocyte and macrophage-agglutinating activities of the second of the two ALG preparations used in the present experiments. A minor amount of clumping, affecting less than 10% of the cells, occurred in all tubes containing peritoneal macrophages, but no agglutination

TABLE I

Assay of Leukagglutinating Activity of ALG against Mouse Thymocytes and Peritoneal Macrophages

Antigen	Antiserum	Dilutions of ALG (9.75 mg/ml) and NRG (9.0 mg/ml)							
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	Control
Peritoneal cells	NRG	±	±	±	±	±	±	±	±
	ALG	±	±	±	±	±	±	±	±
Thymocytes	NRG	−	−	−	−	−	−	−	−
	ALG	++++	++++	++++	++++	+++	++	−	−

++++, Massive agglutination.
±, Occasional clumps and many pairs.
−, No associated cells.

which could be attributed to specific antibody. It should be noted that the agglutinin titer against thymocytes at 2°C was one tube less than in tests performed at 37°C.

The exact composition of the peritoneal cells used in the agglutination test is unknown. Apart from mature macrophages, which constituted 62% of the cells present, an unknown percentage of the smaller cells in the peritoneal cavity of the mouse may also be macrophages at an immature stage of development. The true lymphocyte content may thus be quite small. Since the antiserum was prepared against thymocytes and shown to be active against thoracic duct lymphocytes, the absence of agglutination among mixed cells of the peritoneal cavity may mean that circulating lymphocytes of the type found in thoracic duct lymph are not well represented in the normal peritoneal cavity of the mouse.

The absence of macrophage agglutinating activity points again to the conclu-

sion that ALG interferes with the passive transfer of immunity through its effect on immune lymphoid cells. Further evidence of this is contained in the following experiment.

The Effect of ALG on Established Immunity in Intact Mice.—After a sublethal infection with *L. monocytogenes* the host's macrophages become conspicuously, but nonspecifically, bactericidal. At this early stage of convalescence, the host is absolutely resistant to a *Listeria* challenge by virtue of the activated macrophages present in the tissues (1). If ALG is devoid of anti-macrophage activity, it would not be expected to influence host resistance at this stage of convalescence.

Surviving mice which had been immunized 7 days earlier with a relatively large immunizing dose of *Listeria* (4.5×10^8) were injected intravenously with large doses of ALG or NRG (5.0 mg protein). They were challenged with *L. monocytogenes* ($5.1 \times 10^4 = 10$ LD_{50}) 24 hr later. A group of unimmunized controls were challenged at the same time. After 15 min, and at 24 and 48 hr, 5 mice from each group were used for estimating viable bacterial counts in spleen and liver.

Fig. 7 shows that ALG had little effect on host resistance when given to actively immunized mice at the height of their response to a sublethal infection. Since resistance at this time is due to the presence of activated macrophages in the host, it follows that ALG does not possess any marked capacity to interfere with the physiological activity of macrophages in vivo. The dose of ALG used in this experiment was 40 times greater than the amount needed to abolish the activity in 10^8 spleen cells (Fig. 3). It seems, therefore, that resistance at this stage of convalescence is vested in cells which are inaccessible or are unaffected by ALG.

Effect of Antilymphocyte Globulin upon the Recall of Anti-Listeria Resistance.—Later in convalescence, *Listeria*-infected mice enter a second phase of resistance to reinfection (1). It lasts for several weeks, during which the animal displays hypersensitivity to *Listeria* antigens; and resistance to reinfection is characterized by a short period of latency before the host's macrophages become reactivated to provide a mechanism for eliminating the parasite. The effect of antilymphocyte serum on this second, or reactivation phase, of acquired cellular resistance was investigated. A preliminary experiment indicated that a single injection of ALG had little effect upon the capacity of previously infected mice to show the characteristic recall of acquired resistance. This finding was consistent with the observation (Fig. 5) that a single injection of ALG did not neutralize committed cells within the spleens of immune donors. It was reasoned that more protracted treatment with ALG might influence residual immunity if the cells responsible beong to a recirculating pool. Migration should bring them in contact with ALG sooner or later.

Animals were immunized intravenously with 1.0×10^8 viable *L. monocyto-*

genes. On days 21 and 28, half of the immunized mice were injected subcutaneously with 0.5 ml of ALG (4.9 mg protein); the remainder received a comparable dose of NRG (4.5 mg protein). Two groups of normal mice were treated similarly. Three days after the second injection, all mice were challenged with *L.*

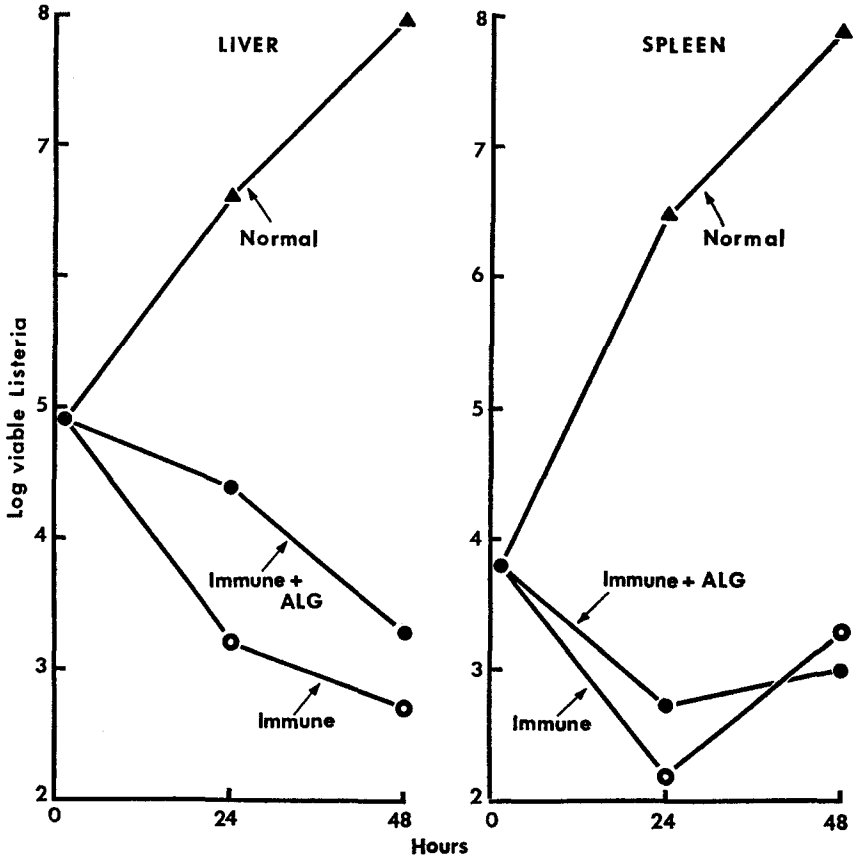

FIG. 7. Growth curves of *L. monocytogenes* in normal (▲) and reinfected mice which were treated on day 6 of the primary infection with 5 mg of NRG (O) or ALG (●) and challenged 24 hr later with 40 LD_{50}'s of virulent *Listeria*.

monocytogenes ($9.5 \times 10^3 = 2$ LD_{50}). After 15 min, and again at 24 and 48 hr, five mice from each group were used for viable counts on spleen and liver.

Fig. 8 shows that 10 days of treatment with approximately 10 mg of ALG had a marked depressive effect on the capacity of convalescent mice to show the characteristic recall of acquired resistance which normally exists in previously infected mice. The effect was more marked in the liver, but the bacterial

growth pattern in the spleen showed that a low level of residual reactivity still persisted in the ALG-treated animals.

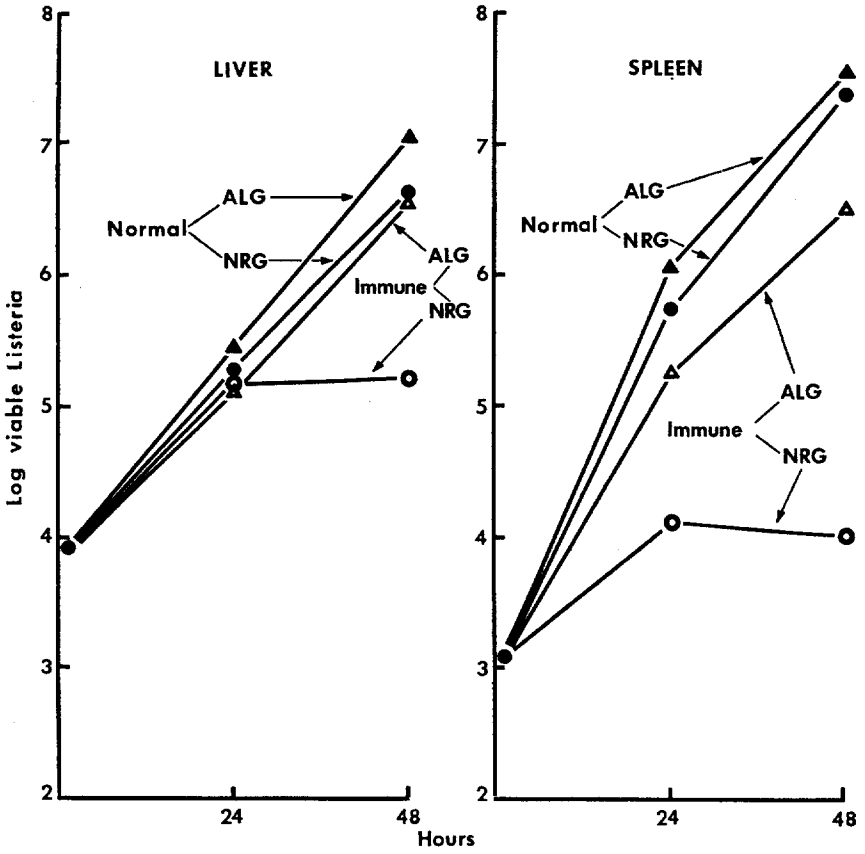

FIG. 8. Growth curves of *L. monocytogenes* in normal and reinfected mice challenged 4 wk after the primary infection. At 10 and 4 days prior to reinfection the animals were injected subcutaneously with NRG or ALG (5.0 mg). Means of 5.

DISCUSSION

Previous studies have shown that the ultimate expression of resistance against *Listeria monocytogenes* and other facultative intracellular parasites depends upon the activation of host macrophages (1). Although an immunological mechanism has been clearly implicated (5), its nature could only be deduced from some circumstantial evidence which seemed to indicate that delayed-type hypersensitivity was somehow involved (6). Since the latter is thought to be mediated by immunologically committed lymphoid cells (3), and is susceptible

to suppression by anti-lymphocyte serum (7), it is logical to apply this reagent to an analysis of the mechanisms which underlie the phenomenon of acquired cellular resistance to infectious disease.

Gaugas and Rees (8) have already shown that anti-lymphocyte serum impairs the development of resistance to two classical intracellular bacterial infections. Chronic treatment of mice shortened the survival time of tuberculous mice and enhanced the multiplication of *Mycobacterium lepraemurium* in mouse tissues. But anti-lymphocyte serum also interferes with antibody production (9, 10, 11), making it difficult to draw conclusions in regard to mechanism from experiments on actively immunized animals.

It was shown in the previous paper (2) that resistance and hypersensitivity to *Listeria* are transferred concurrently with immune lymphoid cells from actively infected donors. Before concluding that delayed-type hypersensitivity and acquired cellular resistance are outward expressions of a common immunological pathway, it was necessary to exclude the possibility that suspensions of immune spleen cells are heavily contaminated with monocyte precursors, and that these are the cells which provide a protective mechanism for recipient animals. It could be reasoned that resistance to infection and the state of delayed-type hypersensitivity are independent phenomena, one resulting from induced changes in cells of the monocyte-macrophage series, and the other from immunological commitment on the part of cells belonging to the lymphoid series. The specificity of ALG provides the means to resolve this question.

It was shown that the absolute protection which can be adoptively conferred with immune spleen cells is totally ablated by the IgG fraction of an antiserum which was raised against mouse thymocytes but showed equal agglutinating activity against thoracic duct lymphocytes. Four lines of evidence indicate that it was devoid of activity against mouse macrophages. (*a*) ALG did not agglutinate mouse macrophages in vitro (Table I). (*b*) When immune lymphoid cells were treated with ALG and washed exhaustively before transfer, they failed to protect (Fig. 6). The immunosuppressive effect of ALG is judged therefore to result from its action on donor cells rather than the phagocytes of the recipient. (*c*) ALG did not interfere significantly with established resistance in recently infected mice. Since resistance in early convalescence is due to the highly activated macrophages which persist for some time after the infection has been overcome (1), the absence of any depressing effect of ALG at the height of established immunity indicates that it has little if any antimacrophage activity in vivo. Since bacteria are cleared rapidly from the blood, it is obvious that the cells which ingest them are accessible to ALG, so that the question of penetration need not be raised. (*d*) Finally, it will be reported in a subsequent paper that the preparations of ALG used in these experiments did not depress the monocyte count in the peripheral blood.

All of the foregoing observations make it reasonable to conclude that ALG

owes its immunosuppressive activity in the system studied to its ability to neutralize the immunologically committed cells in suspensions prepared from immune spleens. Its capacity in this regard is very impressive. The rabbits which provided the antiserum were in no sense hyperimmunized. They had received only two injections of 10^9 thymocytes. The ALG fractions prepared from these animals 9 months after immunization were as potent in lymph-agglutination and immunosuppressive activity as were those obtained 2 wk after immunization. Their capacity to neutralize immune lymphoid cells was greater than would be deduced from their agglutinating activity. A sharp end point, represented by the lowest dilution containing cell aggregates, was obtained in agglutination tests at a dilution of 1:128 (= 75 μg protein/ml), and a cell concentration of 1×10^7/ml. The same preparation at a dilution of 1:80 (120 μg protein/ml) completely suppressed the committed cells in a population of 2×10^8 spleen cells, of which the great majority were lymphocytes. Since 20 times as many cells were used to measure immunosuppressive activity, it follows that this test is more sensitive than agglutination by a factor of 12.

A further indication of the immunosuppressive potency of ALG was the finding that the specific antibody in a single dose of 0.1 ml (0.97 mg total IgG) suppressed most of the protective activity associated with immune cells injected 24 hr later (Fig. 4). During the interval, considerable opportunity must have existed for antibody molecules to be removed from circulation. Even when allowance is made for the fact that extravascular lymphocytes may be inaccessible to ALG, it is still surprising that so much activity persisted in the circulation after 24 hr. There are about 10^7 lymphocytes in circulation and many times this number are delivered to the circulation from the thoracic duct lymph alone (12). It must be reasoned that very few antibody molecules are needed to interfere with the function of committed cells and that the innumerable cells which line the vasculature cannot be very rich in the antigenic determinants represented on the lymphocytes.

Allusion has been made repeatedly to the uncharacterized cells in the spleens of immune donors against which the activity of ALG was measured in these studies. One of the major unsolved problems of cellular immunology concerns identity and properties of the cells which mediate specific cellular hypersensitivity and the associated antibacterial immunity which arises in certain infections. According to the conventional view, the immunocompetent cells which engage in an immune response undergo blast transformation and a succession of divisions from which come committed cells with the morphology of lymphocytes (13, 14). The question of whether or not these cells engage in the synthesis of immunoglobulin remains unanswered because no antibody has been found in serum or cells which will passively sensitize recipients (3) or protect them from infection (1, 2). But they clearly have specific properties which enable

them to perform both of these functions. To do so they must be alive (2) (Fig. 2), and be metabolically intact if they are to perform efficiently (2). It seems, therefore, that the immunosuppressive activity of ALG depends upon its capacity to interrupt the metabolism of immunologically committed cells. Mitomycin C does not appear to be capable of this, since it was shown in the previous paper (2) that a given number of immune cells produced a low but persisting background of resistance which did not increase with time, suggesting that mitomycin C interferes with cell division without disturbing other important metabolic activities. Whatever ALG does to lymphoid cells, it does it with greater efficiency than mitomycin C. It is comparable, in fact, with heat at 56°C (Fig. 2) in its ability to abolish the immunological capabilities of passively transferred cells. This suggests that ALG-treated cells may actually be killed. There was no evidence, however, that they are lysed by the complement levels present in mouse serum. Opsonization and subsequent phagocytosis would be an equally effective way to dispose of sensitized cells. A subsequent paper[1] will present evidence that destruction by phagocytosis is a major factor in the immunosuppression achieved with ALG.

This raises an important point. It has long been argued that ALG does not interfere with an ongoing immune response (11) or with a secondary immune response (10). Although the evidence for this is not good enough to sustain a compelling argument, it has been concluded that ALG is inactive against immunologically committed cells and must therefore owe its immunosuppressive activity to its effect on uncommitted cells before they become engaged in an immune response (11). In other words, the immunocompetent precursors are the cells which are violated by this immunosuppressant. If we regard the cellular mediators of delayed-type hypersensitivity and acquired anti-*Listeria* resistance as the cellular equivalents of specific antibody, these immunologically committed cells are the counter parts of the plasma cells which are said to be unaffected by ALG. Since it is clear from the present studies that the mediators of cellular immunity are highly vulnerable to attack by ALG, we must conclude, either that cells committed to antibody production and those committed to the mediation of cellular hypersensitivity differ in their sensitivity to ALG, or that the evidence is misleading.

The question seems to have been resolved by experiments reported in this paper. When no precautions were taken to avoid a source of artifact, it seemed that immune lymphoid cells could be inactivated in the spleen by treating donors with ALG for 24 hr prior to cell transfer. This finding could not be reconciled with the fact that 10 days of treatment with ALG did not wholly suppress the memory of a previous infection in actively immunized animals (Fig. 8). The experiment was therefore repeated with modifications which led

[1] Sutton, J., and G. B. Mackaness. 1969. Unpublished.

to a more easily interpreted result. When care was taken to leach from spleen slices what ALG was still present in the organ, presumably in blood vessels, the immune cells within the spleen were still fully protective when dispersed and injected into normal recipients. Since immune lymphoid cells which had been treated with ALG in vitro could not be reactivated by repeated washing (Fig. 6), it follows that similar treatment of spleen slices would not restore activity to cells which had been exposed to ALG in vivo. Since cells were still active when taken from donors which had been treated with ALG in a dose 40-fold greater than that required to abolish the protective activity in 2×10^8 spleens in vitro, it follows that ALG does not have ready access to extravascular cells. This finding accords with the conclusion of Denman et al. (4) who claimed, from fluorescent antibody studies, that extravascular lymphocytes within the lymphoreticular tissues are not easily accessible to ALG.

This means that ALG can depress immune mechanisms which are mediated by committed cells only if the cells recirculate. This would bring them in contact not only with ALG but also with the phagocytic elements of the reticuloendothelial system. If the immunosuppressive effects of ALG are due to deletion of cells by phagocytosis, there is an easy explanation of why it takes many days to ablate an existing state of immunological reactivity. The time taken to eliminate all reactive cells would become a function of their recirculation time. It is known that the memory cells which engage in a secondary antibody response enter the circulation (15). There is every reason to believe that those which are concerned in cell-mediated reactions also circulate.

From these considerations it seems that observed differences in the susceptibility of various states of immunity to suppression by ALG are not explained by differing susceptibilities of the cells involved, but by their anatomical location. All cells within the lymphoid series may be equally susceptible, but not equally accessible to ALG.

SUMMARY

The specificity of anti-lymphocyte globulin (ALG) has been used to analyze an immune mechanism which is mediated by immunologically committed lymphoid cells to the apparent exclusion of humoral antibody. Rabbit anti-mouse lymphocyte globulin completely suppressed the immunity which can be passively transferred with *Listeria*-immune lymphoid cells from actively infected donors. When prospective donors were given a single dose of 1.0 mg of ALG, it remained active against immune lymphoid cells transferred 24 hr later; yet immune cells in the spleens of donors could not be inactivated *in situ* by even larger doses of ALG given 24 hr prior to cell harvest. In keeping with this finding, the immunity to reinfection with *Listeria* was not suppressed by a single dose of ALG, indicating that the immunologically active cells in the spleen are not accessible to intravenously administered ALG. On the other

hand, protracted treatment with ALG did abolish most of the memory of a previous infection in intact animals. From this and other evidence, it was concluded that immunologically committed cells are vulnerable to attack by ALG only if they circulate. While in circulation, they make contact both with ALG and the phagocytic elements of the reticuloendothelial system which appear to be responsible for their destruction.

Four lines of evidence indicated that the suppression of anti-*Listeria* resistance with ALG depends upon destruction of immune lymphoid cells and not to any action it has on host macrophages. It is possible to infer from this that immunity to *L. monocytogenes* depends upon a two cell system in which the donor lymphoid cells provide the immunological reactivity to the organism and recipient macrophages provide the mechanism through which resistance is expressed. Accompanying papers provide additional support for this view, and reasons for believing that delayed-type hypersensitivity and acquired cellular resistance are mediated by the same population of immunologically committed lymphoid cells.

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