Effects of Antilymphocyte Serum (ALS) on the Induction of Lymphocytic Leukemia in Mice (35278)

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The suppression of immune reactions has helped to analyze their role in the induction and control of neoplastic growths. The frequency of those neoplasms which contain virus-specific transplantation antigens (TA) usually is increased strikingly in animals rendered immunologically defective by neonatal thymectomy, treatment with ALS, and in some instances by X-irradiation (1). On the other hand, immune deficiencies do not always influence the frequency or latent period of neoplasms known to contain tumor specific TA; for example, chemically-induced primary fibrosarcomas and mammary tumor virus-induced tumors of the mouse appear not to be susceptible to ALS or to neonatal thymectomy (1).

Heterologous antilymphoid sera have been shown to be effective suppressants of cell-mediated immune responses such as skin graft or tumor rejection and of delayed hypersensitivity reactions (2). Processes that lead to humoral antibody production are also sensitive to ALS but may have unusual time-dependent relationships to antigen administration (3). ALS has been reported to increase the frequency and shorten the latent period of neoplasms induced by polyoma, adeno-12, and murine sarcoma (MSV) virus (4–9) and to strikingly influence the induction of certain lymphocytic neoplasms in resistant adult mice treated with Moloney leukemia virus (MLV); in fact, one regimen of treatment with ALS produced localized reticulum cell sarcomas (10), a neoplasm observed with high frequency in man bearing kidney transplants and given immunosuppressants, including ALS [see (11)].

In many of the tumor systems, referred to above, cell-mediated immune responses elicited by tumor specific transplantation antigens (TSTA) appear to be involved; however, preliminary evidence suggests a role of humoral antibodies in susceptibility and resistance of neoplasms induced by the RNA leukomogenic-sarcomagenic viruses (12).

In the present study the relationship was explored between immunologic reactivity, using ALS as an immune suppressant, and the induction and repression of lymphocytic neoplasms in adult BALB/c mice infected with the murine leukemogenic virus, MLV (Moloney). Different regimens of treatment with ALS were employed. Attempts to reverse the effects of ALS were made using adoptively transferred syngeneic sensitized and nonsensitized lymphoid cells at different concentrations and timing. In addition, preliminary studies were done on the influence of ALS on *in vivo* replication of MLV and on the induction of anti-MLV neutralizing antibodies.

Materials and Methods. Mice. Pathogen-free (PF) mice of the BALB/c inbred strain were used throughout. For the most part 12-week-old mice ordinarily resistant to the leukemogenic effects of MLV were employed, unless otherwise stated.

Virus. MLV (Moloney leukemia virus) was obtained from Germ Free Products, Inc.; the virus preparation used was a 1 g/ml equivalent of spleen. Usually 0.1 ml of 10^{-1} concentration was inoculated intraperitoneally. The infectivity titer of the original virus preparation as determined by syncytial cell formation with XC cells and mouse embryo (ME) cells infected with virus dilutions (*vide infra*) (13) was $1 \times 10^{4.5}$/ml.

Sensitized and non-sensitized lymphoid cells. Twelve-week-old BALB/c mice were immunized once a week for 3 weeks with an *in vitro* propagated MSV(MLV) virus. Lymphoid cells of splenic origin were used for adoptive transfer from 2 weeks to 2 months

TABLE I. Influence of Different Regimens of ALS-Treatment on MLV-Induced Lymphocytic Neoplasms in 12 Week BALB/c Mice.

Group	No. of expts.	Treatment with ALS at days:	Total ALS (ml)	No. with leukemia/no. of mice		Latent period (months)
I a	6	−7, +7	0.2	28/41	(70%)	3.2 (2.2–4.0)
b	1	−7, +7	0.2	7/8		3.0 (2.8–3.3)
c	1	−3, −1	0.2	4/6		4.0 (3.5–4.5)
d	2	−7, +7, +9, +11, +13, +15	0.6	8/8	(87%)	2.8 (1.5–3.8)
e	2	−7, −4, −2	0.3	15/16		3.5 (3.0–5.0)
f	1	−7, −4, −1, 0	0.4	5/8		2.5 (2.0–3.5)
g	1	−11, −9, −7	0.3	8/8		2.7 (2.0–3.5)
h	1	+7, +10, +13	0.3	1/8		4.0
i	1	+14, +17, +20	0.3	2/8	(19%)	5.0, 7.0
II	8	MLV only	—	2/73	(2.7%)	4.0, 5.0
III	5	ALS only	0.2–0.6	1/48	(2%)	3.0

after the last immunization. The concentration of this pseudotype virus, inoculated subcutaneously in 0.2-ml volumes was 1×10^4 ffu/ml.

Age-matched, non-treated BALB/c mice provided nonsensitized lymphoid cells (spleen). Dissociated, washed lymphoid cells were inoculated intravenously at the times indicated in the tables.

Virus titers and virus neutralizing antibody. MLV infectivity titers were determined at various periods in pooled spleen extracts (2 spleens at each period) of BALB/c adults treated with ALS prior to, and after MLV infection and in a group not treated with ALS. The stock MLV used for infection was diluted 20-fold. Plates of secondary NIH Swiss ME cells (3.5×10^5/plate) were inoculated with various dilutions of 20% spleen extracts. The cells were trypsinized and subcultured on day 6 and again on day 9 at which time XC cells were added (8×10^5/plate) for cocultivation (13). The reciprocal of the highest dilution of a spleen extract showing the characteristic syncytial cell formation in 2–3 days was taken as the infectivity titer of MLV contained in the extract.

MLV neutralizing antibodies were determined by the inhibition of focus formation by pseudotype virus MSV(MLV) on ME cells. Antiserum was pooled (8 mice/group), inactivated at 56° for 30 min, followed by incubation for 1 hr at 37°. This mixture was inoculated in 0.4-ml amounts into duplicate plates of secondary ME cells. After a 3-hr adsorption period, medium was added and foci were determined after 7 days.

ALS preparations. Six separate preparations of ALS were produced in New Zealand rabbits following the procedure of Levey and Medawar (2). In four preparations BALB/c thymocytes were used, CBA in one preparation and SJL thymic cells in one preparation. Absorption with mouse RBC in a volume of 1:5 was followed by decomplementation before use. All preparations were observed to be effective *in vivo*; agglutination titers against thymocytes ranged from 1:512 to 1:2048 and a striking lymphopenia was found 48 hr after ALS inoculation.

Results. Twelve-week-old BALB/c mice are seen to resist the leukomogenic effects of intraperitoneally injected MLV, Group II, Table I. The different schedules of subcutaneously-injected ALS provided a striking sensitivity to the leukemogenic virus provided at least one treatment of ALS was administered prior to virus (Table I). The reduced effects of ALS administered beginning 7 days after virus infection (Group Ih and i) has been observed also in this laboratory using MSV(MLV) in the induction of solid tumors in BALB/c mice. Generalized lymphocytic leukemia was detected as early as 1.5 months after MLV infection in these

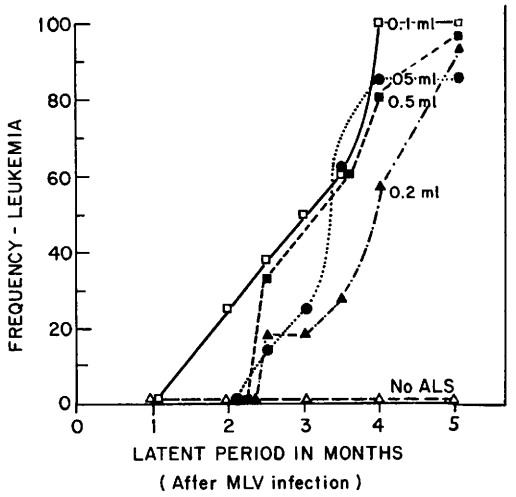

FIG. 1. Effect of different regimens of ALS on MLV-induced generalized lymphocytic leukemia. BALB/c mice 12 weeks of age at beginning of treatment. ALS given subcutaneously at amounts shown on days −7, −4, −1, and 0. MLV was inoculated intraperitoneally at day 0. Age to necropsy approximately 2 weeks later than latent period.

adult mice and death followed usually in 2 weeks. ALS alone (Group III) was ineffective; previously normal rabbit serum (NRS) was observed not to influence MLV induction of lymphocytic leukemia (14).

All six ALS preparations used in this study were found to be immunosuppressive.

It is of interest to note that a minimal total dosage of ALS, 0.2 ml given in divided 0.1 ml injections, was quite effective in producing susceptibility to MLV. Figure 1 shows that there were no substantial differences in frequency of MLV-induced leukemia or in latent period in 12-week-old BALB/c mice administered ALS over a range of concentrations from 0.05 ml × 4 to 0.5 ml × 4. Although other investigations have shown a response to antigen related directly to the number of injections of ALS, the present results probably signify that the lowest dosage used here, 0.05 ml × 4 was optimal for the necessary immune depression required for MLV leukemogenesis and that this cannot be increased with increased levels of ALS.

An unexpected finding in these studies (not shown in the tables) was the lack of response to the leukemogenic effects of MLV in ALS-treated BALB/c mice 6 to 8.5 months of age at the beginning of treatment. Fifteen BALB/c mice followed until necropsy did not develop the characteristic lymphocytic leukemias observed in the 12-week-old treated mice.

If ALS is inactivating or removing those lymphoid cells concerned with the establishment of immune competence, it should be possible to replace those components removed by ALS and thus restore the immune response and, as a consequence, prevent leukemogenesis. This has been accomplished successfully in restoring the capacity to reject allogeneic skin grafts (2) and in restoring the response to sheep erythrocytes (15). Table II shows that intravenously inoculated lymphoid cells of splenic origin from 12- to 20-week-old syngeneic BALB/c donors were capable of restoring the capacity to resist the oncogenic effects of MLV provided adoptive transfer was done in the period from 3 to 10 days after the last ALS treatment. Presumably, lymphoid cells inoculated at 2 days or earlier were inactivated by residual ALS while it was too late for those immunocompetent cells administered at 14 days to prevent emergence of transformed cells. Dosage of lymphoid cells as well as time was a critical factor in successful adoptive transfer; not shown in Table II is the lack of restorative capacity of 10×10^6 lymphoid cells transferred during the sensitive period 3 to 10 days after the last ALS injection.

These results, reestablishing resistance to the leukemogenic effects of virus by adoptive transfer of immunologically competent syngeneic lymphoid cells support the concept that the immunosuppressive effect of ALS is directly responsible for the observed increased susceptibility.

It was expected that lymphoid cells from "immunized" syngeneic donors would be more effective than lymphoid cells from nonimmunized donors in reestablishing resistance to MLV leukemogenesis. This indeed has been the experience in adoptive transfer experiments in mice bearing neoplasms with polyoma-specific tumor antigens and with carcinogen-induced neoplasms (16, 17). On the contrary, putatively sensitized lymphoid

TABLE II. MLV-Induced Leukemia in ALS-Treated BALB/c Mice.[a]
ALS given on days —7, —4, —1, and MLV on day 0.

Group	A		B	
	No. with leuk./ total no.	Latent period (months)	No. with leuk./ total no.	Latent period (months)
Nonsensitized lymphoid cells	8/40	4.1	23/28	3.5
Sensitized lymphoid cells	20/21	1.8	—	
None	23/32	2.8	19/21	2.5

[a] Intravenous inoculations of 25–60 × 10^6 lymphoid cells of splenic origin were made in the period from 3 to 10 days after the last ALS treatment in A group, whereas inoculations were given in group B at less than 3 days or at 14 days after ALS. Totals of 5 separate experiments.

cells from BALB/c donors may have enhanced leukemogenesis (Table II).

The virus used for immunization, MSV (MLV) is known to provide adequate immunity against the transplantation of an MLV-induced transplantable leukemia containing TSTA and against a transplantatable MSV(MLV)-induced hemangiosarcoma (18). Previous work has also shown that MSV and MLV share common antigens. Sensitized lymphoid cells used in this study were always obtained from hyperimmunized syngeneic donors from 1 to 4 months after the last immunization.

In at least one system, detecting cytotoxicity of H-2 antibody *in vitro*, there has been noted a decreased cell-mediated cytotoxicity, using spleen cells from hyperimmunized mice, that paralleled an increase in titer of humoral antibody in these same donors; these data suggest a suppressing effect by antibody on the cell-mediated response (19). Attempts are now being made in our system to study the possible effect of enhancing or suppressing antibodies in recipients of "sensitized" lymphoid cells.

The reduced capacity of spleen cells from "sensitized" BALB/c donors to reverse the ALS effect may indeed represent a decrease in the numbers of reactive cells in the spleen due to accumulation of "transformed" cells thus resulting in a dilutional effect of sensitized cells. This would hardly account, however, for the apparent lowered latent period in recipients of putatively sensitized lymphoid cells.

Preliminary studies have been made in attempts to define the mechanism of action of ALS. MLV infectivity in spleen extracts and levels of anti-MLV antibodies were determined over a period of time in several groups of BALB/c with differing regimens of ALS

TABLE III. MLV-Neutralizing Antibodies in Sera of MLV-Infected BALB/c Mice Treated with ALS Prior to (—) or After (+) Virus.[a]

Group (days at treatment)	No. with leuk./no. of mice	Latent period (months)	Neutralizing antibody at: week: 5 ffu[b]	5 % Inhib.	7 ffu	7 % Inhib.	9 ffu	9 % Inhib.
ALS (—7, —4, —1)	8/8	3.4 (3–5)	105	38.9	103.5	40.5	134.5	21.5
ALS (+14, +17, +20)	2/8	4.5 (3.5–5)	17.5	98.9	1.5	99.9	1.5	99.9
No ALS	0/8	—	10.5	99.4	0	100	5	97.1

[a] All mice inoculated with MLV at day 0. There were no differences among groups at 1 and 3 weeks after infection with MLV.

[b] Focus forming units (mean)/0.4 ml of MSV(MLV) antibody (diluted 1:10). Mixture inoculated after 1-hr incubation into duplicate plates of ME cells. The virus-diluent mixture (control) gave 172 ffu/0.4 ml (av of 3 plates); % inhibition = [(172—ffu sample)/172] × 100.

TABLE IV. MLV Infectivity Titers of Spleen Extracts of ALS-Treated BALB/c Mice.[a]

Group (days at treatment)	week:	MLV infectivity titers of spleen extract at: 2	4	6	8	10
ALS (−4, −2, 0)		10^5	10^6	10^5	10^6	10^4
ALS (+7, +9, +11)		10^3	10^5	10^4	$10^{5.5}$	10^3
No ALS		10^1	10^3	$<10^1$	$<10^1$	$<10^1$

[a] All mice were inoculated with MLV at day 0.

and MLV treatment. As shown in Table III, a close relationship was found between susceptibility to leukemia and titers of neutralizing antibody detected by *in vitro* neutralization of MSV(MLV) focus formation (20); increased susceptibility in ALS-treated mice correlates with reduced anti-MLV antibodies.

Table IV records the high LV titers paralleling low neutralizing antibody titers in the leukemia susceptible group given ALS before leukemogenic virus. These results are of interest since ALS is known not to influence antiviral antibody induction nor induction of neoantigens in animals infected with some DNA oncogenic viruses. Inhibition of the growth of transplanted DNA virus-induced tumors or repression of primary tumors in the autochthonous host are known to be cell-mediated phenomena (1) and the present results may signify a more important role of humoral antibody in suppression of RNA virus-induced neoplasms.

Discussion and summary. The immune deficit created in 12-week-old BALB/c mice by as small a dose of ALS as 0.2 ml, in divided doses, is sufficient to provide susceptibility to the leukemogenic effects of MLV. ALS alone at various dosage levels was found not to be leukemogenic.

The immunosuppressive effect of ALS in all probability allows the survival of cells converted to neoplasia by virus; these cells are so highly antigenic that they would be eliminated in the normal animal. MLV is known to induce resistance against the transplantation of lymphoma cells carrying the corresponding antigen in BALB/c mice. Thus, as with DNA virus-induced neoplasms immune suppression and tumor antigens of the transplantation type appear to be major determinants in tumor induction with an RNA virus, MLV. That this is so is shown by the lack of increased susceptibility in those animals rendered tolerant at birth to virus or to new virus-specified cellular antigens (14). In this situation immunosuppression as observed is expected to have a negligible influence.

The reversal of susceptibility in ALS-treated BALB/c mice by adoptive transfer of syngeneic adult normal lymphoid cells probably represents replacement of those immune elements removed by ALS. Critical factors in the reversal were both timing and size of the adoptive transfer cells. The increased frequency and lowered latent period of ALS-treated recipients of "sensitized" lymphoid cells probably represents immunologic enhancement but this remains to be determined.

ALS appears to remove selectively or inactivate those cells concerned with the establishment and maintenance of immunocompetence but to be relatively ineffective against cells of lymphoid origin susceptible to transformation by MLV from the normal to the neoplastic state.

ALS administration prior to infection with leukemogenic virus inhibited antiviral antibody but provided leukemia induction, whereas ALS administered after MLV was largely ineffective. This most likely signified that a major effect of ALS is upon antigen-sensitive cells [see (21)]. The precise role of antiviral antibody, however, has not been determined.

The model systems described lend themselves to studies of the mechanisms concerned with susceptibility and resistance to lymphoid neoplasms induced in the primary host.

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