Cytotoxic Activity of Lymph Node Cells from Mice Treated with Immunosuppressive Antilymphocyte Serum[1] (36220)

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(Introduced by Leonard J. Cole)

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Although antilymphocyte serum (ALS) has been shown to prolong the survival of allografts (1, 2) and to reduce the graft-versus-host reactivity of lymphoid cells (3), we have previously reported that lymph node cells from mice treated with immunosuppressive ALS have significant local reactivity in the skin of the X-irradiated hamster (4, 5). Since that local reactivity could be due, in part, to the elaboration of cytotoxic substance(s) following ALS-induced lymphocyte transformation (6–9), we have studied the cytotoxic activity of ALS-exposed lymph node cells in both *in vivo* and *in vitro* systems and report here the significant elaboration of cytotoxic substance(s) by mixtures of histoincompatible lymph node cells obtained from mice treated with immunosuppressive ALS.

Materials and Methods. Inbred Balb/c mice, C3H mice, and a strain of Golden Syrian hamsters were used. Six- to 10-day-old (Balb/c $\times$ C3H) F_1 hybrid mice were raised using the above breeder mice. Horse anti-mouse lymphocyte serum (HAMLS) was produced in a 680 kg horse injected intradermally with 1×10^9 Balb/c lymph node cells in complete Freunds adjuvant, followed at 14 day intervals with intravenous injections of 1×10^9 similar cells in normal saline solution; a bleeding obtained during the sixth week of immunization was used in our study. Normal horse serum (NHS) was also obtained, and both sera were heat-inactivated at 56° for 30 min.

The lymphocytotoxicity titers of the sera were determined by a modification of a method of Spooner and Sell (10). The immunosuppressive potency of each serum was determined by its capacity to prolong the survival of C3H skin allografts on 20 treated Balb/c recipients (11). Recipient treatment consisted, in each case, of injections of 0.25 ml of the appropriate serum intraperitoneally (ip) on days -1, $+1$, $+3$, and $+5$; skin allografting was performed on day 0. Skin allografts were considered rejected when less than 10% of the allografted skin remained soft and pliable. Statistical analysis of the data was performed by a method of Litchfield (12).

Cell donors were either untreated mice or treated mice injected with the serum preparations noted. Mouse cells for intradermal (id) injection into X-irradiated hamsters were obtained from the cervical, axillary, and inguinal lymph nodes of the mouse donors on the day following the completion of serum administration.

Local reactions caused by lymphocyte populations in the skin of the X-irradiated hamster were assessed according to a technique similar to that previously described by Ramseier and Streilein (13). Hamsters were placed in Plexiglas dosimetry cages and were subjected to 1500 R whole-body X-irradiation (irradiation factors: 250 kVp; dose rate, 20 R/min in free air; distance, 1 m; HVL, 1.9

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TABLE I. Local Reactivities of Serum Preparations and Lymph Node Cells in the Skin of the X-Irradiated Hamster.[a]

Saline, serum preparation, or lymph node cell population injected[b]	Treatment of the cell donor(s)	No. of skin reactions	MRI[c] (± 1 SEM)
Saline	No cells injected	25	0.02 (± —)
NHS[d]	No cells injected	13	0.00 (± —)
HAMLS[e]	No cells injected	13	0.03 (± —)
Single population of 10×10^6 hamster cells	Untreated	8	0.28 (± 0.08)
Single population of	Untreated	50	0.59 (± 0.03)
10×10^6 Balb/c or	NHS-treated[d]	22	0.56 (± 0.08)
10×10^6 C3H cells	HAMLS-treated[e]	28	1.60 (± 0.11)
Double populations	Untreated	25	1.00 (± 0.08)
(mixtures) of 5×10^6	NHS-treated[d]	11	1.00 (± 0.11)
Balb/c and 5×10^6 C3H cells	HAMLS-treated[e]	14	2.31 (± 0.13)

[a] Hamster received 1500 R (48 hr prior to injection).
[b] All preparations consisted of total volumes of 0.1 ml.
[c] Mean reactivity index (see Materials and Methods).
[d] Normal horse serum.
[e] Horse antimouse lymphocyte serum.

mm Cu). Forty-eight hours after irradiation, the hamsters were injected id with 0.1 ml of a freshly prepared lymph node cell suspension in Hanks' balanced salt solution (HBSS). Lymph node cells injected were either single strain populations (cells from a single donor strain consisting of either 10×10^6 Balb/c cells or 10×10^6 C3H cells in 0.1 ml of suspension) or double strain populations (cells from the two different mouse strains consisting of 5×10^6 Balb/c plus 5×10^6 C3H cells present together in 0.1 ml of suspension). Double strain populations were mixed *in vitro* prior to injection. All experiments were internally controlled by the injection of each hamster with both experimental and control populations. As many as nine injection sites on each hamster were utilized. Hamsters were killed 48 hr after injection, skin areas bearing the injection sites were removed, and the sizes of the reaction sites were recorded (mm of induration). The reactivity index of each intradermal reaction was calculated by dividing each individual reaction value by the mean reaction value caused by the injection of control double populations of normal cells as previously reported (5); the mean reactivity index (MRI) was obtained by averaging those individual reactivity indices.

In addition, lymph node cells obtained from NHS-treated and HAMLS-treated Balb/c mice were labeled with ^{51}Cr using a method previously described by Martin (14). 10×10^6 of those ^{51}Cr-labeled cells (present in 0.1 ml of suspension) were injected id into X-irradiated hamsters as described above. Forty-eight hours after injection, the hamsters were sacrificed, and the injection sites were excised. The radioactivity of those sites was determined in a Gamma spectrometer (Nuclear Chicago Corporation) in order to ascertain the clearance of cells from the injection sites.

Finally, 5 ml aliquots containing either: (a) 50×10^6 Balb/c or C3H lymph node cells (obtained from untreated, NHS-treated, or HAMLS-treated mice); or (b) mixtures of 25×10^6 Balb/c plus 25×10^6 C3H lymph node cells (obtained from untreated, NHS-treated or HAMLS-treated mice) were incubated at 37° in 5% CO_2 for a period of 48 hr in sterile Eagle's medium containing 2X Eagle's amino acids (Grand Island Biological Co., Grand Island, NY) (15). After incubation, the suspensions were ascertained to be bacteria-free and were centrifuged at 2000 rpm for 15 min. The cellular pellet was resuspended in 0.5 ml of sterile, distilled water. After 45 min, it was brought to final volume

TABLE II. Clearance of ^{51}Cr-Labeled Balb/c Lymph Node Cells from Injection Sites in the Skin of the X-Irradiated Hamster.[a]

Treatment of cell donor	10×10^6 ^{51}Cr-labeled Balb/c lymph node cells		Hamster skin site 48 hr after labeled cell injection		^{51}Cr (cpm; in %) remaining in skin site after 48 hr
	$\overline{\text{cpm}}$[b] (± 1 SEM)	No. of specimens	$\overline{\text{cpm}}$[b] (± 1 SEM)	No. of specimens	
Normal horse serum	19,390 (± 623)	14	12,770 (± 127)	13	65.9
Horse antimouse lymphocyte serum	39,220 (± 536)	15	4680 (± 112)	14	11.9

[a] Performed as noted in Materials and Methods.

[b] $\overline{\text{cpm}}$ = (cpm of individual specimen − mean cpm of background)/(No. of specimens).

of 1.0 ml by the addition of 2X phosphate buffered saline. Most cellular debris was removed by centrifugation at 4000 rpm for 90 min followed by 0.22 μ filtration. The same procedure was performed on identical cell populations prior to incubation. Those aliquots were then tested for direct lymphocytotoxicity (without heterologous complement) against Syrian hamster lymph node cells.

Results. The HAMLS had a lymphocytotoxicity titer of 1:6400 against Balb/c lymph node cells; the NHS was not lymphocytotoxic.

Median effective times (with 95% confidence limits) of skin allograft survival for untreated, NHS-treated and HAMLS-treated mice were 9.6 (9.1–10.2), 9.8 (9.2–10.4), and 17.3 (15.9–18.8) days, respectively.

Table I outlines the local reactivities of serum preparations and lymph node cells in the skin of the X-irradiated hamster. The mean reactivity indices (MRI) of saline, NHS, HAMLS, single populations of hamster cells and single populations of Balb/c or C3H cells (untreated or NHS-treated) showed negligible reactivities. HAMLS yielded a nonspecific increase in the MRI of single and double populations of mouse lymph node cells (mean reactivity indices of 1.60 and 2.31, respectively). As previously reported, the MRI of double populations of cells from HAMLS-treated mice was significantly greater than the MRI of double populations of cells from untreated mice, with mean reactivity indices of 2.31 and 1.00, respectively (5).

Regardless of the donor treatment (untreated, or HAMLS-treated), the double (mixed) cell populations caused intradermal reactions that were consistently characterized by the histological appearance of local intradermal necrosis, edema, and mononuclear cell infiltration.

Table II presents data dealing with the clearance of ^{51}Cr-labeled lymph node cells from the intradermal injection sites. The cells obtained from the HAMLS-treated mice were more rapidly cleared from the injection sites than were the cells obtained from NHS-treated mice.

Table III presents the lymphocytotoxicity titers of mouse lymph node cell extracts against the inbred hamster cells. Following the 48 hr incubation period, the extracts obtained from the mixed cell populations showed significant cytotoxic activity against the hamster lymph node cells; the lymphocytotoxicity titers of those extracts obtained from untreated, NHS-treated, and HAMLS-treated cells were 1:32, 1:16, and 1:16, respectively. The supernatant medium was not cytotoxic.

Discussion. We have previously reported that lymph node cells obtained from mice treated with immunosuppressive ALS showed no decrease in local reactivity when tested in the skin of the X-irradiated hamster (4, 5). Present data augment those findings in showing that mixtures of histoincompatible lymph node cells obtained from mice treated with immunosuppressive ALS are capable of elaborating significant levels of intracellular cytotoxic substance(s) following a 48 hr *in vitro* incubation; this cytotoxic activity appears to

TABLE III. Lymphocytotoxic Activities of Mouse Lymph Node Cell Extracts Against Hamster Lymph Node Cells.[a]

Mouse lymph node cell population(s) used as source of extract	Treatment of lymph node cell donors	Incubation of mouse cells (48 hr, 5% CO_2, 37°)	Lymphocytotoxic titer against hamster lymph node cells[b]
Single populations of Balb/c or C3H cells (10×10^6 cells/ml)[c]	Untreated	−	1:4 or less
		+	Negative
	NHS-treated[d]	−	Negative
		+	1:4 or less
	HAMLS-treated[e]	−	Negative
		+	1:4
Double populations (mixtures) of Balb/c (5×10^6 cells/ml) and C3H (5×10^6 cells/ml)[c]	Untreated	−	1:4
		+	1:32
	NHS-treated[d]	−	1:4
		+	1:16
	HAMLS-treated[e]	−	1:4
		+	1:16

[a] Extracts prepared as noted in Materials and Methods.
[b] Lymphocytotoxic titering performed without exogenous complement.
[c] Suspensions in a total of 5 ml of Eagle's medium (2X amino acids) (15).
[d] Normal horse serum-treated donors.
[e] Horse anti-mouse lymphocyte serum-treated donors.

be nonspecific, as shown by the lymphocytotoxic activity directed against the hamster lymph node cells.

Other investigators have shown that prior exposure to immunosuppressive ALS renders lymphoid cells less capable of causing cytotoxic effects on target cells (16). The data presented here do not contradict those earlier studies, since our present experiments involve: (a) the liberation of intracellular material from the cells under study; and (b) the exposure of third-party cells to that material in a direct lymphocytotoxicity assay; this eliminates the necessity for contact induced cytotoxicity that most earlier studies demanded.

Our studies are in agreement with earlier experiments in raising the possibility that target cell death might be due to reactions other than immunological, *i.e.*, contact with structurally foreign surface sites (17); the lack of specificity in our studies shows that the activities measured might be due to factors other than the usual immunological recognition and reaction.

We postulate that the increased local reactivity of mixed populations of lymph node cells obtained from mice treated with immunosuppressive HAMLS was a result of: (a) normal elaboration of cytotoxic substance(s) during exposure to foreign histocompatibility antigens combined with (b) increased local release of those substance(s) due to more rapid intradermal clearance and lysis of those HAMLS-treated cells.

Summary. A horse antimouse lymphocyte serum was shown to be immunosuppressive in prolonging the survival of C3H skin allografts on Balb/c mice. Despite the immunosuppressive effect of that preparation, however, mixtures of histoincompatible lymph node cells obtained from treated mice showed: (a) an increased local reactivity in the skin of the X-irradiated hamster; (b) a more rapid clearance from those intradermal injection sites; and (c) a significant, *in vitro* production of nonspecific lymphocytotoxic substance(s). We conclude that the liberation of cytotoxic substance(s) through mouse cell lysis is responsible, in part, for the increased local reactivity of lymph node cells obtained from immunosuppressed mice.

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