

Variations in Rabbit Antimouse Lymphocyte Sera Due to Strain Differences and "Nonthymic" Lymphocytes (35554)

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In a previous study it was found that immunization with mouse lymphoid cells of differing origins, *i.e.*, thymus, lymph node, or stimulated lymph node draining a third set allograft, would induce rabbit anti-mouse lymphocyte sera (RAMLS) of differing activity (1). The present report describes further observations of differences in activity of RAMLS if the sera were produced using differing inbred mouse strains as well as lymphoid cell types in the immunizing inocula. Rabbit antisera were raised against thymus (ATS) and lymph nodes (ALS) of three strains of inbred mice differing at major H-2 histocompatibility loci. An antiserum was also made by immunizing with lymph node cells of adult mice in the absence of a thymic system (NTALS), *i.e.*, after thymectomy and X-irradiation, using a modification of the method described by Miller *et al.* (2). In testing the effects of the sera on the prolongation of allograft survival, it was found that donor strain specific ALS and ATS had a greater effect than antisera induced to recipient strains. However, recipient strain specific antisera caused a more marked peripheral blood lymphocyte depletion. Donor specific NTALS minimally prolonged allograft survival, but in all other experiments NTALS caused no significant effect on either skin allograft survival or peripheral lymphocyte counts. Nevertheless, leukoagglutinin activity of NTALS against lymph node cells was equal to that of ALS and ATS.

Materials and Methods. Mice. Inbred male mice of the Ajax (H-2^a), C57BL/6J (H-2^b), DBA/2J (H-2^d) and C3H/HeJ (H-2^k) strains, 8 to 12 weeks of age were obtained from Jackson Memorial Laboratory. They were housed in stainless steel cages and fed

food and water *ad libitum*. In conducting the present research, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

Skin grafts. Skin allografts were performed as described by Martinez *et al.* (3) and used extensively in our laboratory. The grafts were read daily by 2 of the investigators (J. M. and E. S.). The mice were picked out at random without knowledge of which animal or group was involved. Graft survival was expressed as the mean survival time (MST \pm SE) of groups of 12 to 30 mice. Each experiment was performed on two occasions and was analyzed statistically by *t* tests.

Lymphocyte counts. Peripheral lymphocyte counts were performed on tail blood by standard clinical methods. The data were analyzed statistically by the Kruskal-Wallis one-way analysis of variance using ranks (4).

X-Irradiation. One thousand rads whole body X-irradiation were administered with a 250 kv Westinghouse X-ray machine using a 1 mm Al and 0.25 mm Cu filter. Left inguinal and popliteal lymph nodes were removed surgically from each mouse 1 day previously. During X-irradiation, 2-mm thick lead shields were placed over the left hind limbs. Dosimetry calculations using a Victoreen ionization chamber and lithium fluoride capsules indicated no more than 30 rads X-irradiation under the shield. This technique has been shown by morphologic studies to ablate temporarily the lymphoid tissue, except for the bone marrow of the shielded

TABLE I. Leukoagglutination of Thymus and Lymph Node Cells by ALS, ATS, and NTALS.

Sera	Ajax		C57BL/6	
	lymph node	Ajax thymus	lymph node	C57BL/6 thymus
Anti-Ajax ALS	64 ^a	64	128	64
Anti-Ajax ATS	64	1024	64	512
Anti-C57BL/6 ALS	64	64	64	64
Anti-C57BL/6 ATS	64	512	64	1024
Anti-C57BL/6 NTALS	64	64	64	64
NRS	0	0	0	0

^a Reciprocal of tube dilution.

limb, without producing mortality (5).

Heterologous antilymphocyte sera. The antisera were raised against C57BL/6J, Ajax and C3H/HeJ mice in New Zealand white male rabbits weighing 3 to 3.5 kg following the technique of Gray *et al.* (6). Anti-Ajax and Anti-C57BL/6 ALS and ATS were produced in groups of 5 rabbits. Anti-C57BL/6 NTALS was raised in 3 rabbits. An emulsion of lymphoid cells in complete Freund's adjuvant containing 100×10^6 cells/rabbit was equally divided and inoculated in each foot pad (0.25 ml/foot pad); and 28, 29, and 30 days later, intravenous injections of 100×10^6 cells in Hanks' solution were given. Seven days after the last injection the animals were bled. NTALS was raised as follows: Thymectomies were performed on groups of eighty 8-week-old C57BL/6 mice using the technique of Sjodin *et al.* (7). Two weeks later 1000 rads of whole body X-irradiation was administered while the left hind limbs were shielded, 1 day after removing the ipsilateral inguinal and popliteal nodes. Four weeks later each mouse was injected intraperitoneally (ip) with 0.1 ml of typhoid-paratyphoid vaccine (TPT) containing 50 million typhoid and 12.5 paratyphoid organisms emulsified in complete Freund's adjuvant. After 21 days, a similar injection without adjuvant was given. Seven days later the mice were bled, sacrificed, and inspected for completeness of thymectomy. As an additional check a few lymph nodes of these mice were studied histologically. Suspensions of axillary, inguinal, and mesenteric lymph nodes from animals with complete thymectomies were emulsified in complete Freund's adjuvant and inoculated into rabbit foot pads (100×10^6 cells/rabbit). Intravenous injections

were performed using "nonthymic" lymphoid cells from other C57BL/6 mice which were thymectomized, X-irradiated, and immunized with TPT, so as to furnish these cells 28, 29, and 30 days after the initial rabbit inoculations. Normal rabbit serum (NRS) was obtained by bleeding four untreated rabbits. The sera were stored at -70° , and were thawed, pooled, and inactivated at 56° before use. Mice were injected intraperitoneally with 0.1-ml doses on day +2 or days +2 and +4 after receiving allografts.

Leukoagglutinations, immunoelectrophoresis, and typhoid antibody determinations. Leukoagglutinations were performed in triplicate as modified from the method of Amos and Peacocke (8). Immunoelectrophoresis was performed as described by Scheiddeger (9). Typhoid paratyphoid anti-H and anti-O examinations were performed by standard clinical methods (10).

Results. "Nonthymic" mice. Lymph nodes of thymectomized X-irradiated mice immunized with TPT contained increased numbers of plasma cells and germinal centers, but were devoid of small lymphocytes in the deep paracortical or thymic dependent areas (Fig. 1). Cells from these lymph nodes were used to induce NTALS. The sera of these mice showed flocculation titers to typhoid H and O antigens equal to that of mice similarly treated, but without thymectomy. Serum immunoelectrophoretic patterns were normal.

Leukoagglutinations. Leukoagglutination titers of anti-C57BL/6 ALS, ATS, and NTALS as well as anti-Ajax ALS and ATS were equal (1:64) when tested with lymph node lymphocytes of either strain (Table I). There was greater reactivity of anti-C57BL/6 and anti-Ajax ATS when tested

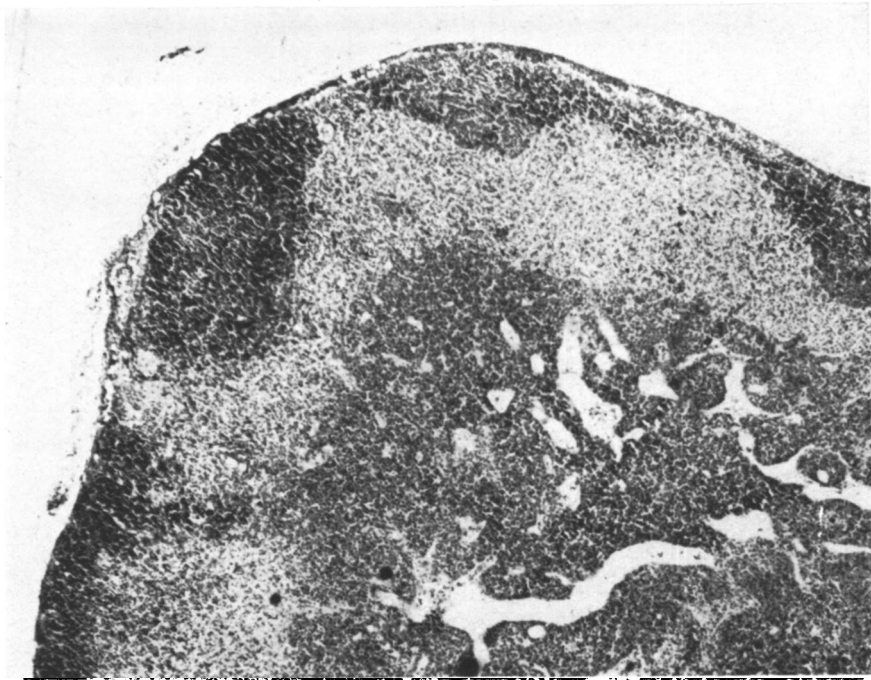


FIG. 1. Peripheral lymph node of a C57BL/6 mouse which was thymectomized, X-irradiated, and then immunized with TPT. The paracortical or deep cortical areas were depleted of small lymphocytes while cortical germinal centers and medullary plasma cells were present.

with thymic cells of either strain as opposed to lymph node cells, confirming other studies in which absorptions were performed (1, 11).

Skin allografts and lymphocyte counts. Donor strain specific ALS and ATS caused greater prolongation of allograft survival than recipient strain specific antisera, while recipient strain specific antisera caused a greater decrease in peripheral lymphocyte counts. In C57BL/6 recipients of Ajax allografts (Table IIa and b) anti-Ajax ATS in one and two 0.1-ml doses ip had a significantly stronger effect ($p = 0.01$) than anti-C57BL/6 ATS (MST of 13.7 ± 0.4 and 16.0 ± 0.4 vs 9.5 ± 0.2 and 14.0 ± 0.4 , respectively). The same was true but to a lesser extent when anti-Ajax ALS was compared with anti-C57BL/6 ALS. There was a significant difference ($p = 0.01$) when the means of groups given either one or two doses were combined (MST of 9.7 ± 0.2 and 11.5 ± 0.2 vs 8.6 ± 0.2 and 10.6 ± 0.2). In the converse experiment also, in Ajax recipients of C57BL/6 skin allografts (Table IIc and d) ATS raised against the C57BL/6 strain was

more effective than anti-Ajax ATS in both one and two doses (MST of 13.3 ± 0.2 and 18.2 ± 0.7 vs 10.5 ± 0.2 and 11.2 ± 0.2). The same effect was seen to a lesser extent with ALS (12.6 ± 0.5 and 14.7 ± 0.6 vs 10.6 ± 0.3 and 10.1 ± 0.3).

By contrast, 24 hr after either one or two injections, the peripheral blood lymphocytes were more depleted, when anti-C57BL/6 antisera were given to C57BL/6 recipients, and anti-Ajax sera to Ajax recipients (Table IIa, b, c, and d). These findings were supported by following the lymphocyte counts between 3 and 13 days after allografting. In Fig. 2, lymphocyte counts of groups of C57BL/6 mice injected with either anti-C57BL/6 ALS or ATS were combined and compared during the 2-week period after treatment with counts of mice injected with either anti-Ajax ATS or ALS. Also included are the responses to NTALS, NRS, and uninjected mice. In Fig. 3 the same data are presented in Ajax mice. Both the extent and duration of the differences between the effects of strain specific antisera were highly significant ($p =$

TABLE II. Allograft Survival and Absolute Lymphocyte Counts.

Strain		No./group	Treatment and no. of injections ^a	MST (days \pm SE)	Mean absolute lymphocyte count \pm SE ^b
Donor	Recipient				
a					
Ajax	C57BL/6	37	—	8.0 \pm 0.2	8115 \pm 709
		20	NRS 1	7.6 \pm 0.1	5980 \pm 363
		19	NTALS 1	6.6 \pm 0.1	5042 \pm 513
		17	C57BL/6 ALS 1	8.6 \pm 0.3	4140 \pm 433
		29	C57BL/6 ATS 1	9.5 \pm 0.2	2443 \pm 265
		18	Ajax ALS 1	9.7 \pm 0.2	5142 \pm 470
		29	Ajax ATS 1	13.7 \pm 0.4	3700 \pm 270
b					
Ajax	C57BL/6	20	NRS 2	9.1 \pm 0.2	8521 \pm 791
		19	NTALS 2	7.7 \pm 0.2	5745 \pm 594
		20	C57BL/6 ALS 2	10.6 \pm 0.3	2920 \pm 317
		28	C57BL/6 ATS 2	14.0 \pm 0.4	1510 \pm 183
		20	Ajax ALS 2	11.5 \pm 0.2	4932 \pm 479
		24	Ajax ATS 2	16.0 \pm 0.4	3261 \pm 448
c					
C57BL/6	Ajax	32	—	9.9 \pm 0.2	4549 \pm 382
		18	NRS 1	8.8 \pm 0.2	4378 \pm 115
		18	NTALS 1	10.8 \pm 0.3	4003 \pm 235
		16	C57BL/6 ALS 1	12.6 \pm 0.5	3056 \pm 167
		22	C57BL/6 ATS 1	13.3 \pm 0.2	2763 \pm 178
		16	Ajax ALS 1	10.6 \pm 0.3	2468 \pm 240
		25	Ajax ATS 1	10.5 \pm 0.2	1698 \pm 122
d					
C57BL/6	Ajax	18	NRS 2	9.1 \pm 0.2	4178 \pm 382
		15	NTALS 2	10.2 \pm 0.3	3828 \pm 467
		13	C57BL/6 ALS 2	14.7 \pm 0.6	2248 \pm 180
		19	C57BL/6 ATS 2	18.2 \pm 0.7	2151 \pm 232
		17	Ajax ALS 2	10.1 \pm 0.3	2152 \pm 188
		26	Ajax ATS 2	11.2 \pm 0.2	1385 \pm 172
		15	C ₃ H/Hej ATS 2	13.3 \pm 0.3	1941 \pm 161
		e			
C57BL/6	DBA/2	26	—	10.2 \pm 0.2	7625 \pm 431
		15	Ajax ATS 1	13.6 \pm 0.6	3539 \pm 307
		18	C ₃ H/Hej ATS 1	12.7 \pm 0.3	3486 \pm 239
		17	C57BL/6 ATS 1	16.8 \pm 0.6	3908 \pm 291
f					
Ajax	DBA/2	12	—	9.8 \pm 0.3	6901 \pm 373
		16	C57BL/6 ALS 2	14.7 \pm 0.3	2609 \pm 200
		14	NTALS 2	10.5 \pm 0.4	5022 \pm 496

^a Each injection was 0.1 ml of serum ip.

^b 18 mice/group 24 hr after the last injection of antiserum.

0.001) in both strains. NTALS and NRS had little if any effect.

The MST of C57BL/6 skin on DBA/2 mice injected with donor specific ATS (anti-C57BL/6) was somewhat more prolonged over groups injected with anti-Ajax

and anti-C₃H (MST of 16.8 \pm 0.6 compared with 13.6 \pm 0.6 and 12.7 \pm 0.3, $p = 0.01$). There was no difference in lymphocyte depleting effects of the sera, none of which were raised against DBA/2 strain (Table IIe).

In C57BL/6 mice there was no significant

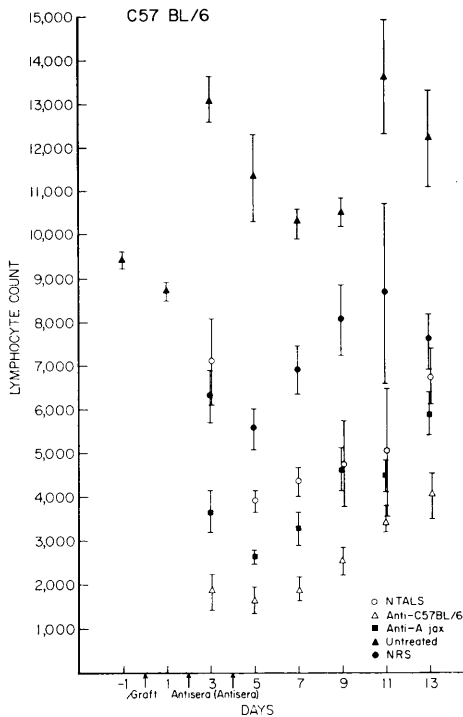


FIG. 2. Peripheral blood lymphocyte counts of C57BL/6 mice which received Ajax skin allografts on day zero (arrow). The mice then were given 0.1 ml of antisera (arrow) on day +2, or on day +2 and +4. (○) NTALS = the response to "nonthymic" ALS; (Δ) anti-C57BL/6 = the response to anti-C57BL/6 ALS and ATS; (\blacksquare) anti-Ajax = the response to anti-Ajax ATS and ALS; (\bullet) NRS = response to normal rabbit serum; and (\blacktriangle) = the response of untreated mice. Each point represents the mean and standard error of at least 6 mice.

difference in the effects of NTALS and NRS in one and two doses (Table IIa and b) on either allograft acceptance (MST of 6.6 ± 0.1 and 7.7 ± 0.2 compared with 7.6 ± 0.1 and 7.7 ± 0.2) or peripheral lymphocyte counts (5042 ± 513 and 5745 ± 594 compared with 5980 ± 363 and 8521 ± 791). In Ajax mice NTALS caused a slight prolongation in allograft survival ($p = 0.01$) compared to the effects of NRS 10.8 ± 0.3 and 10.2 ± 0.3 compared with 8.8 ± 0.2 and 9.1 ± 0.2), but no difference in lymphocyte counts (4003 ± 235 and 3828 ± 467 vs 4378 ± 115 and 4178 ± 382). With a nonspecific donor recipient strain combination, *i.e.*, DBA/2 mice receiving Ajax allografts, there

was no effect of NTALS, while treatment with anti-C57BL/6 ALS caused significant allograft prolongation and peripheral lymphocyte depletion (Table IIc).

Discussion. Histocompatibility antigens in mice have long been known to induce antibodies across species barriers as observed by Gorer in 1938 (12). However, observations on the strain specific effects of antilymphocyte sera have not been consistent. Gray *et al.* (6) did not find specificity for the strain of origin if ALS raised in rabbits against mouse lymph nodes was tested by leukoagglutination. Levey and Medawar (13) noted no specificity while Brent *et al.* (14) presented conflicting evidence. Ogburn *et al.* (15) and Miller *et al.* (1) demonstrated that absorptions with lymphoid cells of nonspecific strains could bring out strain specificity *in vitro*. Shorter and Elveback (16), using ATS raised in response to the recipient strains, showed some strain specific effects on

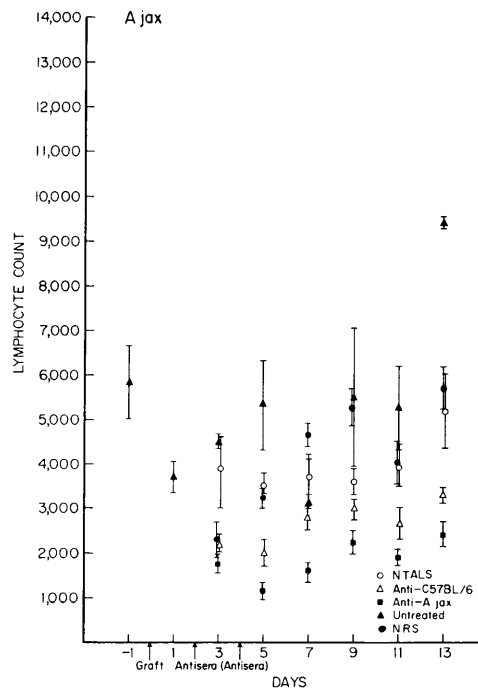


FIG. 3. Peripheral blood lymphocyte counts of Ajax mice which received C57BL/6 allografts on day zero (arrow). The mice were given 0.1 ml of antisera (arrow) on day +2, or day +2 and +4 (see Fig. 2 for key). Each point represents the mean and standard error of at least 6 mice.

allograft prolongation but no specific effect on peripheral lymphocyte depletion. In the present work using four different strains and three types of heterologous antisera, *i.e.*, ATS, ALS, and NTALS, there was evidence of strain specificity without absorption, when tested for effects on allograft survival. Contrary to the observations of Shorter and Elveback (16), however, the prolongation was significantly more pronounced when antisera specific against the donor strain were used. Also, there was a significantly greater depletion of peripheral lymphocytes after administration of recipient strain antisera. One explanation of the effects on graft survival might possibly be that the effectiveness of recipient specific antisera was dissipated in reactions against antigenic sites other than those important in facilitating allograft acceptance. However, this does not explain why donor specific antisera (anti-C57BL/6) were more effective than nonspecific antisera (anti-Ajax and anti-C₃H) in DBA/2 strain recipients. More plausible perhaps is that enhancement antibodies were present (17), although enhancement has ordinarily been described with allogenic rather than xenogeneic sera. As enhancement antibodies have been observed only during a finite period after immunization, while at other times sera may be hyperimmune (18), the time of bleeding and method of immunization of the rabbit might explain the controversial observations. Histocompatibility antigens therefore might have either facilitatory or inhibitory effects, although the primary action of ALS appears to be directed against the small lymphocyte (19, 20). Demonstrating these effects may be difficult also because of the many antigens controlled by the H-2 alleles (21) with "minor" histocompatibility antigens conceivably playing a role.

The significantly stronger effect of strain specific antisera on peripheral lymphocyte depletion in the present work might be because our observations were over a prolonged period, between 24 hr and 12 days after treatment, rather than at an earlier time (16).

The question of thymus versus lymph nodes as the source of lymphoid antigen for heterologous antisera has been extensively ex-

amined (22, 23), but the results have been inconsistent. In the present work, lymph nodes depleted of paracortical or "thymic" lymphocytes (Fig. 1) evoked antisera (NTALS) with much reduced effect on allograft survival and peripheral lymphocyte depletion, compared with ALS and ATS, despite the fact that leucoagglutinating activities of all three sera against lymph node lymphocytes were equal. This is consistent with the thesis that the primary action of RAMLS is directed against the thymic derived lymphocyte, although other antigens in the immunizing inocula may also be active. To further support this absorption studies carried out previously with thymocytes and lymph node cells have indicated specificity in these sera (1). NTALS is presently being further characterized in a variety of *in vivo* and *in vitro* tests in order to evaluate its specificity.

Summary. Heterologous antimouse lymphocyte sera were evoked by injecting rabbits with normal mouse thymuses and lymph nodes (ATS and ALS), as well as lymph nodes of mice thymectomized and X-irradiated, and then immunized with typhoid-paratyphoid vaccine (NTALS). Antisera were raised against C57BL/6, Ajax, and C₃H inbred mouse strains. The effects were tested on skin allograft survival and peripheral lymphocyte counts. Allograft survival was more prolonged in recipients injected with antisera raised against the donor strains, while peripheral lymphocyte counts were more depressed if the antisera were directed against the recipient strains. While there was minimal prolongation of allograft survival if NTALS was raised against the donor strain, there were no effects of NTALS in any other strain combination, and no significant depression of the lymphocyte counts. Leukoagglutinin titers of ALS, ATS, and NTALS were equal when tested against mouse lymph node lymphocytes. It is suggested that enhancement could have been a factor in the prolongation of allograft survival when antisera were directed against the donor strain.

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