IMMUNOSUPPRESSIVE AND GRAFT-REJECTING ANTIBODIES IN HETEROLOGOUS ANTI-LYMPHOCYTIC SERUM

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Heterologous anti-lymphocyte serum (ALS)[1] is widely used as an immunosuppressive agent, and may represent an important intermediate stage in the endeavor to achieve a narrowing of the target, affected by immunosuppression. Two questions arise in this context; the first concerns the specificity of immunosuppressive antibodies and the second concerns the mechanism by which such antibodies exert their central effect on the immune system.

ALS contains antibodies which appear to have specificity for cell populations which are not the primary target of immunosuppression (1, 2). Some antibodies of this type can be demonstrated in vitro, but seem to have little effect in vivo (3, 4); others may have in vivo activity, but have so far only been detected by their general toxicity in the host and not by a target-defined activity. Thus the development of techniques for the discrimination between immunosuppressive and toxic antibodies is a crucial requirement for the functional purification of ALS.

ALS appears to cause inactivation of circulating lymphocytes and it is tempting to assume that this inactivation is due to cytotoxic antibody and thus results in immunosuppression (5, 6). However the immunosuppressive potency of ALS does not correlate with its content in agglutinating and cytotoxic antibodies directed against host lymphocytes (7–9). In fact, ALS preparations can be potent immunosuppressive agents and yet be virtually devoid of lymphocytotoxic antibodies (8). This may be due to an in vivo process of destruction or inhibition which does not involve cell lysis (3, 10, 11). Indeed, suppression by allotype antibodies, which shows some analogies with ALS-mediated suppression, has been found to be independent of late acting complement components and hence of cell lysis (12).

In an attempt to analyze the role of cytotoxic antibodies, we have compared the survival times of allografts on ALS-treated strains of C5 (MuB1)-deficient and C5 (MuB1)-competent mice (13–16). These survival times would be different were cell lysis involved in ALS action. In an initial series of experiments, allograft survival times were found to be longer when the ALS-treated animals

[1] *Abbreviations used in this paper:* ALS, anti-lymphocyte serum; SRBC, sheep red blood cells.

were C5 deficient rather than competent and this suggested that immunosuppression by ALS did not require the C5-mediated lysis of lymphocytes by cytotoxic antibody. In subsequent experiments, attempts were made to determine why graft-survival times were shorter in the C5 competent group of animals. It was found that this was because ALS, in addition to having immunosuppressive properties, contained graft-rejecting activity which acted directly on the graft and was enhanced when C5 was available.

Materials and Methods

Animals Used.—Inbred mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. Male animals, 6–10 wk of age, of the following strains, were employed: A/J, B10.D2-old Sn/J, B10.D2-new Sn/J, CBA/J, C57BL/6J, DBA/1J, DBA/2J, and RF/J. Among these animals, the following lack MuB1 (13, 14) which is the C5 component of complement (15, 16): B10.D2-old Sn/J, A/J, RF/J, DBA/2J.

Normal Horse Serum.—Obtained from Dr. S. Wilson (Fraser Laboratory, Connaught Laboratories, University of Toronto, Toronto, Canada).

MuB1-Positive and Negative Sera.—Animals were bled into tubes kept in ice water. The blood was kept at 2°C for 2–4 hr. Sera were separated by centrifugation at 2°C and were kept frozen. Such serum pools were obtained from the Jackson Laboratory, or were prepared in our laboratory.

MuB1 Antisera (13).—Antisera were obtained by immunizing female mice of strain AHe/J with C57L/J ♀ sera incorporated in complete Freund's adjuvant. The mice were bled from the tails; a pool was prepared from bleedings taken over 237 days after 8–17 injections.

Preparation of ALS.—Sera, raised in horses (922-29, HAMTS-Tech B), were prepared by Dr. G. Lamoureux (Institute of Hygiene, Laval-des-Rapides, Quebec, Canada) and were obtained from the Medical Research Council as part of their ALS project. For preparation of ALS 922-29, a horse was repeatedly injected with a mixture of thymus and lymph node cells from C57BL/6J mice. The animal received 10^9 cells subcutaneously on days 0, 3, 6, and 9, and 10^9 cells intravenously on days 10, 14, and 21. The horse was bled on day 29. ALS HAMTS-Tech B was prepared in the same way but the immunizing inoculum only contained C57BL/6J thymus cells and the subcutaneous injections were given in Freund's adjuvant. Another equine ALS, the British Research standard for anti-lymphocyte serum (in vivo use), 68/65, was obtained from Dr. S. G. Anderson (Division of Biological Standards, National Institute for Medical Research, Mill Hill, London, England). Antisera raised in rabbits, R30/31 and 30, were obtained from Dr. D. A. L. Davies (Searle Research Laboratories, High Wycombe, Bucks, England) and from Dr. E. M. Lance (The Hospital for Special Surgery, New York) respectively. R30–31 and 30 were prepared by repeatedly immunizing rabbits with a membrane fraction of mouse thymus cells. PA was obtained by the immunization of a rabbit with polyoma cells. The cells were from a tumor, raised in a C57BL/6J animal and carried by serial passage; 4×10^8 cells in Freund's adjuvant were injected on day 0 into the footpad of a rabbit. The same cell-number was injected on day 14 and the rabbit was bled on day 21. PA was not immunosuppressive, but was cytotoxic and agglutinating for thymus cells. R30/31 and 68/65 were freeze-dried; other sera were stored at −20°C. Prior to use, all sera were heated at 56°C for 1 hr. In some experiments, the antisera were absorbed with an equal volume of packed sheep red cells, as indicated in the text. Though not strictly accurate, general usage sanctions the use of the term ALS to refer to sera raised by immunization with thymus cells alone or a mixture of thymus and lymph node cells.

Single Diffusion-Ring Test (17, 18).—MuB1 antisera (0.38 ml) and glycine buffer (1.12 ml) were mixed at 56°C and added to glycine agarose (1.5 ml). The carefully mixed fluid was poured

into the agar diffusion plate and allowed to set. For this purpose Immuno-Plates supplied by Hyland Laboratories, Los Angeles, Calif., were used. A series of 24 evenly spaced wells (diameter: 3.7 mm) were punched into the gel and were filled with antigen to the rim. To establish suitable conditions for the test, various dilutions of a standard serum were incubated for varying periods at 20.8° + 0.2°C; the shortest incubation time which gave reproducible results was finally selected. The conditions chosen for the assay were as follows: the agar diffusion plates were sealed, kept for 4 hr at 20.8° + 0.2°C and were then photographed in the Cordis immunodiffusion camera (Cordis Corp., Miami, Fla.). Photographs were projected and the ring-widths of the opaque antigen antibody precipitin zones were evaluated by comparison with a calibration curve in which ring-widths were plotted against the logarithm of various concentrations of a standard preparation.

Cytotoxicity of ALS in the Presence of Complement.—2 ml of a 1:64 dilution of ALS pool 922-29 was added to an equal volume of phosphate-buffered saline (pH 7.25–7.35) containing 10% decomplemented C57BL/6J mouse serum and 1.5×10^7 C57BL/6J thymus cells per milliliter. After 1 hr at 37°C, 0.2 ml samples of the mixture of cells and antisera were placed in separate test tubes and 1.5 ml of undiluted serum or serum diluted with buffer (as indicated in Fig. 2) was added to each tube. The serum used was obtained from guinea pigs and from B10.D2-old Sn/J and new mice. After 30 min at 37°C the mixture was gently centrifuged. The supernatant was discarded, 0.2 ml of buffer containing 10% decomplemented syngeneic mouse serum and 0.3 ml of a 1:1000 dilution of trypan blue in buffer were added to the sediment and gross agglutinates were then broken up by gentle pipetting. The percentage of cells stained with trypan blue was determined by counting in a hemocytometer 15 min later. At least 200 cells were counted on each occasion.

Skin Grafting.—The method employed and the criteria used for assessing graft rejection have been previously described (19).

Immunization with Sheep Red Blood Cells (SRBC) and Determination of Hemagglutinin Titers.—SRBC from freshly bled sheep were stored in Alsever's solution at 4°C and were always used within a week of collection. Before use they were washed three times in 0.9% NaCl and suspended in TC199 (Difco Laboratories, Inc., Detroit, Mich.) before injection. Each mouse received 0.1 ml of a 10% suspension of SRBC by intravenous injection. Hemagglutinin titers were determined on serum obtained 7 days later using a previously described method (20). The titer recorded was the dilution in the last well in which the cell patterns differed from the buttons of cells present in the control wells. All titers have been recorded in terms of $\log_2$ of their reciprocals.

RESULTS

Serum C5 Levels after ALS Treatment.—Administration of ALS is accompanied by small, though significant changes in the serum levels of C5 (Fig. 1). Thus a relatively late acting complement component is affected by events which follow treatment with ALS.

Effect of Cytotoxic Antibody on Lymphocytes in the Absence of C5.—Fig. 2 shows the uptake of trypan blue by ALS-treated thymus cells in the presence of MuB1-negative and MuB1-positive serum. Cytoxicity was observed when guinea pig serum or MuB1-positive mouse serum was used but not when MuB1-negative serum served as a source of complement. Similar results were obtained in another experiment in which still larger amounts of serum were used as the source of complement.

Thus serum from mice that have C5 can promote the uptake of trypan blue

by lymphoid cells coated with cytotoxic antibody, whereas serum from mice deficient in C5 cannot. Hence, there is no in vitro "by-pass" to compensate for the absence of C5, though one cannot exclude the possibility that an in vivo by-pass exists.

Survival Times of Allografts on ALS-Treated MuB1-Positive and Negative Mice.—To test the effect of C5 on ALS-mediated immunosuppression we

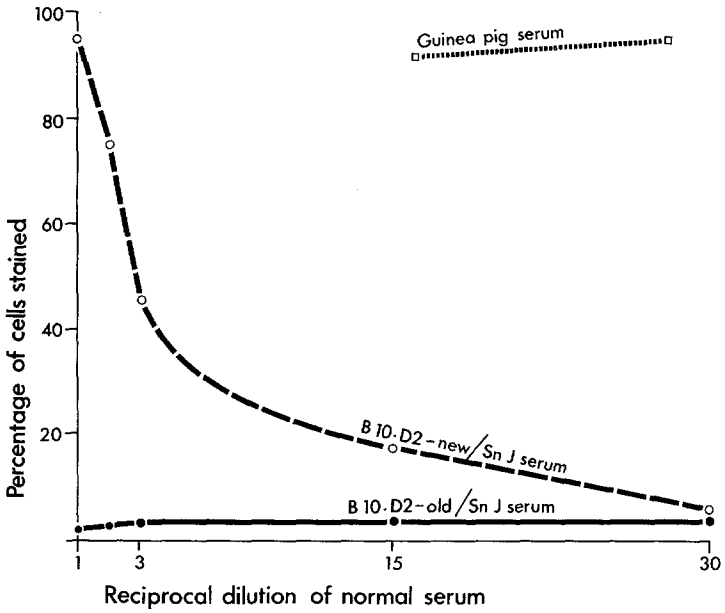

FIG. 1. The cytotoxic effect of ALS in the presence of serum from B10.D2-old Sn/J and B10.D2-new Sn/J. ALS pool 922-29 was added to thymus cells from C57BL/6J mice. Normal serum from C5-positive mice (B10.D2-new Sn/J), from C5-negative mice, or from guinea pigs served as the source of complement. After 30 min at 37°C, uptake of trypan was determined. The percentage of cells stained with trypan blue was plotted as a function of the quantity of the added normal serum.

transplanted skin across an H-2 barrier to mice which were known to lack C5. Graft survival times on untreated mice were compared with those found when mice received normal sera or ALS. Graft retention on untreated coisogenic MuB1-positive and MuB1-negative animals was identical and was not significantly affected by the administration of normal horse or rabbit serum.

Graft survival times were prolonged by ALS treatment. They were longer when ALS was given to C5-deficient rather than to C5-competent animals (Table I). This was found with different ALS preparations of both horse and rabbit origin (Table I). The effect was observed with MuB1-positive and MuB1-

negative donors of various H-2 genotypes (Table II). The magnitude of the effect depended on the dose schedule of ALS administration (Table III). The period of graft retention in C5-deficient ALS-treated animals was reduced by the administration of serum from C5-competent mice (Table IV). On the basis of these observations, it appeared reasonable to assume that ALS contained

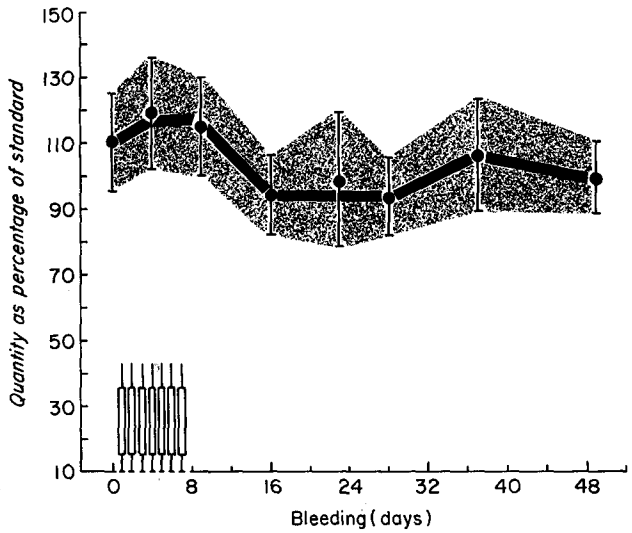

FIG. 2. Change in the concentration of MuB1 (C5) after the administration of ALS. Male mice (C57BL/6J), 9 wk old, were given daily subcutaneous injections with ALS HAMTS-Tech B as indicated. Injections are indicated by syringe symbols above the horizontal ordinate. The mice were bled on days 0, 4, 9, 16, 23, 28, 37, and 49; MuB1 concentration was determined by radial diffusion (18) and related to a standard (100%). The heavy line shows the change of MuB1 concentration with time, the stippled area corresponds to 2 SD. A comparison of the MuB1 content in bleedings taken between days 0 and 9 with those taken between days 16 and 28 or days 37 and 49 showed a significant decrease in MuB1 ($P < 0.1$). This change is also significant if the level before injections (day 0) is compared with concentrations on days 4 and 9 ($P = 0.05$), on days 16, 23, and 28 ($P < 0.01$) or on days 37 and 49 ($P < 0.01$). An initial increase in MuB1 concentration is seen after the first days of ALS injection (comparing MuB1 concentration on day 0 and day 4; $P = .01$).

two populations of antibodies (or one population acting on two targets) of which one had an immunosuppressive effect and did not require the complete complement system and the other had a peripheral and graft-rejecting effect which depended on the late acting complement components. As will be seen from Table III, differences between graft retention in complement competent and deficient animals appeared to be reduced when ALS administration was prolonged. The question therefore arose whether involvement of the complete complement system was an absolute requirement for the graft-rejecting component

TABLE I

Greater Graft Prolongation by ALS in C5-Deficient Mice

Serum administered				Recipient strain	Graft survival		
Serum donor	Specificity	Designation	days of s.c. injections*		Days × number of animals	Mean ± SD	Comparison‡
—	—	—	—	B10.D2-old	9 × 1, 10 × 3, 11 × 8, 12 × 2, 13 × 5	11.4 ± 1.2	$P>0.10$
				B10.D2-new	9 × 1, 10 × 1, 11 × 7, 12 × 9	11.3 ± 0.8	
Horse	Normal	TBO2925–2929	−7 to −1	B10.D2-old	10 × 4, 11 × 8, 12 × 2	10.9 ± 0.6	$P>0.10$
				B10.D2-new	10 × 5, 11 × 7, 12 × 1	10.7 ± 0.6	
Rabbit	Normal		−7 to −1	B10.D2-old	10 × 5, 11 × 6, 12 × 1	10.7 ± 0.6	$P>0.10$
				B10.D2-new	10 × 5, 11 × 9, 12 × 1	10.7 ± 0.6	
Horse	ALS	922–29	−7 to −1	B10.D2-old	15 × 2, 18 × 5, 20 × 1, 21 × 1, 23 × 2, 24 × 1	19.3 ± 3.1	$P<0.002$
				B10.D2-new	12 × 1, 13 × 4, 15 × 2, 16 × 4	14.4 ± 1.5	
Horse	ALS	HAMTS-Tech B	−7 to −1	B10.D2-old	15 × 1, 16 × 1, 17 × 5, 18 × 1	16.8 ± 1.1	$0.002<P<0.02$
				B10.D2-new	11 × 1, 12 × 2, 15 × 5, 16 × 2	14.2 ± 1.7	

Rabbit	ALS	R30/31	+1, +5	B10.D2-old	20 × 2, 21 × 1, 24 × 2, 25 × 2, 27 × 2, 28 × 1, 73 × 2	32.3 ± 18.4	*0.002<P<0.02*
				B10.D2-new	13 × 2, 14 × 1, 18 × 1, 19 × 2, 20 × 1, 21 × 1, 23 × 1, 24 × 1, 26 × 1	19.4 ± 4.2	
Horse	ALS	68/65	−7 to −1	B10.D2-old	13 × 1, 14 × 2, 15 × 4, 16 × 1, 17 × 2	15.1 ± 1.2	*P = 0.02*
				B10.D2-new	12 × 1, 13 × 5, 14 × 2, 15 × 1, 16 × 1	13.6 ± 1.6	

C5-deficient (B10.D2-old/SnJ) and C5 possessing (B10.D2-new/SnJ) male mice were given several injections (0.25 ml subcutaneously) of ALS before or after transplantation with skin from male A/J mice.

* Day 0 being the day of transplantation. s.c., subcutaneous.

‡ Mann Whitney U-test. Significant differences are shown in italics.

of ALS to exert its action, or whether it could be effective in the absence of the complete complement system. To answer this question, donors as well as recipients were injected with ALS and graft survival was measured as a function of the ALS dose administered to the donor. It will be seen from Table V that, under these circumstances, the immunosuppressive effect of ALS could be com-

TABLE II

The Survival of Skin Grafts From Different Donors on C5-Deficient (MuB1-Negative) and C5-Competent (MuB1-Positive) Recipients

Donor			Recipient	Treatment with ALS*	Graft survival		
Strain	H-2	MuB1			Days × number of animals	Mean ± SD	Comparison‡
A/J	H-2^a	—	B10.D2-old	None	9 × 1, 10 × 3, 11 × 8, 12 × 2, 13 × 5	11.4 ± 1.2	*P*>0.10
			B10.D2-new		9 × 1, 10 × 1, 11 × 7, 12 × 9	11.3 ± 0.8	
			B10.D2-old	−7 to −1	15 × 1, 16 × 1, 17 × 5, 18 × 1	16.8 ± 1.1	*0.002<P<0.02*
			B10.D2-new		11 × 1, 12 × 2, 15 × 5, 16 × 2	14.2 ± 1.7	
RF/J	H-2^k	—	B10.D2-old	None	10 × 5, 11 × 2, 12 × 1	10.5 ± 0.7	*P*>0.10
			B10.D2-new		10 × 5, 11 × 2	10.3 ± 0.4	
			B10.D2-old	−7 to −1	12 × 2, 19 × 3, 20 × 1, 22 × 2, 24 × 3, 26 × 1	20.3 ± 4.3	*0.002<P<0.02*
			B10.D2-new		10 × 2, 11 × 2, 12 × 2, 13 × 1, 14 × 2, 18 × 1, 20 × 1, 23 × 2	14.7 ± 4.5	
CBA/J	H-2^k	+	B10.D2-old	None	10 × 4, 11 × 1, 12 × 2	10.7 ± 0.9	*P*>0.10
			B10.D2-new		10 × 4, 11 × 2, 12 × 1	10.6 ± 0.7	
			B10.D2-old	−7 to −1	10 × 1, 12 × 1, 19 × 1, 20 × 1, 21 × 1, 22 × 1, 23 × 3, 26 × 1, 34 × 1	21.2 ± 6.1	*0.002<P<0.02*
			B10.D2-new		10 × 2, 11 × 2, 12 × 2, 15 × 2, 16 × 2, 18 × 1, 19 × 1, 21 × 1, 22 × 1	14.9 ± 3.9	

Recipients were male animals of strain B10.D2-old/SnJ (MuB1-negative, H-2^d) and B10.D2-new/SnJ (MuB1-positive, H-2^d).

* Days at which subcutaneous (s.c.) injections with 0.25 ml of ALS (HAMTS-Tech B) were given, day 0 being the day of transplantation.

‡ Mann-Whitney U-test. Significant differences are shown in italics.

pletely eliminated, both in MuB1-positive and in MuB1-negative recipients. It follows that the graft-rejecting component of ALS can decrease graft sur-

TABLE III

Graft Retention in Complement-Competent (B10.D2-New Sn/J) and Complement-Deficient Mice (B10.D2-Old Sn/J) as a Function of the Dose Schedule of ALS Administered

Designation of ALS preparation	Dose schedule Days of s.c. injections*	Strain of recipient male animals	Graft survival		
			Days × number of animals	Mean ± SD	Comparison‡
922-29	−7 to −1	B10.D2-old	15 × 2, 18 × 5, 20 × 1, 21 × 1, 23 × 2, 24 × 1	19.3 ± 3.1	*P<0.002*
		B10.D2-new	12 × 1, 13 × 4, 15 × 2, 16 × 4	14.4 ± 1.5	
922-29	−5 to −1	B10.D2-old	11 × 1, 13 × 1, 14 × 1, 16 × 2, 17 × 2, 18 × 3, 19 × 2, 20 × 2	16.9 ± 2.5	*P<0.002*
		B10.D2-new	10 × 3, 11 × 5, 12 × 1, 13 ×7, 15 × 2	12.1 ± 1.5	
922-29	+1, +5	B10.D2-old	11 × 8, 12 × 5, 13 × 4, 14 × 3	12.1 ± 1.1	*P>0.10*
		B10.D2-new	10 × 4, 11 × 4. 12 × 6, 13 × 3, 14 × 1	11.6 ± 1.2	
922-29	−7 to −1, +1, +5	B10.D2-old	10 × 2, 13 × 2 ,17 × 2, 19 × 1, 20 × 1, 22 × 1, 27 × 1	16.8 ± 5.2	*P>0.10*
		B10.D2-new	11 × 1, 13 × 1, 15 × 1, 16 × 1, 17 × 4, 19 × 5, 20 × 1	17.0 ± 2.3	
R30/31	+1, +5	B10.D2-old	20 × 2, 21 × 1, 24 × 2, 25 × 2, 27 × 2, 28 × 1, 73 × 2	32.3 ± 18.4	*0.002<P<0.02*
		B10.D2-new	13 × 2, 14 × 1, 18 × 1, 19 × 2, 20 × 1, 21 × 1, 23 × 2, 24 × 1, 26 × 1	19.4 ± 4.2	
R30/31	+5	B10.D2-old	11 × 1, 12 × 2, 13 × 1, 14 × 2, 15 × 4, 16 × 1, 17 × 1, 18 × 1, 21 × 1	14.9 ± 2.6	*P>0.10*
		B10.D2-new	11 × 2, 13 × 5, 14 × 2, 15 × 1, 16 × 4, 17 × 2, 18 × 1	14.5 ± 6.4	

Mice were given several s.c. injections of ALS before and/or after transplantation (0.25 ml/s.c. injection) and were grafted with skin from A/J male mice.

* 0 being the day of transplantation; s.c., subcutaneous.

‡ Mann Whitney U-test. Significant differences are shown in italics.

vival times, though much less effectively, in the absence of the complete complement system.

Rejection of Syngeneic Grafts from ALS-Treated Donors.—The effect of ALS on syngeneic grafts was examined as the final test for the postulated presence

TABLE IV

The Effect on Graft Survival of Passive Administration of MuB1-Positive and MuB1-Negative Sera

Recipient strain	Treatment		Graft survival		
	ALS	Normal serum	Days × number of animals	Mean ± SD	Comparison*
B10.D2-old	922–29	DBA/2J	14 × 3, 15 × 3, 16 × 4, 17 × 2, 18 × 1, 20 × 2, 38 × 1	17.6 ± 5.5	*0.02<P<0.05*
B10.D2-old	922–29	C57BL/6J	13 × 2, 14 × 1, 15 × 2, 16 × 1	14.3 ± 1.1	
B10.D2-old	HAMTS-Tech B	C57BL/6J (heated)	11 × 2, 18 × 1, 19 × 2, 21 × 2, 22 × 2, 23 × 1	18.7 ± 2.3	*0.02<P<0.05*
B10.D2-old	HAMTS-Tech B	C57BL/6J	11 × 4, 12 × 1, 16 × 2, 17 × 1, 19 × 1, 20 × 1	14.4 ± 3.4	
DBA/2J	HAMTS-Tech B	C57BL/6J (heated)	15 × 2, 16 × 1, 17 × 3, 18 × 2, 19 × 6, 21 × 2, 23 × 1	18.4 ± 2.1	*0.002<P<0.02*
DBA/2J	HAMTS-Tech B	C57BL/6J	11 × 1, 13 × 4, 14 × 2, 15 × 2, 16 × 1, 17 × 2, 18 × 3, 19 × 1, 20 × 1	15.5 ± 2.5	
DBA/2J	HAMTS-Tech B	DBA/1J (heated)‡	14 × 1, 15 × 1, 16 × 1, 17 × 1, 19 × 2, 20 × 4, 21 × 2, 22 × 1, 23 × 1, 24 × 1	19.4 ± 2.8	*0.02<P<0.05*
DBA/2J	HAMTS-Tech B	DBA/1J	12 × 3, 14 × 2, 16 × 2, 17 × 2, 18 × 2, 19 × 2, 20 × 3	16.5 ± 2.9	

Male, 8-wk old animals of the C5-deficient strain, B10.D2-old/SnJ or DBA/2J, were injected s.c. daily from day −7 to the day of rejection, with 0.1 ml of normal or heated (56°C, 1 hr) sera from MuB1-positive C57BL/6J and MuB1-negative DBA/2J mice. They were also injected s.c. with 0.25 ml of heated (56°C, 1 hr) ALS from days −7 to −1. On day 0, the animals received a skin transplant from a male 8 wk old A/J donor.

* Mann Whitney U-test. Significant differences are shown in italics.

‡ After day 17, animals were injected with C57BL/6J, heated and unheated respectively.

in ALS of graft-rejecting antibody. C5-deficient and C5-competent donors were treated with ALS and transplantation was carried out on untreated recipients of the same genotype as that of the donors. In these circumstances, a graft from

TABLE V

Injection of Donor and Recipient With ALS

Dilution of ALS given to donor	Injection given to recipient	Recipients: B10.D2-old Exp. No.	B10.D2-old Graft survival (days × number of animals)	B10.D2-old Mean ± SD	B10.D2-new Exp. No.	B10.D2-new Graft survival (days × number of animals)	B10.D2-new Mean ± SD
1:1	ALS	1	11 × 6, 12 × 1, 13 × 1	11.4 ± 0.7	8	11 × 6, 12× 1, 13 × 1	11.4 ± 0.7
1:10	ALS	2	10 × 2, 11 × 1, 12 × 1, 14 × 1, 15 × 1, 17 × 1, 18 × 1, 20 × 1	14.1 ± 2.4	9	10 ×1, 11 × 6, 12 × 1, 13 × 1	11.2 ± 0.8
1:50	ALS	3	13 × 3, 14 × 3, 16 × 1, 17 × 1	14.3 ± 1.4	10	11 × 1, 12 × 3, 13 × 1, 14 × 5	13.0 ± 1.1
1:100	ALS	4	10 × 1, 12 × 1, 15 × 4, 16 × 1	14.0 ± 2.0	11	10 × 4, 11 × 4, 14 × 1	10.9 ± 0.7
1:250	ALS	5	14 × 1, 15 × 3, 16 × 2, 18 × 1	15.6 ± 1.2	12	12 × 2, 13 × 3, 15 × 2, 16 × 1	13.6 ± 1.4
1:1000	ALS	6	15 × 1, 16 × 4, 19 × 2, 22 × 1	17.4 ± 2.2	13	10 × 3, 11 × 1, 13 × 2, 14 × 1, 15 × 1	12.0 ± 1.9
None	ALS	7	15 × 2, 18 × 5, 20 × 1, 21 × 1, 23 × 2, 24 × 1	19.3 ± 3.1	14	12 × 1, 13 × 4, 15 × 2, 16 × 4	14.4 ± 1.5
1:1	—		11 × 10	11.0 ± 0.0		11 × 10	11.0 ± 0.0
1:10	—		10 × 7, 12 × 2	10.4 ± 0.8		10 × 9, 11 × 1	10.1 ± 0.3
None	—		9 × 1, 10 × 3, 11 × 8, 12 × 2, 13 × 5	11.4 ± 1.2		9 × 1, 10 × 1, 11 × 7, 12 × 9	11.3 ± 0.8

Comparison between*	Probability‡	Comparison between*	Probability‡	Comparison between*	Probability‡
1 and 2	*P*>0.10	8 and 9	*P*>0.10		
1 and 3	*P<0.002*	8 and 10	*P<0.02*	1 and 8	*P*>0.10
1 and 4	*0.014<P<0.02*	8 and 11	*P*>0.10	2 and 9	*P*>0.10
1 and 5	*P<0.002*	8 and 12	0.36>*P*>0.32	3 and 10	*P*>0.05
1 and 6	*P<0.002*	8 and 13	*P* = 0.43	4 and 11	*0.002<P<0.02*
1 and 7	*P<0.002*	8 and 14	*P<0.002*	5 and 12	*P = 0.02*
2 and 7	*P<0.02*	9 and 14	*P<0.002*	6 and 13	*P = 0.00*
3 and 7	*P<0.002*	10 and 14	0.05<*P*<0.10	7 and 14	*P<0.002*
4 and 7	*P<0.002*	11 and 14	*P<0.002*		
5 and 7	*P<0.02*	12 and 14	*P*>0.10		
6 and 7	*P*>0.10	13 and 14	*P<0.05*		

The recipients (B10.D2-old/SnJ and B10.D2-new) were subcutaneously injected on days −7 to −1 (0 being the day of transplantation) with 0.25 ml of ALS 922-29. The donors (A/J♂) received injections of 0.25 ml of ALS 922-29 on days −7 to −1. The concentration of the ALS given to the donors was different in different groups. ALS was heated to 56°C for 1 hr before injection.

* Mann Whitney U-test.

‡ Significant differences are shown in italics.

a treated MuB1-negative donor to an untreated syngeneic MuB1-negative recipient was retained throughout the period of observation. However, a graft from an MuB1-positive donor to an untreated syngeneic recipient was rejected in a proportion of animals in a relatively short period (Table VI).

We have, so far, come to the conclusion that the potency of the graft-rejecting component of ALS depends on the intact complement system. If the potency of the immunosuppressive moiety of ALS depends on the cooperation of late acting complement components, it does so to a lesser degree, and it was not unambiguously demonstrable in the present series of experiments.

TABLE VI

The Effect on Graft Survival of ALS Administration to the Donor of a Syngeneic Graft

Donor and recipient		Treatment of donor*		Treatment of recipient*	Graft survival (days × number of animals)
Strain	MuB1				
B10.D2-old	—	none		none	>143 × 10
B10.D2-old‡	—	0.1 ml C57BL/6J	(−7 to −1)	0.1 ml C57BL/6J (−7 to rejection)	>118 × 9
B10.D2-old	—	0.25 ml HAMTS-Tech B	(−7 to −1)	none	>215 × 9
B10.D2-old	—	0.25 ml HAMTS-Tech B	(−7 to −1)	none	>187 × 8
B10.D2-old	—	0.25 ml HAMTS-Tech B	(−7 to −1)	none	>150 × 7
B10.D2-old‡	—	0.25 ml HAMTS-Tech B 0.1 ml C57BL/6J	(−7 to −1) (−7 to −1)	0.1 ml C57BL/6J (−7 to rejection)	12 × 2, >118 × 9
B10.D2-old‡	—	0.15 ml C57BL/6J	(−7 to −1)	0.15 ml C57BL/6J (−7 to rejection)	>83 × 8
B10.D2-old‡	—	0.25 ml HAMTS-Tech B 0.15 ml C57BL/6J	(−7 to −1) (−7 to −1)	0.15 ml C57BL/6J (−7 to rejection)	11 × 2, 13 × 2, 14 × 1, >83 × 7
B10.D2-old‡	—	0.25 ml normal horse serum 0.15 ml C57BL/6J	(−7 to −1) (−7 to −1)	0.15 ml C57BL/6J (−7 to +14 and then weekly)	>54 × 10
DBA/2J	—	0.25 ml HAMTS-Tech B	(−7 to −1)	none	>131 × 16
DBA/1J	+	none		none	>110 × 14
DBA/1J	+	0.25 ml normal horse serum	(−7 to −1)	none	>75 × 12
DBA/1J	+	0.25 ml HAMTS-Tech B	(−7 to −1)	none	11 × 12, 16 × 1, >131 × 6
DBA/1J	+	0.25 ml HAMTS-Tech B	(−7 to −1)	none	11 × 4, 12 × 2, >110 × 8
DBA/1J	+	0.25 ml ALS 30	(−7 to −1)	none	11 × 6, >110 × 6
C57BL/6J	+	0.25 ml HAMTS-Tech B	(−7 to −1)	none	15 × 1, 16 × 1, >29 × 6
C57BL/6J	+	0.25 ml PA	(−7 to −1)	none	14 × 2, 15 × 2, 16 × 1, 17 × 1, 18 × 1, >30 × 2

Animals were male and 8 wk of age. ALS was given to the donor before transplantation of his skin. In complement-competent (C5-positive or MuB1-positive) animals this led to graft rejections; this was not observed in complement-deficient (C5-negative or MuB1-negative) animals unless they were given MuB1-positive serum. Rejections were not observed in control experiments in which MuB1-positive donors and recipients were given normal horse serum and in which MuB1-negative donors and recipients were given MuB1-positive serum.

* Number in brackets shows day of injections, day 0 being the day of transplantation.

‡ MuB1-negative recipient and donor given MuB1-positive serum.

SRBC-Antibody Production by C5-Deficient and Competnet Mice.—The effect on the humoral rather than the cellular component of the immune system was chosen to further investigate the importance of late acting components in ALS-mediated immunosuppression. We utilized a system in which there was no possibility of ALS affecting the peripheral antigenic stimulus: the response to SRBC by mice pretreated with ALS which had been absorbed with an equal volume of packed SRBC before use.

The injection of ALS, freed of SRBC antibody, reduced the production of hemagglutinating antibody to the same extent in complement-deficient and complement-competent congenic mice (Table VII). Thus the late acting comple-

TABLE VII

ALS-Mediated Depression of the Response to Sheep Red Cells in Complement-Competent and Deficient Congenic Mice

Mice received	Serum given on days	Strain of mice injected with sheep erythrocytes	Number of animals having a hemagglutinin titer* of									
			0	2	3	4	5	6	7	8	9	>9
—	—	B10.D2-new										8
—	—	B10.D2-old										8
NHS	−4, −3, −2, −1	B10.D2-new										8
NHS	−4, −3, −2, −1	B10.D2-old										8
ALS	−1	B10.D2-new										8
ALS	−1	B10.D2-old										8
ALS	−2, −1	B10.D2-new						1	2	3	1	
ALS	−2, −1	B10.D2-old							1	6	1	
ALS	−3, −2, −1	B10.D2-new		1	1		6					
ALS	−3, −2, −1	B10.D2-old			2	3	3					

Mice were given one to four daily subcutaneous injections of 0.5 ml of normal horse serum (NHS) or equine ALS (pool 922–29) and, 24 hr after the last injection, were injected intravenously with 0.5 ml of a 2% suspension of sheep erythrocytes in 0.15 M NaCl.

* Titers expressed in terms of $\log_2$ of their reciprocals.

ment components did not appear to be involved in the ALS-mediated suppression of the immune response to SRBC. We next approached this problem in an experimental arrangement which allowed us to confine the ALS target to spleen cells (21). Previous experiments have shown that the decrease in immunological competence of spleen cells following in vitro incubation with ALS is directly proportional to the immunosuppressive potency of the ALS used and not to its content of lymphocyte-agglutinating and cytotoxic antibodies (6, 19–21).

In Vivo Antibody Production by Spleen Cells after In Vitro Incubation with ALS.—ALS was decomplemented and absorbed with an equal volume of packed SRBC before use. Normal spleen cells (18×10^7) from C5-competent and deficient mice were incubated in 1 ml of ALS922-29, undiluted or diluted with TC199, as indicated in Table VIII. After 30 min at 37°C the mixture of

spleen cells and ALS was diluted with TC199 (7 ml) and centrifuged. The sediment was washed once, resuspended in a mixture of SRBC and TC199, and injected intravenously into mice given cyclophosphamide (250 mg/kg body weight) 7–8 hr previously. There were eight mice in each experimental group

TABLE VIII

Antibody Production by Spleen Cells From C5-Deficient and Competent Donors After Their In Vitro Incubation With ALS 922–29

Exp. No.	Spleen cells		Recipients		Hemagglutinin titers* 7 days after transfer of cells	
	Strain of donor	Incubated in	Strain	Treatment	Mean	95% confidence limits of the means
1	none	—	B10.D2-old	none	11.4	10.5–12.3
	none	—	B10.D2-new	none	11.2	10.0–12.4
	none	—	B10.D2-old	CY‡	2.2	1.8– 2.6
	none	—	B10.D2-new	CY‡	2.8	2.0– 3.6
	B10.D2-old	—	B10.D2-old	CY‡	10.6	10.0–11.2
	B10.D2-new	—	B10.D2-new	CY‡	10.9	10.2–11.3
2	B10.D2-old	Medium	B10.D2-old	CY‡	9.8	9.0–10.6
		ALS(1:128)		CY‡	10.2	9.4–11.0
		ALS(1:32)		CY‡	5.5	4.6– 6.4
		ALS(1:8)		CY‡	2.0	1.8– 2.2
		ALS(1:1)		CY‡	1.2	0.8– 1.6
3	B10.D2-new	Medium	B10.D2-new	CY‡	11.2	9.8–12.6
		ALS(1:128)		CY‡	10.4	9.2–11.6
		ALS(1:32)		CY‡	6.2	5.2– 7.2
		ALS(1:8)		CY‡	1.4	0.4– 2.4
		ALS(1:1)		CY‡	0.2	0.0– 0.8
4	B10.D2-old	Medium	B10.D2-new	CY‡	10.0	9.1–10.9
		ALS(1:32)	B10.D2-new	CY‡	4.9	4.2– 5.6
		Medium	B10.D2-old	CY‡	9.7	9.0–10.4
		ALS(1:32)	B10.D2-old	CY‡	5.0	4.9– 5.1
		ALS(1:32)	B10.D2-old	CY,‡ B10.D2-new serum§	5.0	4.3– 5.7
5	B10.D2-new	Medium	B10.D2-new	CY‡	9.4	8.5–10.3
		ALS(1:32)	B10.D2-new	CY‡	5.8	4.3– 6.3
		Medium	B10.D2-old	CY‡	9.8	8.9–10.7
		ALS(1:32)	B10.D2-old	CY‡	5.9	5.0– 6.8
		ALS(1:32)	B10.D2-old	CY‡, B10.D2-new serum§	5.6	4.9– 6.3

Donors and recipients of spleen cells were congenic C5-deficient (B10.D2-old Sn/J) and C5-competent (B10.D2-new Sn/J) 8-wk old male mice. Recipients were treated with cyclophosphamide (CY) or were left untreated and were given SRBC (0.1 ml of a 10% suspension) alone or in combination with 2×10^7 spleen cells. The spleen cells were incubated either in ALS (922–29), previously heated at 56°C and absorbed with SRBC, or they were incubated with medium TC 199. The ALS, used for incubation, was undiluted or was diluted with medium TC 199. 7 days after the transfer, the animals were bled and anti-SRBC agglutination titers were determined.

* Titers expressed in terms of $\log_2$ of their reciprocals.

‡ Cyclophosphamide (250 mg/kg body weight) was injected intraperitoneally 6–8 hr before injection of sheep erythrocytes and spleen cells.

§ Fresh, C5 containing serum from B10.D2-new Sn/J mice was injected intravenously immediately after the injection of spleen cells. Each mouse received 0.9 ml of serum.

and each animal received 0.5 ml of TC199 containing 2×10^7 syngeneic spleen cells and 0.1 ml of a 10% suspension of SRBC. Hemagglutinin titers were determined on serum obtained 7 days later. If mice were treated with cyclophosphamide, their response to red cells was reduced from a hemagglutination titer of 11 ($\log_2$ of the reciprocal titer) to a titer of 2–3. A titer of 11 was found in the cyclophosphamide-treated recipients of SRBC after transfer of 2×10^7 normal spleen cells from either C5-deficient or C5-possessing donors (experiment 1, Table VIII). If the donor cells were incubated with undiluted ALS, or with ALS diluted 1:8, their restorative capacity was destroyed. If they were incubated in a 1:32 dilution of ALS, restoration was 50–60% of that obtained with untreated cells. This inhibitory effect of ALS did not depend on the complement competence of donor or recipient (experiments 2 and 3, Table VIII) and was not affected by the administration of serum from MuB1-positive animals (experiments 4 and 5, Table VIII). It follows that immunosuppression, in this system, is not significantly affected by the late acting components of the complement system.

DISCUSSION

Our observations are compatible with the view that ALS can destroy grafts and can suppress the immune response and that the former effect is promoted by late acting complement components while the latter is not dependent on them. Questions which arise in this context concern the validity of the experimental model used, the existence of two distinct types of activities, and the identity or independence of the molecules responsible for these two antagonistic activities.

It is clear that B10.D2-old and -new mice may differ in respects other than those already known. Both strains originate from a cross between C57BL/10 and DBA/2, both have H-2^d, but only B10.D2-old is deficient in MuB1 (Hc°, Herzenberg et al., 14), and thus has at least one, but possibly more than one DBA/2-chromosome fragment in addition to the one found in the B10.D2-new strain (22, 23). It was for this reason that we did not rely on the difference between graft retention in the two congenic strains of ALS-treated animals, but verified them by the transfer of C5-containing sera to deficient mice (Tables IV, VI). This transfer led to shortened graft survival and thus provided a crucial argument which was independent of the similarity or difference of the congenic strains and which led to the conclusion that complement was a factor in the observed differences in graft survival. However, the shorter graft-survival times, found when the recipients of ALS possessed C5, might have been due to some unknown complement-dependent interference with ALS-mediated immunosuppression. The strongest argument against such a view and in favor of graft-rejecting factors in ALS is based on the reduced survival of allografts from ALS-treated donors on ALS-treated recipients, and on the rejection by an

untreated recipient of a syngeneic graft from an ALS-treated donor. On the basis of these findings, it seems quite clear that ALS contains a graft-rejecting substance and that it can be detected and probably assayed by the rejection of an ALS-treated graft transferred to an untreated syngeneic C5-competent recipient.

Levey and Medawar (10) have shown that antisera having immunosuppressive properties can be raised by immunizing rabbits with mouse epidermal cells and this indicates some degree of cross-reactivity between skin and lymphocytes. In addition, Cohen et al. (24) have shown that in vitro incubation of mouse skin in rabbit anti-mouse skin serum can result in rejection when the skin is subsequently grafted on syngeneic animals. Hence it is not surprising that ALS can be shown to have graft-rejecting properties. However, our findings appear to be at variance with those reported by Guttman et al. (25) who have concluded that prolongation of allograft survival by ALS is largely due to a direct action of ALS on the graft. Their observations, though unconfirmed (26), are not necessarily at variance with those described in this paper. Guttman et al. (25) investigated the effect of ALS on the survival of kidney allografts in rats, and thus differences in the grafted tissue and in the species might explain the differences between their findings and ours.

The results presented in this paper strongly suggest that immunosuppression by ALS is not dependent on late acting complement components. If the contrary were true, it should have been possible to find longer allograft-survival times in ALS-treated MuB1-positive rather than negative animals. The reverse occurred and we therefore thought it unlikely that suppression of the cellular immune response by ALS could depend on the presence of late acting complement components. However, this conclusion was far from compelling as long as two opposing ALS effects were in operation: immunosuppressive and graft-rejecting activities.

Therefore, in the next series of experiments mice were challenged with SRBC after pretreatment with ALS which had been fully absorbed with SRBC prior to use. In such a system, any direct interaction between ALS and the antigen can be excluded. In agreement with Barth and Carroll (27) it was found that ALS was equally efficient in suppressing the humoral antibody response to SRBC in C5-deficient and competent animals.

In a final series of experiments, spleen cells were incubated in vitro with different dilutions of ALS. The washed cells were mixed with SRBC and were injected into mice rendered immunologically anergic by treatment with cyclophosphamide. In previous experiments (21) it had been shown that incubation with ALS decreases the immunological capabilities of spleen cells and that this decrease was directly proportional to the immunosuppressive potency of the ALS, but not to its content of lymphocyte-agglutinating and cytotoxic antibodies. The present experiments show that the ability of ALS to decrease the

immunological competence of spleen cells does not depend on the presence of C5. It is conceivable that ALS functions in this system by interfering with the homing of cells and that it may, for this reason, appear to be complement independent. These considerations led us to an experimental arrangement, in which only 50% of the capacity of the cell population was inhibited by ALS. Clearly this partial inhibition reflected concentration differences in the distribution of ALS among cells involved in antibody formation. A proportion of these cells, which carried less than the suppressive dose of ALS, would be inactivated by complement if such inactivation were to occur. The absence of increased immunosuppression in complement-competent individuals furnishes another strong argument against the involvement of late acting complement components.

The conclusion that ALS has a graft-damaging as well as an immunosuppressing activity is relevant to the use of ALS in clinical transplantation. For the present we have tacitly assumed that the graft-damaging and the immunosuppressive antibody are not identical but this remains to be explored experimentally and is clearly crucial to any practical extension of our findings (cf. 28). The methods employed in the foregoing analysis can provide a tool for the future resolution of this problem.

SUMMARY

Skin allografts survived longer on ALS-treated, complement-deficient (C5 negative) recipients than on ALS-treated, complement-competent (C5 positive) recipients. Administration of C5-positive serum to C5-negative, ALS-treated recipients resulted in reduced graft survival. A percentage of grafts from ALS-treated, C5-positive donors was rejected when transferred to untreated syngeneic recipients; this was not observed when C5-negative, syngeneic animals served as ALS-treated donors and untreated recipients. It was concluded that ALS has graft-rejecting properties which are promoted by late acting complement components. Unlike ALS-mediated graft rejection, ALS-mediated immunosuppression appeared to be independent of the late acting complement components.

The effect of ALS on the humoral response to sheep erythrocytes was examined in complement-deficient and complement-competent mice. Immunosuppression was determined by ALS treatment of C5-competent and C5-deficient mice and also by transfer of in vitro ALS-treated spleen cells from C5-negative and C5-positive donors to cyclophosphamide-treated recipients. The ability of ALS to depress the humoral response to sheep cells and to decrease immunological competence of spleen cells was the same in the presence as in the absence of C5.

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