FURTHER EXPERIMENTS ON THE ACTION OF ANTILYMPHOCYTIC ANTISERUM

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The experiments described in this paper are intended to throw further light on the problem of how antilymphocytic serum (ALS) exercises its remarkable power to prolong the life of homografts.[1–8] Two questions have preoccupied us: (*a*) do the descendants of a lymphoid population made immunologically inert by the administration of ALS remain inert for one or more cellular generations? (*b*) Does ALS act primarily on peripheral lymphocytes rather than on central lymphocytes in the nodes and spleen? The answer to the first question provides a critical test of the "blindfolding" hypothesis,[3] according to which ALS acts by surrounding lymphocytes with a protein coat that interferes with antigenic recognition. An affirmative answer to the second question might go some way towards explaining why ALS seems to be particularly effective in suppressing the immunological reactions that respond least well to conventional immunosuppressive agents, viz. the hypersensitivity reactions (including the homograft reaction) that are mediated through peripheral lymphocytes.[9] In the outcome, the results cast grave doubt on the blindfolding hypothesis, while leaving open the possibility that ALS acts by exciting some kind of sterile immune response. They support the belief that central lymphocytes are slower to respond to ALS than lymphocytes in circulation.

Materials and Methods.—Antisera active in mice and guinea pigs were raised in New Zealand White rabbits by the simple methods already described.[3, 4] The mice belonged to local sublines of strains A, CBA, and C57Bl, and the guinea pigs to a Hartley closed colony, the Heston strain (a subline of Wright strain 2), and Wright strain 13. As hitherto, ALS was assayed in mice by determining the mean or median survival times of A-strain tail skin grafts on male CBA mice which received 0.5 ml ALS 2 days (+2) and 5 days (+5) after grafting. ALS active in guinea pigs was tested by its power to obliterate the normal lymphocyte transfer (NLT), reaction[10] when injected into the recipient on the day before and on the day of cell transfer. Such antisera can greatly prolong the life of skin homografts in guinea pigs, but this property was not made the basis of a systematic assessment of potency.

All normal sera and antisera were heated to 56° for 30 min as a matter of routine. The route of injection was invariably subintegumentary. All antisera raised against guinea pig lymphoid cells were exhaustively absorbed with washed red cells and with acetone powders or fresh, repeatedly washed triturated matter prepared from guinea pig kidneys and lungs.

Immunological Competence of Lymphoid Populations from Mice Chronically Treated with ALS.—The immunological competence of CBA splenic cells was tested by their ability to cause splenic enlargement after intravenous injection into adult hybrid mice of the composition CBA × C57—an application of the principle first established by Simonsen.[11] Each member of a panel of not less than 8 hybrid mice received 10^8 or 2×10^8 isolated splenic cells, and their own spleens were weighed 7 days later. Table 1 shows that splenic cells from normal CBA mice caused a twofold to threefold enlargement of the spleens of the hybrid recipients. No enlargement was caused by splenic cells from mice treated beforehand with ALS (0.4 ml followed by 4 × 0.2 ml at 2-day intervals ending 1 or 2 days before transfer), and by this test they must be judged incompetent. When normal rabbit serum

TABLE 1

ENLARGEMENT OF SPLEENS OF ADULT (CBA × C57) HYBRID MICE CAUSED BY I/V INJECTIONS SEVEN DAYS BEFOREHAND OF CBA SPLENIC CELLS FROM UNTREATED DONORS OR FROM DONORS TREATED WITH ALS OR NRS

Expt.	No. mice	Donor treatment	No. cells injected (× 10^6)	Mean spleen weight at 7 days (mg)	Weight range	Weight ratio
151A	8	ALS	200	119	80–141	1.1
151B	8	Nil	200	221	88–334	2.0
151C	8	—	Nil	112	86–148	1.0
162A	8	ALS*	100	113	79–144	1.1
162B	8	Nil	100	198	134–240	1.9
162C	11	—	Nil	106	85–137	1.0
172A	8	NRS*	100	161	88–300	2.0
172B	8	Nil	100	225	140–354	2.8
172C	8	—	Nil	82	62–96	1.0
178A	10	NRS*	100	157	97–222	1.9
178B	10	Nil	100	204	97–315	2.4
178C	11	—	Nil	83	63–119	1.0

* Sera absorbed with CBA erythrocytes.

(NRS)—native or absorbed—was substituted for ALS, the degree of splenic enlargement secured was slightly less than that produced by the same number of normal unmodified splenic cells. It is relevant here that both ALS and NRS cause gross enlargement (see Table 2, col. 6) and increase the cellular populations of the spleens of the cell *donors*.[12] Even with absorbed sera, this enlargement is due in part to a proliferation of hematopoietic elements,[13] an empty magnification in terms of immunological potency. This may account for the fact that splenic cells from NRS-treated mice were slightly less effective than normal splenic cells.

Recolonization of Mice Rendered Unresponsive by ALS.—It has been clearly shown by Gray *et al.*[2] that mice made unresponsive by injections of ALS (and bearing skin homografts as evidence that they are so) can, like tolerant mice,[14] be restored to ordinary immunological capability by the inoculation of lymphoid cells from normal or specifically presensitized mice. The experiments illustrated in Figure 1 show the tempo at which the restoration of capability can occur. Three groups of 11 CBA mice were given 0.5 ml ALS 2, 5, and 8 days after the transplantation of A-strain tail skin. On the 20th day, two of the three groups received injections of, respectively, 200×10^6 mixed splenic and node cells from normal CBA mice, and 145×10^6 mixed splenic and regional lymph node cells from CBA mice which had been grafted bilaterally with A-strain skin 2 weeks beforehand. The grafts on mice that received sensitized cells, normal cells, and no cells enjoyed a further mean lifetime of 5.3 ± 1.8, 10.1 ± 2.5, and 15.5 ± 3.3 days, respectively. The same principle is illustrated by Figure 2, in which two sets of CBA males (17 and 19, respectively) were recolonized on the 25th day with 200×10^6 normal thymocytes or 200×10^6 sensitized splenic cells. The two sets of grafts survived for mean additional periods of 16.9 ± 3.1 and 5.9 ± 2.8 days, respectively. Thymocytes were evidently ineffective.

Recolonization of Irradiated Mice with Lymphoid Cells from ALS-Treated Donors.—It was felt that a more informative variant of the experiment just described would be to recolonize irradiated mice with splenic and myeloid cells from normal, ALS- or NRS-treated donors. The experiment was based on the presumption that, upon being introduced into an environment largely depopulated by irradiation, the injected cells would probably undergo one or more cell divisions.

TABLE 2

RECOLONIZATION OF IRRADIATED (900 R) CBA MICE WITH SPLENIC AND MARROW CELLS FROM NORMAL, ALS-TREATED OR NRS-TREATED CBA DONORS, FOLLOWED BY SKIN GRAFTING FROM A-STRAIN DONORS AS TEST OF IMMUNOLOGICAL COMPETENCE

Expt.	No. mice	Donor treatment	No. cells ($\times 10^6$) Spleen	Marrow	Hypertrophy factor*	Time gap† (days)	Skin Graft Survival (days)‡ MEL ± SD	Range
86A	5	ALS	100	5	2.5	19	26.9 ± 2.2	22–35
86B	5	Nil	100	5			17.9 ± 1.2	16–18
122A	14	ALS	90	1.5	1.6	9	30.1 ± 8.1	21–49
122B	15	Nil	90	1.5			17.9 ± 2.7	13–22
129A	13	ALS	100	1	3.1	9	47.1 ± 21.9	25–97
129B	13	Nil	100	1.5			17.7 ± 4.9	11–30
149A	6	ALS	20	2	4.1	7	51.8 ± 11.2	38–67
149B	10	Nil	20	2			25.4 ± 5.8	14–33
157A	10	Nil	100	3	—	9	17.4 ± 2.1	14–20
157B	8		25	3			19.0 ± 2.6	15–22
157C	9		6	3			22.7 ± 5.3	15–31
158A	10	ALS§	100	9	1.8	7	34.6 ± 5.7	35–44
158B	12	Nil	100	9			18.3 ± 2.3	14–22
165A	8	Nil	100	3	—	9	13.1 ± 1.7	10–16
165B	8		25	3			16.9 ± 2.7	13–23
165C	9		6	3			16.2 ± 1.9	13–18
166A	7	ALS§	100	8	—	7	26.6 ± 6.0	18–39
166B	5		25	8			29.7 ± 9.5	19–46
166C	7		6	8			36.1 ± 13.6	15–57
170A	9	NRS§	100	0.5	2.9	8	19.8 ± 4.3	17–27
170B	7	Nil	100	0.5			21.1 ± 4.8	17–27
175A	17	NRS§	100	1.5	2.8	10	18.4 ± 4.0	13–30
175B	17	Nil	100	1.5			21.8 ± 7.3	14–45

The course of the repopulation of irradiated mice after the inoculation of 100×10^6 splenic cells from ALS-treated donors was followed by examining spleens and nodes at weekly intervals after transfer. Mean weights of spleens recolonized with ALS-treated spleen cells were 63 mg at 7 days and 87 mg at 14 days, the mean nucleated cell count being 53×10^6 (~50% small lymphocytes) per spleen at 7 days, and 84×10^6 (~40% small lymphocytes; many blast forms) at 14 days. Histological analysis confirmed that the spleens were still perceptibly depleted at 14 days, though not to a degree which could account for the total remission of homograft reactivity.

* Ratio of spleen weights, treated:untreated donors, at time of recolonization.
† Between recolonization and transplantation of skin graft.
‡ Control value (untreated mice): 11.6 ± 1.3 days.
§ Serum fully absorbed with CBA red cells.

The cell donors were CBA females which received either no treatment, or a course of injections of ALS or NRS (0.4 ml followed by 4×0.2 ml at 2-day intervals ending 1 or 2 days before cell transfer). Such a course of treatment with ALS has been shown (above) to abolish any degree of immunological competence that can be revealed by the hybrid splenic enlargement test. The recipients, CBA males, were exposed to 900 r whole-body irradiation in a Co^{60} source 1 day before reconstitution. The numbers of cells transferred are shown in Table 2. To record the revival of immunological competence, A-strain tail skin was grafted on the irradiated hosts 7–10 (on one occasion, 19) days after recolonization. It is clear from Table 2 and Figure 3 that cells from ALS-treated donors are much slower to restore competence than cells from normal or NRS-treated mice (Fig. 4). Table 2 shows that 100×10^6 splenic cells from ALS-treated donors were in all cases less active than one-sixteenth of that number of normal splenic cells. The flatness of the dose-response relationship may betoken a rapid proliferation of the newly introduced cells, or may indicate that the marrow cells, injected in constant numbers, made a disproportionately large contribution to the regenerant population.[15]

These results (Table 2) are compatible with the hypothesis that the progeny of ALS-treated cells are relatively or absolutely incompetent for one or more cell generations (see *Discussion*).

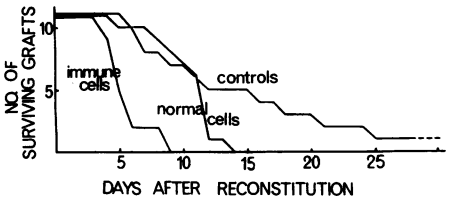

FIG. 1.—Survival curves of A-strain skin grafts on ALS-treated CBA mice after reconstitution with normal or presensitized CBA lymphoid cells on the 20th day after grafting.

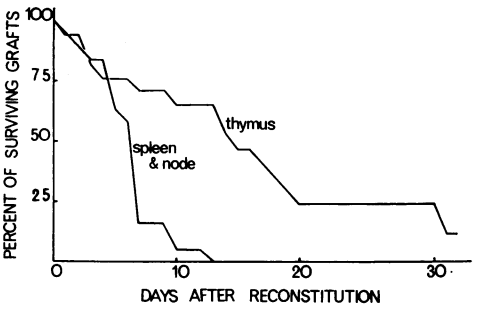

FIG. 2.—See Fig. 1: experiment of similar design comparing presensitized lymphoid cells with thymocytes. Reconstitution on 25th day after grafting.

Effect of ALS on the Sensitivity Excited by Allogeneic Lymphoid Cells.—The transplantation immunity engendered by the intravenous or intraperitoneal injection of isolated lymphoid cells, though quick to arise, is also quick to decay,[16] and it is generally regarded as much feebler than that which is provoked by solid vascularized homografts such as homografts of skin. The sensitivity aroused by such injections should therefore, in theory, be easy to abolish by injections of ALS. Table 3 and Figure 5*a* show that the opposite is the case.

These results are compatible with the belief that ALS is less well able to oppose immunization by antigens which (like lymphoid cells[17]) travel directly to lymphoid centers, than to oppose immunization mediated through peripheral mechanisms.[18]

Effect of ALS on Lymphocyte Transfer Reactions in Guinea Pigs.—It has already been shown[3] that, alone among all immunosuppressive agents so far tested, ALS has the power to abolish all episodes of the normal lymphocyte transfer reaction.

Lymphocyte transfer reactions were carried out by the techniques described in Brent and Medawar.[10] Typically, sets of 10^7 Heston lymph node cells were injected intradermally into Hartley or Strain 13 guinea pigs which, in addition to any

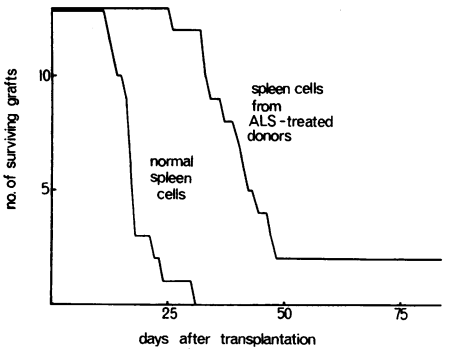

FIG. 3.—Expt. 129 (Table 2): survival curves of A-strain skin grafts on irradiated CBA mice which had been recolonized by normal CBA splenic cells or by splenic cells from ALS-treated donors. Contrast Fig. 4.

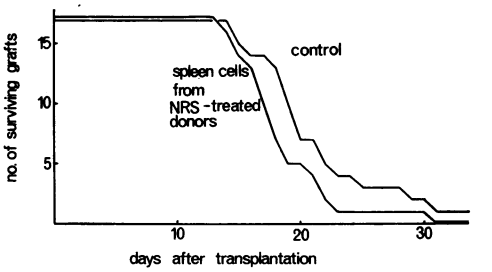

FIG. 4.—Expt. 175 (Table 2): as in Fig. 3 except for replacement of ALS by NRS.

TABLE 3

RELATIVE INABILITY OF ALS TO COUNTERACT SENSITIVITY AROUSED IN CBA MICE BY I/V INJECTIONS OF 5×10^6 (CBA × A) HYBRID SPLENIC CELLS, AS TESTED BY RATE OF REJECTION OF A-STRAIN SKIN GRAFTS

Scheme 1

Expt.	Day 0 A.M.	Day 0 P.M.	Day +1	MST (days)
147	Cells	Graft	—	≪8
	Cells	Graft	1.0 ml ALS	$9\frac{1}{2}$
	—	Graft	1.0 ml ALS	$22\frac{1}{2}$
152*	Cells	Graft	—	≪8
	Cells	Graft	1.0 ml ALS	$9\frac{1}{2}$
	—	Graft	1.0 ml ALS	24

Scheme 2

Expt.	Day −5	Day −4	Day 0	MST (days)
147	Cells	—	Graft	≪8
	Cells	1.0 ml ALS	Graft	<8
	—	1.0 ml ALS	Graft	$20\frac{1}{2}$
156	Cells	—	Graft	≪8
	Cells	1.0 ml ALS	Graft	<8
	—	1.0 ml ALS	Graft	$18\frac{1}{2}$

CBA mice were sensitized by the intravenous injection of 5×10^6 CBA × A hybrid splenic cells and handled according to two experimental schemes. In Scheme 1, the sensitizing cells were injected in the morning, A-strain skin was grafted in the afternoon, and an injection of 1.0 ml ALS administered on the following day. In Scheme 2, the sensitizing cells were given a day before and the skin graft transplanted 4 days after the injection of ALS. The survival time of the skin grafts can be taken as a measure of the hosts' sensitivity. It is clear that ALS administered according to either of these schemes was almost totally unable to abrogate the sensitivity excited by 5×10^6 CBA × A splenic cells. Nevertheless, Fig. 5*b* shows that if some time is allowed to pass between the sensitizing injection and the administration of ALS, the state of sensitivity can be abolished: in this experiment the sensitizing cells were injected 2 weeks before grafting (−14), and four injections of 0.5 ml of a rather weak antiserum were administered on days −3, −1, +1, and +3, respectively.

* See Fig. 5*a*.

other treatment, had been exposed to 600 r whole-body irradiation in a Co^{60} source 24 hours before cell transfer.

Transfer reactions are greatly intensified by presensitizing the lymphocyte donor against "transplantation antigens" characteristic of the guinea pig into which they are injected. The best results were obtained by using cells from the regional lymph nodes of Heston guinea pigs which had rejected Strain 13 skin grafts or skin grafts from the Hartley into which the cells were to be transferred. So far as present evidence goes, however, the differences between normal (NLT) and immune (ILT) lymphocyte transfer reactions are merely quantitative: the latter are pitched higher at all stages, but only because a larger number of cells is engaged in the immunological performance.[10] Many experiments of the kind illustrated by Figure 6*a* show that all episodes of the ILT reaction can be inhibited by the injection of as little as 0.5 ml/kg ALS into the lymphocyte *recipient* on the day before and on the morning of cell transfer. In the experiment in Figure 6*a*, the guinea pigs were grouped into trios consisting of two Hartleys and one Heston. Skin from both Hartleys was grafted upon both sides of the Heston, and when these grafts had broken down, pooled cells from the regional lymph nodes were injected into the two Hartleys, of which one only had received ALS, though both had been irradiated according to the usual routine.

At these dosage levels, strong antisera injected into the lymphocyte recipients can reduce all episodes of the NLT reaction to an imperceptible level. Dosages of this magnitude and timing were, however, quite ineffective when given to the lymphocyte *donor*. In the experiment shown in Figure 6*b*, a just perceptible inhibition of the ILT reaction was achieved by injecting 2.0 ml/kg ALS into the sensitized cell donor on the three days before transfer and on the day itself. In this experiment

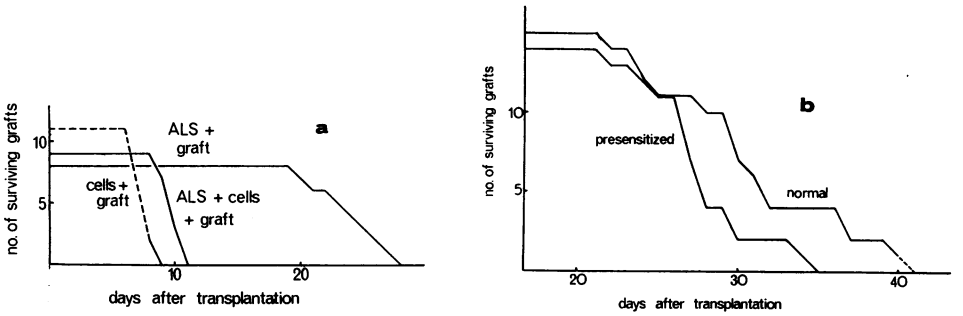

FIG. 5.—(*a*) Expt. 152 (Table 3): survival curves of A-strain skin grafts on CBA mice, illustrating relative inability of ALS to prevent sensitization by 5×10^6 (CBA × A) lymphoid cells injected one day before administration of ALS. (*b*) Expt. 48. Contrast (*a*): in this experiment the lymphoid cells were injected 14 days before the administration of a larger dose of ALS.

and others of the same design, each trio of animals consisted of one Hartley and two Hestons. Both Hestons were sensitized by Hartley skin, but only one received injections of ALS. Cells derived from the regional lymph nodes of the normal and ALS-treated Hestons were inoculated into opposite sides of the Hartley skin donor.

Only prolonged administration of ALS on the scale necessary to make skin homografts fully acceptable to the lymphocyte donor will deprive its lymph node cells of the power to excite transfer reactions. Such a course of administration was adopted in the experiment shown in Figure 7, which contrasts the performance in Strain 13 guinea pigs of normal Heston lymph node cells, and lymph node cells from Heston donors which had received a total of 7 injections, twice weekly, of ~5 ml/kg ALS. The lymphocyte donors carried fully surviving Strain 13 skin homografts as evidence of their inability to mount a homograft reaction.

Discussion.—Significance of recolonization experiments: It is clear that splenic cells from CBA mice given a total of 1.2 ml ALS over a period of a week have greatly reduced immunological capabilities, and are less well able than normal cells to restore competence to CBA mice depopulated of lymphoid cells by irradiation.

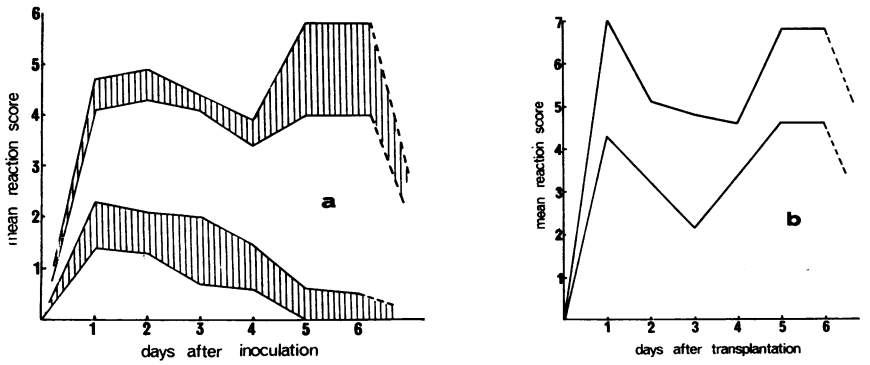

FIG. 6.—(*a*) Immune lymphocyte transfer reactions (scored in arbitrary units) raised in Hartley guinea pigs by presensitized Heston lymph node cells. *Lower band:* recipients given 0.5–1.25 ml/kg ALS on day before and on day of cell transfer. *Upper band:* controls. (*b*) As in (*a*), but ALS (4 × 2.0 ml/kg) given to lymph node cell *donors* on the 4 days before transfer (*lower line*).

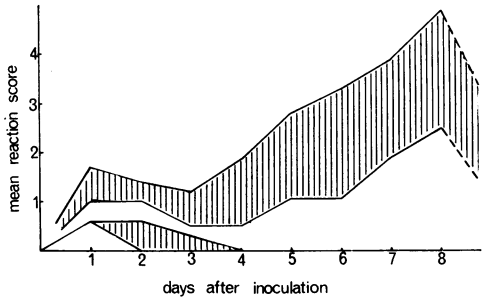

FIG. 7.—NLT reactions raised in Strain 13 guinea pigs by normal Heston cells (*upper band*) and by cells from Hestons which had received 7 injections of ~5 ml/kg ALS twice weekly before transfer (*lower band*).

The simplest interpretation of this phenomenon is that the capabilities of lymphoid cells from ALS-treated donors remain impaired for one or more cellular generations. If this is indeed so, the "blindfolding" hypothesis[3] cannot be a sufficient explanation of how ALS brings about its immunosuppressive action. The idea that induced nonreactivity may persist through several cellular generations fits well with evidence that treatments which retard or hasten the turnover of lymphoid populations will prolong or curtail, respectively, the life of homografts.[4] It is specially relevant that the incapacity produced by ALS can persist over a period of rapid growth: Jooste[19] has shown that when CBA mice are given 0.05 ml ALS on the day of birth and 0.10 ml ten days later, A-strain tail skin homografts transplanted on the 21st day of life last two or three times longer than they do on mice which receive NRS instead.

The ability of normal and (*a fortiori*) of presensitized cells to restore competence to mice deprived of it by treatment with ALS two or three weeks beforehand shows that ALS does not impose a long-lasting impediment on the immunological process. If, therefore, a proliferating lymphoid population is relatively incompetent, it is because the cells themselves are relatively incompetent, not because immunosuppression is being exercised anew, or because their environment cannot sustain an immunological performance.

The primary target of ALS: It is a well-attested fact that lymphoid cells injected by a systemic pathway "home" to central lymphoid organs.[17] The inability of ALS to prevent sensitization by even so few as 5×10^6 allogeneic lymphoid cells tempts us to suggest that ALS may act in the first instance on *peripheral* lymphocytes, and on central lymphoid organs only in so far as they liberate cells into and recapture them from the peripheral circulation.[20] Such a mechanism would explain why ALS seems specially well able to control reactions transacted by peripheral lymphocytes and refractory to conventional immunosuppressive agents.

This interpretation is ostensibly supported by our evidence that ALS readily suppresses lymphocyte transfer reactions in guinea pigs when injected into the cell recipients, but requires prolonged administration if it is to be effective after injection into the cell donor. Lymphocytes after intradermal transfer are peripheral with respect to ALS injected into their recipients, but the lymph nodes from which they were extracted are "central" with respect to any ALS injected into their donor. This distinction remains valid even if, in this context, ALS should prove to act merely by killing the cells engaged in the transfer reactions. It is at all events clear that ALS can subvert the reaction against homografts at dosage levels which do not prostrate the orthodox immunological defences.

Summary.—Splenic cells from CBA mice which have received injections of 1.2 ml ALS spread over a week are immunologically incompetent, and are much less well able than normal cells to restore competence to CBA mice depopulated of lymphoid

cells by irradiation. For these and other reasons it is argued that ALS-induced impairment of the immunological capability of lymphoid populations persists through at least one cellular generation.

In spite of its profound effectiveness with skin homografts, ALS is almost incapable of preventing sensitization by low doses of lymphoid cells injected intravenously. It is suggested that ALS acts primarily on peripheral lymphocytes, affecting lymph nodes only in proportion as their population is recruited from circulating cells. With some reservations, this interpretation is supported by the fact that ALS inhibits lymphocyte transfer reactions in guinea pigs much more effectively when injected into the recipients than when injected into the cell donors.

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