REVIEW

Immunological Effects of Heterologous Antilymphocytic Sera –

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Introduction

Antisera to white blood cells were discovered in 1899 (1) and were the subject of sporadic and unsystematic research up to the year 1956. The initial results obtained by workers following Metchnikoff's pioneering work (e. g. 2–11) have recently been reviewed by others (12, 13) and will not be dealt with here. Suffice it to mention that it was established that the antisera against white blood cells were shown to be species-specific, could cause either agglutination or lysis of the white blood cells in vitro depending on whether complement was present, and that a more specific anti-*lymphocytic* serum (ALS) could be prepared which, when administered in vivo, caused lymphopenia in the recipient.

Application of antisera against white cells to solve immunological problems was initiated by *Humphrey* (14, 15), who showed that antisera to neutrophils would effectively suppress the Arthus-type reaction in guinea pigs, and inspired *Inderbitzin* (16) to demonstrate that administration of rabbit antilymphocyte serum (ALS) could suppress the tuberculin sinsitivity in guinea pigs.

The final critical evidence for the relationship between circulating lymphocytes and related cells, and lymphoid tissue on the one hand, and immunological phenomena (i. e. antibody formation, delayed hypersensitivity reactions and allograft rejection) on the other, has only been found during the last decade (17). With the upsurge of importance of transplantation and related mechanisms, not only for research workers but also in the clinical field, it is not surprising that the possibility of employing anti-lymphocytic antiserum (ALS) as an immunosuppressive agent would be investigated.

The initial investigations of *Woodruff* and his coworkers confirmed the lytic effect ·
of ALS on lymphocytes (18, 19) but they felt discouraged from using ALS as an immunosuppressive agent in allotransplantation because of the inability of ALS to cause a prolonged profound lymphopenia which they considered necessary (20). Also, the results of *Waksman* et al. (21), who attempted to protect first-set skin allografts by using ALS, were not very satisfactory, though not completely negligible.

Further work of *Woodruff* and his team (22, 23) and results achieved by *Paul Russell* and his coworkers (24, 25, 26) and *Sir Peter Medawar* and his colleagues (27, 28), clearly indicated that the use of ALS hat great promise in the field of transplantation and other immunological phenomena. Technical difficulties involved in transplantation were being mastered at a rate which outstripped the ability to control the immunolo-

gical rejection of the graft; and this increased the chances of employing ALS as an immunosuppressant in the clinical field. This has lead to an outburst of literature on the subject during the last four to five years. This article will be mainly concerned with results published up to March 1969.

It has been demonstrated that ALS can suppress certain immunological reactions, viz. allo- and xenograft rejection, antibody formation, delayed hypersensitivity reactions and manifestations of autoimmune reactions, as will be described in detail later. Initial trials of ALS as an immunosuppressant in human transplantation have been promising and have shown that doses of other toxic immunosuppressive agents could be diminished when used in conjunction with ALS (29).

Nevertheless, these findings could leave many researchers and clinicians with the illusion that the use of ALS has brought the final answer to the control of immune reactions, and that ALS could be used indiscriminately in humans. This, however, is definitely not the case. Many problems still remain unresolved and the value of administering ALS for immunosuppression in human transplantation is still controversial and speculative.

It is therefore the aim of this review to describe results obtained by many research teams on the in vitro and in vivo effects of ALS, stressing not only those results that qualify ALS as a possible immunosuppressive agent for human transplantation, but also indicating the negative results which emphasize the need for still further basic research on the properties of ALS, and concluding with a note of caution that research on ALS has not yet reached the stage where clinicians can feel safe to use it on a routine basis in human patients.

In this article reference will be made to the so-called "activity" of ALS; this means the ability of ALS antibodies to suppress immunological reactions, be they graft rejection, humoral antibody formation or delayed hypersensitivity reactions.

ALS as an immunosuppressive agent

Preparation methods

Heterologous antilymphocytic serum is prepared by immunizing members of one animal species with lymphoid cells from another. Various animal species are used for raising ALS by different research groups: they include horses, goats, rabbits, guinea pigs, sheep, ducks and geese. ALS has in fact been prepared against an even wider variety of animals, including mice, rats, guinea pigs, rabbits, dogs, pigs, monkeys, chimpanzees and human beings. The antigen sources include any of the lymphoid tissues of the body, as well as lymphoblasts from long-term tissue cultures (30), and the preparation methods vary from just two intravenous immunizing doses (31) to a hyperimmune serum obtained by the use of antigen and adjuvant. The reader is referred to the article of *James* (32) for a detailed discussion and comparison of the different parameters mentioned above which are outside the scope of this article.

Because of all the variable parameters mentioned above, it is understandable that different and even contradictory results are claimed by different research groups. It has been shown that the deletion of adjuvant in the preparation method gives rise to a less toxic antiserum (33, 34).

Choice of ALS donors

The choice of animal in which to raise ALS also influences the results obtained. The rabbit has been claimed as superior to the horse for ALS production against rat, monkey and human lymphoid cells. This is due either to a higher immunosuppressive effect per volume unit (35, 36, 37) or to a reduction in toxic side-effects (38). *Martin* and *Miller* (39) explain the former by suggesting that rabbits might be particularly good producers of the necessary type of antibody, whereas *Monaco* (40) maintains that it could be due to the higher concentration of circulating IgG in horses as compared to rabbits, and that the addition of the "new antibodies", which also fall within the IgG fraction (see below), is less "diluted" in rabbits than in horses.

Sheep have been dismissed as poor donors by some workers (41), but *Halpern* et al. (42) maintain the opposite in the dog system, and *Gelfand* et al. (43) in that of rats. *Jooste* and her colleagues (44) found chickens and ducks incapable of producing "active" (i. e. immunosuppressive) serum in mice (this antiserum has no cytotoxicity in the presence of mouse complement), but a duck anti-rabbit ALS could protect a skin allograft in rabbits.

Closely related species give less "effective" ALS in monkeys (45). *Kinie* and *Simmons* (46) found no prolongation of allograft survival with iso-antisera in mice. *Taub* (47) found some prolongation of an A-strain skin graft in CBA mice by using an ALS obtained by immunizing C57/Ks mice against CBA thymocytes. Thus these experiments cannot be completely explained by the enhancement phenomenon described by *Kaliss* (48), since this is obtained by an iso-antiserum directed specifically against antigens of the donor of the graft.

Antigens for the preparation of "active" ALS

Although lymphoid cells are regarded as the most appropriate antigens for an immunosuppressive serum, it has been shown that this ability is not limited to whole live lymphocytes as such. "Active" ALS was also obtained using membrane fractions of thymocytes (49, 50, 51). Furthermore, different degrees of immunosuppression have been obtained with antisera by using epidermis cells, L-cells (49) or mouse-embryo fibroblasts (52). The short prolongation of skin grafts in mice using anti-epidermal cells as antigen, found by *Brent* and coworkers (53), could be explained by an inadequate immunization schedule. Furthermore, it has been mentioned that immunosuppressive antisera have been raised by Dr. *E. B. Harber* using *Salmonella typhi*-antigen (quoted from *Woodruff*, 54), (*Salmonella typhimurium* and mouse-liver cells share a common antigen 55), and in the Cleveland clinic using brain tissue as antigen (*Shorter* et al., 34).

In vitro studies have also indicated that antisera prepared by using non-lymphoid tissue as antigen either gave results corresponding to ALS in suppressing ameboid movements of leukocytes (6), or did not give results similar to stimulation lymphocyte transformation (56, 57). Absorption studies confirmed that ALS produced by using lymphoid cells as antigen gave rise to antibodies directed against antigens also present in other tissues, e. g. liver, kidney, striated muscle etc. (58–61).

Comparison of antisera prepared by using cell-free extracts of lymph nodes, with thymocytes used as antigens, led *Turk* and *Willoughby* (62) to believe that, whereas

both had a peripheral effect, the latter had a stronger central effect (on lymph nodes). Furthermore, thymocytes have been claimed as superior antigens to lymph node cells for an "active" ALS (35, 63, 64, 65). Claims for a thymus-specific antigen have been made (66), or a higher concentration on thymocytes of mouse-specific lymphocyte antigens, when compared to lymph node cells (67) as well as a specific precipitating antibody directed to lymphocytes (68).

Even though *Besredka* (2) showed loss of leukocytotoxicity of ALS after the antigen (lymph nodes) had been treated with heat and alcohol, *Jooste* et al. (44) found no loss of immunosuppressive activity after the thymocyte antigens had been heated at 48.5° C. for 20 minutes.

Possible storage of the antigen without diminishing its capacity to stimulate ALS formation would be an asset, especially in the clinical field, but *Jooste* et al. (44) found that storage of the antigen in dimethylsulphoxide (DMSO) at –79° C had given rise to an ALS inferior to the controls. *Nossa* et al. (69), however, report obtaining "good" ALS by using lymphoid cells from a cadaver stored for three months. The use of tissue-culture lymphocytes as antigens should also be mentioned in this respect (30).

It is worth mentioning that antisera have also been prepared by using other white blood cells as antigens. Detailed analysis of the results obtained will not be dealt with here, but the reader is referred to the original articles (3, 4, 5, 14, 70, 71).

Controls in experiments

In experimental studies on ALS, different controls have been employed, e. g. no serum treatment, saline administration, sera from non-immunized ALS donors (i. e. "normal" serum) or an antiserum obtained after immunization by kidney and liver cells to obtain heterophile antibodies in the control serum (10, 59).

Characterization of the ALS antibody

It is obvious that the "activity" of ALS will be found in the antibody moiety of the serum in as much as it is obtained by active immunization of the donor and can be removed from ALS by adequate absorption with lymphoid cells (21, 28, 50). As has been indicated above, however, ALS consists of a variety of antibodies directed against many antigens on different cells, either arising as a side-effect in obtaining the ALS, or due to previous immunizations of the donor by other antigens.

Intensive studies have been made to establish the localization of the "active fraction" of antilymphocytic serum. The methods for obtaining the different fractions of the serum will not be dealt with here, since they have been discussed in detail by *James* (32). It has been conclusively established that the activity is in the globulin moiety of ALS obtained after ammonium sulphate precipitation (ALG) (60, 72, 73, 74).

IgM fraction

It has been indicated that neither the IgM-ALS (19s fraction of ALS) nor the 2-mercaptoethanol (2ME) reduction product thereof contains lymphagglutinating activity in vitro (53), nor immunosuppressive activity in vivo (75); it does, however, contain the red blood cell antibodies (36, 37, 76, 77).

IgG fraction

It has been clearly shown that the "activity" of ALS is in the immunoglobulin G fraction of ALS (IgG or 7s γ-globulin) (13, 50, 78), which will be termed as ALS-IgG in this article. However, *Starzl* and coworkers (38) have stated that the T-equine globulin can also contain activity, thus confirming an earlier observation of *James* and *Medawar* (75). As suggested by *James* (32), this finding could also be explained, by supposing that loss of activity could be due to denaturation of the proteins (and hence also of the antibodies) during the purifiying process of the horse-ALS.

Most of the results obtained by the use of "whole" ALS have been confirmed by application of ALG or ALS-IgG. These results will not be discussed separately, but will be considered together with those obtained using ALS as such.

Reduction of rabbit anti-mouse ALS-IgG with 2ME caused no loss of activity (35, 75) but similar treatment with horse anti-mouse ALS-IgG could not protect a subsequent skin allograft in mice, pointing once again to the possible importance of the T-equine globulin (IgA) moiety of horse ALS (75).

Fragments of the IgG molecule

Further fragmentation of the IgG molecule has been carried out with the aid of proteolytic enzymes, to the F(ab')$_2$-, Fab-fragments, with the destruction of the Fc fragment; no immunosuppressive activity has been found in either of these fragments prepared from ALG. The F(ab')$_2$ fragment agglutinates lymphocytes in vitro (36, 37, 60, 79, 80, 81), causing an increased uptake of ^{3}H-labeled thymidine and uridine (79, 81, 82) but does not cause lymphocytolyses (37, 80). Although *Guttman* et al. (80) obtained immunosuppression after in vivo administration of this fragment (renal transplants in rats), other workers have not been able to suppress antibody formation, skin graft rejection nor a graft-versus-host (GHV) reaction (13, 36, 37, 82-87). *Ogborn* et al. ware also unable to cause suppression of transferred lymph node cells by using papain fragments of the IgG molecule (88).

Although the Fab fragment binds to lymphoid cells, as shown by using the Coombs-test (36), no "positive" results could be obtained when compared to the results obtained by either IgG or F(ab')$_2$ fragments in vitro or in vivo (36, 37, 79, 81, 82). It thus seems that the presence of the Fc fragment, shown to bind complement (89), is necessary for positive results after in vivo administration (82, 87). Compare the fact that duck anti-mouse ALS, which does not bind complement to a measurable degree, is unable to cause lymphopenia after in vivo administration (44, 90). One must bear in mind, however, that other properties of the Fc fragment, i. e. membrane attachment, immunogenicity or membrane transfer, might also be important (87).

Specific antilymphocytic antibody

Although the active antibody or antibodies of ALS are for the most part in the IgG fraction, other antibodies, irrelevant and possibly detrimental to the immunosuppressive activity of ALS, are also recovered in this fraction. *Woodruff* and coworkers (91) demonstrated that more than 95% of antibodies present in their prepared pools of ALS did not bind to lymphocytes. The specific anti-lymphocytic antibodies

can be obtained by absorption to lymphocytes, followed by elution at low pH. Not much has been published about this aspect, but some indications do exist (*Lance,* personal communication, 52, 92) that the isolation of such a biologically active fraction might be achieved.

In vitro effect of ALS, ALG and ALS-IgG

Effect on lymphocytes

A variety of antibodies are present in ALS, ALG or ALS-IgG, directed against white blood cells as such, or against lymphocytes specifically. These can be measured and demonstrated by different techniques.

Immunofluorescence and isotopic studies have demonstrated that ALS antibodies attach to lymphocytes (27, 81, 93). ALS causes inhibition of the motility of, and phagocytosis by, white blood cells (58). After complement has been inactivated by heating the ALS or ALS-IgG at 56 °C for 20 minutes, it agglutinates leukocytes (24, 37, 53, 60, 74, 79, 93–103). Under correct circumstances in tissue culture it causes lymphocyte-blast-transformation, and increased uptake of ^{3}H thymidine and uridine into cellular RNA and DNA (37, 56, 57, 79, 81, 102, 104–107). In this test, *Holt* et al. mention that complement should be absent (56). *La Via* et al. (108) have also demonstrated cell transformation in human *thymocytes* incubated with ALS. The suppressive effect of ALS on *antigen*-induced lymphocyte transformation is not complement-dependent (109). ALS binds complement in the presence of the appropriate antigen (eg. thymocytes and lymphocytes) in vitro (5, 8, 110, 111) and accordingly it has been demonstrated that ALS and ALS-IgG will cause lysis of lymphocytes in the presence of complement (3, 8, 9, 37, 53, 93, 94, 97, 104, 112). ALS also inhibits lymphocyte-mediated cytolytic reactions on target cells in vitro (113) and has the ability to opsonize lymphocytes; thus it enhances phagocytosis of these lymphocytes by macrophages in vitro (65).

Effect on red blood cells

Freshly prepared ALS contains varying amounts of red blood cells agglutinins and lysins, depending on the amount of red blood cells in the immunizing antigen pool, and cross-reactivity between red blood cells and leukocyte antigens. ALS, ALG and ALS-IgG can be freed from these antibodies by appropriate absorption with red blood cells, without loss of immunosuppressive activity of the antiserum, (e. g. 112, 114 and many others), as accepted by all research workers today. Thus removal of red blood cell antibodies has become part of the routine in the preparation of ALS in all laboratories.

In vivo effect of ALS, ALG or ALS-IgG

It was demonstrated by the earliest workers in this field that ALS is largely species-specific (2, 6, 11, 24, 97, 110 and others) although it has been found that anti-human ALS is also active in chimpanzees and rhesus monkeys (115).

The importance of deciding on the schedule of ALS administration in vivo in correlation with subsequent antigenic stimulation is accepted; e. g. *Berenbaum* (116) demonstrated that, for suppression of antibody production, ALS administration

should take place either before or simultaneously with antigen administration. For protection of allografts, many workers administer ALS before grafting (25, 35, 63, 72, 117) but ALS administration following grafting is also effective (31).

Comparing the latter results, with the enhancing effect of a sublethal whole body X-irradiation on the immunosuppressive activity of a subsequent ALS administration (see below), it will be noted that in both instances ALS was administered during a phase of lymphoproliferation. It can thus be concluded that ALS prevents or suppresses proliferation of competent lymphoid cells.

Different routes of administration are followed by the various research groups, and this fact also influences the effect of the ALS in the recipients (10, 35, 112, 118). Because of the safety of a subcutaneous (s.c.) and intraperitoneal (i.p.) route of administration, these routes are widely used, although an intravenous route is preferred in humans by some clinicians because of the side-effects caused by s.c. or i.p. administration (see below).

The regulation of chronic administration is important, for a daily dose (i.v. or s.c.) which is too high has been shown to lead to atrophy of the lymphoid tissue and subsequent death of the animal (63, 72, 98). Furthermore, regulation of chronic administration, i.e. either a regular administration with short intervals or administration of larger doses with longer intervals in between, has an effect on the occurrence of antibodies against the IgG or other serum fragments with a subsequent loss of efficacy or allergic side-effects as will be indicated later (119).

Transplantation

The successful application of ALS, ALG or ALG-IgG as an immunosuppressive agent has been demonstrated in skin allografts in mice, rats, pigs, guinea pigs, chimpanzee, rhesus monkey as well as on human beings (21, 22, 23, 24, 26, 31, 35, 36, 37, 46, 49, 63, 72, 75, 79, 97, 100, 105, 109, 115, 119, 120, 121, 123). *Ptak* et al. (122) have shown that skin-graft survival is more prolonged by administration of ALS and *Bordetella pertussis* (which increases the circulating lymphocyte pool, importance will be shown later) than by ALS alone.

Prolongation of graft survival has also been achieved· in renal transplantation in dogs (40, 98, 117, 118, 124-128), rats (80, 129, 130, 131) and humans (34, 38, 115, 127, 132 and others), and in liver transplantation in dogs (117, 118, 127). Prolongation of murine cardiac grafts (free-grafting technique) has also been achieved by administration of ALS (133).

In the above-mentioned cases, the ALS antibodies were administered to the *recipient* of the graft. *Guttman* and his colleagues claim some prolongation of rat renal transplants by administration of ALS to the *donor* of the graft (80, 129), but *Cerilli* et al. were unable to confirm these findings in the dog (134).

ALS also has the ability to protect a second-set skin graft if administration is carried out about the time of grafting (21, 27, 28, 72). *Levey* and *Medawar* demonstrated that if the interval between the last ALS administration and grafting was lengthened, prolongation of the second-set graft was not obtained, but "memory to the antigen"

was wiped out, i. e., the animal did not revert to a sensitized state, as would be expected, but to a virgin state, as if contact with the specific antigens had never occured (49). This "wiping out of memory" has been confirmed by others (116, 124, 135, 136, 137).

ALS also has the ability to protect xenografts, as was shown by different workers on skin xenografts in mice (31, 72, 138), and human tumor xenograft in hamsters (139).

Delayed hypersensitivity reactions

The immunosuppressive activity of ALS is not limited to the field of organ and tissue transplantation. Thus it has been shown that ALS suppresses delayed-type hypersensitivity to tuberculin in guinea pigs (16, 21, 114), as well as to purified diphtheria toxoid (21). The skin reaction to bovine serum albumin (BSA) was suppressed in rats by ALS administration (140) and that to trychophytin, monilia and mumps virus in human beings (100, 132). Furthermore, inhibition against contact hypersensitivity to dinitrochlorobenzene (DNCB) in chimpanzees (109) as well as in guinea pigs was achieved, together with partial suppression of the non-specific reaction to turpentine (62, 141).

Virus and parasite infections

ALS treatment, alone or in conjunction with adult thymectomy, increased the oncogenicity of polyma virus (142, 143, 146), adenovirus-type 12- (144, 145, 146), Moloney leukemogenic virus (147), and *Rauscher* leukemogenic virus (148). The course of the disease following peripheral herpes-simplex or vaccinia virus administration was worsened by prior ALS treatment (146), but mice were protected against lymphocyte choriomeningitis after intra-cerebral LCM virus innoculation (149, 144, 145) as well as against the effect of 17-D-yellow fever virus (144).

The primary immune response to the nematode *Nippostrongylus brasiliensis* was also suppressed in mice by ALS treatment (150).

Tumor cells

It was found that mice treated with ALS would accept Hep-2 and Hela cells sufficiently long enough to enable them to proliferate and form visible tumors (151, 152).

Autoimmune manifestations

Interest has also been taken in the ability of ALS to suppress or delay the onset of some autoimmune processes. Experimental allergic encephalomyelitis was suppressed in rats by ALS treatment (21, 77), and the antiserum has also been shown to suppress *Freund*'s adjuvant polyarthritis in rats (153), and to delay the appearance of Coombs positive hemolytic anemia in the NZB mice strain (73, 106). It could not, however, prevent the onset of the spontaneous kidney disease in B/W mice (154). It has been recently reported that ALS had a suppressive effect on Systemic-Lupus-Erythematosis in a woman (69), as well as effecting improvement in two patients with dermatomyositis and one with temporal arteritis (155). These claims require confirmation.

Tolerance

Administration of ALS also assisted in the temporary induction of specific immune tolerance in adult mice but, due to the regeneration of the recipient's own cells, this state of tolerance could not be maintained permanently (137, 156).

Potentation or weakening of the effect of ALS

The immunosuppressive effect can be aided by different mechanisms such as sublethal doses of x-rays (450 R) in mice before ALS administration, the opposite effect was obtained if the x-irradiation was done after ALS administration (49). A hypersecretory function of the adrenal gland, and hence a higher corticosteroid concentration in the blood, can have a lymphocytolytic effect (157, 158). Even though ALS administration was found to cause a rise in the blood corticosteroid concentration in mice. *Gray* et al came to the conclusion that this was due to cellular destruction by ALS, and did not cause lymphopenia (97) per se. The immunosuppressive effect of ALS was further increased by the administration of hydrocortisone acetate (49, 120), drainage of the thoracic duct (22, 23) and adult thymectomy (25, 26, 100, 139, 151, 159, 160, 161). The latter finding has been somewhat controversial since Starzl did not find any potentation of the effect of ALS in a human being by a previous thymectomy (127), nor did the experiments of *Levey* and *Medawar* (49) support this postulate, although they did find some of their longest skin survivals in the thymectomized mice which had also been treated with ALS.

ALS has also been shown to have a synergistic effect when used with immunosuppressive drugs, such as azothioprine, prednisone, bromodeoxiuridine and thioguanine. These are employed in the clinical immunosuppression, and the doses of these drugs can be reduced when used in conjunction with ALS (120, 127, 132).

Humoral antibody formation

Administration of ALS, ALG or ALS-IgG in appropriate doses can suppress the primary antibody production to a number of antigens in various species, e. g. to sheep red blood cells in mice (26, 72, 103, 116, 162) and rats (36, 37, 79, 153), to alumprecipitated BSA in rats (37, 79, 153, 164) in mice (165) and to *Salmonella-II*-antigen mixed with *Freund*'s adjuvant (24). However, ALG was unable to prevent antibody production to certain humoral antigens, e. g. Keyhole limpet hemocyanin in rats (162), and the suppressive effect on a secondary humoral antibody immune response is slight (24, 26, 36, 72, 135, 163).

Antibody production to ALS fractions

Heterologous ALS, ALG or ALS-IgG are themselves antigens, and can thus elicit antibody formation in the recipient. The results of the various research groups differ, but it is worth mentioning here that in the times of anti-tetanus antisera administration to humans, the recipients of the horse serum could be divided into three groups; those which react quickly, moderately quickly or fail to react. In the last mentioned group no immune elimination was observed for about 40 days after administration (166).

In several series of studies no antibodies to IgG or albumin were detected after ALS administration (31, 35, 97, 103). The occurrence of antibodies can be prevented by high doses of ALS (167); if they are formed, the immunosuppressive effect of ALS is obliterated (119, 131, 133).

It has been demonstrated by different workers that prior induction of tolerance to rabbit IgG prevented sensitization to subsequently administered ALS, reduced the dose necessary for immunosuppression, and prolonged graft survival in mice to a greater extent than administration of ALS without previously induced tolerance (151, 168, 169).

On the other hand, antibody production to the IgG fraction *has* been found in ALS-treated mice (60, 118, 153, 169, 170) and has been reported to have had *no* detrimental influence on the immunosuppressive effect of ALS (31, 153). Rabbit antimouse IgG appears to be more antigenic than the IgG prepared from normal rabbit serum (171, 172) and accordingly immune elimination of isotopically (^{131}I) labeled NRS-IgG has been found after intensive ALS treatment in animals (79, 80, 129, 173).

Standardization of the immunosuppressive activity of ALS

Every batch of ALS prepared differs from the next in immunosuppressive activity. This is due not only to species but also to individual differences in the ability to react to antigenic stimulation, even though the route of antigen administration is the same (44, 174). The necessity of being able to demonstrate the "activity" per unit volume of each ALS is readily appreciated, and many techniques have been tried to this end.

In vitro methods

These depend on the in vitro effects of ALS on lymphocytes described above. The methods determine the lymphocyte agglutinating or lysis titre of ALS, the ability of ALS to inhibit rosette formation of sheep red blood cells around spleen cells (175, 176) the employment of mixed lymphocyte cultures (177), as well as determining the opsonization titer as described by *Greaves* and his colleagues (65).

In vivo methods

In vivo methods depend on the peripheral lymphopenia obtained by in vivo administration of ALS (22, 112, 144, 160, 161, 170 and others); the ability of ALS to inhibit the clinical and histological manifestation of choriomeningitis after the inoculation of LCM virus in one-week-old ICR mice (144); or the use of tumor production by Hela cells after ALS treatment (152). The research group of Sir *Peter Medawar* at *Mill Hill* have determined the mean survival times of skin allografts in a group of eight to 12 mice, after the subcutaneous injection of a standard volume of ALS on the second and fifth day after grafting. This method measures the immunosuppressive activity of the ALS directly, and is thus an appropriate standardization procedure. It is easily applied to small laboratory animals but is obviously of no clinical use. The finding that antihuman ALS can protect skingrafts in chimpanzees and rhesus monkeys, however, does present a ray of hope (115).

In vitro versus in vivo methods

An in vitro standardization method is, of course, the ideal, but no simple relationship between the "activity" measured in vitro and immunosuppressive effect in vivo has been satisfactorily demonstrated (39, 176, 178). The correlation between immunosuppression and the inhibition of rosette formation (176) or opsonization titers (65) seem the most promising at present.

"Active" sera can be obtained with just one immunizing dose of antigen. Further antigen administration, (especially with the use of adjuvants), yields more potent ALS, but there are indications that it can also lead to the formation of more irrelevant passenger-antibodies which can either restrict the activity of ALS or have to be removed by absorption before the ALS can be used (44, 50, 74).

Effects of ALS on cell-mediated as compared with humoral immune responses

It has been demonstrated that ALS has a suppressive influence on some humoral as well as cell-mediated immune responses, but a higher dose of ALS is necessary for the former than the latter (21). With doses of ALS able to protect skin allografts, mice could still produce antibodies to *Salmonella-H* antigens, and to specific H-2 antigens of the donor (35, 119). The latter finding has, however, been disputed by Monaco and Franco (179). These divergent results can perhaps be ascribed to the use of different techniques for demonstrating the presence of specific H_2-antibodies.

It has been concluded by many workers that ALS has a preferential immunosuppressive effect on cell-mediated immune responses (28, 84, 146, 148, 149, 180). The finding of selective cell-mediated immune suppression is further supported by the survival of mice for long periods under ALS treatment, indicating that the animals are still able to combat many microbial infections. (It must be remembered, however, that ALS treatment enhances the susceptibility of mice to certain mycobacterial infections [168]).

The effect of ALS on peripheral circulating lymphocytes

Many workers have concluded that the primary effect of ALS is on the peripheral circulating lymphocytes. *Levey* and *Medawar* (27, 28) demonstrated that doses of ALS which could protect a skin allograft were unable to prevent sensitization by a low dose (5×10^6 cells) (181) of intravenously administered allogeneic lymphoid cells. These cells have been shown to home to lymph nodes (182), whereas sensitization by skin grafts is achieved by circulating lymphocytes (12, 183).

The ability of ALS to suppress the normal lymphocyte transfer test more easily when it is administered to the recipient of the transferred cells than to the donor (lymph node cells are used for transfer), (27, 28, 31), as well as results of other types of experiments (32, 106, 122, 184, 185, 186) have all supported this conclusion. Furthermore, lymphocyte depletion in the paracortial areas of lymph nodes and periarteriolar region of follicles of spleens has been found by different workers (62,

187). The idea that these areas are "thymus-dependent" (188) is still controversial, but it is generally accepted that these areas do represent the areas occupied by recirculating lymphocytes which probably play a role in cell-mediated immune responses (189).

Effect of ALS on the cells of the recipient

As has been mentioned above, it can be expected that administration of ALS, ALG or ALS-IgG will have side-effects on other cells of the recipient due to cross-reacting antibodies or "passenger antibodies" present in the antiserum. Functional impairment by ALS was found on specific types of macrophages (190), as well as cross-reactivity with epidermis cells (53), but especially on other members of the hematolymphoid series (191). Side-effects on other leukocytes differ between workers and could be influenced by their preparation schedules of the ALS. Thus some workers found no demonstrable effect on circulating neutrophils (20, 21, 70), while others reported cytolytic activity to granulocytes (192) with a resulting "shift to the left" (118). A temporary neutrophilia has also been observed (5, 10, 112, 114, 126).

Eosinophilia and complete basopenia was observed after ALS administration by *Wilhelm* et al. (114).

It is obvious, however, that the primary effect of ALS is on lymphocytes. Nearly all workers (with the exception of *Mitchell* et al. [124]) agree that some degree of lymphopenia is found in recipients after ALS administration. Changes in lymphocytes visible with the electron microscope due to contact with ALS, have been described by *Land* and his coworkers (193). Opinions differ on the importance of this lymphopenia for immunosuppression. Some workers regard it as essential (21, 120, 194) whereas others claim that immunosuppression is not dependent on the temporary lymphopenia produced by ALS administration (23, 28, 31, 35, 49, 53, 60, 98, 124, 127, 163, 178, 195, 196 and many others). Some investigators also state that the lymphopenia is followed by hyperplasia of lymphoid tissue (2, 4, 5, 8, 10, 50, 100, 105, 117, 167). The possible significance of the lymphopenia in terms of the mode of action of ALS will be discussed later.

The immunological competence of ALS-treated lymphocytes

Whatever the effect of ALS on lymphocytes, be it purely lytic or stimulating, it still remains very important to establish whether lymphocytes present in the body after ALS treatment are immunologically competent.

A standard method of determining the immune competence of the cells is the employment of the graft-versus-host reaction (GVH) as described by Simonsen (197). This is based on the ability of the administered cells to react to the "foreign" antigens of the recipient.

The immune system of the recipient is rendered incompetent by one of the following methods:

1. a high dose of ionizing irradiation; or,

2. choice of the recipient in the neonatal stage of life when its immune system has not yet developed sufficiently; or,

3. using F_1 descendants confronted by parental cells.

The reaction is measured by the occurrence of secondary disease and resultant death of the recipient, as found in the first two systems mentioned, or splenomegaly obtained in the last mentioned. The degree of the reaction is proportional to the antigenic diversity between the administered donor cells and the cells of the host.

Using these criteria, administration of an adequate amount of ALS was found to suppress or obliterate the immunological abilities of-the lymphoid cells present in the donor after ALS, ALG or ALS-IgG administration (27, 53, 100, 141, 191, 195, 198–203). When central lymphoid tissue (e.g. spleen) was used to obtain cells for this test, *Brent* et al. (53, 200) showed that a least 24 hours had to elapse after ALS administration before removal of the spleens, if these results were to be obtained. It is remarkable that the recipients which survived the GVH reaction were not found to be chimeras when tested later (100, 141), thus confirming the finding of *Lance* and *Medawar* (137) that permanent tolerance could not be achieved with the aid of ALS.

There has been some speculation as to the possible effect of ALS on the descendants of initially treated lymphoid cells. Both *Levey* and *Medawar* (27, 28, 50) and *Allen* et al. (64) have used a system in which lymphoid cells from ALS-treated mice were transferred to lethally irradiated syngeneic recipients. These cells were thus brought into a milieu devoid of other mature lymphoid cells, and were accordingly stimulated to rapid division. These recipients were found to be immunologically immature after cellular proliferation had taken place and the recipient's lymphoid tissues had been reconstituted with donor cells. *Jooste* (204) found that mice which had received ALS treatment at the age of one to ten days were still immunologically incompetent when tested, based on their ability to reject a skin allograft at the age of 21 days. In the period between the last ALS administration and the grafting of skin, these mice had doubled in weight, indicating rapid cellular proliferation.

It is thus tempting to speculate on the immunosuppressive effect of ALS being transferred through at least one cell generation, but these results can also be explained by specific precursor cells having been depleted (64), or by effects on long-lived lymphocytes, active in immunological systems.

Localisation of ALS-antibodies in the recipient

The localisation of the antibodies present in ALS after in vivo administration has been studied by different authors. The results are not all in agreement, but the differences can be related directly to the different preparation methods, each leading to the formation of a whole spectrum of antibodies with different binding affinities (205). The determination of antibody localization is done by radioactive or fluorescent labeling of the antibody. Results have been obtained which point to the preferential localization of the antibodies in the thymus (206) or preferentially in other lymphoid tissues (e.g. spleen and lymph nodes) and bone marrow (184, 207). Significant further

localization on striated muscles, bone and GI tract has also been found, indicating cross-antigenicity between these tissues and the thymus and lymph nodes, where the highest localization was found (206).

Histological lesions in ALS recipients

Histological lesions found in animals after ALS, ALG or ALS-IgG, differ between research groups (4, 21, 23, 31, 32, 35, 60, 63, 97, 100, 105, 106, 170, 195 and many others). These differences can be related to the different types of antibodies and other passenger elements present in the antisera used by various groups. The real causive effect of the "active" fraction of ALS will not be demonstrated until ALS is freed from these other elements, and until such time as the significant histological effects due to the immune suppressive portion of ALS will not be evaluated. These effects will not be evaluated. These effects will not be discussed in detail since they have been documented by James (32). One striking resemblance found by different workers, however, has been the depletion of lymphocytes in the paracortical areas of lymph nodes and splenic follicular periarteriolar zones, as mentioned earlier (62, 115, 187).

Histological findings in grafts protected by ALS, ALG or ALS-IgG

The histological findings reported on grafts protected by ALS, ALG or ALS-IgG also differ from group to group and can be related to the different methods of preparing the ALS, as discussed in the previous chapter. These have also been dealt with by James (32) and can be summarized as an illustration of a whole spectrum of stages in the different steps in the process of rejection. It has been noted, however, that intensive infiltration of lymphocytes in kidney allotransplants can occur before any clinical signs of rejection appear (127, 132).

Mode of action of ALS, ALG, ALS-IgG

The precise mode of action of the immunosuppressive ability of ALS has not yet been established. The answer to this question will not be of mere academic value, but also of great importance to the clinical application of this material. Knowledge of the underlying mechanism will no doubt lead to the evaluation of the optimal procedures for clinical application and indeed to whether clinical application is feasible at all.

Different theories have been put forward to explain the "modus operandi" of ALS – all of which explain some of the results obtained and leave others unexplained.

Cytotoxic theory

The well-documented ability of ALS to cause lysis of lymphocytes was one of the first explanations suggested. As described above, however, the importance of lymphopenia for graft survival is not generally accepted (31, 36, 49, 60, 77). Other experimental methods which cause an even more pronounced peripheral lymphopenia, e. g. extracorporeal irradiation of blood and a similar depletion of lymphocytes in central lymphoid tissue allocated to circulating lymphocytes, do not cause significant allograft

survival (208). It has been shown however that the presence of the Fc fragment of the IgG molecule is necessary for immunosuppression and the ability for complement binding has been allotted to this section (see above).

One is thus led to the conclusion that if lymphocytolysis were the answer, it would involve the depletion of a certain "group" or "class" of lymphocytes, be they functionally or antigenically different or distributed according to age or degree of immune competence (10, 13, 37, 85, 105, 141, 165, 209).

Blindfolding theory

The observation that lymphoid cells are coated by ALS in vitro (see above) led *Levey* and *Medawar* (31) to this hypothesis. Coating of lymphocytes would have a "blindfolding effect" and would thus prevent recognition of antigens, either on the afferent or efferent pathways of the immune response, (102, 109, 125, 191).

The hinderance of histocompatibility typing with isoantisera after treatment of lymphocytes with ALS (45) and return of GVH-reaction ability after in vitro treatment of cells with 0.5% trypsin (53, 200) also supports this theory. But the findings that F(ab')₂ fragments coat lymphocytes in vitro but do not have immunosuppressive activity in vivo (see above) are not explained. It should be noted that the Fc fragment, regarded as necessary if the IgG molecule is to be "immunologically active," also has the membrane-attachment characteristic allotted to it (87).

The possible mechanism of the coating of the *graft* as such by ALS, as described by *Guttman* et al. (129) is not supported by *Cerilli* et al. (134). But the former results have also been explained as being due to the "elimination of highly immunogenic passenger leukocytes by ALS, following grafting" (130).

Enhancement

Enhancement (48) has been suggested as the explanation of graft survival after ALS administration (129, 210, 211), but the findings of *Taub* (47) described above, and those of *Levey* and *Medawar* (28) in which it was demonstrated that A-strain skin grafts on CBA mice could be protected equally well by an anti-CBA-ALS and an anti-A-ALS, are left unexplained by this hypothesis.

Tolerance

The question arises as to whether specific immunological tolerance is not perhaps the mechanism by which ALS protects an allograft. Chimerism has been achieved in mice possessing genetically determined macrocytic anemia treated with ALS and infused with allogeneic hemopoietic cells (212), and also after adult thymectomy and ALS treatment in other "normal" mice (99, 213). The effect of ALS has, however, been found to be insensitive to so-called "strong" and "weak" histoincompatibilities in allotransplantation (49, 50) and the elucidating experiments of *Lance* and *Medawar* (137, 156) in which they endeavored to induce specific immune tolerance by the use of ALS, were only successful for a limited period of time. There after the cells of the host became competent again and graft rejection took place.

It has been shown, however, that ALS *can* assist in the induction of specific immunological tolerance (50, 99). ·

Competitive antigen theory

Heterologous ALS is itself an antigen in its recipient, in which it also has a preferential affinity for lymphocytes. It is thus conceivable that it could act as an obligate antigen with an advantage over other antigens, and thus cause preoccupation of the immunological competent cells (50, 196). *Halpern* et al. (214) have demonstrated similar findings by high doses of heterologous proteins and *Dresser* has shown heterologous immunoglobulins to be strong antigens (as opposed to immunogens) (50) in their ability to cause immunological paralysis. This would explain the inability of ALS to prevent antibody formation to itself, due to the fact that lymphocytes by ALS-IgG would be phagocytosed by macrophages (as shown by the opsonization test of *Greaves* et al. [65]) and thus stimulate antibody formation against it. The wiping out of immunological memory (see above), however, cannot be explained in this way.

The possible thymus-dependence of the action of ALS

As shown above, some authors have shown the superiority of thymocytes as antigens for ALS production and that adult thymectomy potentiates the effect of ALS. It is thus not surprising that theories were suggested in which the thymus was stressed as an important organ in the action of ALS. The precise role of the thymus in immunological phenomena is still a somewhat controversial matter, but it has been suggested that ALS inhibits the humoral factor of the thymus (63, 140) or that cells bearing "thymus" specificity or being thymus-dependent are selectively destroyed or inactivated (39, 62, 65, 159, 215, 216). It has also been claimed that recovery of immunological competence is thymus-dependent (100, 160).

Differential effect on certain lymphocytes

In summarizing different theories of the mode of action of ALS, it can be suggested that ALS has a differential effect on certain lymphocytes. This "effect" (be it destruction, immobilization, coating, preoccupation or some other mechanism) will prevent these cells from taking part in the subsequent immune response and "immunosuppression" will thus be achieved.

The "certain" lymphocytes might be any of the following, as suggested by different authors (see "cytotoxic theory"): "long-lived lymphocytes" (see above) (106, 177, 217), certain "clones of immunologically committed cells" (167, 186) or "cells influenced by the thymus" (65, 159) and others mentioned in previous paragraphs.

Some of these above-mentioned characteristics have been alotted to the same group of cells, i.e. being different characteristics of the same cells (65, 136). They have not been unequivocally grouped together here as being so. Cellular kinetic studies have advanced considerably in the last few years and some light has been shed on different problems regarding lymphocyte kinetics. Nevertheless, many gaps in an understanding of the overall mechanisms still exist and, even though there is a temptation to ignore these and postulate interrelationships (which may very well be proved quite correct in the future), the author feels that at the present stage of our knowledge some reservations are still necessary.

Clinical application of ALG or ALS-IgG

As explained in the introduction, the aim of this review is to relate the findings of experimental results achieved in research done by different groups on ALS. A detailed analysis of clinical results thus falls outside the scope of this review, but has been dealt with by others (29, 32).

As mentioned previously, it is quite understandable that the possibility of using ALS as an immunosuppressive agent in human transplantation would soon be realized and, appreciating the dangers associated with such a decision, it was the American surgeon *Thomas E. Starzl* who first introduced ALS to this field. Since then, the use of ALS has spread to different prominent transplantation teams such as places in America, England, Europe, South Africa, etc. (32). The results obtained by the use of ALS, ALG or ALS-IgG have been described in the foregoing sections in which an overall review was given on the in vivo effects of the ALS antibody and will not be dealt with separately here. The results obtained by the use of ALS in human renal transplantation are difficult to analyze because ALS has been used in conjunction with immunosuppressive drugs and that it is also necessary to have a large number of long-term results available. On the whole the reports of initial trials are encouraging and the hopes attached to the clinical use of ALS are high.

Detrimental side effects of ALS application

Without seeming to be unduly pessimistic a summary of the possible detrimental side-effects, as indicated in laboratory animals and noticed in patients treated with ALS, will be given in order to emphasize that the use of ALS cannot be accepted without due appreciation of these possible complications.

Patients receiving intramuscular or subcutaneous injection of ALS experienced pain, sometimes necessitating the use of narcotics, with associated erythema, induration, tenderness and itching at the site of injection (37, 60, 100, 115, 132, 218). It was found, that the intensity of the pain diminished with later injections (115, 132) or could be avoided by using the ALS preparation method described by *Levey* and *Medawar* (31, 34), or by using rabbits as donors of ALS instead of the usual horses (38).

The occurence of fever at some stage during treatment has been shown, accompanied by tachycardia (29, 100, 115, 132), as well as other systematic complications, e. g. hypotension, air hunger, anaphylactic shock, etc. (32). Another trouble-some side-effect to ALG therapy has been thrombocytopenia (32, 38, 219). The inability of ALG to prevent antibody formation to itself has also been indicated in humans (100, 132) and the dangers attached to this are well appreciated. Furthermore, unnecessary induction of antibody production to passenger elements present in the ALG have been obtained by the lack of isolation of the relevant immunosuppressive "active fraction" of ALG. Unfortunately immunosuppressive activity was lost by purification of horse antihuman-IgG by *Starzl* et al. (38) and, as described previously, this could be due to the loss of the T-equine fraction of the globuline as explained by them or else due to denaturation of the proteins during the isolation procedures (32).

As already described, certain experimental results also imply due caution in the clinical use of ALS. Some of these results of ALS administration are:

1. The obliteration of immunological memory which might result in the loss of effect of earlier active immunization against certain diseases,
2. the enhancement of susceptibility to mycobacterial infections in mice which might cause reactivation of quiescent TB, as has been found in corticosteroid therapy (168),
3. the enhancement of virus infection found in mice (see above), dogs (124) and monkeys (195, 203), (findings not to be ignored),
4. the oncogenic dangers related to suppression of the recipients' immune response is shared by other immune suppressive drugs. Reference has already been made to work showing potentiation of oncongenic viruses in experimental animals, under "virus and parasite infection"; abnormal mitotic figures and giant cells have been found in guinea pig bone marrow cells exposed to ALS (220), as well as malignant lymphomas in renal transplant patients who had received ALG (221).

Furthermore the absence of a guaranteed in vitro standardization method for measuring the immune suppressive potency of the ALG, has made it impossible to determine the amount of ALG necessary. This could lead to an "overdose" or "underdose" of ALG being administered.

The unknown mode of action of this immunosuppressive drug is also a hazard to the effective clinical application of ALG.

Conclusion

ALS has proved to be an experimental research tool of considerable value. Although many problems remain unanswered, studies with ALS have already broadened our understanding of host reactions against grafts, infections and tumors. Extrapolation to the clinical field is hazardous.

Stressing all the negative results attached to ALS administration in human transplantation has not been aimed at condemnation of its clinical use. The intention is purely to emphasize that, although promising results have been obtained with the use of ALS and although the achievement of an innocuous potent immunosuppressive agent is desperately needed in transplantation today, further basic research on the effects, characteristics and mode of action of ALS, (even on the whole immunological system as such) is essential before ALS, ALG or ALS-IgG can be regarded as giving even a provisional answer to the control of immunological rejection phenomena.

Acknowledgements

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ABSTRACTS

FISH, J. C., H. E. SARLES, A. R. REMMERS, K. TYSON, C. CANALES, G. BEATHARD, M. FUKU-SHIMA, ST. RITZMANN, W. LEVIN (Univ. of Texas Med. Branch, Galveston, Texas): Circulating Lymphocyte Depletion in Preparation for Renal Allotransplantation. Surg. Gynec. Obstet. 128 (1969), 777–787.

The thoracic duct functions as the single major pathway to blood for immunologically active cells and protein. External diversion of thoracic duct lymph suppresses immunologic activity and prolongs survival of transplanted foreign tissues.

In this report, observations in 12 patients with chronic renal failure receiving prolonged thoracic duct lymph dialysis against tap water to induce lysis of lymphatic lymphocytes revealed a profound lmphocyte depletion in blood, lymph, and lymphoid tissues. Five of these patients received six cadaveric renal transplants. No other form of immunosuppressive therapy was used in the post-transplant period for 19 to 78 days. After this period, maintenance therapy was begun with azathioprine and prednisone. One renal allograft underwent hyperacute rejection in an AB0 incompatible match. All other renal allografts were transplanted in AB0 compatible recipients. One kidney transplant was removed after 78 days because of a persistent urinary fistula. The other four renal allografts have good renal function four to 13 months after transplantation. The first patient has not received azathioprine during the period of the past four months.

Immunoglobulin levels in blood and lymph remained normal during circulating lymphocyte depletion. The maintenance of immunoglobulin levels, while depressing cellular immunity with lymphocyte depletion, lends further support to the concept of separate cellular and humoral immune systems in man.

Although the precise nature of the immunologic deficiency resulting from external thoracic duct lymph diversion remains unclear, it appears from this and other studies that the longer thoracic duct lymph is diverted prior to and after transplantation the more difficult it becomes for the host to recognize and reject the transplant. Overwhelming infection is not prominent in this form of immunosuppression so that protacted periods of lymph drainage are feasible. In patients with renal insufficiency, removal of nitrogenous wastes and elimination of circulatory congestion may be accomplished at the same time. Further investigations should reveal the best timing for external thoracic duct lymph diversion, the exact fractions of lymph to be removed and retained, and the best combined regimen with other immunosuppressive technics.

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GLEISSNER, P., F. ASANTE, G. E. SCHUBERT (Path. Inst. d. Univ. Tübingen): Congenital Pulmonary Lymphangiectasia – Report of 4 Cases. (Angeborene pulmonale Lymphangiektasien.) Dtsch. med. Wschr. 94 (1969), 1987–1990.

About 20 cases of congenital pulmonary lymphangiectasia have been reported in the literature. The authors encountered within a period of two years four newborns in whom a post mortem diagnosis of congenital pulmonary lymphangiectasia was made. Therefore the question if the disease is indeed as rare as the literature may indicate.

Four cases, all newborns, are described. One was a stillborn, the others died within