PRODUCTION AND CHARACTERIZATION OF ANTILYMPHOCYTIC SERA TO GUINEA PIG LEUKOCYTES

by

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ABSTRACT

Antilymphocytic serum (ALS) is effective in prolonging graft survival but it also has the drawback of interfering with other immune responses of recipients. It was the purpose of this investigation to produce a specific antiserum directed only against the so called "sensitized" lymphocyte or its products that are responsible for homograft rejection.

Amana strain guinea pig recipients were sensitized by orthotopic application of full thickness skin grafts from a single Rockefeller strain guinea pig donor. After the recipients had rejected the homograft around day 10, they were tested for sensitivity by a second graft from the same donor. Only those animals which demonstrated a white graft reaction were used as cell donors. Lymphocytes were obtained from the thoracic duct by cannulation. Lymph node cells were collected by the teasing of nodes regional to the graft. Peripheral blood lymphocytes were obtained by passing heparinized blood through a column of siliconized glass beads and then layering the blood over a mixture of sodium diatrizoate and methylcellulose. Lymphocytes from these sources were used for the immunization of rabbits. Each rabbit received lymphocytes from a single source. Each antiserum was adsorbed at 37 C for varying periods with 10^6 lymphocytes per ml

from untreated Amana guinea pigs until they showed little or no reactivity against lymphocytes from untreated animals.

The unadsorbed and adsorbed antilymphocytic sera were tested for in vitro activity by the lymphocytotoxicity, lymphagglutination, complement fixation, the mitogenic index and opsonic index tests against normal and homologous sensitized guinea pig lymphocytes. The unadsorbed sera showed activity similar to those observed by other workers. The adsorbed sera showed negligible effect in vitro against normal lymphocytes. However, they did show low titer activity against homologous sensitized lymphocytes. These sera were tested for their in vitro activity against guinea pig red blood cells (RBC) by the hemagglutination technique and for the presence of antibodies to guinea pig serum proteins by the precipitin test. Results indicated that unadsorbed ALS had high titer activity whereas the adsorbed sera had little or no activity. The immunosuppressive property of unadsorbed and adsorbed ALS was found to be in the IgG fraction.

The ability of these antisera to prolong skin homografts was studied. Amana strain guinea pigs were injected intraperitoneally with either unadsorbed or adsorbed ALS every day from day -3 to day +3 and then every other day until day 19. On day 0 all recipients were challenged with grafts from the original Rockefeller donor. As

expected the normal rabbit serum recipients rejected the grafts on approximately day 10. All recipients receiving unadsorbed ALS showed graft survival from 12 days or more. The adsorbed ALS recipients showed consistently better graft survival. In this case the grafts lasted longer, that is from day 15 up to indefinite survival.

The species specificity of the adsorbed ALS was tested. Amana strain recipients were injected with adsorbed sera on the regular schedule and on day 0 challenged with grafts from the original Rockefeller donor, an indifferent Rock donor, an indifferent Amana donor, a Hartley strain donor and an autograft. Results indicated that adsorbed ALS prolonged both the original Rockefeller graft and the indifferent Rockefeller graft. These recipients consistently rejected the indifferent Amana graft and the Hartley grafts.

Results of in vitro titrations of both unadsorbed and adsorbed ALS failed to correlate with in vivo graft survival prolongation. Adsorbed sera appeared not to influence immune responses other than the specific target response appreciably, thus giving an immunosuppressant with high specificity and a minimum of undesirable drawbacks.

CHAPTER 1

INTRODUCTION

Metchnikoff, in 1899 (1), demonstrated that guinea pig antisera against lymph node and spleen cells from rabbit or rats agglutinated and killed polymorphonuclear leukocytes of the cell donor species. Subsequently, a number of studies on the in vitro properties of similar antisera were reported. Besredka (2) observed that the cytotoxic effect of guinea pig anti-leukocytic sera disappeared on heating at 56 C for 30 min. Christian and Leen (3) immunized rabbits with rat spleen and lymph node cells and produced antisera with toxicity for rat leukocytes which could be measured by cessation of cellular amoeboid movement. They noted that immunization with rat kidney, liver, or cardiac muscle preparations also yielded leukocyte cytotoxins. Pappenheimer (4), in 1917, injected rabbits intravenously with suspensions of rat thymus cells and human tonsil lymphocytes and produced antisera containing cytotoxic and leukoagglutinating antibodies for donor lymphocytes. Heating of the sera at 56 C for 30 min removed the cytotoxic effect but the leukoagglutinating activity was preserved. Furthermore, adsorption of the sera to remove hemagglutinins did not affect cytotoxic or leukoagglutinating properties.

In vivo effects of antileukocyte sera have been reported. Flexner (5) found lymphoid hypoplasia and a minor degree of cell death in the germinal centers of lymph nodes from guinea pigs and rabbits treated with heterologous anti-lymph node serum. Chew and Lawrence (6) showed that daily administration of rabbit anti-guinea pig lymph node serum to guinea pigs caused a fall in total blood lymphocyte counts. Cruickshank (7) rendered rats lymphopenic by administration of anti-rat lymphocyte serum. He showed further that complement was utilized when such serum was incubated with rat lymphocytes. Splenectomy of recipient rats did not alter the effect of the serum on circulating lymphocytes. He also found little histologic evidence of cell damage in the lymph nodes and spleen.

In 1956, Interbitzen (8) demonstrated that antilymphocyte serum inhibited tuberculin sensitivity in guinea pigs. His observations were confirmed and extended by Wilhelm, Fisher and Cooke (9) and Waksman, Arbuoys and Arnason (10). Thus, the role of antilymphocyte serum in inhibiting cell mediated immune responses was established.

The first studies involving anti-lymphocyte serum in tissue transplantation studies were done by Woodruff and Foreman (11). They produced lymphopenia in rats with a crude rabbit anti-rat lymphocyte serum, but they failed to show prolongation of homograft survival. Later, utilizing a specific anti-lymphocyte serum Woodruff and Anderson (12) were able to show prolongation of homograft survival

in rats. They found that the effects of anti-lymphocyte serum and thoracic duct fistulae were additive in producing maximal graft life. The involvement of lymphocytes in the homograft rejection reaction was suggested by Brent, Brown and Medawar (13). They presented evidence indicating that the reaction provoked by homografts of skin in their hosts is a cellular rather than a humoral mechanism.

On the other hand, the possible role of humoral antibodies in graft rejection has been shown by Stetson and Demopoulous (14) and discussed by Kretschmer and Perez-Tamayo (15). A similar role of serum antibodies was reported by Steinmuller (16). Halesz and Orloff (17) reported transplacental transfer of a factor that can cause accelerated rejection of a first set homograft in rabbits. From these data, it is still unclear whether cellular or humoral factors or both are involved in graft rejection.

Whatever the graft class, there are two fundamental aspects of the rejection process. First, there is the necessity for recognition of foreign tissue by the host, the so called afferent arc of the process described by Feldman (18). Once recognition occurs, there is an efferent arc response which is a complex array of defense reactions. In the mitral encounter between host and graft, there is an aggregation of lymphocytes, monocytes and neutrophils around thin-walled venules three to four days after placement of the tissue. Presumably, this is because of the time needed to elaborate a capillary connection between

recipient and transplant (19). How inflammation is produced can be explained by the example of the transplantation antigens H-s in mice and HL-A in man. These are complexes of protein, carbohydrates and lipids which reside on the surfaces of cells. They distinguish one individual from another within the same species. They represent the foreignness of the graft and start inflammation. In vitro, when lymphocytes are incubated with homologous cells bearing such transplantation antigens, the lymphocyte, or a fraction of them almost immediately undergo a series of both chemical and structural changes (20). In the first hour, there are cytoplasmic and nuclear rearrangements that affect the lysosomes, ribosomes, RNA, DNA, and histones. Within 24 hours the activated lymphocytes prepare for mitosis and by 48 hours they divide. During this period the activated cells enlarge and acquire the cytologic features of undifferentiated elements (transformation). The whole process of in vitro cellular transformation is associated with graft destruction.

Ways to avoid rejection have been studied extensively. There have been three main methods employed to accomplish this. The first of these is the matching of donor and recipient. That is, if the donor's tissues are antigenically the same as the recipient's, there are no macromolecular marks for the host to detect as foreign and therefore, no response. Such a situation exists in inbred lines of rodents. In man, it is exemplified by monozygotic twins. It is now possible to

test, to a limited extent the antigenic composition of homologous individuals by analyzing their leukocytic antigens with a panel of antisera (21).

The second avenue of approach is to modify the graft so that it is disguised and unrecognized by the recipient as foreign. This may be done by treatment which eliminates certain antigens from transplanted tumors (22), and skin grafts. Additionally, grafts are exposed to one or more antigens from the donor (23) in an attempt to induce antigenic gain. These procedures have been regarded with disfavor for man, due to the complex nature of the human HL-A locus and the multitude of antigenic differences between homologous individuals. Also, one can cover antigenic differences so that the host is unable to detect foreign antigens by the phenomenon of "enhancement." Hyperimmune serum against donor antigens presumably "fixes" to these antigens and prevents their being "seen" by the recipient of the graft. In this sense the graft has been modified. The problem with these sera is that they prolong survival of normal tissues only a few days beyond the expected survival time.

Third, the popular approach to the problem of rejection is to suppress the response of the host. This can be achieved by immunological tolerance (24), but there is a problem of graft versus host reactions, whereby the recipient can be injured, often fatally. The immune response can also be suppressed by the reduction of total

lymphoid mass, circulating lymphocytes or both. This has been thoroughly investigated and documented by James (25). It can be achieved by whole body X-irradiation, extracorporeal irradiation of the blood, nitrogen mustard treatment and administration of cortisone acetate and other immunosuppressive drugs (26, 27), thoracic duct fistula (28, 29) and antilymphocytic serum. All of these agents help to prolong graft survival for varying lengths of time beyond the normal survival period. Presumably, reduction of lymphoid elements reduces the number of immunocompetent cells and thereby decreases by simple quantitative changes the number of effector elements which can either attack the graft or synthesize specific macromolecules to do so (30).

Antilymphocytic serum (ALS), as its name implies, is an antiserum prepared in one species against the lymphocyte of another. The methods of preparation of ALS are documented by James (25). ALS has been tried as an immunosuppressant agent for the prolongation of skin graft survival by various investigators. They have studied the various physiological, biological, and immunological aspects of ALS prepared in a variety of animal species, including mice, rats, guinea pigs, rabbits, dogs, monkeys, human beings (25, 31), horses, goats, and cows (32).

ALS has been used to enhance skin homograft survival in a variety of animal species, such as mice (33, 34, 35), monkeys (36) and human beings (37). Various sources of cells have been used to

produce antilymphocytic serum. These are lymph node cells, peripheral blood lymphocytes, thymocytes, thoracic duct lymphocytes and sub-cellular fractions of cells from lymphoid and non lymphoid origin (38, 39). Lance, Ford, and Ruskiewicz (40) report the use of sub-cellular fractions of lymphocytes as sources of antigens for the production of antilymphocytic serum. They purified the antigen by extracting the membrane sub-component from whole cells (41). This fraction gave rise to potent antisera which were non toxic, did not require adsorption with erythrocytes, and which did not show the decline in potency with repeated immunization as reported by Jooste et al. (42) and Perper et al. (43).

ALS has been used to delay the rapid destruction of renal homotransplants in both rats (44) and dogs (45, 46, 47). It has also been used in renal transplants of human beings but only in conjunction with steroids and other immunosuppressive drugs (48, 49, 50).

Suppression of humoral antibody formation also has been investigated. Heterologous ALS was used to suppress the primary immune response of mice (33, 51, 52), and rats (53, 54, 55) to sheep erythrocytes. It has been shown to suppress the primary immune response of rats to diptheria toxoid (10), of mice to Salmonella II antigen incorporated with Freund's adjuvant (58), and of rats to alum precipitated BSA (57, 58). James demonstrated (59) that it suppresses humoral antibody formation only if given prior to antigenic stimulation,

whereas it has no marked effect on the secondary immune response (33, 34, 55, 58).

Monaco, Wood, and Russell (60) demonstrated that both humoral antibody response and the skin allograft reaction are suppressed as a result of "central failure" of the immune response. The results of Allen, Friedman, and Mills (61) indicated that rabbit sera with specificity directed towards mouse lymphoid cells, affect not only specific antibody forming cells, but can also reduce the number of precursor cells capable of responding to antigen in a new environment. Barth, Hunter, and Southworth suggested that specific types of macrophages also may be functionally impaired by ALS (62). James (63) reported that pretreatment of hooded female rats with horse anti-rat lymphocytic IgG suppresses and/or delays their primary humoral antibody activity to alum precipitated BSA.

Other areas which have been investigated are the species specificity of antilymphocytic antibody (64), the effects of ALS on sensitized lymphoid tissues (65), the influence of chemical (66), and enzymological treatment (67, 68) on the activity of the antilymphocytic antibody molecule and the antigenicity of the heterologous protein (69, 70).

Since heterologous antilymphocytic globulin is an antigen itself, its pathological and immunological effects have been studied. Turk (71) has shown that there are certain antibodies in heterologous ALS

which react with non specific tissue antigens, e.g., antigens in the basement membrane of the renal glomerulus and tubules and cause extensive tissue damage. This is thought to be caused by soluble immune complexes containing humoral antibodies and complement. These antibodies have an increased importance, since it is thought that the lesions that occur in the rejection crisis in patients who have received organ transplants are due to the action of humoral antibodies reacting with complement and not due to cell mediated immunological processes (72). Focal venous thrombosis in man has been shown to develop after the infusion of erythrocyte adsorbed anti-rabbit thrombocyte sera and appeared to be initiated by the degeneration of leukocytes (73). Barth, Friedman, and Malmgren (74) reported the depression of the interferon system, as well as the immune response which contributed to the increased susceptibility of ALS treated animals to viral infections and viral oncogenesis.

Sheagreen, Barth, Edelin, and Malmgren (75) reported that the injection of antiserum to mouse lymphocytes impaired clearance from the blood stream of colloidal carbon and microaggregated human serum albumin in mice. Intravenous injection of ALS produced immediate, profound and prolonged blockade of the reticulo-endothelial-system (RES).

The effects of ALS on other immune responses have been studied. The immune response to contact allergy to

2,4-dinitrochlorobenzene, to diptheria toxoid, experimental allergic encephalomyelitis has been shown by Waksman et al. (10). Turk and Willoughby demonstrated that anti-thymic sera suppressed the effects of contact allergy in the guinea pig (76). Starzl et al. (77) and Monaco et al. (37) demonstrated the disappearance of expression of delayed hypersensitivity to a number of antigens by the use of ALS in human renal homotransplantation. The lymphocyte transfer phenomenon has been shown to be inhibited by ALS (35), the graft versus host reactions in F-1 hybrid recipient mice has been shown to be uninducible by ALS treatment by Boak, Fox, and Wilson (78). ALS is also effective in suppressing Freund's adjuvant induced polyartheritis in rats (53), and Coomb's positive hemolytic anemia in NZB mice (79).

It is evident from analysis of the above data that ALS has a generalized immunosuppressive effect on both humoral and cell mediated immunity, and the total effect dependent upon such factors as dosage, route of injection, animal species and strain.

The purpose of these studies was to prepare and characterize antilymphocytic sera which were specific for and reactive with cells involved in a particular immunologic process, allograft rejection.

CHAPTER 2

MATERIALS AND METHODS

Animals

Outbred albino male and female guinea pigs of the Amana, Rockefeller and Hartley strains from the colonies of the Department of Microbiology, University of Arizona were used. The animals weighed 500 to 900 gm and were housed in separate cages in an environment where temperature (74^{o} F $\pm$ 2^{o} F) and relative humidity (50% $\pm$ 5%) were controlled. They were given antibiotic free Purina guinea pig chow and tap water supplemented with 0.3% ascorbic acid ad libitum. Cabbage was provided daily.

Male albino New Zealand rabbits weighing $6\frac{1}{2}$ to 8 lbs were employed to produce antilymphocytic serum. They were housed individually in a controlled environment and were fed Purina rabbit chow and tap water as above. Cabbage was provided daily. As a prophylactic measure all incoming rabbits were treated for 10 days with Terramycin in their drinking water (Oxytetracycline HCl, soluble powder, 100 mg/gallon of water, Pfizer & Co., N. Y., N. Y.) before use.

Skin Grafting Procedure

Skin grafting was done according to the techniques of Siebeling (80). Skin donor and recipient guinea pigs were anesthetized with an intrabdominal injection of Sodium nembutal 30 mg/kg body weight. Ether was used supplementally when required. The abdominal area of the skin donor and the right and left dorso-lateral chest walls of the recipient guinea pigs were clipped and shaved free of hair. Clipped areas were scrubbed with 2% amphyl followed by 70% alcohol.

Abdominal skin measuring about 2 x 16 cm was removed aseptically from guinea pig donors. The skin deficit in the donors was closed by 11 mm Michelle wound clips. All skin grafts removed were full thickness and were placed in Eagle's Minimum Essential Medium (Grand Island Biological Company, Grand Island, N. Y.). Excessive adipose and connective tissues were trimmed away from the under surface of the skin strips. Each skin strip was then cut into 1 cm squares. These grafts were stored surface down in sterile Petri plates containing Eagle's MEM.

Recipient guinea pigs of the Amana strain received two sensitizing skin grafts from the donor. The grafts were fitted orthotopically on the dorso-lateral aspect of the rib cage just posterior to the scapula. The graft beds on the recipients were prepared by surgical removal of a square cm of full thickness skin. Care was taken during excision to keep the panniculus dorsum on the graft bed intact, thereby

ensuring that its capillaries and lymph vessels would be available for nourishing and healing of the donor skin graft. The skin grafts were held firmly in place on the graft beds by a strip of 1" Blenderm surgical tape (Blenderm, 3-M Co., St. Paul, Minnesota) applied directly over the grafts and the surrounding areas. No sutures were employed. The surgical tape provided adequate pressure to prevent slippage and dehydration of the graft. It also permitted visualization of the graft. Sterile gauze pads were placed immediately over the taped skin grafts and the recipients were taped with 2" Blenderm surgical tape over the gauze pads for safety and protection of the grafts.

After the fourth post surgical day the grafts were inspected grossly each day. Change in color, integrity of the epidermis and dermal layers and ability to produce bleeding upon scraping with a scalpel blade were noted.

Upon complete rejection of the first set of skin grafts, the recipient guinea pigs were tested for sensitivity by another application of a single skin graft from the original donor. Only those animals which showed a "white graft reaction" (81) were subsequently used as a source for sensitized lymphocytes.

Tissue Culture Media

Minimum Essential Medium (MEM) Eagle's base with glutamine (Grand Island Biological Co., Grand Island, N. Y.) was used for the processing of grafts, cell collections, washings, etc.

Collection of Cells

Thoracic duct cells were collected from a group of guinea pigs showing a white graft reaction by the method of Bollman, Cain and Grindlay (82) with certain modifications. Guinea pigs were anesthetized with Sodium numbutal (30 mg/kg body weight) and controlled with ether for anesthesia whenever necessary. The abdominal area was clipped and shaved by means of an electric shaver. The surgical area was cleansed by means of 2% amphyl followed by the application of 70% alcohol. This was let air dry. A mid line incision was made and another extending from the mid line anteriorly to the medial border of the left quadratus lumborum muscle posteriorly. A pack of sterile gauze was placed in such a way that it pushed the stomach, liver, and intestines back and to the right and exposed the left portion of the diaphragm, the aorta, and the left adrenal gland and the kidney. Sometimes 0.1 ml of a 0.5% solution of Evans blue dye was injected through a 27 gauge needle in the liver. This helped in localizing the lymph channels which get dyed slightly blue in color.

With the use of a blunt dissecting probe an opening was made in the retroperitoneal cavity over the quadratus lumborum approximately 0.5 cm cephalad to the supra renal artery. With the help of the blunt probe the adipose tissue was dissected away and exploration was made till the aorta was exposed. Posterior to the aorta runs the thoracic duct which was about 0.5 to 1.0 mm in diameter and was

embedded in loose connective tissue and fat. The duct was picked up by means of the blunt probe and held in place. By means of a sharp 13 gauge needle which was passed through the abdomen at approximately the level of the xiphoid process a plastic cannula 0.3 mm (Clay Adams Inc.) in diameter was threaded through this needle and then the needle was pulled out leaving the plastic cannula in place. A small longitudinal incision was made on the anterior surface of the thoracic duct by means of a 27 gauge needle and the cannula was passed into the duct about 0.5 to 1.0 cm and held in place. The cannula was filled with a dilute solution of sodium heparin (Heparin, sodium, 10 mg/ml, U.S.P., Rabin Winters Pharmaceuticals, El Segundo, Calif.) and as the lymph flowed out it was collected in sterile siliconized glass tubes. Lymph fluid was collected from 30 to 90 min and then centrifuged at 1500 rpm for 15 min at 4 C. The supernatant fluid was discarded and the cells were washed twice with Eagle's MEM, pooled and resuspended and total, differential, and vital counts were performed. One billion cells were used for the immunization of the rabbits.

Lymph node cells were obtained by sterile surgical excision of regional draining supra scapular lymph node from guinea pigs showing a white graft reaction. These nodes were teased over a sterile wire gauze (40 mesh) coated with Monocote E (Armour Laboratories, Kanakakee, Illinois) and the wire gauze was held over a sterile pestle and tissue culture media used to wash the cells off the wire mesh.

These cells were washed twice with Eagle's MEM and resuspended in this medium and total, differential, and vital counts were made. One billion cells were used for the immunization of the rabbits.

Peripheral blood was also obtained from Amana strain guinea pigs showing a white graft reaction. Blood was obtained by means of cardiac puncture and sodium heparin added to it (10 units/ml of blood). The heparinized blood was passed through a siliconized glass column containing siliconized glass wool and #100 superbrite glass beads (3-M Co., St. Paul, Minnesota). The flow rate was adjusted to about 6 drops a minute. This method insured the removal of a large percentage of glass adhering cells. The first ml was discarded and the rest collected in siliconized glass tubes. The column effluent containing mostly red cells and mononuclear cells was layered over a mixture of methylcellulose (Methocel-Dow Chemical Co. Lot 042477, type MC-premium, visc. 25 cps) and sodium diatrizoate (Sodium diatrizoate, Winthrop Laboratories, Park Ave., N. Y., N. Y.) according to the method of Hulliger and Blazkovec (83). The erythrocytes settle to the bottom and the mononuclear cells are left in the supernatant fluid as a white suspension. The supernatant fluid was centrifuged at 225 x g for 15 min at 4 C. The cell button was resuspended and washed twice with Eagle's MEM containing 25 units/ml of sodium heparin. After the final wash the cells were resuspended in Eagle's MEM and

total, differential, and viable counts were made. One billion cells were subsequently used for the immunization of the rabbits.

Cell Counting

Total and viable white cell counts were made on all cells used for the immunization of rabbits employing standard techniques on the Spencer Bright-Line Hemocytometer. Viability was determined by the dye exclusion technique of Pappenheimer (84). Differential cell counts were done by a supra vital stain technique employing Janus Green and Neutral Red according to the technique outlined by Gurr (85).

Glassware

All glassware (glass column, pasteur pipettes, syringes etc.) were siliconized with a 2% solution of Dow Corning fluid (Dow Corning Corp., Midland, Michigan) in chloroform and cured at 300 C for 30 min. The 13 gauge needle used for the glass column was treated with Monocote E (Armour Laboratories, Kanakakee, Illinois). The glass wool was siliconized using Dow Corning #1208 vapor phase siliconizing mixture (Dow Corning Corp., Midland, Michigan). The vapor of this mixture was passed through the glass wool for 1 min and then quickly flushed out with air. The glass wool was washed 10 times with water heated for 30 min at 110 C, soaked in water for 30 min, rinsed 5 times with distilled water and finally air dried in a hot air

oven. The glass wool used was Pyrex fiber #3950 (Corning Glass Works, Corning, N. Y.).

The glass beads were #100 superbrite (3-M, St. Paul, Minnesota). Glass beads were washed twice with hemosol, rinsed 6 times with tap water, acid rinsed, and then re-rinsed 6 times with tap water, 3 times with distilled water, 3 times with double distilled water and finally air dried in a hot air oven. All glassware were sterilized in an autoclave prior to use.

Immunization of Rabbits

Rabbits were immunized with a sub-cutaneous injection of one billion washed lymphoid cells in MEM in a total volume of 4 mls, 0.2 ml in each site on the back. Three weeks later the rabbits were test bled and a preliminary cytotoxicity titer determined. In all cases boosting was necessary which was accomplished by an intravenous injection of 1×10^7 washed lymphoid cells of homologous origin. Seven to ten days after the last injection the rabbits were exsanguinated and the sera collected and stored at -20 C until further use.

Adsorption of Sera

Antilymphocytic sera from each rabbit was divided into equal portions. One half was used as unadsorbed ALS and the other half was adsorbed with lymphocytes from untreated animals from the same source as used for the immunization of the rabbits (homologous

origin). The adsorption was performed by taking 1 ml of two fold dilutions of ALS heated at 56 C for 30 min and incubating it with one million cells of homologous origin in a 37 C water bath in siliconized test tubes for varying periods of time. After each time interval the tubes were centrifuged at 1500 rpm at 4 C for 15 min. The supernatant sera was aspirated by means of a pasteur pipette and lymphocytotoxicity and lymphagglutination tests performed with lymphocytes from untreated and allograft sensitized animals of homologous origin. Curves were drawn on the respective titers of each dilution at different time intervals and the optima calculated to be the dilution of ALS that is adsorbed at 37 C for a specific time period which removes most of the cytotoxicity against lymphocytes from untreated animals. Sera were thus adsorbed according to this optima and collected and stored at -20 C until further use. These sera were henceforth called adsorbed ALS.

Serum Protein Determinations

Determination by ultraviolet absorptions was performed by the crude protein estimation by reading the absorbance of the sample at 260 nm and 280 nm according to the method of Warburg and Christian (86) using a Bausch and Lomb spectrophotometer 600. Standard protein controls were included.

Determination by the Folin-Ciocalteau Method

Further estimation of protein were performed by the method outlined in the Manual of Microbiological Methods (87) using the Folin-Ciocalteau phenol reagent and reading absorbance at 660 nm. The sensitivity range was between 7 to 70 μg of protein and the error was 3 to 5%. Total protein was estimated by using a BSA standard curve.

Cellulose Acetate Electrophoresis

The Gelman Rapid chamber #51100 (Gelman Instrument Co., Ann Arbor, Michigan) and Sepraphore III cellulose polyacetate strips (1" x 6 3/4", Lot 1392, Gelman Instrument Co., Ann Arbor, Michigan) were employed to study the electrophoretic patterns of unadsorbed and adsorbed sera according to the method of Briere and Mull (88). Ponceau S was employed as a general protein stain. The different bands were eluted and protein determinations were made on each band according to the method outlined by the manufacturer.

Disc Electrophoresis

Disc electrophoresis in polyacrylamide gel on the Canalco Disc Electrophoresis apparatus was performed according to the procedure of Davis and Ornstein (89). Samples were electrophoresed at 5 mamp/tube for 30 min and stained with amido schwartz.

Immunoelectrophoresis

Immunoelectrophoresis was carried out using the LKB model 6800 A immunoelectrophoresis apparatus. Veronal buffer pH 8.6 ionic strength 0.1, supporting a 50 milliamp constant current for 1 hour was employed for the separation of samples. After incubation with antiserum, staining was accomplished with 0.6% amido schwartz in an acid alcohol solution.

Gamma Globulin Separation by Column Chromatography

Sera were taken and applied to a G-200 Sephadex column (Sephadex, Pharmacia Fine Chemicals, Piscataway, New Jersey) according to the method of Flodin and Killander (90) in a glass column with an inside diameter of 2.5 cm, a variable height and an outlet mixing chamber of less than 0.1% of the column volume. The Sephadex G-200 was appropriately swollen and packed as a slurry in one operation without layering. Constant head pressure not exceeding 15 cm was maintained throughout the operational procedure.

Samples applied to the column were eluted in a 0.1 M tris-HCl buffer pH 8.6, at a flow rate of about 15 ml an hour. Fractions were collected employing the Warner-Chilcott model 1205 DB fraction collector with a 10 ml volumetric siphon. The samples were scanned for absorbance in a Bausch & Lomb Spectronic 600, ultraviolet spectrophotometer at 260, 280, and 360 nm. The Beckman model 1005

Potentiometer Recorder was attached to the Spectronic 600 which monitored the ultraviolet and visible spectrums and graphed the characteristics of each sample.

The gamma globulin fraction was concentrated by prevaporation in a fisking tubing at 4 C and adjusted to a specific protein concentration. This whole gamma globulin fraction was further fractionated using a DEAE-Sephadex column employing DEAE-Sephadex A-50 coarse (Sephadex, Pharmacia Fine Chemicals, Piscataway, New Jersey), according to the method of Baumstark, Laffin, and Bardawil (91). The 7 S fraction was purified by re-application of the concentrated fraction to a similar column twice. The purity and identification of the fractions were determined by immunoelectrophoretic analysis.

Fluorescent Antibody Analysis

The purified 7 S gamma globulin was tagged with fluorescein isothiocyanate and applied on a very thin smear of lymphocytes from untreated and allograft sensitized animals according to the procedure outlined by Holborow and Johnson (92). The stained preparation was finally mounted in 10% glycerine in buffered saline pH 7.0 and observed under the microscope using a high pressure mercury vapor lamp (Ernest Leitz GMBH Wetzlar, Lampenhaus 250, Germany).

Lymphagglutination

Lymphagglutination tests were performed according to the method of Greaves et al. (93). 0.1 ml of two fold dilutions of unadsorbed ALS were added to each ml of a lymphocyte suspension containing 1 x 10^6 cells in Eagle's MEM. The sera were heated at 56 C for 30 min prior to use. After incubation for an hour at 37 C in a water bath the tubes were placed in an ice bath and samples taken from it and counted in a Spencer line hemocytometer using phase contrast microscopy. The number of free unaggregated cells were determined, the percentage agglutination being calculated as 100 x (T -F)/T where T is the total number of cells counted, F is the total number of free cells. Titers were expressed as the reciprocal of the highest dilution of sera giving more than 20% agglutination. Control values were in the range of 5 to 10% agglutination. Tests were performed with lymphocytes from untreated and allograft sensitized animals of homologous origin simultaneously.

Lymphocytotoxicity

The test was performed by adding 0.1 ml of two fold dilutions of specific unadsorbed or adsorbed heat inactivated ALS to a suspension of lymphocytes such that 0.8 ml of the suspension contained 1 x 10^6 cells. 0.1 ml of guinea pig serum containing two full units of complement was added as a source of complement to make a total

volume of 1 ml. The test was performed in siliconized glass tubes, and, after the addition of complement the tubes were incubated in a 37 C water bath for 1 hour with intermittent shaking. After incubation the test tubes were plunged into an ice bath. Samples were taken from each tube and total and percent viability was determined using the dye exclusion technique employing 0.4% trypan blue in Hank's Balanced Salt Solution. The percent viability was expressed as the total number of viable cells after incubation divided by the total number of viable cells before incubation times 100. Titers were expressed as the reciprocal of the highest dilution of the sera giving more than 20% cytotoxicity as compared to the values obtained with normal rabbit serum.

Complement Fixation Tests

The complement fixation tests were performed according to the procedure of McKee and Jeter (94) as follows: to 0.2 ml of heat inactivated two fold dilutions of sera was added 0.2 ml of a suspension of lymphocytes containing 2.5×10^5 cells and 0.2 ml of 2 full units of complement (guinea pig serum). The mixture was incubated in a 37 C water bath for 30 min and then 0.4 ml of sensitized sheep red blood cells were added as the indicator system. The serology tubes were shaken and then re-incubated in the 37 C water bath for 30 min. The complement fixation titer was expressed as the reciprocal of the

highest dilution of sera showing no lysis of the sensitized red blood cells. Controls were included with each serum to show anticomplementary activity if any.

Mitogenic Index

Suspensions containing 1×10^6 lymphoid cells were prepared in Eagle's MEM with glutamine + 20% heat inactivated fetal calf serum + penicillin 100 units/ml + streptomycin 100 μg/ml + sodium heparin 10 units/ml + 1.7 mg/ml $NaHCO_3$.

0.1 ml of heat inactivated unadsorbed or adsorbed ALS in two fold dilutions was added to duplicate 1 ml suspensions of lymphocytes prepared as above. These were carried out in Tissue Culture test tubes with Morton closures employing tissue culture techniques according to Ling (95). With each experiment some tubes were included which contained PHA (Phytohemagglutinin, Burroughs Wellcome and Co.) or PPD (Purified Protein Derivative, DIFCO) or just control tubes with a 0.1 ml of the above media. The cultures were incubated at 37 C in a 5% CO_2 humidified atmosphere for 4 to 6 days. After incubation the cultures were centrifuged at 150 xg for 5 min and the supernatant fluid discarded. The lymphocytes were resuspended and washed twice in sterile 0.15 M NaCl and finally resuspended in 0.2 ml of the same sterile saline. Cell viability was determined before and after the experiment by the trypan blue dye exclusion technique of Pappenheimer (84).

Smears of each culture were made in duplicate utilizing single cell preparations on ultra clean microscope slides and the preparation was stained by the Giemsa stain.

Differential count of the number of blastoid cells was made according to the technique of Coulson and Chalmers (96). One thousand mononuclear cells were counted and recorded in three categories, 12 μ or more were classified as blastoid, 8 to 10 μ as transitional, and below 8 μ as small lymphocytes with the help of a calibrated micrometer scale. The number of blastoid cells in the 1000 mononuclear count was expressed as the percentage of transformed cells. Net percent transformation was calculated as the difference between the total average percent transformation in cultures when antisera was added and the total average percent transformation in cultures in the tubes containing normal rabbit serum. Titers were expressed as the highest dilution of ALS unadsorbed or adsorbed which gave more than 10% net transformation.

Opsonic Index

Peritoneal macrophages were obtained 72 hours after injecting untreated Amana strain guinea pigs with 20 mls of sterile mineral oil intraperitoneally. The cells were collected in sterile Eagle's MEM containing 20% heat inactivated fetal calf serum, penicillin, streptomycin, and sodium bicarbonate. The cells were washed twice by

centrifugation at 125 x g at 4 C for 10 min each time and then resuspended in fresh media. Total, viable, and supravital counts were done on this washed sample. Cells were then diluted as to contain 1 x 10^6 cells/ml and 0.4 ml of this suspension was put on an ultra clean cover slip and Permount (#48205 -H-568, Braun Chemical Co., Los Angeles, California) was applied to the edges of the cover slip and the cover slip was attached to the top of a sterile plastic Petri plate. The plastic Petri plate contained a sterile filter pad on the bottom soaked in 3% $NaHCO_3$ in sterile water. These cultures were incubated at 37 C in a 5% CO_2 humidied atmosphere for 24 hours. After incubation the cover slips were observed for macrophage adherence, uniformity, and sterility.

Lymphocytes were obtained from untreated and allograft sensitized Amana strain guinea pigs and washed twice with Eagle's MEM with centrifugation at 125 x g at 4 C for 10 min each time. They were resuspended and total, viability, and supravital counts were made. They were then diluted such that each ml contained 1 x 10^6 viable lymphocytes. To 0.4 ml of this suspension was added 0.1 ml of the appropriate heat inactivated ALS dilution in duplicate cultures, in siliconized glass tubes. These cultures were incubated at 37 C for 30 min in a 5% CO_2 atmosphere. After incubation this lymphocyte suspension was added to the macrophage containing cover slips which was gently tapped off the Petri plate cover. This was incubated in a fresh

sterile Petri plate in a humidified atmosphere containing 5% CO_2 at 37 C for 2 hours. The cover slip was inverted and observed under the phase contrast microscope. The number of lymphocytes attached to each macrophage was noted and 1000 macrophages were counted at random. The ratio between lymphocyte and macrophage was determined and the titer of ALS was expressed as the reciprocal of the highest dilution giving 50% adherence of lymphocytes.

Precipitin Interfacial Test

The ring or interfacial precipitin test was carried out by layering two fold dilutions of fresh guinea pig serum over unadsorbed and adsorbed ALS. The test was performed in 4 mm tubes. Precipitates at the interface were read at 30, 60, and 90 min and recorded. Saline layered over guinea pig serum and ALS layered over normal pre-bleed rabbit serum were included as controls.

Hemagglutination Test

Direct hemagglutination tests were performed according to the method outlined by Campbell et al. (97). Blood from untreated Amana strain guinea pigs was collected in equal volumes of Alsever's solution and stored 3 days before use. The blood was then centrifuged at 125 x g at 4 C for 10 min, the supernatant fluid was discarded and the buffy coat removed. The guinea pig red blood cells were washed 3 times

with sterile buffered saline pH 7.2. A 0.5% suspension of these red blood cells was then made in the buffered saline pH 7.2.

0.5 ml of a 0.5% cell suspension was added to two fold dilutions of heat inactivated ALS and the tubes were incubated in a 37 C water bath for 60 min. Pre-bleed normal rabbit serum and saline were used as controls. After incubation positive hemagglutination was recorded as clumping or a homogenous layer of erythrocytes and negative hemagglutination recorded as the formation of a bead or button at the bottom of the test tube. Titer was expressed as the reciprocal of the highest dilution of ALS showing positive hemagglutination.

Depression of In Vitro Spleen Cell Hemolysis

The test was performed according to the technique of Klesius (98). Guinea pigs were immunized with 1×10^9 washed sheep red blood cells. Spleen cells obtained from these animals were washed twice with MEM and finally resuspended in fresh MEM so as to contain 1.4×10^8 cells per ml. Culture plates were made such that each culture plate contained 2 mls of this cell suspension. Unadsorbed or adsorbed ALS were added to triplicate culture plates of these spleen cells. Normal rabbit serum and saline were used as controls. Fresh guinea pig serum was added as a source of complement. After incubation of these cultures at 37 C in a 5% CO_2 humidified atmosphere, the cells were centrifuged at 125 x g for 10 min and hemolysis of the supernatant

fluid was read at O.D. 541 nm in a Coleman Jr. Spectrophotometer.

Lymphopenia Studies

Lymphopenia studies were carried out by taking blood samples from Amana strain guinea pigs receiving 1 ml of unadsorbed or adsorbed ALS, from day -3 to day +3 every day and from then on every other day up till day 19. Blood samples for leukocyte counts were taken by nicking the ear vein of the recipient by means of a sterile lancet. Two hours after the first ALS injection the first experimental blood sample was taken. Prior to this sampling, base line readings on leukocyte counts were established with each animal. Blood smears were made in duplicate and stained with Giemsa blood stain and a differential count done on a 1000 white blood cells. These were expressed as average percentage.

Similar studies were also made with Amana strain guinea pigs receiving both ALS and skin allografts, normal rabbit serum and untreated controls.

Prolongation of First Set Skin Allografts by ALS

Guinea pigs of the Amana strain were injected intraperitoneally with 1 ml of a 1:2 or 1:3 dilution of unadsorbed or adsorbed ALS in 0.15 M NaCl every day from day -3 to day +3 and then every other day till day 19. On day 0 the ALS recipients were grafted with skin from

homologous Rockefeller strain guinea pig(s) according to the procedure described before. Six guinea pigs were used for each serum tested. The grafts were observed daily and the rejection of each graft noted. With each experiment a control animal was included who received pre-bleed normal rabbit serum instead of ALS and another animal who received no treatment.

Prolongation of First Set Skin Allografts and Response of Recipients to BSA

Amana strain guinea pigs were treated with unadsorbed or adsorbed ALS on a schedule similar to the above and skin grafted with a Rockefeller guinea pig serving as the skin donor on day 0. Also on day 0 they were injected with 5 mg of BSA in incomplete Freund's adjuvant in the nape of the neck. The rejection time of the grafts were recorded and on day 21 the animals were bled and sera was collected and tested for precipitins against BSA. Control animals received normal rabbit serum or nothing (used as an untreated control).

Prolongation of First Set Skin Allografts and Response of Recipients to Sheep Red Blood Cells (SRBC)

Amana strain guinea pigs were treated with ALS and grafted as above on day 0 with skin from a homologous Rockefeller strain donor. The recipients also were injected intradermally with 0.1 ml in each site of washed SRBC containing 1×10^9 red blood cells. On day 21 the animals were bled and sera collected and the hemagglutination titer

determined using a technique described previously. One control group received pre-bleed normal rabbit serum and the other control group was used as untreated. Titers were expressed as the reciprocal of the highest dilution of guinea pig serum showing positive hemagglutination.

Depletion of In Vivo Complement

Amana strain guinea pigs were taken and treated with unadsorbed and adsorbed ALS and skin grafted as above. These recipients were bled intracardially on various days before and after the treatment and their sera were analyzed for complement activity by the method of McKee and Jeter (94). One control group received pre-bleed normal rabbit serum and the other control group was used as an untreated control.

Determination of the Specificity of Adsorbed ALS

Transplantation Specificity

Transplantation specificity of the unadsorbed and adsorbed ALS was performed by taking recipient Amana strain guinea pigs and injecting them with ALS according to the schedule described before. On day 0 five different grafts were applied on each recipient guinea pig. One graft was from the original Rockefeller strain donor guinea pig which was used for the sensitization of the lymphocyte donors, this

was termed as the homologous Rockefeller skin graft. The second was an autograft which was applied to insure technique. The third graft was from a Hartley strain guinea pig, an indifferent allograft. The fourth was a graft from a Rockefeller strain donor which was neither a sibling or twin to the homologous Rockefeller strain skin donor, this was termed the heterologous Rockefeller skin donor. The fifth graft was from an Amana strain guinea pig which was similarly neither a sibling or a twin to the recipient Amana strain guinea pig, and this was termed as the heterologous Amana strain graft. The first three grafts were applied on one side of the guinea pig on the back whereas the last two were applied on the other side of the guinea pig on the back. The rejection time for each graft was noted and the specificity of each unadsorbed and adsorbed ALS in terms of its prolongation of its homologous and/or heterologous grafts noted.

In addition Rockefeller strain guinea pigs were grafted with Amana strain guinea pig skin and treated with the same ALS unadsorbed and adsorbed according to the same schedule and the rejection time in each case was noted.

Second Set Response

A group of Amana strain guinea pigs were grafted with skin from a homologous Rockefeller strain guinea pig and the grafts were allowed to reject in the normal fashion. After complete rejection had

occurred they were re-grafted with skin from the same homologous Rockefeller donor but this time they were treated with either unadsorbed or adsorbed ALS on a schedule as before. The second set response of these recipients was studied daily and the rejection time in each case was noted.

Other Cell Mediated Phenomenon

Groups of Amana strain guinea pigs were treated with either unadsorbed or adsorbed ALS according to the established schedule as before and on day 0 they were shaved on the nape of the neck with an electric shaver and painted with a 2% solution of 2, 4-dinitrofluorobenzene in ethyl alcohol on the shaved area. The animals were painted daily for 6 days and on day 10 were skin tested for sensitivity with a 0.1% solution of 2, 4-dinitrofluorobenzene in olive oil. Twenty-four hours later the skin reactions were read and recorded according to the procedure outlined by Burger (99).

CHAPTER 3

RESULTS

In Vitro Characterization of Antilymphocytic Sera

In vitro characterization of unadsorbed and adsorbed ALS was carried out using lymphocytes from untreated and allograft sensitized guinea pigs. The lymphocytes used for the titrations were from the same strain and collection sources as those employed for producing the antilymphocytic sera.

The results of lymphocytotoxicity studies are shown in Table 1. Unadsorbed sera against both sensitized and normal lymphocytes collected from all three sources (peripheral blood, thoracic duct and lymph nodes) showed titers ranging from 64 to 2048. No significant differences were observed between titers obtained with the use of lymphocytes from untreated and homologous sensitized animals. After adsorption it is evident that there is stronger reactivity of the sera with sensitized cells as compared with those with normal cells. ALS made against lymphoid cells from untreated animals lose all their in vitro activity after adsorption. Lymphocytotoxicity tests using fresh guinea pig serum as the source of complement gave the most consistent

TABLE 1. Comparison of Lymphocytotoxicity Titers of Unadsorbed and Adsorbed Antilymphocytic Sera.

	Sera	Unadsorbed Antigen		Adsorbed Antigen	
		N[1] cells	S[2] cells	N cells	S cells
1.	ALS(NTDL)[3]	128	64	0	0
2.	ALS(NPBL)[4]	128	128	2	0
3.	ALS(SLNC)[5]	64	64	0	2
4.	ALS(SLNC)	64	128	0	4
5.	ALS(SLNC)	64	64	2	8
6.	ALS(SPBL)[6]	256	256	2	32
7.	ALS(SPBL)	2048	2048	256	512
8.	ALS(SPBL)	512	1024	4	32
9.	ALS(SPBL)	256	128	8	16
10.	ALS(SPBL)	128	256	16	32
11.	ALS(STDL)[7]	128	128	0	16
12.	ALS(STDL)	128	256	8	32
13.	ALS(STDL)	128	128	4	16
14.	ALS(STDL)	64	128	0	8
15.	ALS(STDL)	128	256	0	16
16.	ALS(STDL)	256	256	0	16

[1] N cells = Lymphocytes from untreated animals.

[2] S cells = Lymphocytes from allograft sensitized animals.

[3] ALS(NTDL) = ALS against normal thoracic duct lymphocytes.

[4] ALS(NPBL) = ALS against normal peripheral blood lymphocytes.

[5] ALS(SLNC) = ALS against sensitized lymph node cells.

[6] ALS(SPBL) = ALS against sensitized peripheral blood lymphocytes.

[7] ALS(STDL) = ALS against sensitized thoracic duct lymphocytes.

results. When rabbit serum was used as the source of complement variable results were obtained.

The results of lymphagglutination tests are shown in Table 2. There was no significant difference between titers obtained with unadsorbed sera using lymphocytes from untreated and allograft sensitized animals. This was true regardless of the source of the cells. The various sera showed titers ranging from 64 to 2048. Significant differences are observed in the titers obtained with the adsorbed sera. These sera show consistently higher titers with homologous allograft sensitized cells than with cells from untreated animals, the titers ranging from 0 to 8 with cells from untreated animals and 0 to 128 with cells from homologous allograft sensitized animals. Lymphagglutination tests performed using cells from peripheral blood or thoracic duct were consistent, but when lymph node cells were used intermittent shaking was necessary otherwise cells would clump and would therefore yield inconsistent results.

The results of the complement fixation titers are shown in Table 3. No significant difference was observed between titers obtained with the use of cells from untreated or homologous allograft sensitized cells in the case of unadsorbed sera. The titers ranging from 64 to 2048. In the case of adsorbed sera consistent higher titers were obtained with the sensitized cells as compared to cells from normal animals. The titers ranged from 0 to 8 with the use of cells

TABLE 2. Comparison of Lymphagglutination Titers of Unadsorbed and Adsorbed Antilymphocytic Sera.

	Sera	Unadsorbed Antigen		Adsorbed Antigen	
		N[1] cells	S[2] cells	N Cells	S cells
1.	ALS(NTDL)[3]	64	64	0	0
2	ALS(NPBL)[4]	128	128	0	0
3.	ALS(SLNC)[5]	128	128	0	8
4.	ALS(SLNC)	32	128	0	4
5.	ALS(SLNC)	64	64	2	16
6.	ALS(SPBL)[6]	1024	2048	8	128
7.	ALS(SPBL)	256	512	4	64
8.	ALS(SPBL)	256	256	2	32
9.	ALS(SPBL)	512	1024	4	128
10.	ALS(SPBL)	512	2048	2	16
11.	ALS(STDL)[7]	256	256	2	16
12.	ALS(STDL)	128	256	2	8
13.	ALS(STDL)	128	256	2	32
14.	ALS(STDL)	128	512	0	16
15.	ALS(STDL)	256	256	0	8
16.	ALS(STDL)	128	256	2	16

[1] N cells = Lymphocytes from untreated animals.

[2] S cells = Lymphocytes from allograft sensitized animals.

[3] ALS(NTDL) = ALS against normal thoracic duct lymphocytes.

[4] ALS(NPBL) = ALS against normal peripheral blood lymphocytes.

[5] ALS(SLNC) = ALS against sensitized lymph node cells.

[6] ALS(SPBL) = ALS against sensitized peripheral blood lymphocytes.

[7] ALS(STDL) = ALS against sensitized thoracic duct lymphocytes.

TABLE 3. Comparison of Complement Fixation Titers of Unadsorbed and Adsorbed Antilymphocytic Sera.

	Sera	Unadsorbed Antigen		Adsorbed Antigen	
		N[1] cells	S[2] cells	N cells	S cells
1.	ALS(NTDL)[3]	128	64	0	0
2.	ALS(NPBL)[4]	512	256	2	0
3.	ALS(SLNC)[5]	128	256	2	16
4.	ALS(SLNC)	64	128	2	16
5.	ALS(SLNC)	128	64	2	32
6.	ALS(SPBL)[6]	1024	1024	2	64
7.	ALS(SPBL)	1024	1024	8	64
8.	ALS(SPBL)	512	512	8	64
9.	ALS(SPBL)	1024	512	8	64
10.	ALS(SPBL)	1024	2048	8	16
11.	ALS(STDL)[7]	512	256	4	32
12.	ALS(STDL)	256	512	2	32
13.	ALS(STDL)	256	256	4	16
14.	ALS(STDL)	512	512	2	32
15.	ALS(STDL)	512	256	4	16
16.	ALS(STDL)	256	512	2	32

[1] N cells = Lymphocytes from untreated animals.

[2] S cells = Lymphocytes from allograft sensitized animals.

[3] ALS(NTDL) = ALS against normal thoracic duct lymphocytes.

[4] ALS(NPBL) = ALS against normal peripheral blood lymphocytes.

[5] ALS(SLNC) = ALS against sensitized lymph node cells.

[6] ALS(SPBL) = ALS against sensitized peripheral blood lymphocytes.

[7] ALS(STDL) = ALS against sensitized thoracic duct lymphocytes.

from untreated animals and 0 to 64 with homologous allograft sensitized cells. Several unadsorbed sera gave a typical Neisser and Wechsberg phenomenon (100) at low dilutions (1:2 to 1:8). Only two of the adsorbed sera showed this phenomenon and both of them at a 1:2 dilution of antisera. Both these adsorbed sera were against thoracic duct lymphocytes.

The results of the mitogenic index test are shown in Table 4. No significant difference between titers obtained with normal and sensitized cells was observed when unadsorbed sera was used, but significant difference was seen in the case of adsorbed sera. Unadsorbed sera showed a titer of 64 to 2048, whereas adsorbed sera showed a titer ranging from 0 to 16 with normal cells and 0 to 128 with sensitized cells. Mitogenic index studies showed that the maximum response using adsorbed ALS occurred by day 4, but, the response using unadsorbed ALS occurred after day 5 regardless of the source of the cells. The optimum mitogenic response was observed in the case of cells stimulated with undiluted PHA (Phytohemagglutinin). PPD (Purified Protein Derivative) had no effect whatsoever on either lymphocytes from untreated or allograft sensitized animals. Lymph node cells gave very poor viability, four days after culture as judged by the trypan blue dye exclusion technique, even when the media was supplemented with 20% autologous heat inactivated guinea pig serum. The peripheral blood lymphocyte cultures retained 60 to 70% viability

TABLE 4. Comparison of Mitogenic Indices of Unadsorbed and Adsorbed Antilymphocytic Sera.

	Sera	Unadsorbed Antigen		Adsorbed Antigen	
		N[1] cells	S[2] cells	N cells	S cells
1.	ALS(NTDL)[3]	128	128	0	0
2.	ALS(NPBL)[4]	256	256	4	0
3.	ALS(SLNC)[5]	64	128	0	16
4.	ALS(SLNC)	16	64	2	64
5.	ALS(SLNC)	128	128	2	32
6.	ALS(SPBL)[6]	256	512	8	64
7.	ALS(SPBL)	512	512	8	128
8.	ALS(SPBL)	512	1024	4	128
9.	ALS(SPBL)	1024	2048	2	64
10.	ALS(SPBL)	512	1024	4	128
11.	ALS(STDL)[7]	256	512	16	128
12.	ALS(STDL)	128	256	8	64
13.	ALS(STDL)	128	256	4	64
14.	ALS(STDL)	256	512	2	64
15.	ALS(STDL)	128	512	0	32
16.	ALS(STDL)	128	256	0	32

[1] N cells = Lymphocytes from untreated animals.

[2] S cells = Lymphocytes from allograft sensitized animals,

[3] ALS(NTDL) = ALS against normal thoracic duct lymphocytes.

[4] ALS(NPBL) = ALS against normal peripheral blood lymphocytes.

[5] ALS(SLNC) = ALS against sensitized lymph node cells.

[6] ALS(SPBL) = ALS against sensitized peripheral blood lymphocytes.

[7] ALS(STDL) = ALS against sensitized thoracic duct lymphocytes.

six days after culture, the thoracic duct lymphocytes retained 65 to 70% viability and the lymph node cells were consistently below 60%.

The results of the opsonic index test are shown in Table 5. Similarly, it was observed that unadsorbed sera showed similar titers when titered against cells from untreated and allograft sensitized animals. The titers in this case ranged from 8 to 1024. In the case of the adsorbed sera surprisingly not much difference was noted with either cells from untreated or allograft sensitized animals. The titers ranged from 0 to 8 in the case of cells from untreated animals and 0 to 16 in the case of cells from allograft sensitized animals. This test was difficult to perform. It required 6 to 7 cover slip preparations of macrophages to get 1 or 2 that had the required number of macrophages attached to it which could be subsequently used. Another big source of error was the inability to determine whether the lymphocytes were attached to the macrophage or lying below it or intracellular. It was found that a lymphocyte/macrophage ratio of 3/1 corresponded with 50% opsonization. The titer was taken as the reciprocal of the highest dilution of ALS giving these values.

The hemagglutination and precipitin titers of the various unadsorbed and adsorbed antilymphocytic sera are shown in Table 6. ALS prepared against peripheral blood lymphocytes gave the highest hemagglutination titers whereas ALS prepared against thoracic duct lymphocytes gave the lowest hemagglutination titers. This was due to the

TABLE 5. Comparison of Opsonic Indices of Unadsorbed and Adsorbed Antilymphocytic Sera.

	Sera	Unadsorbed Antigen		Adsorbed Antigen	
		N[1] cells	S[2] cells	N cells	cells
1.	ALS(NTDL)[3]	ND[4]	ND	ND	ND
2.	ALS(NPBL)[5]	ND	ND	ND	ND
3.	ALS(SLNC)[6]	16	32	0	2
4.	ALS(SLNC)	8	32	0	0
5.	ALS(SLNC)	32	128	2	2
6.	ALS(SPBL)[7]	128	256	4	4
7.	ALS(SPBL)	128	512	8	8
8.	ALS(SPBL)	256	256	8	16
9.	ALS(SPBL)	256	512	4	16
10.	ALS(SPBL)	512	1024	8	8
11.	ALS(STDL)[8]	64	256	4	8
12.	ALS(STDL)	64	128	4	8
13.	ALS(STDL)	64	128	8	16
14.	ALS(STDL)	128	256	2	8
15.	ALS(STDL)	ND	ND	ND	ND
16.	ALS(STDL)	ND	ND	ND	ND

[1] N cells = Lymphocytes from untreated animals.

[2] S cells = Lymphocytes from allograft sensitized animals.

[3] ALS(NTDL) = ALS against normal thoracic duct lymphocytes.

[4] ND = Not Done.

[5] ALS(NPBL) = ALS against normal peripheral blood lymphocytes.

[6] ALS(SLNC) = ALS against sensitized lymph node cells.

[7] ALS(SPBL) = ALS against sensitized peripheral blood lymphocytes.

[8] ALS(STDL) = ALS against sensitized thoracic duct lymphocytes.

TABLE 6. Hemagglutination and Precipitin Titers of Antilymphocytic Sera.

	Sera	Hemagglutination		Precipitin	
		Unadsorbed	Adsorbed	Unadsorbed	Adsorbed
1.	ALS(NTDL)[1]	4	0	100	2
2.	ALS(NPBL)[2]	64	4	32	4
3.	ALS(SLNC)[3]	16	2	16	0
4.	ALS(SLNC)	16	2	16	0
5.	ALS(SLNC)	32	4	8	0
6.	ALS(SPBL)[4]	64	2	32	0
7.	ALS(SPBL)	128	4	16	0
8.	ALS(SPBL)	64	4	32	2
9.	ALS(SPBL)	32	2	64	2
10.	ALS(SPBL)	64	2	32	0
11.	ALS(STDL)[5]	8	0	100	4
12.	ALS(STDL)	4	2	64	2
13.	ALS(STDL)	8	0	64	0
14.	ALS(STDL)	4	0	100	4
15.	ALS(STDL)	8	0	64	0
16.	ALS(STDL)	8	2	64	2

[1] ALS(NTDL) = Antilymphocytic serum prepared against thoracic duct lymphocytes from untreated animals.

[2] ALS(NPBL) = Antilymphocytic serum prepared against peripheral blood lymphocytes from untreated animals.

[3] ALS(SLNC) = Antilymphocytic serum prepared against lymph node cells from allograft sensitized animals.

[4] ALS(SPBL) = Antilymphocytic serum prepared against peripheral blood lymphocytes from allograft sensitized animals.

[5] ALS(STDL) = Antilymphocytic serum prepared against thoracic duct lymphocytes from allograft sensitized animals.

contamination of red blood cells in the immunizing dose of peripheral blood lymphocytes. Most hemagglutination activity was adsorbed out after incubation of ALS with washed erythrocytes from untreated guinea pigs. ALS prepared against thoracic duct lymphocytes gave the highest precipitin titers against guinea pig serum proteins. Most of the precipitins were also adsorbed out by incubation of ALS with guinea pig red blood cells.

Immunofluorescence studies showed fixing of fluorescein tagged unadsorbed gamma globulin to the surface of lymphocytes regardless of the source of the lymphocytes. Fluorescein tagged adsorbed ALS gamma globulin fixed very poorly to lymphocytes from either allograft sensitized or untreated animals.

Analysis of the disc electrophoresis data merely showed quantitative differences in the amount of gamma globulin in adsorbed and unadsorbed antilymphocytic sera.

In Vivo Characterization of Antilymphocytic Sera

Lymphopenia studies were carried out with both unadsorbed and absorbed ALS. Unadsorbed ALS consistently showed a sharp drop in the peripheral leukocyte count 1 hour after administration. This drop in leukocyte count reached a maximum depression at about 3 hours and subsequently stabilized at about 10 to 17% below the original values. This occurred about 5 to 10 hours after the first injection of

ALS. Groups of guinea pigs were injected intraperitoneally with 1 ml of either unadsorbed or adsorbed ALS. Blood samples were taken hourly for a 24 hour period. Figure 1 shows the average leukocyte counts of such a study.

Spleen cells from guinea pigs immunized with sheep red blood cells when cultured in vitro showed hemolytic activity upon addition of fresh guinea pig complement. This system was used to study the effect of ALS on these cultured cells. Prior to the addition of guinea pig serum, appropriate dilutions of unadsorbed and adsorbed ALS were added to triplicate cultures of these spleen cells. Normal rabbit serum and saline were used as controls. Unadsorbed ALS consistently showed O.D. (541 nm) values of less than 0.3 whereas adsorbed ALS, normal rabbit serum and saline controls showed values of O.D. (541 nm) 0.5 or better.

Repeated injections of unadsorbed ALS according to the routine schedule showed that it took longer to reach a stable leukocyte count. After day 9 there was a net decrease of about 25% which remained stable for 48 hours. After this period the leukocyte counts would start returning back to original values before ALS administration. The normal leukocyte count was reached in all cases by day 25. This was found with all unadsorbed sera tested.

Repeated injections of adsorbed ALS showed a completely different blood picture. No marked change was observed throughout the

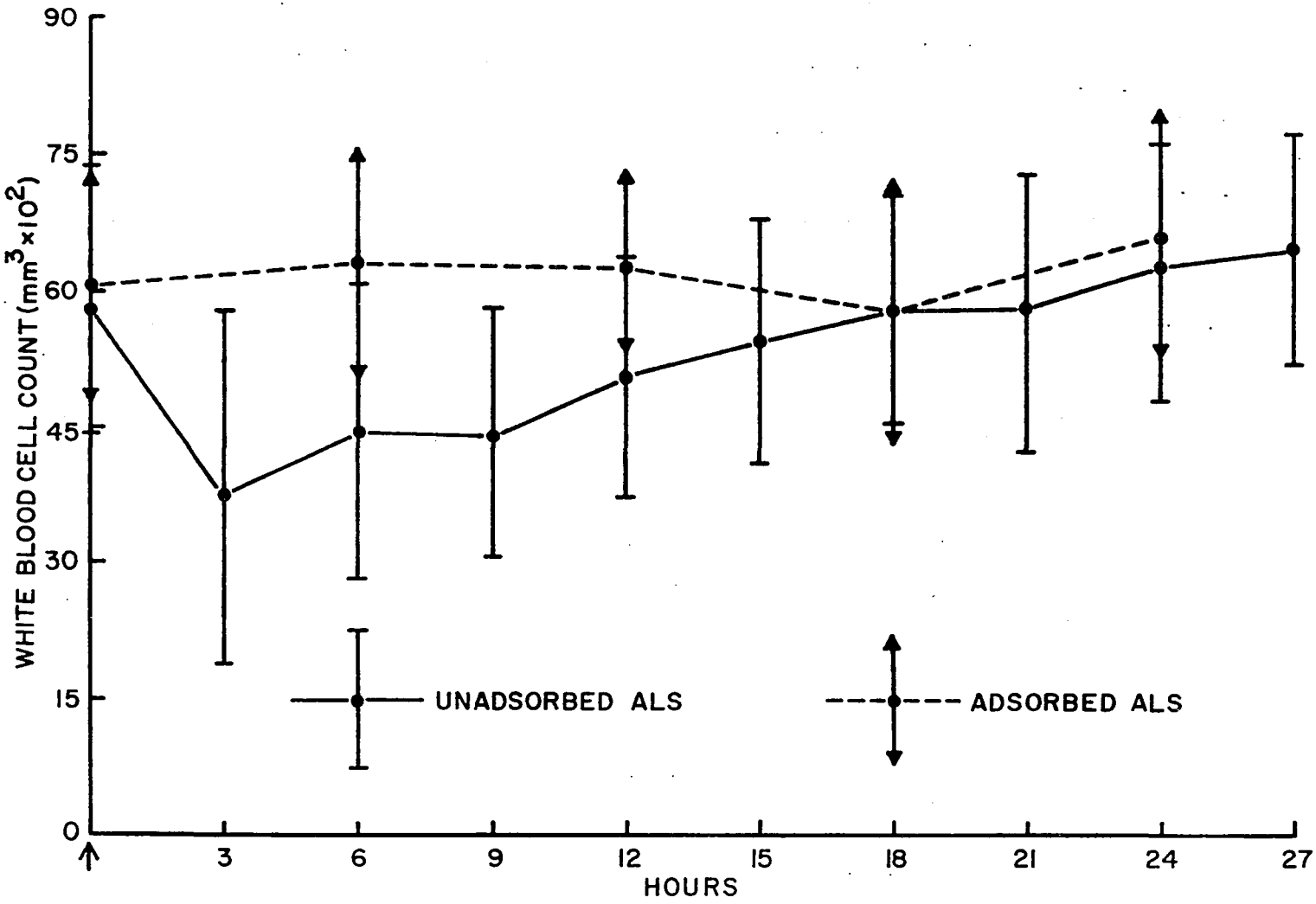

Fig. 1. Twenty-four Hour Blood Leukocyte Counts of Guinea Pigs Receiving a Single Injection of Unadsorbed and Adsorbed Antilymphocytic Serum.

course of adsorbed ALS administration. Figure 2 demonstrates the blood leukocyte counts of guinea pigs receiving unadsorbed and adsorbed ALS according to the routine schedule.

Table 7 shows the average and net survival times of Rockefeller strain grafts on guinea pigs injected with unadsorbed and adsorbed ALS. The data represent the average values for 6 guinea pigs. All unadsorbed ALS showed a net prolongation of skin graft survival. This ranged from 3 to 13 days except in the case of ALS #7. ALS #7 was prepared against allograft sensitized peripheral blood lymphocytes. This serum had very high in vitro activity but showed no net prolongation of skin graft survival. It demonstrated that in this case in vitro activity as measured had little or no significance as to its in vivo activity. Unadsorbed ALS to allograft sensitized lymph node cells showed net prolongation of skin grafts from 2 to 6 days beyond the normal rejection time whereas after adsorption of these sera with lymph node cells from untreated animals the net survival time was increased to between 5 to 9 days. Unadsorbed ALS to allograft sensitized peripheral blood lymphocytes showed a net prolongation from 5 to 11 days (except serum #7) whereas after adsorption the net survival time increased from 10 days to indefinite survival in the case of serum #6. Serum #7 which showed no net prolongation in the unadsorbed state showed a dramatic increase to 10 days net prolongation after adsorption. Unadsorbed ALS to allograft sensitized thoracic duct lymphocytes showed

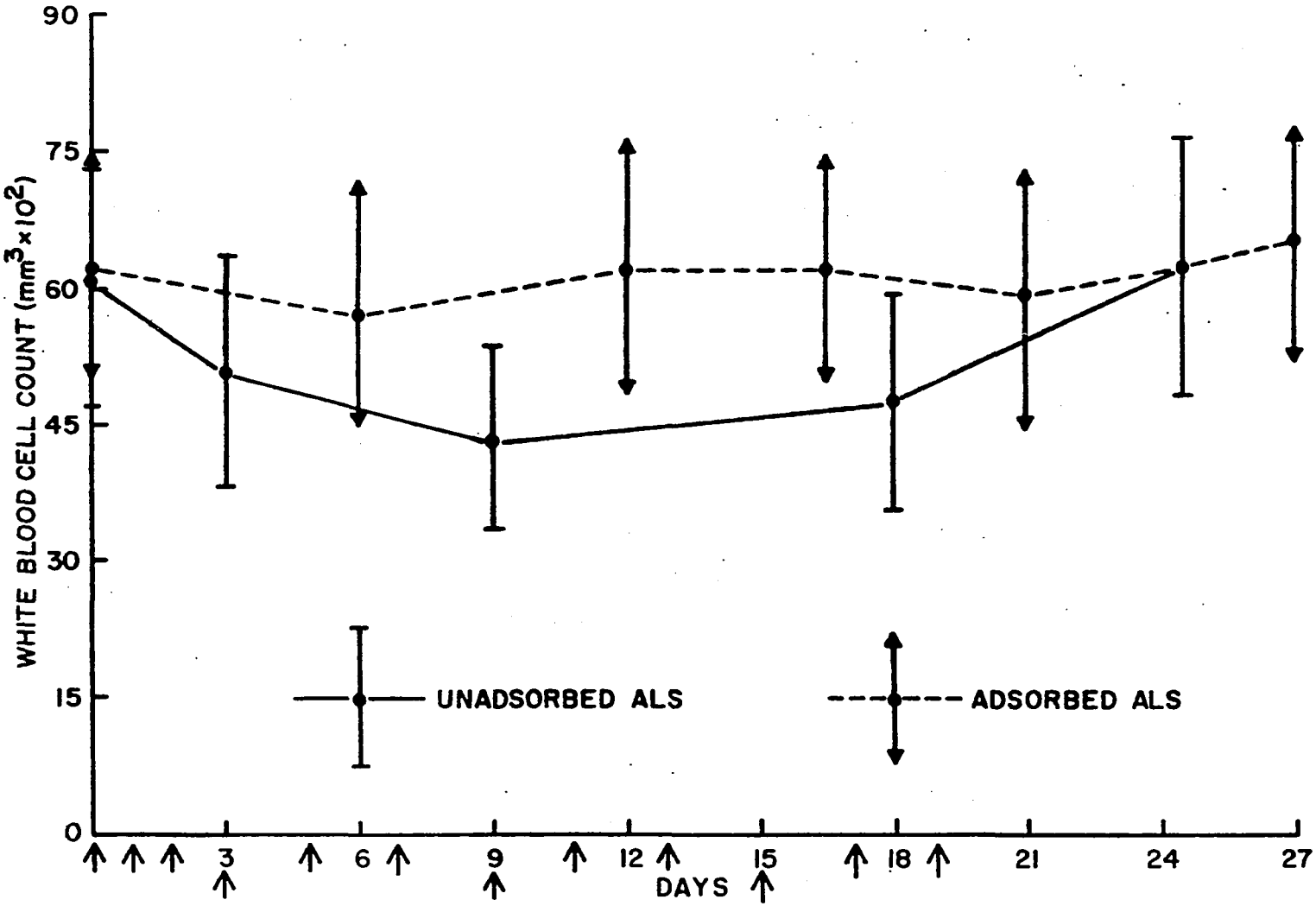

Fig. 2. Blood Leukocyte Counts of Guinea Pigs Receiving Unadsorbed and Adsorbed Antilymphocytic Serum on the Regular Schedule.

TABLE 7. Skin Graft Survival After Administration of Unadsorbed and Adsorbed Antilymphocytic Sera.

		Unadsorbed Sera			Adsorbed Sera		
		Average Total Graft Survival (Days)	Net Graft Survival (Days)	Range Net Survival (Days)	Average Total Graft Survival (Days)	Net Graft Survival (Days)	Range Net Survival (Days)
1.	ALS(NTDL)[1]	15	5	4--6	10	0	0
2.	ALS(NPBL)[2]	13	3	2--5	9	0	0
3.	ALS(SLNC)[3]	15	5	4--6	19	9	7--12
4.	ALS(SLNC)	16	6	4--9	18	8	7--11
5.	ALS(SLNC)	12	2	1--4	15	5	4--7
6.	ALS(SPBL)[4]	20	10	7--12	I.S.[5]	--	-----
7.	ALS(SPBL)	10	0	0	20	10	9--11
8.	ALS(SPBL)	15	5	4--6	26	16	15--17
9.	ALS(SPBL)	21	11	10--12	28	18	17--19
10.	ALS(SPBL)	20	10	8--12	27	17	16--18
11.	ALS(STDL)[6]	16	6	5--8	27	17	16--20
12.	ALS(STDL)	18	8	7--10	24	14	13--17
13.	ALS(STDL)	20	10	8--13	I.S.	--	------
14.	ALS(STDL)	20	10	9--11	I.S.	--	------
15.	ALS(STDL)	15	5	4--7	19	9	8--11
16.	ALS(STDL)	23	13	12--14	I.S.	--	------
17.	Normal rabbit serum	12	2	0--3	11	1	0--2
18.	Untreated	10	0	0	0	0	0

[1]ALS(NTDL) = ALS against thoracic duct lymphocytes from untreated animals.
[2]ALS(NPBL) = ALS against peripheral blood lymphocytes from untreated animals.
[3]ALS(SLNC) = ALS against lymph node cells from allograft sensitized animals.
[4]ALS(SPBL) = ALS against peripheral blood lymphocytes from allograft sensitized animals.
[5]I.S. = Indefinite survival to date.
[6]ALS(STDL) = ALS against thoracic duct lymphocytes from allograft sensitized animals.

net survival time between 5 and 13 days whereas after adsorption the net survival time increased from a minimum of 9 days to indefinite survival in the case of 3 out of the 6 sera made against thoracic duct lymphocytes. Unadsorbed ALS prepared against lymphocytes from untreated animals showed net prolongation of 3 to 5 days but adsorption of these sera with lymphocytes from untreated animals removed the graft prolonging activity of these sera. Normal rabbit sera also showed some graft prolonging activity.

The immunosuppresive activity was found in the 7 S gamma globulin fraction of both unadsorbed and adsorbed ALS. Guinea pigs were injected with this 7 S gamma globulin fraction of adsorbed and unadsorbed ALS according to the routine schedule and showed similar net survival times. In each case the protein concentration was adjusted to 10 mg/ml. Table 8 demonstrates the graft survival time of guinea pigs injected with 7 S gamma globulin fraction of the few ALS tested.

Loss of weight in guinea pigs receiving unadsorbed ALS and its 7 S gamma globulin fraction was consistently observed though no such weight loss was seen in guinea pigs receiving adsorbed ALS or the 7 S fraction of adsorbed ALS.

Data here suggested that the best source of immunosuppressive antilymphocytic sera is thoracic duct lymphocytes but similar results can be obtained with peripheral blood lymphocytes depending perhaps

TABLE 8. Skin Graft Survival After Administration of 7S* Gamma Globulin Fraction of Unadsorbed and Adsorbed Antilymphocytic Sera.

		7S Gamma Globulin (Unadsorbed Sera)			7S Gamma Globulin (Adsorbed Sera)		
		Average Total Graft Survival (Days)	Net Graft Survival (Days)	Range Net Survival (Days)	Average Total Graft Survival (Days)	Net Graft Survival (Days)	Range Net Survival (Days)
1.	ALS(NTDL)[1]	17	7	5--9	10	0	0
2.	ALS(NPBL)[2]	15	5	4--6	10	0	0
3.	ALS(SLNC)[3]	15	5	4--6	18	8	7--9
4.	ALS(NLNC)[4]	14	4	3--5	10	0	0
5.	ALS(SPBL)[5]	15	5	4--6	24	14	12--16
6.	ALS(STDL)[6]	20	10	9--11	I.S.[8]	--	------
7.	NRS[7]	10	0	0	10	0	0

*7S Gamma blobulin separated and purified and adjusted to 10 mg/ml protein concentrate and administered according to regular schedule.

[1]ALS(NTDL) = ALS against thoracic duct cells from untreated animals.

[2]ALS(NPBL) = ALS against peripheral blood lymphocytes from untreated animals.

[3]ALS(SLNC) = ALS against lymph node cells from allografted sensitized animals.

[4]ALS(NLNC) = ALS against lymph node cells from untreated animals.

[5]ALS(SPBL) = ALS against peripheral blood lymphocytes from allograft sensitized animals.

[6]ALS(STDL) = ALS against thoracic duct lymphocytes from allografted sensitized animals.

[7]NRS = Normal rabbit serum.

[8]I.S. = Indefinite survival.

on the purity of the immunizing dose. Lymph node cells did not seem to be a good source of antigen for good immunosuppressive sera production, suggesting some differences in the antigen makeup and constituency of different lymphoid cells.

It was also observed that if a group of animals receiving one particular ALS were chosen such that their body weight varied from 450 to 1000 gms, the net survival time of grafts applied to them from a single donor, varied extremely. The larger animals would show survival times much less than those shown by the smaller animals. Therefore after this initial observation all animals receiving ALS were chosen such that their body weight had minimal variation (550 to 670 gms).

Table 9 shows the response of allografted guinea pigs to sheep red blood cells and BSA while undergoing ALS treatment. Though not enough data were collected, it is evident that unadsorbed ALS inhibited the primary immune response of allografted guinea pigs to sheep red blood cells whereas adsorbed ALS and normal rabbit serum had very little effect. The mean titer of animals receiving adsorbed ALS was 64 whereas those receiving unadsorbed ALS and normal rabbit serum was 4, significant change.

In the case of the primary immune response of allografted guinea pigs to BSA some recipients of adsorbed ALS, normal rabbit serum and controls showed no precipitin activity. Thus it is shown

TABLE 9. Effect of Antilymphocytic Serum Treatment on the Primary Immune Response of Guinea Pigs.

Immunizing Agent	Antigen					
	Titers with Sheep Red Blood Cells			Titers with Bovine Serum Albumin		
	Mean	Range	Distribution	Mean	Range	Distribution
1. Unadsorbed ALS	4	0-8	0	2	0-4	0
			8			0
			4			0
			4			4
			2			0
			6			8
2. Adsorbed ALS	64	16-128	100	80	0-128	0
			32			80
			64			100
			64			100
			128			80
			16			128
3. Normal rabbit serum	64	16-128	100	100	0-256	0
			32			0
			80			128
			16			256
			32			128
			128			80
4. Untreated control	128	64-256	64	100	0-256	0
			128			64
			256			128
			128			64
			128			32
			128			256

again that guinea pigs are poor precipitin producers. The mean titer of animals receiving unadsorbed ALS was 2 as compared to 80 shown by animals receiving adsorbed ALS, 100 shown by animals receiving normal rabbit serum and 100 shown by untreated control animals. This does point out the depression of the primary immune response of animals receiving unadsorbed ALS.

Depletion of In Vivo Complement Activity

Complement activity was shown to be depressed throughout the course of unadsorbed ALS administration but returned to normal levels one week after treatment was stopped. In the case of adsorbed ALS, normal rabbit serum and controls no appreciable change in complement activity was observed throughout the course of administration of these sera. The normal values of the recipient guinea pigs ranged between 300 to 350 units of complement (CH_{100}) in 1 ml of guinea pig serum before administration of ALS. After administration of unadsorbed ALS the values ranged between 250 and 300 units of complement in 1 ml of recipient guinea pig serum. After the administration of adsorbed ALS the values were between 290 and 350, not significantly different from the normal values. Values were determined as an average of 6 guinea pigs for each serum tested.

The adsorbed ALS were subsequently tested for their specificity in the prolongation of allograft skin survival. Table 10 shows the

TABLE 10. Specificity of Adsorbed Antilymphocytic Sera in Graft Survival.

	Sera	Day of Rejection of Skin Graft			
		Original Rock Graft	Indifferent Rock Graft	Indifferent Amana Graft	Hartley Graft
1.	ALS(NTDL)[1]	10	10	11	9
2.	ALS(NPBL)[2]	11	10	10	10
3.	ALS(SLNC)[3]	20	21	11	10
4.	ALS(SLNC)	18	18	11	9
5.	ALS(SLNC)	15	15	10	10
6.	ALS(SPBL)[4]	I.S.[5]	I.S.	9	9
7.	ALS(SPBL)	23	22	10	10
8.	ALS(SPBL)	26	27	11	9
9.	ALS(SPBL)	27	27	10	10
10.	ALS(SPBL)	21	20	9	10
11.	ALS(STDL)[6]	28	28	10	10
12.	ALS(STDL)	25	24	11	10
13.	ALS(STDL)	I.S.	I.S.	10	9
14.	ALS(STDL)	I.S.	I.S.	10	10
15.	ALS(STDL)	27	27	10	9
16.	ALS(STDL)	I.S.	I.S.	10	10
17.	NRS[7]	12	11	12	12
18.	Untreated control	10	10	9	10

[1] ALS(NTDL) = ALS against thoracic duct lymphocytes from untreated animals.
[2] ALS(NPBL) = ALS against peripheral blood lymphocytes from untreated animals.
[3] ALS(SLNC) = ALS against lymph node cells from allograft sensitized animals.
[4] ALS(SPBL) = ALS against peripheral blood lymphocytes from allograft sensitized animals.
[5] I.S. = Indefinite survival to date.
[6] ALS(STDL) = ALS against thoracic duct lymphocytes from allograft sensitized animals.
[7] NRS = Normal rabbit serum.

survival time of grafts from the original Rockefeller strain donor, indifferent Rockefeller strain donor, an indifferent Amana strain donor, and a Hartley strain donor. Adsorbed ALS showed significant prolongation of skin grafts from both the original and indifferent Rockefeller strain donor, but did not show any prolongation of graft survival in the case of an indifferent Amana donor or the Hartley donor. Net survival times of the Rockefeller strain grafts were very much similar to the ones reported in Table 7. Adsorbed ALS was shown therefore to be strain specific for Rockefeller strain guinea pig skin and not for Amana or Hartley strain guinea pig skin. Normal rabbit serum again showed some net prolongation.

Unadsorbed ALS did not have any significant effect on the second set response of guinea pigs, in some cases it even decreased the rejection time by a day or two. Adsorbed ALS had no effect at all on the second set response of the doses administered.

Adsorbed ALS showed no inhibition of the response of guinea pigs sensitized to 2, 4-dinitrofluorobenzene. This further indicated the specificity of the adsorbed sera in terms of its in vivo effect of only affecting cell mediated immune responses of the recipients of Rockefeller allografts.

CHAPTER 4

DISCUSSION

These data show that heterologous antilymphocytic serum can be made highly specific towards the target cells which are essentially responsible for graft rejection. The nature of the target antigen at present is a basis of speculation. Perhaps it is pertinent to quote Dr. Macleod (101) who stated, "It is unlikely that heterologous ALS is a definitive answer to the problem of transplant rejection. It is a shot gun approach where a rifle is needed. The bullet should be directed against that fraction of lymphocytes that have become sensitized to these antigens and should not effect the non sensitized cells."

The various in vitro data suggest that there is stronger in vitro activity of adsorbed ALS with homologous sensitized lymphocytes than with lymphocytes from untreated animals. This implies that the sensitized lymphocytic population of cells is in some way different from that of untreated animals, and that this difference can be recognized by specific antibodies present in adsorbed ALS. The effective differences must be located at the surface of the lymphocytes because most of the in vitro data deal with surface properties of the cells. The sensitivity elements must be antigenic in nature so that they can

give rise to specific antibodies against themselves, and present in sufficient quantity. These can be postulated to be transplantation antigens, specific transfer factor or both.

The various in vitro tests performed failed to correlate with the in vivo immunosuppressive properties of both unadsorbed and adsorbed ALS. It has been stated by Bach, Dardenne, and Fournnier (102) that opsonic index is the best in vitro correlate of the immunosuppressive properties of ALS. This was not found to be true with the various sera tested during the course of this investigation.

One of the sera (sera #7) prepared against peripheral blood lymphocytes had high in vitro activity but very poor immunosuppressive properties in terms of graft survival prolongation. After adsorption, there was a considerable increase in the in vivo immunosuppressive properties. This suggests that unadsorbed ALS has a complex of antibodies, some of which might interfere at times with the immunosuppressive properties of ALS. These antibodies could be non specific in the sense that they could be directed against lymphocytes which are not sensitized, other cells, guinea pig serum, proteins, etc.

ALS produced only transient lymphopenia in recipients and this confirmed the results of Levey and Medawar (35). Adsorbed ALS did not appreciably depress the blood leukocyte counts and this could be due to the removal of the cytotoxic properties of unadsorbed ALS directed against lymphocytes.

The indefinite survival of Rockefeller allografts by the use of adsorbed ALS was surprising. It may be that tolerance is produced, or that there is complete and permanent neutralization of the transplantation antigens. It is possible that the specific ALS may inactivate among the total lymphocyte population the particular few cells which bear transfer factor directed against the specific donor's antigens and thereby may intercept their interaction with the target cells in the graft. This interpretation, if correct, suggests that an antibody to neutralize transfer factor is a more highly selective approach to the problem of transplantation.

Levey and Medawar (103, 104) describe a property of ALS as it produces "erasure of memory" in homograft systems. The work of Monaco, Wood, and Russell (105) has established the fact that the injection of ALS weakens the "second set" response through which an animal rapidly and violently rejects a second homograft when it has had prior experience of a first. Erasure of immunologic memory is, however, more than the weakening of this pre-existing sensitivity. It means that ALS has the power to restore an animal to a state of virgin reactivity, a state in which it behaves as if it had never met with or reacted against homograft antigens before. Obviously, this property of ALS has considerable importance for such a clinical application as the treatment of autoimmune diseases. If autoimmune diseases represent a reaction to self constituents which behave as if

they were foreign antigens, then any agent capable of abolishing recognition of endogenous antigens could provide the basis for cure instead of treatment, that is merely symptomatic or palliative.

The mechanism of action of ALS has been the object of great debate. The present generally accepted concept of the action of ALS was first formulated by Lance in 1967 (106). The concept was that ALS acts primarily on lymphocytes belonging to the long lived thymus dependent, recirculating pool of small lymphocytes, that is on the cell population generally considered to be responsible for homograft rejection and other cell mediated immunities. There is now direct evidence that the lymphocyte population of animals chronically exposed to ALS is essentially short lived, the long lived cells being depleted or eliminated (107).

In the testing of the primary immune response of ALS recipients to sheep red blood cells and BSA, inconclusive results were obtained, especially the response of ALS recipients to BSA. This was a poor choice of antigen to study the immune response in the guinea pig and a more effective antigen would probably be a bacterial antigen. Further investigation is therefore necessary in order to determine the efficacy of ALS treatment on the immune response of ALS recipients.

It is evident from the data that a specific antilymphocytic serum directed against the socalled "sensitized" lymphocytes, their membrane associated antigens or their products can be produced. This

adsorbed ALS has remarkable immunosuppressive properties without any discernible effect on both humoral and cell mediated immune responses. This is an ideal answer to long term graft survival, for, though unadsorbed ALS as shown by Lance and Medawar (108) if given in proper low doses has marked in vivo immunosuppressive properties but insignificant effect on the humoral response of recipients, it still depresses the other essential cell mediated immune responses of the recipients. Whereas the adsorbed ALS prepared during the course of this investigation has the property of abrogating the specific immune response of the recipient against allograft sensitized cells towards which it is directed. It leaves other functions of the recipients immune mechanism relatively unaltered.

If it is not too vivacious and gregarious, I would like to quote a surgeon who had the following to say about American and British surgical research: "Transplantation research is the seductive blonde of surgical research but from the point of view of survival of our societies and our species, the study of social problems and their solutions are far more important. The latter may be more important at the moment but I am not sure the people concerned with transplantation are in the best position to be helpful. We do not know yet how to transplant a kind heart into an angry rebel, for example."

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