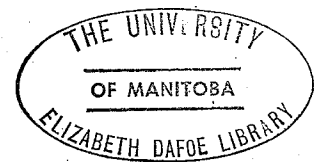


ON THE MECHANISM OF ACTION OF ANTILYMPHOCYTIC SERUM (ALS):
As studied by the Reverse Immune Cytoadherence Technique (RICA)

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ABSTRACT

Ellen Diane Anderson

ON THE MECHANISM OF ACTION OF ANTILYMPHOCYTIC SERUM (ALS):

As studied by the Reverse Immune Cytoadherence Technique (RICA)

The Reverse Immune Cytoadherence Technique is used to study the effect of ALS in vitro on normal mouse spleen cells. This technique demonstrates the presence of γ -globulin-like receptors on the surface of a proportion of these spleen cells as indicated by the formation of rosettes. Treatment of the spleen cells with ALS results in the inhibition of this rosette formation. Inhibition occurs over a wide range of dilutions of ALS and is maximal at a certain dilution. It is totally lacking in undiluted ALS and in highly diluted ALS.

ALS serum is fractionated into its 7S and 19S components and the inhibitory effect of each one, or of various combinations of the two, is studied. 7S or 19S alone inhibit only at high concentrations with maximal inhibition occurring when the undiluted fraction is used. When 7S and 19S are reconstituted in serum proportions, inhibition is maximal with undiluted 7S plus 19S and loss of inhibition occurs only in highly diluted samples. Varying the proportions in which 7S and 19S are recombined indicates that there is a very narrow range of proportions over which inhibition can occur and that maximal inhibition occurs at serum proportions.

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LITERATURE REVIEW AND INTRODUCTION

Since the immunosuppressive nature of Antilymphocytic serum was discovered, intensive investigations have been carried out on ALS in an attempt to (1) discover the mechanism of action of ALS, (2) develop an in vitro assay system for ALS which would correlate well with its immunosuppressive capacity in vivo, and thus facilitate the clinical usage of ALS, and (3) shed further light on the mechanism of the immune responses using ALS as an experimental tool. The failure so far to devise an in vitro test that measures the activity of ALS in vivo is the main impediment to current progress in the investigation and use of ALS.

The in vivo treatment of mice with ALS has been found to prevent or delay the development of cell-mediated immune responses such as homograft rejection, delayed hypersensitivity reactions, and graft versus host reactions (1, 2). This occurs with both first and second set reactions and it is this second set immunosuppression that distinguishes ALS from all other immunosuppressive agents so far studied. It has also been found that such treatment is capable of suppressing the primary humoral response to certain antigens although the secondary humoral response can be depressed only slightly (1, 2). In general, there is much greater efficacy at suppressing the cellular responses, both primary and secondary, than the humoral responses.

ALS has been found to have an effect localized to the periarteriolar areas of the spleen, and the paracortical areas of the lymph nodes (3, 4,

5, 6). These areas become depleted of their small lymphocytes, and replaced by reticulohistiocytes. The depleted regions are those which consist of the thymus-dependent pool of long-lived small lymphocytes, and which have previously been shown to be the sites of lymphocyte proliferation during cell-mediated immune responses. These findings thus explain the ability of ALS to suppress the cellular immune responses. The hypothesis has been made that ALS may act firstly and selectively by depleting the recirculating pool of long-lived, small lymphocytes present in the peripheral circulation, and acts on the lymphoid organs only indirectly, when they release lymphocytes into the circulation (4, 7). This would further explain why ALS so effectively suppresses reactions transacted by peripheral lymphocytes, i.e. the cellular immune responses.

Experimental evidence has shown that lymphocytes which are relatively sessile will be protected, because ALS penetrates lymphoid organs poorly, if at all (4, 8, 9). Using Cr51-labelled lymphocytes, it has been shown that once cells have homed to the lymphoid organs they become relatively resistant to ALS. On the other hand, recent evidence indicates that ALS does not act solely on the long-lived, recirculating small lymphocytes. It can also act on the short-lived, recirculating small lymphocytes while they are present in the circulation (8, 9). A persisting depletion of the short-lived cells is not observed in the circulation, and is probably the result of the relative rapidity with which new short-lived small lymphocytes can be produced to replace depleted ones. In fact, it has been noted that the amounts of short-lived small lymphocytes in the circulation may actually increase during

ALS treatment, suggesting that short-lived small lymphocytes are possibly being produced to offset the effect of the depletion of the long-lived variety.

The persistence of the recirculating pool of short-lived small lymphocytes during ALS treatment can explain the histological findings that the small lymphocytes of the lymph and splenic follicles, and of their germinal centers, remain unaffected by ALS (4, 5, 6). These lymphocytes are of the short-lived variety. It is these short-lived small lymphocytes which are believed to be derived from the bone marrow without influence by the thymus, and which are involved in the humoral immune responses (10). Thus the relative inefficiency of ALS in suppressing humoral immune responses can be explained (4, 11). It is a result of the inability of the ALS to influence and destroy the short-lived, small lymphocytes of the lymphoid organs. This is further evidenced by experiments in which unsensitized or sensitized cells from lymphoid organs were treated with ALS in vitro and subsequently injected into irradiated, allogeneic hosts (11). The result was a decrease in the humoral responsiveness of these irradiated hosts, as compared to hosts given cells treated with NRS or untreated. Both primary and secondary humoral responses were depressed. Thus, when ALS was able to gain access to the lymphocytes of the lymphoid organs, immunosuppression was able to occur.

It has been further found that the antigens whose humoral antibody responses can be depressed by in vivo administration of ALS are those which require the synergistic proliferation of both thymus-

derived, antigen-reactive lymphocytes and bone marrow-derived, antibody-producing lymphocytes for their antibody production (12, 13). ALS suppresses by acting primarily on the thymus-derived cells, i.e. on the more readily depleted lymphocytes, without affecting the bone marrow-derived cells. Thus, injection of thymus cells can restore to normal the ability of ALS-treated animals to respond to antigen.

A number of mechanisms have been postulated to explain the activity of ALS. The defining property of 'activity' is the power to prolong the life of skin homografts. One of the earliest postulates described is that of cytotoxicity, which states that the level of immunosuppression by ALS is entirely dependent on the degree of lymphoid depletion produced by the ALS. Antisera capable of prolonging skin homografts survival are also capable of producing lymphopenia, after a brief course of antiserum, which is sustained for some days (14). A refinement of this hypothesis suggests that the mode of action of ALS is by the destruction of a critical subpopulation of lymphocytes (15). The primary action of ALS involves a rapid and drastic depletion of the long-lived, recirculating pool of small lymphocytes from the circulation and from the lymphoid organs (14, 15).

However, it has been found that cytotoxicity titers, in vitro, do not regularly correlate with the effect of ALS on graft survival in vivo. Although cytotoxicity appears to be one factor involved, it is not sufficient. Although all active antisera are cytotoxic, not all cytotoxic antisera are active (16). Cytotoxicity titers also indicate that only at high concentrations of the ALS will cytotoxicity be a factor, and that, as the concentration of ALS in the circulation

falls with time, it will cease to be the predominant mechanism.

Another postulated mechanism of action of ALS is that of blindfolding. It holds that lymphocytes become coated with ALS antibody, thus rendering them immunologically ineffective. The recognition of antigen is prevented. Immunofluorescent procedures have shown that lymphocytes exposed to ALS in vitro do become coated with antibody. However, it has also been shown that the descendants of affected lymphocytes remain unreactive for several generations and thus blindfolding does not appear to be the entire story.

A further postulate is that ALS acts by causing a sterile activation of lymphoid cells (17). The cells undergo blast transformation and cell division, without accompanying specific immunological performance. This hypothesis is supported by the known ability of ALS to stimulate lymphocytes to transformation and thymidine uptake in vitro. However, $F(ab')_2$ of ALS-IgG can also stimulate lymphocytes to transformation and thymidine incorporation in vitro, and it is not immunosuppressive in vivo (18). Thus, sterile activation also does not appear to be the entire story.

Recently, it has been postulated that ALS acts as an opsonin (19). The ALS antibody opsonizes a proportion of the lymphocytes, rendering them highly susceptible to phagocytosis by macrophages of the liver. Those lymphocytes that are not opsonized are free to localize in the lymphoid organs.

Numerous in vitro assays have been developed in an attempt to discover one which will correlate well with in vivo immunosuppression.

The first ones developed, hemagglutination, lymphoagglutination, and cytotoxicity titers, have all been shown to not accurately reflect in vivo immunosuppression.

An in vitro assay has been developed that measures the stimulation of thymidine incorporation by lymphocytes incubated in vitro with ALS. It has been found that the relationship between dosage of ALS and stimulation is not a simple one. The greatest stimulation is obtained with small quantities of ALS and inhibition of this stimulatory effect is obtained as ALS levels increase above and decrease below this level (20). Further evidence is required, however, to show the extent to which this stimulation will correlate with in vivo immunosuppression.

A recently developed assay measures the ability of Cr51-labelled lymphocytes treated with ALS in vitro to home to the lymphoid organs, upon injection into normal, allogeneic host, i.e. it measures the opsonizing effect of ALS (9, 21). The localization ratio, i.e. the ratio of liver to spleen uptake of radioactivity, at a 1:1000 dilution of ALS is determined. It has been found that a significant correlation exists between in vivo skin graft prolongation and the effect of 1:1000 ALS on cell localization. The greater the immunosuppressive capacity, the greater the shift of cells into the liver away from the spleen.

Another in vitro assay for ALS involves the determination of the inhibition of spontaneous rosette formation by lymphocytes treated with ALS (22, 23). Cells from unimmunized mice can form rosettes when mixed with sheep red blood cells. ALS, upon incubation with these mouse lymphocytes, inhibits this spontaneous rosette formation. The exact mechanism of inhibition is unknown. However, a good correlation

exists between the rosette inhibition titer, i.e. the highest dilution of ALS at which inhibition still occurs, and the effect of ALS on skin graft prolongation in vivo.

By employing the Reverse Immune Cytoadherence Technique (RICA), the action of ALS on lymphocytes in vitro is further studied. The RICA technique detects the presence of γ -globulin-like receptors on the surface of normal mouse lymphoid cells. A hybrid antibody is used which consists of one anti-mouse γ -globulin site and one anti-ferritin site. Ferritin-coated sheep red blood cells can then form rosettes around lymphocytes carrying such γ -globulin receptors via this hybrid antibody bridge. Treatment of mouse lymphoid cells with ALS results in an inhibition of this rosette formation. The maximal dilution giving an inhibition of rosette formation is extremely high for the ALS tested. It is suggested that this inhibition of rosette formation may reflect, in vitro, the true mechanism of action of ALS in vivo. ALS may act by blocking the γ -globulin-like receptors which, according to accepted dogma, represent the antigen receptors of immunologically competent cells. Thus, the cells are rendered incompetent. This being the case, it would also be a reliable in vitro assay of the immunosuppressive capacity of ALS antiserum.

MATERIALS AND METHODS

I. Immunoelectrophoresis

The method of Grabar and Burtin (24) was used. Glass slides of dimensions 25x76 mm were coated with 2 ml of 0.5 % Noble agar melted in distilled water. These were dried in an oven at 80°C for 4 hours. The coated slides were then layered with 3 ml of 2.0 % Noble agar melted in 0.025 M barbital buffer, pH 8.5. After allowing solidification of the agar, troughs and wells were cut as desired. The substances to be electrophoresed were added to the wells and the slides placed in the electrophoretic apparatus and run at 14 ma with 70 to 75 volts for three and a half hours. The slides were then removed and the troughs filled with the appropriate antisera. They were then placed in a moist chamber at room temperature overnight to allow diffusion of the proteins and formation of the resultant precipitation lines. The slides were then washed in 0.9 % saline for 24 hours to remove non-precipitated proteins. They were desalted with distilled water for 8 hours and then dried overnight at room temperature. The dried slides were stained with amido black (1 gm / 1000 ml sodium acetate buffer) for 10 minutes and destained using a solution of acetic acid : methanol : water (150:750:750 volume) for 10 minutes.

II. Ouchterlony gel diffusion

Slides were coated as described above for immunoelectrophoresis

and were layered with 3 ml of 1.5 % Noble agar melted in 0.15 M saline. After solidification of the agar in a moist atmosphere, wells were cut in the agar slides. These were then filled with the appropriate antigen or antibody solutions and the slides were allowed to sit overnight at room temperature in a moist atmosphere, to permit diffusion of the proteins and precipitin line formation. Washing and staining were done as described for immunoelectrophoresis.

III. Sephadex G 200 gel filtration

The packing and running of the Sephadex G 200 columns followed the method of Pharmacia Ltd. (Montreal, Canada). The gel was allowed to swell in a buffer solution for three days at room temperature. Occasionally during this time, decanting of the buffer and stirring were carried out. To pack a column, a gel-buffer mixture of 1:1 in volume was used. This was evacuated to remove excess of dissolved air, and the evenly dispersed mixture was poured slowly and gently down the side of the column until the column was filled. Care was taken to keep the outlet at the same level as the top of the gel slurry (i.e. pressure equals zero). When approximately 10 cm of gel bed was packed, and thereafter, the outlet was lowered below the top of the gel slurry to maintain a pressure of one tenth of the packed bed length. Excess buffer was removed and replaced with fresh gel-buffer mixture before the previous addition had completely settled, in order to prevent the formation of boundaries. When the column was fully packed, a sample applicator was applied to the top of the gel to prevent disturbing the

packed gel during sample addition. Equilibration was carried out for 24 hours at 10-15 cm of pressure. Blue dextran was passed through the column to check on the homogeneity of the packing. This was done using a pressure of 2-3 cm, as was the case with all subsequent samples.

IV. Mice

Inbred BALB/c mice (female) from Jackson Laboratories, Bar Harbor, Maine, were used in all the experiments. C57BL mice were used in the transplantation experiments as skin donors.

V. Preparation of hybrid antibody and the RICA technique

The method followed for preparation of hybrid antibody and for the RICA technique was that described by Paraskevas et al (25) and by Lee (26).

1. Hybrid antibody preparation

Antisera against mouse γ G-myeloma protein and against horse spleen ferritin were prepared in rabbits. The rabbit anti-mouse γ G antibody, (a γ G), was purified using mouse γ G-myeloma protein aggregated with bis-diazotized benzidine (BDB). Tested by Ouchterlony gel diffusion and by immunoelectrophoresis, it was found to react with mouse γ G-globulin and with Bence Jones protein. It cross-reacted with other mouse γ -globulins through their L chains.

The rabbit anti-ferritin antibody, (aFe), was purified by precipitation with horse spleen ferritin and subsequent dissociation. The Ouchterlony gel diffusion technique confirmed the anti-ferritin activity.

The purified aFe and a γ G were separately digested with pepsin to yield F(ab')₂ (5S) fragments, which were isolated by gel filtration on a Sephadex G 100 column. The two 5S fragments were mixed and reduced with 2-Aminoethanethiol Hydrochloride (Matheson, Coleman, and Bell, Norwood, Ohio), yielding F(ab') fragments. The reducing substance was removed by passing the mixture through a AG50 WX4 cation exchange resin (mesh 100-200, BioRad Laboratories, Richmond, Calif.). The reduced preparation was then reoxidized by bubbling oxygen through it and the dimerized F(ab')₂ fragments separated from any F(ab') by passage through a Sephadex G 100 column.

To isolate aFe-a γ G recombinants only, the F(ab')₂ reoxidized sample was absorbed first with BDB-aggregated ferritin and then with BDB-aggregated mouse γ G-myeloma protein. The preparation contained only aFe-a γ G hybrid molecules as indicated by Ouchterlony gel diffusion on which it precipitated with a mixture of ferritin and mouse γ G-myeloma protein, but not with either one alone.

2. RICA technique

Suspensions of normal spleen cells from female BALB/c mice, at a concentration of 2×10^6 spleen cells per ml, were prepared in Hanks Balanced Salt Solution. To 0.5 ml (i.e. 1×10^6 spleen cells) were added 0.1 mg of aFe-a γ G hybrid antibody and 50 μ l of 2.5 % ferritin-coated sheep red blood cells (approximately 100 sheep RBC's / spleen cell). This was mixed well and incubated overnight at 4°C. In every experiment a control was set up with no hybrid antibody.

VI. Rabbit immunization and collection of ALS

Normal mouse thymocytes were prepared for injection as follows. Thymus cells were teased apart with forceps into Hanks BSS and then strained through a wire mesh. To insure single cell suspensions, the mixture was then aspirated several times with a needle and syringe. The cell suspension was then washed three times with Hanks BSS, centrifuging at 500 rpm at 4°C for 10 minutes. This was to insure the absence of mouse γ -globulin.

One rabbit, (R1Thy), was injected intramuscularly with approximately 1×10^8 thymocytes in a volume of 1.0 ml, consisting of 0.5 ml Hanks BSS plus 0.5 ml complete Freund's adjuvant (Difco). Five weeks later, and on two consecutive days, the rabbit was injected intravenously with approximately 2.5×10^8 thymocytes in a volume of 1.0 ml of Hanks BSS. One week later the rabbit was bled by heart puncture.

A second rabbit, (R6Thy), was injected intravenously with approximately 2.5×10^8 thymocytes in a volume of 1.0 ml of Hanks BSS on two occasions at two week intervals. One week after the second injection the rabbit was bled by heart puncture.

The blood from each rabbit was allowed to stand at room temperature for 2 hours and the sera collected by centrifugation at 3000 rpm for 15 minutes. The sera were heated to 56°C for 30 minutes to destroy complement, and then stored at -20°C in 5 ml aliquots. This was to avoid repeated thawings of a single sample. The sera were checked by immunoelectrophoresis and by Ouchterlony gel diffusion for the presence of rabbit anti-mouse γ -globulins. No precipitating antibodies were detected. A portion of the R6Thy antiserum was absorbed with BDB-aggregated

mouse γ G-myeloma protein to further insure the absence of binding anti-mouse γ G-globulins.

A normal rabbit was also bled, and its serum similarly treated, to be used as a control.

VII. Determination of lymphoagglutinating and cytotoxic titers of the ALS

The procedure of Martin and Miller (21) was used. A single cell suspension of normal mouse thymocytes was prepared (the same as above for immunization). After washing, the cells were suspended in Hanks BSS at a concentration of approximately 25×10^6 cells / ml. Doubling dilutions of the antiserum to be checked, i.e. RLThy and R6Thy, were made in Hanks BSS, and using 0.1 ml volumes. To each dilution was added 0.1 ml of 1:10 guinea pig serum and 0.2 ml of the thymocyte suspension. Incubation was carried out at 37°C for 60 minutes. The dilutions were then chilled in ice. A portion of each dilution was added to trypan blue, in which viable cells exclude the blue dye while non-viable cells become stained with the dye. These samples were then examined under a microscope using a hemocytometer. The first dilution at which no more than approximately 50 % of the cells became stained was considered as the cytotoxic titer, while the highest dilution at which agglutination was detectable was considered as the agglutinating titer.

VIII. Determination of hemagglutination titers for ALS

A microtechnique was employed to determine the hemagglutination

titers of the antisera, R1Thy and R6Thy. Capillary tubes were marked at a level equivalent to a 10 lambda capacity. Doubling dilutions of each antiserum were made in a 0.15 M phosphate-saline buffer, pH 6.4, and using 10 lambda volumes. A portion of each dilution was added to a microcapillary tube. At the surface of each dilution in these tubes was added a small amount of a 2.5 % suspension of mouse red blood cells suspended in the same buffer. The samples were allowed to stand at room temperature for 30 minutes. The tubes were then read for agglutination using a magnifying glass and the last dilution at which agglutination could be seen was considered the hemagglutination titer.

IX. The Homing technique using Cr51-labelled mouse thymocytes

The method used was that of Martin and Miller (21). Thymuses were removed from normal mice with care to avoid contamination with mouse red blood cells. A single cell suspension was prepared in 1:100 Minimal Essential Medium diluted in Balanced Salt Solution, and with 10 % calf serum which had previously been heat inactivated. The cells were washed one time and resuspended to a concentration of 2×10^8 cells per ml. Ten microcuries of Cr51 were added per 1 ml of cell suspension, and incubation at 37°C for 30 minutes was carried out. The cell suspension was chilled and the cells washed 2 times with the above medium.

The Cr51-labelled thymus cells were suspended to a concentration of approximately 10^8 cells / ml; and then incubated at 37°C for 30 minutes with various dilutions of either ALS antiserum, R1Thy or R6Thy, or with NRS. The cells were then chilled, washed once to remove excess ALS or

NRS, and resuspended to a concentration of 10^8 cells / ml. 0.2 ml of suspension (2×10^7 cells) was injected into each of a series of mice intravenously. Four hours later the mice were sacrificed and their livers and spleens removed, minced, and diluted with saline in counting tubes. The amount of radioactivity was determined in a Nuclear Chicago Gamma Ray Counter. The localization ratios at the various dilutions of ALS or NRS were determined by dividing the amount of radioactivity in the liver by that amount in the spleen (TABLE I; figure 2).

X. Skin grafting

The method followed was that of Billingham and Medawar (27). On three consecutive days a series of 6 mice (BALB/c), for each of the three sera tested, were each injected with 0.25 ml of RLThy, R6Thy, or NRS intraperitoneally. On the third day of injection, called day 0, skin was grafted from C57BL mice onto the BALB/c mice. The area on which the skin graft was to be placed was shaved, the mice put to sleep with an intraperitoneal injection of Nembutal, and a patch of skin carefully cut away from the shaven area. Care was taken to avoid breaking the blood vessels. A piece of C57BL mouse skin, which had been carefully scraped free of fatty tissue and also shaven to remove all hair, was placed over the prepared area on a BALB/c mouse. This was then sprayed with Aeroplast Dressing (Parke-Davis, Detroit, Michigan) to sterilize the area and to hold the skin graft in place. Upon drying, gauze was applied and then bandages. Bandages were reapplied as necessary to prevent the mice from scratching at and destroying the

transplanted skin.

On day 7 the mice were again injected intraperitoneally with 0.25 ml of the appropriate antiserum and the bandages and gauze were removed from the mice. The progress of the skin grafts was followed thereafter to check for prolongation of survival in ALS-treated mice (figure 1).

XI. Histological and hematological studies on ALS

A series of 3 mice for each antiserum tested were each injected intraperitoneally on 3 consecutive days with 0.25 ml of either RLThy or NRS. Seven days after the third injection the mice were sacrificed and their blood collected for hematology studies, which included hematocrits, hemoglobins, red blood cell counts, white blood cell counts, and differentials. At the same time, their spleens, thymuses, and lymph nodes (cervical) were collected for tissue sectioning and histological studies. The tissues were fixed in 3 % formalin, and pieces of each were embedded in paraffin wax from which tissue sections were cut. The tissue sections were stained with hematoxylin-eosin and mounted (TABLE II; figure 3).

XII. Preparation of 7S- γ G and 19S- γ M antibody globulin fractions from ALS

A sample of R6Thy was applied to a Sephadex G 200 column for separation of the γ M from the γ G fraction. The γ M was eluted with the void volume, and the γ G in the second peak. The resulting peaks were

each concentrated and the γ M and the γ G peaks were each made up to original serum concentrations. Care was taken to ensure that the portion of the 19S peak concentrated was free of 7S globulin and vice versa.

EXPERIMENTAL

I. Lymphoagglutinating and cytotoxic titers of ALS

The cytotoxic titers, i.e. the first dilution at which no more than approximately 50 % of the mouse lymphocytes become stained, were 1:1024 for R1Thy and 1:256 for R6Thy. The lymphoagglutinating titers, i.e. the highest dilution at which agglutination of the lymphocytes can be detected, were 1:512 for R1Thy and 0 for R6Thy.

II. Hemagglutination titers of ALS

This titer, which measures the highest dilution at which agglutination of mouse red blood cells can be seen, was found to be 1:512 for R1Thy and 1:1024 for R6Thy.

III. Skin graft prolongation by ALS

Results of the experiments with skin grafting showed a definite and considerable prolongation of graft survival in mice treated with ALS, as compared with mice treated with NRS. In the group of 6 mice treated with NRS, one graft was a technical failure. Of the remaining 5, the average time at which graft rejection occurred was day 9 (day 0 being the day on which skin grafting was performed).

In the group of 6 mice treated with R6Thy, five grafts were technically successful and, for these, the average time at which graft rejection occurred was day 30. Thus, the prolongation of graft survival upon treatment with R6Thy was 21 days.

In the final group of 6 mice, which were treated with RLThy, one graft was again a technical failure. All other grafts were successful and, of these, the average time at which graft rejection occurred was day 24. Thus, prolongation of graft survival was 15 days when mice were treated with RLThy.



Figure 1. An example of skin grafts from C57BL mice transplanted onto BALB/c mice, the top being an ALS-treated and the bottom an NRS-treated mouse. The mice were photographed on day 9 and the figure illustrates the neucrotic nature of the skin grafts on the NRS-treated mice in comparison to the normal, healthy appearance of those on the ALS-treated mice.

IV. Results of Homing experiments

Liver to spleen localization ratios were determined for each of the sera under investigation, and at various dilutions of each sera (TABLE I). Using these ratios, curves were plotted on a graph for each of the sera (figure 2). According to Martin and Miller (21), the first part of each of the two ALS curves (RLThy and R6Thy), i.e. that part including dilutions between 1:100 and 1:500, does not appear to be a result of the effect of the true immunosuppressive ALS-antibody on the thymus cells, but rather the result of some other antibody or antibodies present in the ALS antisera. Sera which are not immunosuppressive can still give this part of the Homing curve. It is the part of the curves that falls between dilutions of 1:500 and 1:1000 for both ALS antisera that probably reflects the effect of the true immunosuppressive ALS-antibody on the thymus cells. Within these dilutions, the localization ratios appear to be relatively constant for a given antiserum, and a plateau is formed.

Normal rabbit serum gave results similar to those of Martin and Miller who found that NRS at any and all dilutions gave a localization ratio of approximately 3.5 for mouse thymocytes.

A localization index, defined as the localization ratio of a given ALS divided by the localization ratio of NRS, was determined for each of the two ALS antisera. The localization ratios of the ALS which were used in the determinations were those obtained at a 1:1000 dilution, while the localization ratio of NRS used was 3.25. The localization index of RLThy was 1.77, while that of R6Thy was 2.69. According to

Martin and Miller these results can be interpreted as being indicative of a significant immunosuppressive capacity on the part of both ALS. Also, a comparison of the two indicates that, according to the homing assay, the R6Thy antiserum is more highly immunosuppressive than the R1Thy antiserum.

IV. Results of Homing experimentsTABLE I

DETERMINATION OF LOCALIZATION RATIOS FOR Cr51-LABELLED LYMPHOCYTES
INCUBATED IN VITRO WITH VARYING DILUTIONS OF ALS AND NRS

Dilution of serum used for incubation	Liver radio- activity ct.	Spleen radio- activity ct.	Localization Ratio
RLThy 1:100	8242	603	13.6
RLThy 1:500	1181	170	6.95
RLThy 1:1000	19965	3479	5.74
R6Thy 1:100	24081	1010	23.9
R6Thy 1:500	6971	754	9.25
R6Thy 1:1000	34165	3905	8.75
R6Thy 1:1500	6496	1492	4.35
R6Thy 1:2000	7499	1703	4.39
NRS 1:2	809	232	3.48
NRS 1:2	5459	1814	3.01

NOTE: Each count is the average of two separate counts.

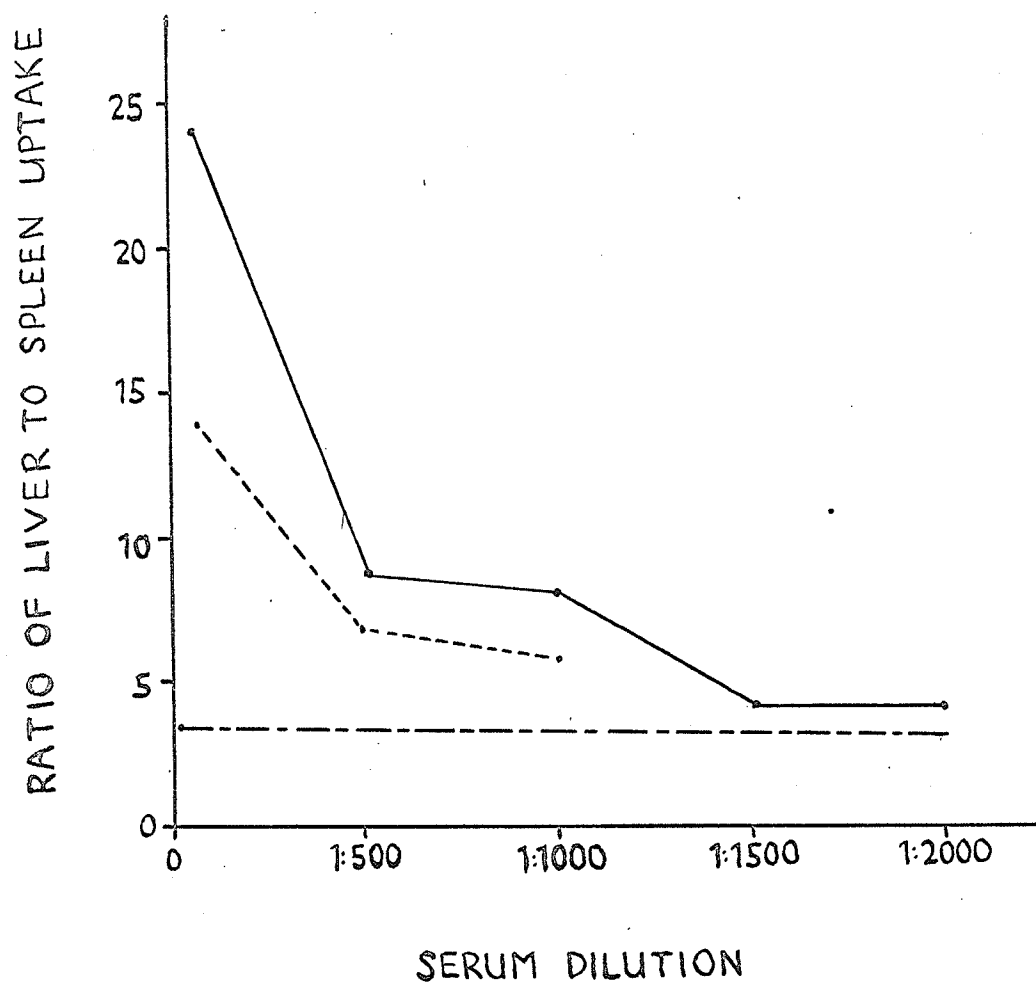


Fig. 2. Graph of liver to spleen localization ratios in BALB/c mice injected with Cr51-labelled thymus cells preincubated with varying dilutions of R6Thy (—), R1Thy (----), and NRS (---). Each point represents the mean of two determinations.

V. Results of histological and hematological studies on ALS

The results of the hematological studies can be seen in TABLE II. As can be seen, in general, both the rbc count and the wbc count were considerably lower in RLThy-treated mice than in NRS-treated mice. However, no deaths occurred in any of the mice treated with ALS in amounts up to 1 ml during the course of our experimental work, and thus, the toxic effects of the ALS were, nevertheless, non-lethal.

The hematoxylin-eosin - stained tissue sections were examined under a microscope. The tissue sections of thymuses had a normal appearance in the case of both the RLThy-treated and the NRS-treated mice. No depletion of small lymphocytes was evidenced.

Upon examination of the lymph node tissue sections, it was found that the NRS-treated mice had lymph nodes of a normal appearance. The lymph node tissue sections from the RLThy-treated mice, on the other hand, showed a very marked depletion of small lymphocytes and replacement by reticulohistiocytes. The area of drastic depletion of lymphocytes was the paracortical region (figure 3, a & b).

The examination of the spleen tissue sections resulted in similar findings to those for the lymph node sections. The NRS-treated mice had spleens with a normal appearance. The spleens of the RLThy-treated mice showed a pronounced depletion of small lymphocytes from the periarteriolar region. Reticulohistiocytes became predominant in the depleted region.

TABLE IIHEMATOLOGICAL STUDIES ON MICE TREATED WITH RLTHY AND NRS

Mouse treated & serum used	Hemato- crit	Hemoglobin gm/100 ml	rbc ct. per cu mm	wbc ct. per cu mm	Lymphocytes per cu mm
RLThy #1	44.5	15.1	7.4×10^6	4950	1831
RLThy #2	46.0	14.6	7.2×10^6	4000	1520
RLThy #3	45.5	15.3	7.4×10^6	6900	3105
NRS #1	49.5	16.9	13.7×10^6	14000	10780
NRS #2	48.5	16.3	10.4×10^6	9000	7560
NRS #3	46.0	15.3	10.9×10^6	13700	8220



Figure 3, a & b. Tissue sections of lymph nodes from NRS-treated (a) and from RLThy-treated (b) BALB/c mice.

VI. Technique of treatment of lymphocytes with ALS in preparation for the RICA technique

Spleens were collected from normal female BALB/c mice. A single cell suspension was prepared by teasing the cells apart with forceps, straining them through a wire mesh, and, finally, aspirating them up and down 3 or 4 times through a needle (#20) and syringe. The cells were suspended in Hanks Balanced Salt Solution, pH 7.4, at 4°C. They were then washed 3 times with the cold Hanks BSS. Centrifugation was carried out at 500 rpm at 4°C for 8 minutes (unnecessarily long or fast centrifugation was avoided to prevent destruction of the spleen cells). A total lymphocyte count was then done and the cells were suspended in Hanks BSS to a concentration of $1-1.5 \times 10^7$ cells / ml. To 1 ml aliquots of these cells in suspension were added varying quantities of the serum (RLThy, R6Thy, or NRS), or of the serum fraction (7S-R6Thy or 19S-R6Thy), whose effect on the cells was under investigation. Thus, in effect, varying dilutions of the sera or fractions of sera were obtained. These cell suspensions were each incubated at 37°C for 30 minutes.

At the 30 minute mark, the treated cell suspensions were placed in an ice bath, washed 1 time with cold Hanks BSS centrifuging as above, and then resuspended in cold Hanks BSS to a concentration of 2×10^6 spleen cells / ml. To 0.5 ml (i.e. 1×10^6 cells) of each of the treated cell suspensions was added 0.1 mg of aFe-a γ G hybrid antibody and 50 μ l of a 2.5 % suspension of ferritin-coated sheep red blood cells.

The mixtures were each mixed well and incubated overnight at 4°C. The following day, the number of rosettes per 1000 lymphocytes was determined for each cell suspension.

For each spleen, whose cells were being treated with the sera, a 1 ml aliquot was left untreated. It was incubated at 37°C for 30 minutes, as were the treated cells, washed, resuspended to the desired concentration, and then mixed with hybrid antibody and Fe-coated sheep red blood cells in the correct proportions. A count of the number of rosettes per 1000 lymphocytes was made. It was against this count that all of the rosette counts of the treated cell suspensions, which originated from the same spleen, were compared to determine the extent of inhibition of rosette formation. This was done to insure reliable results, since the number of rosettes per 1000 lymphocytes for normal mouse spleens varies between 250 to 350, and thus, using an average of 300 for our comparisons would have led to considerable inaccuracies. A final control involved the setting up of a sample without hybrid antibody for every sample set up with hybrid antibody to check for non-specific rosette formation.

VII. Inhibition of rosette formation by ALS

1. Inhibition by ALS whole antiserum

Washed spleen cell suspensions were incubated with varying dilutions of RLThy or of R6Thy whole antiserum. The degree of inhibition of rosette formation which resulted from such treatment was determined for each dilution, and these results were then plotted on a graph

to yield inhibition curves for the two antisera (figure 4). As can be seen, at high concentrations of either of the ALS antisera no inhibition of rosette formation occurred, although the dilution at which RLThy first began to give inhibition was considerably higher (dilution greater than 1:1000) than that at which R6Thy began (dilution greater than 1:50). The amount of inhibition then rose rapidly to a peak at dilution 1:500 for R6Thy and 1:2500 for RLThy, after which the amount of inhibition dropped very slowly over an extremely wide range of dilutions. Inhibition was still evident at dilutions of 1:40,000 for both antisera. The loss of inhibition of rosette formation which occurred between a 1:500 dilution to 0 dilution for R6Thy and between a 1:2500 dilution to 0 dilution for RLThy indicates that at high sera concentrations there is a component(s) present in the antisera which acts as a blocker of the rosette inhibition. This has been diluted out by a 1:500 dilution for R6Thy and by a 1:2500 dilution for RLThy. At dilutions thereafter inhibition can occur.

The sample of R6Thy that had been absorbed with BDB-aggregated mouse γ G-myeloma protein was tested at dilution 1:500. Cells were treated with this dilution of R6Thy-absorbed and the degree of inhibition was determined and compared with the degree of inhibition obtained with the same dilution of R6Thy-unabsorbed. The degree of inhibition was the same in both cases, and, therefore, we are assured that the inhibition which we have observed for the antisera is not the result of binding rabbit anti-mouse γ -globulins.

Normal mouse spleen cells were also treated with varying dilutions of NRS and the degree of inhibition was determined to yield an inhib-

ition curve to be used as a control. It was found that at all varying dilutions of NRS no inhibition of rosette formation occurred.

2. Inhibition by 7S and 19S fractions of R6Thy

i). 7S alone

Spleen cells were incubated with varying dilutions of 7S-R6Thy, which was at a concentration approximately that of 7S in R6Thy whole antiserum. The degree of inhibition at these various dilutions was determined and an inhibition curve was plotted (figure 5). As can be seen from the curve, the maximum amount of inhibition of rosette formation occurred in undiluted 7S-R6Thy and then dropped rapidly until it reached the point of no inhibition. This occurred at a dilution of 1:500 of the 7S-R6Thy, and at all higher dilutions no inhibition occurred. Thus, 7S alone could not reconstruct the inhibition curve obtained with R6Thy whole serum.

ii). 19S alone

As with 7S alone, an inhibition curve was plotted for varying dilutions of 19S-R6Thy (figure 5). Again the maximum amount of inhibition occurred with undiluted 19S-R6Thy, and the degree of inhibition fell to zero at a 1:500 dilution. At all subsequent higher dilutions, no inhibition occurred. Thus, 19S alone could not reconstruct the inhibition curve obtained with R6Thy whole serum.

iii). 7S plus 19S reconstituted in R6Thy serum proportions

Series of 1 ml aliquots of normal mouse spleen cells in suspension were set up. To a single aliquot was added a particular dilution of 7S-R6Thy plus that same dilution of 19S-R6Thy. Other dilutions of the two fractions were added to other aliquots, although the dilution of the two fractions was always the same for a given aliquot. Thus, for example, in one aliquot we had 1:500 7S plus 1:500 19S, in another 1:100 7S plus 1:100 19S, etc. The degree of inhibition at the various reconstituted dilutions was determined. As can be seen from figure 5, the shape of the inhibition curve thus obtained did not, as was expected, exactly reconstruct the curve obtained using R6Thy whole antiserum. As the concentration of the 7S plus 19S fractions increased above that of a 1:500 dilution, i.e. as we moved in the direction of undiluted 7S plus 19S, the degree of inhibition rose continuously, reaching a maximum in undiluted 7S plus 19S. This was exactly opposite to the results obtained with R6Thy whole serum, where the inhibition dropped as the concentration increased above that of a 1:500 dilution. At dilutions greater than 1:500, the curve had a shape resembling that of R6Thy whole serum. These results indicate that the component(s) present in R6Thy whole serum, which blocked the inhibition of rosette formation between a zero dilution to a 1:500 dilution, is no longer present in the 7S plus 19S combined fractions. Thus, it must have been removed by the Sephadex G 200 gel filtration, suggesting that the blocker component(s) may be a small molecular weight substance.

iv). 7S at 1:500 dilution plus 19S at varying dilutions

Series of 1 ml aliquots of normal mouse spleen cells in suspension were set up. To every aliquot was added an amount of 7S-R6Thy which resulted in a 1:500 dilution. Then, varying amounts of 19S-R6Thy were added to each of these aliquots, resulting in a series of dilutions of the 19S. The degree of inhibition of rosette formation was determined for each of these aliquots, and an inhibition curve was plotted (figure 6). The maximum amount of inhibition of rosette formation occurred when both the 7S and 19S were at the same dilution, i.e. 1:500. At dilutions of 19S less than the 1:500 dilution of the 7S, the degree of inhibition dropped rapidly to zero. At dilutions of 19S greater than the 1:500 dilution of the 7S, the degree of inhibition dropped less rapidly, giving approximately half the maximal inhibition at a 1:1000 dilution of the 19S.

v). 19S at 1:500 dilution plus 7S at varying dilutions

Again series of 1 ml aliquots of normal mouse spleen cells in suspension were set up, each containing an amount of 19S-R6Thy resulting in a 1:500 dilution of the 19S. Then, varying amounts of 7S-R6Thy were added to each, yielding a series of dilutions of the 7S. The degree of inhibition at these varying dilutions of 7S was determined, and an inhibition curve was plotted (figure 6). The maximum amount of inhibition of rosette formation again occurred when both the 7S and the 19S were at a 1:500 dilution. At dilutions of the 7S greater than the 1:500 dilution of the 19S, the degree of inhibition dropped rapidly to zero.

At dilutions of the 7S less than the 1:500 dilution of the 19S, the amount of inhibition dropped less rapidly, giving approximately half the maximal inhibition at a 1:250 dilution of the 7S.

3. Inhibition by dialyzed R6Thy whole serum

A sample of the R6Thy whole antiserum was dialyzed overnight against 0.15 M borate-saline buffer, pH 8.0, and was then tested, at various dilutions, for its ability to inhibit rosette formation. An inhibition curve was obtained which was identical to that of non-dialyzed R6Thy. Thus, it appears that the component(s) that blocked the inhibition of rosette formation in R6Thy whole serum was still present in the serum after dialysis. Thus, the blocking component(s), although of a small molecular weight, was not sufficiently small to be dialyzable.

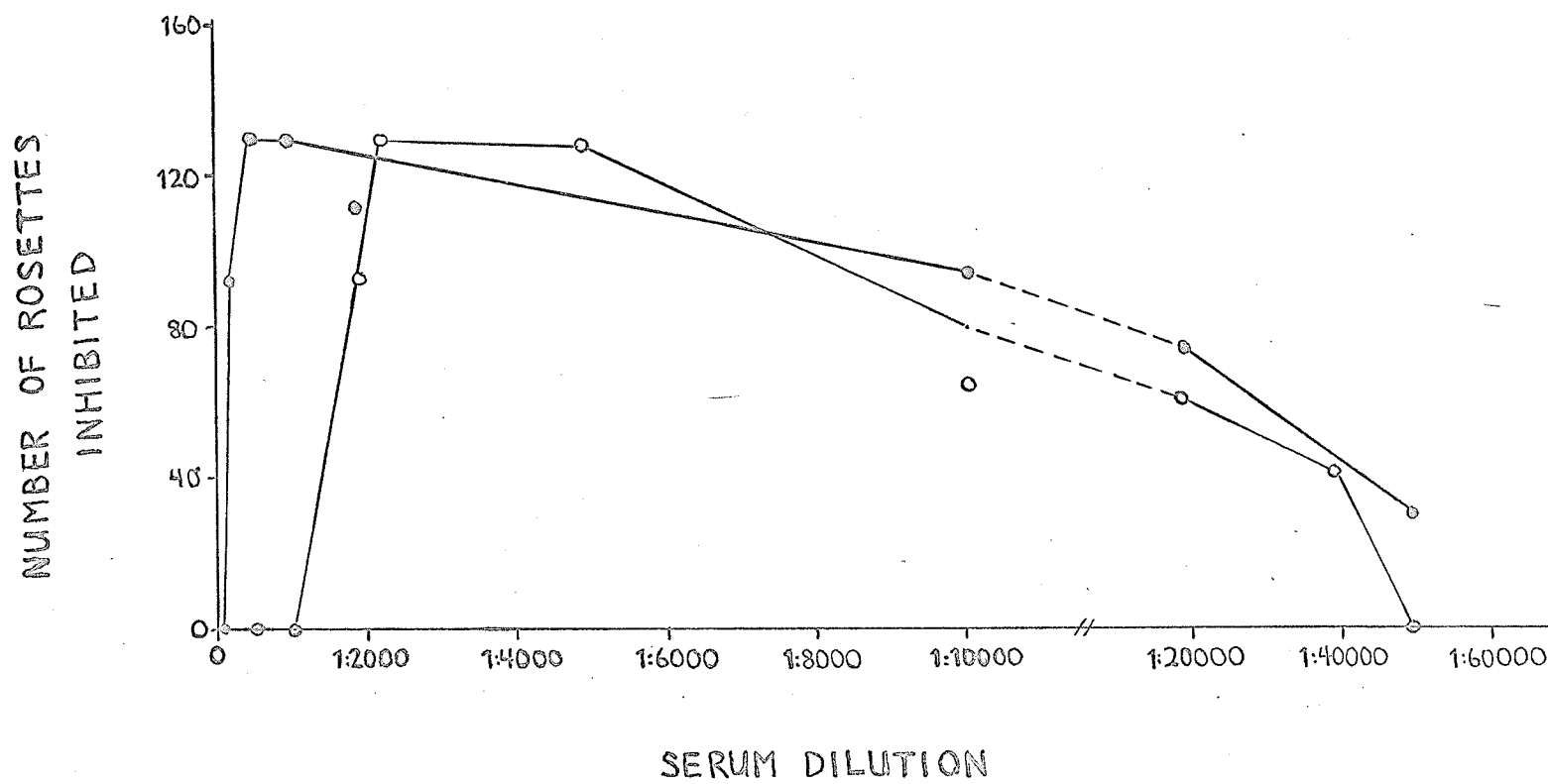


Figure 4. Inhibition curves obtained when normal mouse spleen cells were treated with varying dilutions of either RLThy (○—○) or R6Thy (●—●). The degree of inhibition of reverse rosette formation was determined at the various dilutions and the results were plotted.

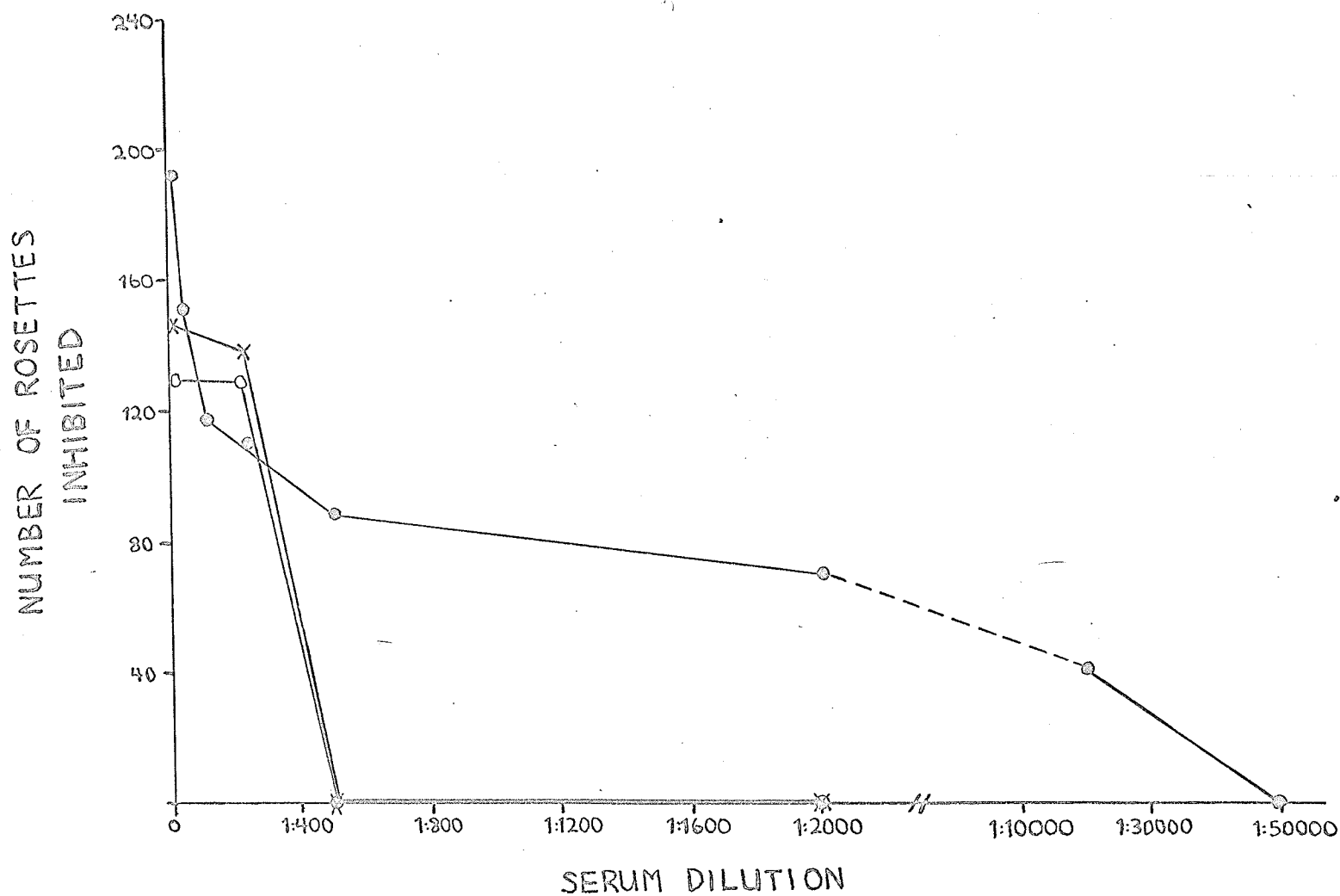


Figure 5. Inhibition curves obtained when normal mouse spleen cells were treated with varying dilutions of either 7S (x—x) or 19S (o—o), or 7S plus 19S reconstituted in R6Thy serum proportions (•—•). Note: 7S = 7S-R6Thy and 19S = 19S-R6Thy.

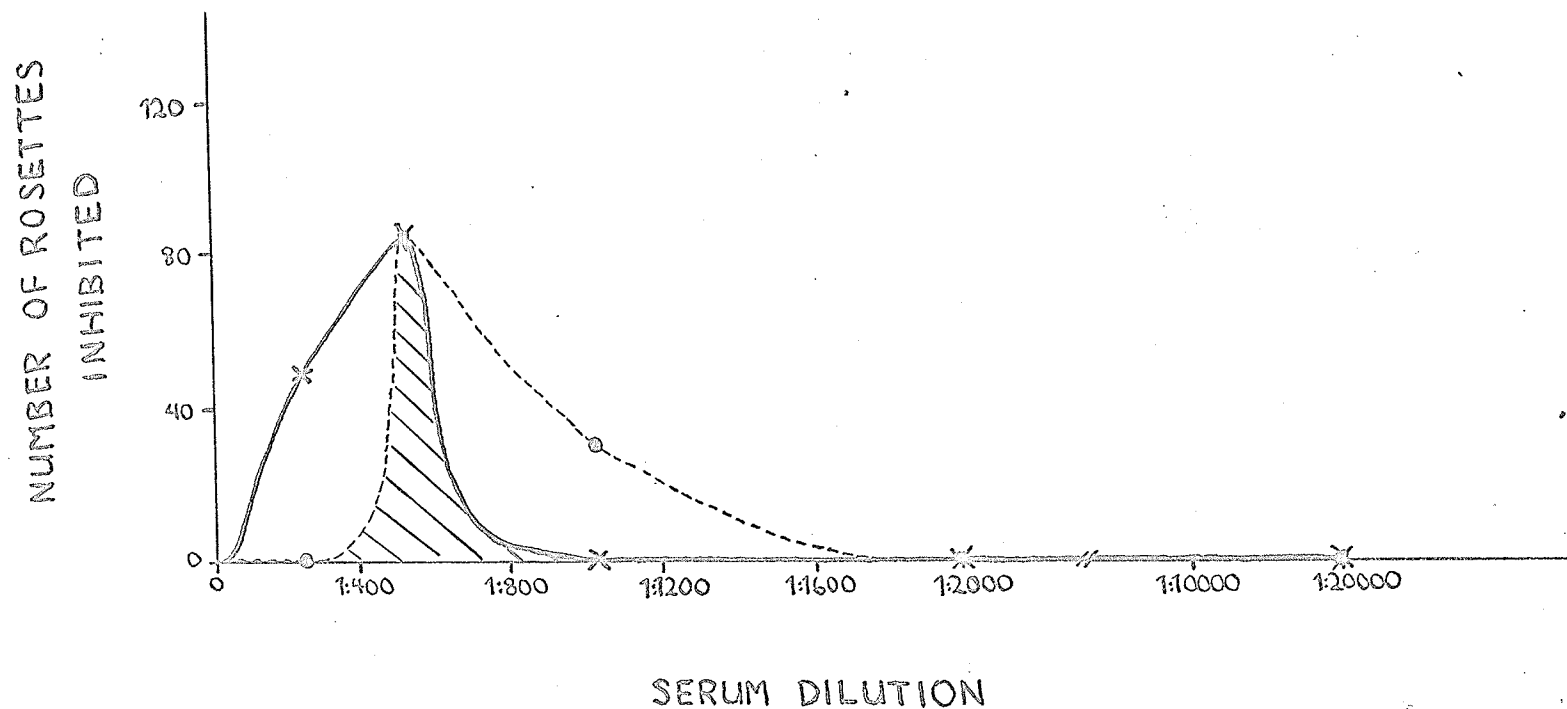


Figure 6. Inhibition curves obtained when normal mouse spleen cells were treated with either 7S at a 1:500 dilution plus 19S at varying dilutions (o----o) or 19S at a 1:500 dilution plus 7S at varying dilutions (x—x).

Note: 7S = 7S-R6Thy and 19S = 19S-R6Thy.

DISCUSSION

As has been previously described, the postulated mechanisms of action of ALS include cytotoxicity, destruction of a critical subpopulation of small lymphocytes, blindfolding, sterile activation, and opsonization of the lymphocytes by ALS. Of these, cytotoxicity has been discredited as the mechanism of action of ALS. Immediately upon injection of ALS into the animal it might occur, but, as the ALS is quickly eliminated from the circulation, it would very soon cease to be a possible mechanism. Blindfolding has been strongly criticized on the basis of results showing that the descendants of affected lymphoid cells remain unreactive for several generations (17). Recent support for a blindfolding mechanism is suggested in the findings of Bach (22, 23) who showed that ALS could inhibit the formation of the spontaneous rosettes that normally form between mouse lymphocytes and sheep red blood cells. Thus, the antigen receptors present on the surface of normal mouse lymphocytes are blocked by ALS. This could result in a loss of their immunological competence. Therefore, a blindfolding of the antigen receptors present on immunologically competent cells might be a mechanism of action of ALS. It remains to be seen which one of the postulated mechanisms, or which combination of them, represents the true mechanism of action of ALS in vivo.

In an attempt to shed further light on the mechanism of action of ALS, the RICA (Reverse Immune Cytoadherence) technique was employed. The RICA technique is used to detect the presence of γ -globulin-like receptors on the surface of normal mouse lymphocytes by the formation of rosettes with ferritin-coated sheep red blood cells. This occurs via a

hybrid antibody carrying one anti-ferritin site and one anti-mouse γ -globulin site. Since it is generally accepted that these γ -globulin-like receptors are in actual fact the antigen receptors of immunologically competent cells, it seemed reasonable to suspect that ALS might cause an inhibition of the reverse rosette formation, as it did with spontaneous rosette formation. With this in mind, normal mouse spleen cells were treated with ALS whole antisera at various dilutions of the ALS. It was found that ALS did in fact block the γ -globulin-like receptors, thus preventing reverse rosette formation. In other words, ALS prevented the binding that normally occurs between the γ -globulin-like receptors and the anti- γ -globulin site of the hybrid antibody. If we extend this, it seems likely that ALS will also prevent the binding between these receptors and their specific antigen. Thus, our findings indirectly support the hypothesis that ALS might act by a blindfolding mechanism, i.e. by a blocking of the antigen receptors of immunocompetent cells.

Normal mouse spleen cells were treated with varying dilutions of R6Thy whole serum or of R1Thy whole serum. The degree of inhibition of reverse rosette formation at these various dilutions was determined and inhibition curves were plotted (figure 4). It was found that, for both sera, no inhibition of reverse rosette formation occurred in undiluted ALS and in low dilutions of ALS. This suggests the presence of a component(s) in whole sera at these low dilutions that blocks the inhibition of reverse rosette formation which normally occurs at higher dilutions of the ALS antisera. Its effect is quickly diluted out. A similar effect was reported by Southworth et al (20) who found

that high concentrations of ALS inhibited the stimulation of thymidine incorporation which normally occurred at higher dilutions of the ALS. The R6Thy whole serum was dialyzed overnight in an attempt to remove this blocker component(s). When the degree of inhibition of reverse rosette formation was determined at various dilutions of the R6Thy-dialyzed, the resultant inhibition curve proved to be identical to that obtained with R6Thy-non-dialyzed. Therefore, the blocker component(s) is of a non-dialyzable molecular weight.

Studies were carried out on the 7S and 19S fractions of R6Thy. These fractions were prepared by passage of a portion of the R6Thy whole serum through a Sephadex G 200 column. The resultant 7S and 19S peaks were each concentrated to approximately their normal serum concentrations. Normal mouse spleen cells were treated with varying dilutions of 7S plus 19S reconstituted in R6Thy serum proportions, and the degree of inhibition of reverse rosette formation was determined and an inhibition curve plotted (figure 5). It was found that there was no longer a blocking of the inhibition with undiluted 7S plus 19S nor with low dilutions of 7S plus 19S. In fact, the maximum amount of inhibition occurred with undiluted 7S plus 19S. Thus, the blocker component(s) can be separated from 7S and 19S globulins by column filtration.

Of interest is the similarity in shape between the inhibition curve for 7S plus 19S fractions of R6Thy reconstituted in serum proportions (figure 5) and the homing curve for R6Thy (figure 2). Both curves show a rapid drop at high concentrations, and then a levelling off at higher dilutions. According to Martin and Miller (21), their homing curves

indicate two mechanisms in action. The first mechanism, occurring at high concentrations where the curve drops rapidly, does not correlate with in vivo immunosuppression. The second mechanism, occurring at higher dilutions where a plateau is formed, does correlate with in vivo immunosuppression. On the other hand, a striking difference exists between the homing and the inhibition curves. While the homing curve falls to the level of the NRS control by a 1:2000 dilution, the inhibition curve for 7S plus 19S of R6Thy reconstituted in serum proportions indicate the presence of inhibition of reverse rosette formation at dilutions as high as 1:30,000. Thus, extremely small amounts of ALS seem to be sufficient to cause an inhibition of reverse rosette formation. Were the blocking or blindfolding of the γ -globulin-like receptors, i.e. the antigen receptors of immunocompetent cells, by ALS a mechanism of action of ALS in vivo, this could explain why the effects of ALS, which is known to be quickly eliminated from the circulation, are long-lasting. Opsonization, as measured by the homing technique, on the other hand, would appear to occur only at dilutions of ALS less than 1:2000. Thus, while opsonization may be a mechanism of action of ALS at higher concentrations, it is not unlikely that another mechanism of action might become prevalent at higher dilutions of ALS. This mechanism of action could possibly be the blocking of the antigen receptors on lymphocytes, i.e. a blindfolding phenomenon.

Attempts were made to reconstruct the inhibition curve obtained with 7S plus 19S reconstituted in serum proportions, using either the 7S or the 19S fraction alone. The curve was not reconstructed (figure 5).

Either 7S or 19S alone inhibit only up to a dilution of 1:500, with the maximum amount of inhibition occurring with the undiluted fraction. At dilutions greater than 1:500, no inhibition of reverse rosette formation occurs. Thus, only when the 7S and 19S are reconstituted in serum proportions does inhibition occur past a 1:500 dilution; in fact, inhibition is still evident at a 1:30,000 dilution using 7S plus 19S. This suggests a synergistic action between 7S and 19S in bringing about in vitro inhibition of reverse rosette formation.

Experiments were done using varying proportions of the 7S to the 19S. In one case, the 7S was held constant at a 1:500 dilution, while the dilution of 19S was varied. In the other case, the 19S was held constant at a 1:500 dilution, while the dilution of 7S was varied. The results show that only within a certain extremely narrow range of proportions of the two fractions does inhibition of reverse rosette formation occur. Maximum inhibition occurs with serum proportions of the two, i.e. with a 1:500 dilution of 7S plus the same dilution of 19S. These results lend further support to a synergistic action between 7S and 19S, at least in vitro.

Thus, the use of the RICA technique to study ALS raises the possibility that the blocking of the γ -globulin-like receptors of immunologically competent cells may be a mechanism of action of ALS in vivo. The blocking of such receptors would render these cells unresponsive to antigen. Supportive evidence would be forthcoming if the degree of inhibition of reverse rosette formation were found to correlate with in vivo immunosuppression as measured by skin homograft survival prolongation.

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