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

As studied by the Reverse Immune Cytoadherence Technique (RICA)

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ABSTRACT

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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 persistance 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 descendents 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 γG-myeloma protein and against horse spleen ferritin were prepared in rabbits. The rabbit anti-mouse γG antibody, (aγG), was purified using mouse γG-myeloma protein aggregated with bis-diazotized benzidine (BDB). Tested by Ouchterlony gel diffusion and by immunoelectrophoresis, it was found to react with mouse γG-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 aγG 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-aγG recombinants only, the F(ab')2 reoxidized sample was absorbed first with BDB-aggregated ferritin and then with BDB-aggregated mouse γG-myeloma protein. The preparation contained only aFe-aγG hybrid molecules as indicated by Ouchterlony gel diffusion on which it precipitated with a mixture of ferritin and mouse γG-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-aγG hybrid antibody and 50 lambda 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 x 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 intraveneously with approximately 2.5 x 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 intraveneously with approximately 2.5 x 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