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STUDIES ON IN VITRO AND IN VIVO IMMUNOLOGICAL PROPERTIES OF HETEROLOGOUS ANTI-MYELOMA AND ANTI-LYMPHOCYTE SERA IN MICE

WILLIAM F. FLYNN, JR

1968

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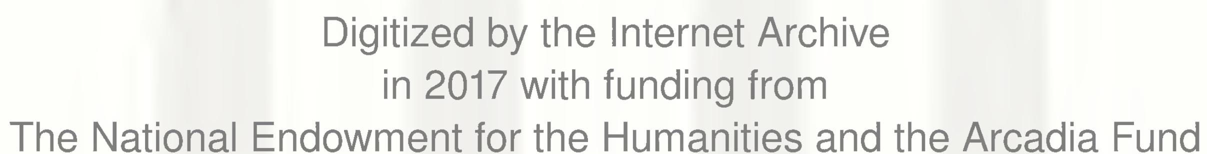
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STUDIES ON
IN VITRO AND IN VIVO IMMUNOLOGICAL PROPERTIES
OF
HETEROLOGOUS ANTI-MYELOMA AND ANTI-LYMPHOCYTE SERA
IN MICE

Thesis presented in partial fulfillment
of requirements for the degree of
Doctor of Medicine

Yale University School of Medicine
Department of Microbiology

1968

William F. Flynn, Jr.

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INTRODUCTION

During the last few years, the interests of the surgeon and the immunologist have been focused on the remarkable immuno-suppressive powers of antilymphocyte serum. Such serum has come into use in experimental and clinical organ transplantation in many series. We shall here discuss what is currently known about its specificity and mode of action.

The work of several early investigators with anti-leucocyte sera has been well summarized by Russell and Monaco (31). In 1937, Chew and Lawrence (6) demonstrated that a serum raised in rabbits against guinea pig lymph node cells produced a fall in total lymphocyte counts when administered to guinea pigs, and that daily injections could maintain lymphopenia up to ten days. Lymphocyte counts were depressed from a level of 4-5,000 to 1,000 or less, but increasing doses could not totally ablate peripheral lymphocytes. They found a generalized hyperplasia of lymphoid tissue, which was also seen after injections of normal rabbit serum. In 1941, Cruickshank (7) demonstrated lymphopenia in rats given rabbit anti-rat lymphocyte serum, and showed that incubation of such serum with rat lymphocytes utilized complement. He also noted lymph node hyperplasia.

In 1956, Interbitzin (12) demonstrated that antilymphocyte serum inhibits tuberculin sensitivity in rats.

Wilhelm et al., in 1958, (36) noted that administration to guinea pigs of rabbit anti-"mononuclear" serum induced a depletion of circulating mononuclear cells, which was correlated with a marked reduction in allergic contact sensitivity. The growing awareness of the role of lymphocytes in depayed hypersensitivity, and, specifically, allograft rejection, led Woodruff (37) to investigate the effects of antilymphocyte serum on allografts of skin and endocrine tissue in rats. These experiments were unsuccessful, but Woodruff has recently admitted (40) that the sera used were not sufficiently potent and the doses too small. In 1961, Waksman et al. (35) reported definite reduction in a variety of reactions of the delayed type after treating guinea pigs with rabbit anti-guinea pig lymphocyte serum. A slight, but definite, prolongation of first set allografts was noted; there was also some histological evidence of slightly delayed rejection of second-set grafts. Repeated injections of the antiserum over several days produced lymphopenia which became less marked with continued treatment. Clear-cut depletion of small lymphocytes in lymph nodes was noted.

McGregor and Gowans (27, 28) showed that chronic depletion of rat small lymphocytes by a thoracic duct fistula led to a marked reduction in various humorally mediated immunologic responses, as well as a definite prolongation of first set allografts. They were unable to affect secondary humoral responses or second set grafts. They could not conclude, however, that lymphocytes are not

involved in the latter reactions, in view of a personal communication from Mithchell and Gowans that a typical secondary response followed the first challenge of rats with diphtheria toxoid occurred, if these rats has previously been given lymphocytes from other, primarily sensitised rats.

Woodruff and Anderson (38, 39) were the first to demonstrate marked prolongation of skin graft survival with antilymphocyte serum; they used a rabbit anti-rat lymphocyte serum, and showed prolonged survival even when distantly related rats were used as donor and recipient. They reported that the lymphocyte count tended to rise during the course of serum treatment. Sacks et al. (32) showed that, in rats given rabbit anti-rat lymphocyte serum, the degree of lymphopenia at 24 hours was dose dependent; furthermore, the lymphopenic effect persisted on long-term treatment. To explain this effect, they suggested, but did not document, that their serum was able to suppress antibody formation against itself. Hemagglutinins and hemolysins present in the serum could be absorbed without interfering with the lymphopenic effect. This has been a consistent observation in reports from other laboratories.

Sacks et al., in the same article, reported gel-diffusion studies that demonstrate precipitin activity of their antilymphocyte serum against a variety of rat tissue homogenates. Precipitin bands were formed against lymphoid tissue, kidney, liver, and (weakly) muscle. A spur was seen on the middle lymph node band that went beyond the

joining kidney band. Two bands were seen with the lymph node homogenate that were not present against other tissues. These data were interpreted to suggest that lymphoid tissue may contain antigens not present on other tissues.

Nagaya and Sieker (29) showed that an antiserum to thymus cells was more effective, as measured by lymphopenia and allograft survival, than antiserum to mesenteric lymph node cells. The results previously observed in rats have been reproduced in mice by Gray et al. (10), Monaco et al. (26), and Levey and Medawar (20,21). These groups demonstrated prolongation of second-set as well as first-set skin grafts.

James (15) has reviewed many current studies demonstrating usefulness of antilymphocyte antibody in protecting renal transplants against rejection, as well as its effect on humoral antibody formation and other immune phenomena. Whole serum and globulin fractions have been immunosuppressive in just about all systems studied, except, notably, formation of secondary humoral antibody (e.g. Monaco et al., 26).

Gray, et al. (10) reported that blood taken from mice one week after the last of 9 injections, over two weeks, of .25 c.c. of rabbit anti-mouse lymphocyte serum, contained little or no activity against rabbit gamma globulin, as compared to high titers in mice given equivalent doses of normal rabbit serum. The control was thought to rule out immunological paralysis as a mechanism, and the authors interpreted the result as showing specific immune suppression,

by their serum, of antibody formation against itself. Lance and Dresser (19), however, showed, in a very elegant experiment studying disappearance rates of isotopically labelled globulins, that antilymphocyte serum could be immunogenic in doses producing potent immunosuppression. In fact, it seemed to be more immunogenic than normal serum. Their data were interpreted to suggest that unresponsiveness in mice to rabbit serum, after prolonged periods of exposure to rabbit anti-mouse lymphocyte serum, may actually represent immunological paralysis. The key point, in order for antilymphocyte serum to maintain unresponsiveness against itself would seem to us to be the use of it in high doses continually; this conclusion is consistent with both protocols. Lance and Dresser's protocol shows immunogenicity of antilymphocyte serum, as measured by rapid elimination of a dose of normal rabbit serum administered several weeks after the injections of antilymphocyte serum; in contrast, Monaco's observation is that a single, intensive course of a potent serum can fail to produce measureable anti-globulin activity when there is no subsequent challenge. Indeed, in a more recent paper, Russell and Monaco (31) have shown that a small injection of their serum, followed several months later by a second injection, will indeed incite formation of anti-globulin.

Starzl et al. (34) have recently shown that human renal allograft recipients treated with anti-lymphocyte serum in their series developed significant precipitin titers against ALS, and that this reaction could be

attenuated by simultaneous use of steroids. Their data also suggest that a spontaneous decline in late host reaction to foreign protein may occur during serum treatment. It is not clear from his data whether this phenomenon represents specific ALS-induced immunosuppression of reaction against itself, or a less specific immune paralysis.

James (15) has reviewed other studies which show in recipients of antilymphocyte serum, either antibodies against donor globulin, rapid "immune" elimination of anti-lymphocyte IgG, or "immune complexes" in transplanted organs. Monaco's serum seems to be among the strongest of those reported so far, at least as measured by persistence of lymphopenia, and suppression of reaction against itself. Perhaps the clinical lesson is that very strong sera or fractions must be produced if ALS is to enjoy clinical usefulness, and, again, that it must be used in high doses without interruption if used at all.

Much has been written about the specificity and mode of action of anti-lymphocyte serum. The obvious assumption from early experiments might be that the serum acts specifically on lymphocytes, and that the mechanism is cell lysis. Recent reports have called into question both of these ideas, however. We shall here attempt to review current studies relating to these two questions. It is difficult to make a complete distinction between specificity and mode of action; some objections pertain to both questions. We shall treat the questions separately, however, even though the discussions will overlap somewhat.

Gray, et al. (10) feel that their serum acts primarily by specific immune lysis of lymphocytes, both central and peripheral. Their serum had strong agglutinating and cytotoxic activity against mouse lymph node cells of several strains, as well as precipitin activity in gel diffusion against mouse lymph nodes, spleen, liver, kidney, and muscle homogenates. As with Sacks's (32) earlier gel diffusion study, serum diffusing against spleen and lymph node homogenates produced some lines which were not present against other tissues. In addition, these authors showed that absorption with mouse liver and kidney removed precipitin lines against these tissues, but left unaltered the precipitin line against spleen and lymph nodes. In contrast, absorption with spleen or lymph node cells removed precipitin lines against all tissues. Cytotoxic activity of their serum against lymph node cells could also be markedly reduced by absorption with spleen or lymph node cells, but not with liver or kidney cells. The same effects were noted (but data not presented) on lymphagglutinin titers. Finally, absorption with lymphoid cells could remove the lymphopenic effect of a diluted sample of serum, whereas absorption with liver cells could not remove this effect. The authors concluded that their serum contained antibodies directed against antigens specific to lymph node cells. Not all their data are conclusive, however, for several reasons. First, the absorptions in their lymphopenia experiment do not seem to have been complete with lymphocytes, nor to have been totally ineffective with liver

cells, at least as measured by absolute lymphocyte levels after treatment; in addition, it seems they only used one animal for each serum tested. Second, they did not titrate cytotoxic antibody, but rather measured the effects of undiluted sera alone. The maximum percent dead cells in their cytotoxic experiment was 55%. Our experiments have shown that meaningful data with cytotoxicity (at least against myeloma cells) require titration with serial dilutions, and that the lowest dilutions with antiserum characteristically show 98-100% cytotoxicity against the cell the serum was raised with. Thus it seems difficult to interpret some of the data of Gray, et al., with the same enthusiasm as the authors do. Nevertheless, the profound and persistent lymphopenia they achieved, as well as their gel-diffusion and lymphagglutinin results, strongly support their conclusions. In a later report, the same group, (31) has shown a picture of a frozen section of a human kidney, which they exposed to rabbit antihuman lymphocyte serum, washed, and covered with fluorescein-labelled goat anti-rabbit gamma globulin antibody; selective adherence of the ALS to lymphocytes in capillary tufts is clearly demonstrated. This adds much weight to their contention that immunization with lymphocytes can induce cell-specific antisera.

Woodruff and Anderson (40) noted uptake of antilymphocyte antibody, using a similar fluorescent technique, by in vitro suspensions of thoracic duct lymphocytes. In this report, they did not give data on similar tests with

non-lymphoid cells, but they gave evidence that their rabbit anti-rat ALS had an insignificant cytotoxic titer against peritoneal macrophages of the rat, as opposed to a high titer against rat lymphocytes.

Evidence has been presented, however, that antilymphocyte serum is not specific in any histological sense. Levey and Medawar (20) have shown that sera prepared in rabbits against mouse L cells and mouse basal epidermal cells exerted a significant prolongation on murine skin allograft survival. None of their sera here was prepared according to the protocol they used for all their antithymic serum. A serum prepared with a number of L cells 5 times the usual number of thymocytes used, with the same protocol of rabbit injections and bleeding, prolonged the mean survival time (MST) of allografts 3.7 days beyond the MST of control allografts, and prolonged none by more than 5.4 days. Antithymic serum administered in the same way, as reported in a different paper (21), prolonged survival of all grafts at least 8.5 days, half the grafts by 18 days, and two (of twenty) by 38 and 52 days, respectively. Their two strongest anti-epidermal sera prolonged the MST by 12.1 and 6.6 days, and no grafts were prolonged by more than 17.4 and 9.4 days, respectively. Fewer cells were used in each injection, but the active sera were harvested after four and five injections, respectively, whereas all their antithymic sera were harvested after two injections. Thus it is impossible to quantitate the difference among the effects of their sera, although

clearly antithymic serum had the most profound effect. It is unfortunate that the authors did not describe the effects of their anti-epidermal and anti-cell sera on peripheral lymphocytes. Nevertheless, the prolongation exerted by these sera on allograft survival is significant, and the authors' contention that ALS is not specific in the histological sense has considerable merit. We shall discuss the point below, in reference to our own experiments.

The antithymus serum of Levey and Medawar (21) had little lymphopenic effect at four hours, after a single injection, but the lymphocyte counts apparently fell further and were recorded at 7 and 14 days as roughly 60-70% of the original level. Although Gray et al. (10) reported a substantial fall in lymphocyte counts at four hours, the percent of original lymphocytes remaining at ten days was not significantly different in their study from that attained by Levey and Medawar.

Several investigators have reported that, during treatment of rats with ALS, lymphocyte depression was not always maintained during prolonged allograft survival (Woodruff and Anderson, 38; Nagaya and Sieker, 30; Anderson, James, and Woodruff, 1; Sacks, et al., 32). Certainly these observations might cast doubt on the specificity of ALS for lymphocytes, or, alternatively, they might call into question the cytotoxic theory of action of ALS (which is discussed below). Indeed, Levey and Medawar have implied (20) that return of peripheral lymphocytes towards normal levels may be irrelevant or even desirable. Interpretations of these data as

disproving specificity of ALS for lymphocytes could be overcome if it could be shown that the return of lymphocytes towards normal during treatment represents a compensatory increase of a sub-population of unsensitized cells, or cells that cannot be sensitized (Levey and Medawar's "sterile activation" theory, discussed below). Similar confusion about lymphocyte levels exists in canine transplantation experiments; again, Monaco's group (25) noted persisting lymphopenia, using material prepared with adjuvant, while Starzl's group (33) reported inconsistent lymphopenia.

Aside from the issue of the actual specificity of ALS, there are several different theories as to its mode of action. These theories have been reviewed in detail by Levey and Medawar (21, 23), Russell and Monaco (31), and James (15). The most prominent are the cytotoxic, blindfolding, competitive antigen, and sterile activation theories.

The first and most obvious theory is that ALS acts essentially as a lymphocyte depleting, or cytotoxic, agent. We have seen that Gray, Monaco, and Russell have strongly advocated this theory. We have cited several papers which show that with some sera lymphopenia need not be marked or sustained during periods of immunosuppression (a fact which is also relevant to the question of specificity). Levey and Medawar (21) have maintained that "the lesser immunosuppressive action" of the thoracic duct drainage experiments of McGregor and Gowans (27, 28), which produced "a

greater lymphocyte depletion [than is observed with ALS] " argues against the cytotoxic theory. McGregor and Gowans observed that thoracic duct drainage significantly prolonged survival of first-set grafts in their non-inbred rats. A much lesser but significant effect was recorded in "distantly related" animals. The authors were unable to prolong second-set grafts, when the course of depletion was begun immediately after first-set rejection. Levey and Medawar (21) got a significant prolongation of second-set graft survival in mice with their antithymic serum, but they began the experiment 14 days after first-set graft rejection, when presumably there may have been more of a decline in quantity and quality of sensitization, or when "memory cells" may have moved from lymph nodes into the more accessible circulation. It seems Levey and Medawar's arguments cannot be accepted until experiments are reported in a single species with parallel courses of thoracic duct drainage and anti-lymphocyte serum administration. Woodruff and Anderson (38, 39) have reported a synergistic effect on rat allograft survival of ALS treatment and thoracic duct drainage. They did not do parallel studies isolating the two techniques, however. Examination of their graphs reveals that a seven day course of ALS (prior to grafting) had a more profound lymphopenic effect than thoracic duct drainage done over five days.

An impressive series of experiments has recently come out of Woodruff's laboratory on the immunosuppressive properties of various fractions of ALS IgG. James (15) has

interpreted the failure of the non-cytotoxic $f(ab')_2$ antibody fragment to produce immunosuppression (1, 14, 16) as favoring the cytotoxic theory. This conclusion, of course, assumes that in vitro cytotoxicity and in vivo cell lysis are equivalent phenomena.

Perhaps selective destruction of a sub-population of lymphocytes in a state of readiness to undertake immune responses, and with proportional sensitivity to antilymphocyte serum, is the mechanism (20, 16), and failure to achieve gross depletion of the lymphocyte population need not in itself nullify the cytotoxic theory (14).

There are several other theories, based on coating of lymphoid cells by antilymphocyte serum without lysis. These are the blindfolding, the competitive antigen, and the sterile activation theories; they tend to overlap somewhat.

Levey and Medawar (22) showed that lymphocytes from CBA mice that had previously rejected skin from C57 mice could lyse C57 fibroblast monolayers in vitro, and that such lysis could be inhibited by prior incubation of the lymphocytes with heterologous antilymphocyte serum. "Blindfolding" of lymphocyte combining sites or recognition units might then, they proposed, explain the in vivo mechanism independent of cytotoxicity. In further work, however, (20) this group found that cells from serum treated donors failed to restore immunological competence to previously irradiated recipients, even though these cells presumably must have undergone several generations of division, and

lost their antibody coat. Russell and Monaco (31) have recently cited unpublished observations by Dr. B. van der Werf in their laboratory, showing that lymphoid cells from ALS treated animals failed to produce the usual graft versus host reaction in newborn recipients, tending to confirm the previous experiment in disproving the blindfolding theory. Russell and Monaco concluded that the population of cells remaining in lymph nodes after serum treatment was definitely incompetent - either from some form of selective destruction of competent cells, or because of converstion of competent cells to an incompetent form. The latter suggestion, of course, is a departure from their previous cytotoxic theories.

Guttman et al. (11) have recently suggested a variant of the blindfolding theory, that the antibody coats the graft tissue, which shares antigens with lymphoid tissue, thus preventing histocompatibility antigen release, or recognition by the lymphocyte of such antigen (a mechanism similar to immunological enhancement). The authors demonstrated that prior treatment of F_1 hybrid rat donors with an antithymic serum leads to a definite uptake of globulin in donor kidney tissue, and that transplantation of kidneys from these animals into parent strain untreated recipients led to definite slowing of rejection. Perhaps Levey and Medawar's anti-epidermal serum (20) might work in part through this mechanism in prolonging survival of allografts of skin. We have seen how Sacks et al., and Gray, et al., have shown by gel diffusion that their rabbit anti-rat lymphocyte sera cross-react with a number of tissues, but

that there are probably specific lymphocyte antigens not shared by other tissues. Iwasaki et al. (13) have maintained that multiple absorptions of their anti-dog lymphocyte serum with liver and kidney cells absorbed out 90% of the agglutinating activity of the serum. They concluded that most of the antigens of the lymphocyte in the dog are represented in other tissues. They failed to show whether there are any lymphocytes in dog kidney and liver parenchyma. The only data they show indicate that a single exposure of their serum to kidney or liver reduces the leukoagglutinin titer from 1:4096 to 1:1024, and this is termed a 75% reduction in titer. They did not test the leukoagglutinin titer of normal horse serum, which is presumably negligible, nor did they test the titer of immune serum absorbed with identical quantities of lymphoid tissue. It is tempting to conclude from these data nevertheless, that most of the antigens on lymphoid tissue are represented on other tissues. However, other data, in the same report, show that absorption of their serum with liver and kidney did not alter its lymphopenic effect.

Cerilli, et al. (5) have recently repeated Guttman's experiment on the treatment of the donor of a kidney with antilymphocyte serum before transplantation, to test the graft-blindfolding hypothesis. Cerilli et al. used dogs instead of rats, and prepared their serum in horses. No effect on graft survival was noted, in contrast to Guttman's findings with inbred rats. The conclusion was that the immunosuppressive effect at the graft site is probably

small, and only noticeable in an inbred animal system. Certainly experiments must be done using sera prepared against tissues other than lymphoid organs in dogs to clarify this point. We have not seen reports, in any system, on the effect of absorption by other organs on in vivo immunosuppressive action of antilymphocyte serum.

The "competitive antigen" theory, in many ways similar to the blindfolding theory, differs in involving a specific and preferential immunological commitment of lymphocytes to anti-lymphocyte antibodies acting as antigens (15). This theory is perhaps supported by the work we have quoted showing that, under proper conditions, antibody to ALS or normal IgG from the species donating the serum may be noted in recipients. James (15) discusses the point further.

The theory of "sterile activation" has been proposed by Levey and Medawar (20). The theory is based on their observations, and those of other, (6,7) that lymphoid hyperplasia and the formation of blast cells occurs in lymphoid organs with certain sera and protocols. (In contrast, Gray, et al. 10, and Waksman et al., 35, reported depletion in their studies. Although Monaco and Russell's group have consistently seen lymph node depletion in all their experiments, they have recently reported (31) "large, foamy lymphoid cells in the periphery similar to many of those left in lymphoid tissue.") Levey proposed that antilymphocyte sera may at least in part act through a sterile activation of lymphoid cell size and growth rates, forestalling

all other immunological commitments. Gräsbeck (9) showed earlier that rabbit anti-human lymphocyte serum can activate in vitro human lymphocytes mitotically. Woodruff et al. (41, 42) have recently shown that horse anti-human lymphocyte IgG can stimulate the uptake of isotopically labelled nucleosides into lymphocyte nucleic acid. Ling et al. (24) have done a similar experiment, and they showed that there is not a constant correlation between transforming antibodies and either cytotoxic or agglutinating antibodies.

There are two major obstacles to the immunosuppressive relevance of blast transformation and its in vivo equivalent, sterile activation. First stand the observations of Woodruff et al. that the same effect is achieved by the divalent $f(ab')_2$ IgG fraction (41); this fraction is non cytotoxic, and has no effect on immune responses, at least in rats (1, 14). Second, complement must be excluded from all in vitro systems using whole immune serum or intact IgG, to get blast transformation; otherwise, lysis occurs (9,24). It is not unreasonable to assume that recipient complement has access to sites of action of heterologous antilymphocyte serum. It would seem that the crucial experiment to determine the immunosuppressive significance of in vitro blast transformation would be to test whether antibody treatment, without complement, can depress immunologic responses of lymphocytes in culture. To our knowledge, no such experiment has been published; until it is, the role of sterile activation in ALS-induced

immunosuppression seems to be in doubt.

Many questions about the specificity and mode of action of antilymphocyte serum are obviously unanswered. Questions about its method of action are certainly of more than academic interest, especially in respect to the rationale for selecting appropriate tests for assaying potency of serum batches. Cytotoxicity in vitro, lymphopenia in vivo, lymphagglutination, or in vitro blast transformation are all possibilities, but it is not yet known which is the best test.

It would seem that the more pressing practical question,however, concerns the specificity of the serum. It seems clear from many papers we have considered, that there are many antigenic determinants on the lymphocyte of all species studied. A key question, as yet not fully answered, is whether there are any antigens peculiar to the lymphocyte. The gel diffusion studies of Gray et al. (10), as well as those of Sacks et al. (32), indicate there may indeed be specific groups on lymphocytes. Iwasaki's serum (13) induced lymphopenia after liver and kidney absorption, but there are other data in his paper in conflict with this observation. Certainly Levey and Medawar (20) attained immunosuppression with anti-epidermal, and to a far lesser extent, with anti-L cell serum. Levey refers (23) to unpublished work of S.V. Joost that rabbit anti-mouse fibroblast serum has a similar effect. But in all of these observations of Levey and Medawar, the immunosuppression achieved by such sera had a much

lesser effect than their own antithymic serum.

It would seem consistent with most reported results that there may be some lymphoid-specific antigens. At any rate, a most important consideration, consistent with most available data, is the possibility that circulating lymphoid tissue is a highly vulnerable target, much more so than any solid tissues, for reasons of either structural weakness, high concentration of antigen (either cell or species specific), or just general physical availability. For any of these reasons, antibodies raised against lymphocytes might be expected to act more quickly and/or effectively against lymphocytes than against tissues not involved in the immune response, even if non-lymphoid tissues should possess potential binding sites.

PURPOSE OF THE EXPERIMENT

While working as a research assistant in the laboratory of Drs. Paul S. Russell and Anthony P. Monaco in the summer of 1966, the author became intrigued with the possibilities for use of antilymphocyte serum in clinical transplantation work. Access at that time to a draft of work later published by Levey and Medawar (20) raised many doubts about the specificity of such sera for lymphocytes. Accordingly, it was thought appropriate to raise a serum against a pure cell line, in the mouse, of non-lymphocytic and non-graft origin, and then to attempt to elicit and characterize any activity of this serum against lymphocytes in vitro and in vivo, comparing this activity with any effect this serum might have on the allograft response.

We selected murine myeloma cells as an appropriate cell line; we received a specimen of the transplantable C3H myeloma X5563, and have maintained this tumor in subcutaneous form in this laboratory. Histology of this tumor reveals a rather well differentiated cell population, resembling closely the appearance of normal plasma cells.

MATERIALS AND METHODS

Animals

White New Zealand female rabbits (about 2.5-3.0 kg.)

were used to prepare all antisera. Inbred adult female mice of the C3H/HeJ strain were used to carry the tumor, and as recipients of all sera and skin grafts. Adult female Balb/c mice were used as graft donors. All mice were obtained from Jackson Laboratories in Bar Harbor, Maine, and were not bred in this laboratory.

Preparation of Myeloma Cells for Immunization

It was found convenient to maintain the tumor in subcutaneous form. About ten days to two weeks after injection of cells into C3H mice under flank skin, a large, firm mass becomes evident. On exposure, these tissue masses are usually white and smooth, with moderate vascularization. For purposes of immunization, tumor cells were pressed through a wire screen by a garlic press into Hank's medium, under sterile conditions, to separate the cells. The method of Gordon et al. (8) was employed to separate out non-myeloma elements (this method was originally described for use with ascites forms of X5563 myeloma, but was found adequate for solid subcutaneous tumors here). Cell suspensions were centrifuged at 600 RPM in a 240 head for 3 minutes, after which all supernatent, and, when necessary, red cell rings, were removed by aspiration. When this procedure had been repeated 10 times, a preparation of 99% myeloma cells was attained.

Preparation of Anti-Myeloma Serum

The protocol of Gray et al. (10) was followed. An appropriate number of cells was suspended in Hank's

medium, and emulsified with an equal volume of complete Freund's adjuvant. Several rabbits received 0.2 ml of the emulsion into each foot-pad, to give a total of $100x10^6$ cells per rabbit. Booster injections of cell suspensions ($100x10^6$ cells again) in Hank's medium were given to each rabbit through an ear vein on three successive days 4 weeks later. The rabbits were bled by cardiac puncture 7 days after the last injection, and on one or two subsequent days in those who survived the first massive bleeding. All blood samples were allowed to clot and stand in the cold (4° C) overnight. Some tubes of immune and normal serum showed evidence of gross hemolysis after clotting. All sera were separated from the clots and pooled, diluting out to unnoticeable color any hemoglobin contamination. All sera were immediately heated to 56° C for 30 minutes, and stored at -20° C until use. No significant hemagglutination activity was found in either the immune or normal pools, and no red cell absorptions were carried out.

A sample of rabbit anti mouse (A/Jax strain) lymphocyte serum, prepared by the same protocol, was kindly donated by Dr. Anthony P. Monaco.

<u>Cytotoxic Antibody Assay</u>

a.) Preparation of Cells

Myeloma cells were harvested from a subcutaneous tumor masses in a manner similar to that described above, but sterile technique was not rigidly observed, and the tumor cells were treated with extra care so as not to damage

cells. Tumor masses were cut to small size with two Bard-Parker #11 scalpel blades, then teased gently to release cells. The suspensions were then either poured through a wire mesh, or through a piece of cotton gauze, to filter out large particles. The cell suspension was allowed to stand for 15 minutes at room temperature to allow smaller clumps to settle, whereupon the supernatant was poured off and used as a source of viable myeloma cells. There was moderate red cell contamination, of course, but it was not felt necessary to separate the two cell populations by centrifugation; indeed, this probably would have caused unnecessary damage of tumor cells.

b.) Cytotoxic Test Procedure

A simplified test described by Boyse et al., (3) and used by Gray et al. (10), was repeatedly attempted without success. This method consists of adding cell suspensions, serum dilutions, and complement directly into vaseline rings on ordinary slides, incubating at 37°, and reading directly the per cent dead (stained) cells. Perhaps our source of lyophilized guinea pig complement was inactive. At any rate, it was found that the vaseline rings tended to melt on the microscope from the heat of the lamp, and frequently all the cells clumped against the vaseline ring. We rarely saw saw a dead cell, and the "simplified" test seemed very unwieldy anyway, so we discarded it, along with the reconstituted lyophilized complement.

Eventually, with the assistance of Dr. Kikuo Nomoto, we achieved significant and reproduceable results using a

modification of a cytotoxic technique described later by Boyse, et.al. (4) All incubations were done in clean, dry small test tubes. To each tube was added .25 c.c. of doubling dilutions of serum to be tested, whereupon 0.1 c.c. containing 10^6 cells was immediately added to each tube. The mixture was allowed to stand at room temperature for 15 minutes to allow fixation of antibodies. Then, to each tube was added 0.1 c.c. of a 1:5 dilution of freshly prepared (within 1 week) guinea pig serum as a source of complement. Tubes were then incubated at 37° for 30 minutes. Immediately before reading each tube, 0.1 c.c. of freshly prepared 0.2% trypan blue was added, and the tube was then gently shaken. A drop of the cell suspension was then placed in a standard white cell counting chamber, and the per cent dead cells (blue stained) was determined. All tubes that were not going to be read soon, when a lot of tubes were to be counted, were put at 4° until about ten minutes before reading. Tubes were read in parallel, i.e., all the tubes of the same dilution, from each series of tubes, were read together, to eliminate bias due to any cell death while tubes were waiting to be read. All results of cytotoxic antibody determinations have been expressed as titration curves of percent dead cells vs. dilution of antiserum used. Controls in every experiment included per cent dead cells in initial sample (diluted in Hank's only, and not incubated), as well as two incubated complement controls, read at the beginning and at the end of all readings, respectively. Normal rabbit serum had no activity

against any cell tested, and it was not felt necessary to titrate it every time a cytotoxic assay was done after the first couple of times. All dilutions were done in Hank's medium with 2% by volume normal rabbit serum.

Absorptions

a.) Cell Suspensions

Sterile cell suspensions were prepared as described above, with both lymphocytes and myeloma cells. Packed cells were mixed with antiserum, 5% cells by volume, suspended, and left overnight. Cells were gently spun down and the procedure was repeated for a total of three times.

b.) Serum Absorptions

An experiment was designed to determine the minimum quantity of C3H serum needed to absorb out all anti-C3H serum protein (or, perhaps, anti-myeloma-produced globulin) activity, from the immune serum. To successive tubes containing 0.5 c.c. of anti-myeloma serum were added aliquots of C3H serum, either straight or diluted representing 0.3 c.c down to 0.01 c.c. After the tubes had stood overnight, a precipitate was visible in all tubes, and addition of more C3H serum to the supernate of all tubes produced no more precipitate. Accordingly it was judged that 2% by volume of C3H serum was adequate to absorb anti-serum protein activity out of anti-myeloma serum, and this was done with a large amount of the serum.

In Vivo Effect on Circulating Lymphocytes

A series of 8 mice was injected with various sera according to the protocol elaborated below, to assay the lymphopenic effects of our anti-myeloma serum, normal rabbit serum, and Dr. Monaco's anti-lymphocyte serum. All blood samples were obtained from animals under light Nembutal anesthesia (0.1 c.c/gm of a 7 mg./ml. solution of Nembutal, administered i.p.). The anesthetized animals were placed under a warm lamp to dilate their tail vessels, and a small sample of blood was drawn from a nick in the tail into a white cell diluting pipette, and diluted 1:20 with 0.1% HCL. Total white cell counts were done in a standard counting chamber. A drop of blood was placed on a glass slide, smeared, and stained with Wrights stain for determination of per cent lymphocytes. Total lymphocyte counts were computed.

Skin Grafting

Ventral abdominal and thoracic skin sections, about 1 cm^2, were taken from donor mice and grafted on the dorsal thoracic wall of recipients, according to the method of Billingham (2). The selection of the respective sites for removal and placement of skin grafts was determined by the need to obtain skin from an area with fewer variations in hair cycles (Dr. Masao Hanaoka - personal communication), and to place grafts where the recipients could not bite or scratch them off. Plaster casts were removed on day 6 (this required anesthesia), and the grafts

were followed daily by visual inspection, until destruction was essentially complete (10% or less of graft surviving). We have not used the same strains as either Monaco and Russell (26, 31), or Levey and Medawar (20, 21). However, all of these investigators have noted, in the same references, that strong histocompatibility differences between donor and recipient mice were easily overcome by anti-lymphocyte serum. Although we have not raised an anti-lymphocyte serum for control, we feel our grafting results are comparable to theirs. Our recipient strain was chosen because of the availability of a myeloma in the same strain (C3H), and our donor strain (Balb/c) was chosen for non-scientific reasons (these mice were available at the time).

RESULTS

Cytotoxic Antibody Studies

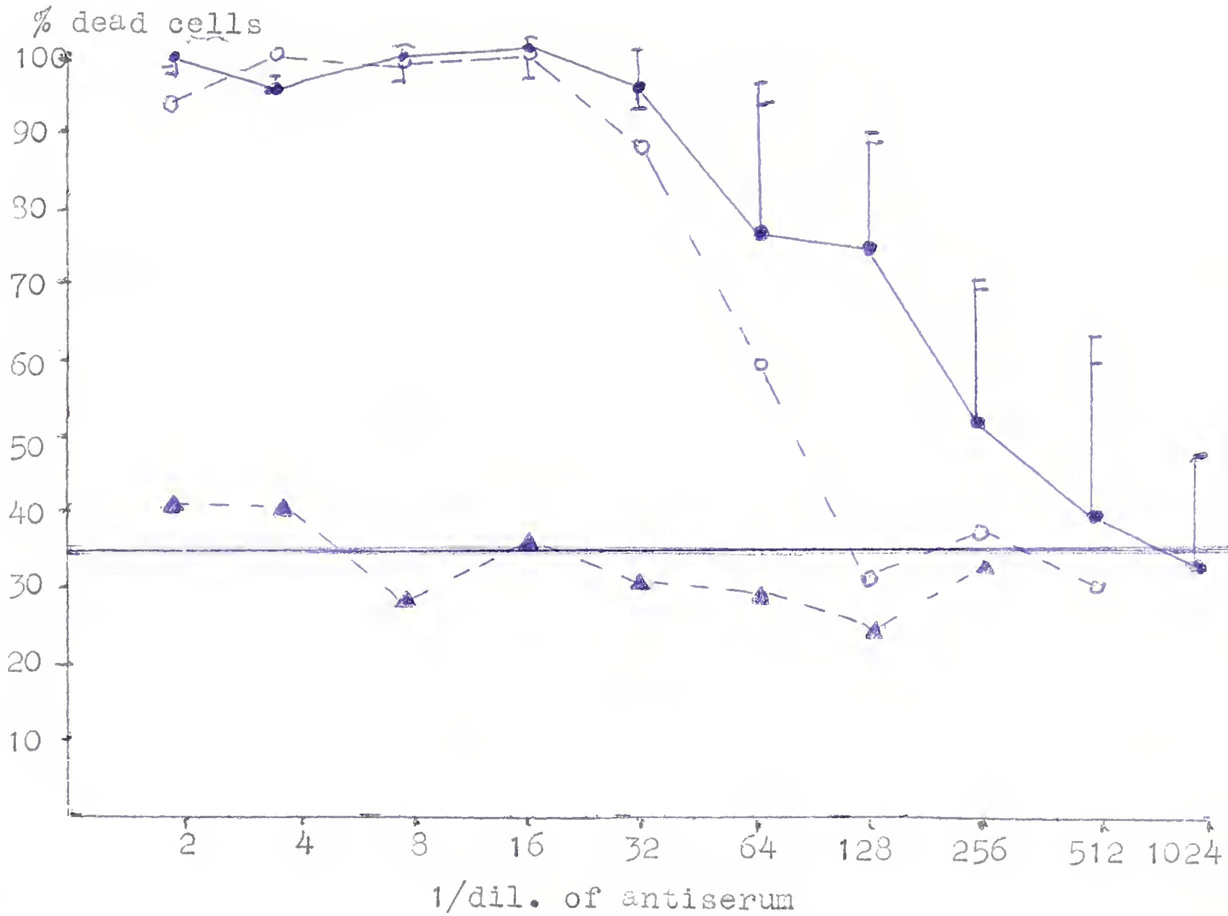
Several attempts at perfecting the technique were tried,with unsatisfactory results. Data from these early experiments will not be presented.

Figure I shows an experiment done with the perfected technique, showing cytotoxic titrations of anti-myeloma serum (AMS), anti-lymphocyte serum (ALS), and normal rabbit serum (NRS), against myeloma cells. The heavy horizontal line represents the percent dead cells in the initial suspension. Initial and final complement controls were in the same range. Clearly, our AMS has a very high cytotoxic titer against myeloma cells. Dr. Monaco's antilymphocyte serum has significant activity against the same cells, but clearly the AMS has much more effect at higher dilutions. Normal rabbit serum has no cytotoxic effects. Points from titrations performed with AMS against myeloma cells on two other occasions are depicted also. The line is drawn through the points attained with the same cell sample used for the single ALS titration,however.

Figure II,depicting an experiment kindly done for us by Dr. Kikuo Nomoto, demonstrates that AMS has a significant but rather low cytotoxic titer against lymphocytes.

Figure III represents cytotoxic titration of AMS done in parallel with samples of the same serum absorbed with lymphocytes (AMS/L), and with myeloma cells (AMS/M). Clearly our absorptions were inadequate, as the absorption

FIGURE I - Cytotoxic activity of AMS, ALS, and NRS against X5563 myeloma cells



Horizontal line at 35% = per cent dead cells in initial cell sample
Initial complement control - 35% dead cells
Final complement control - 25% dead cells

Legend

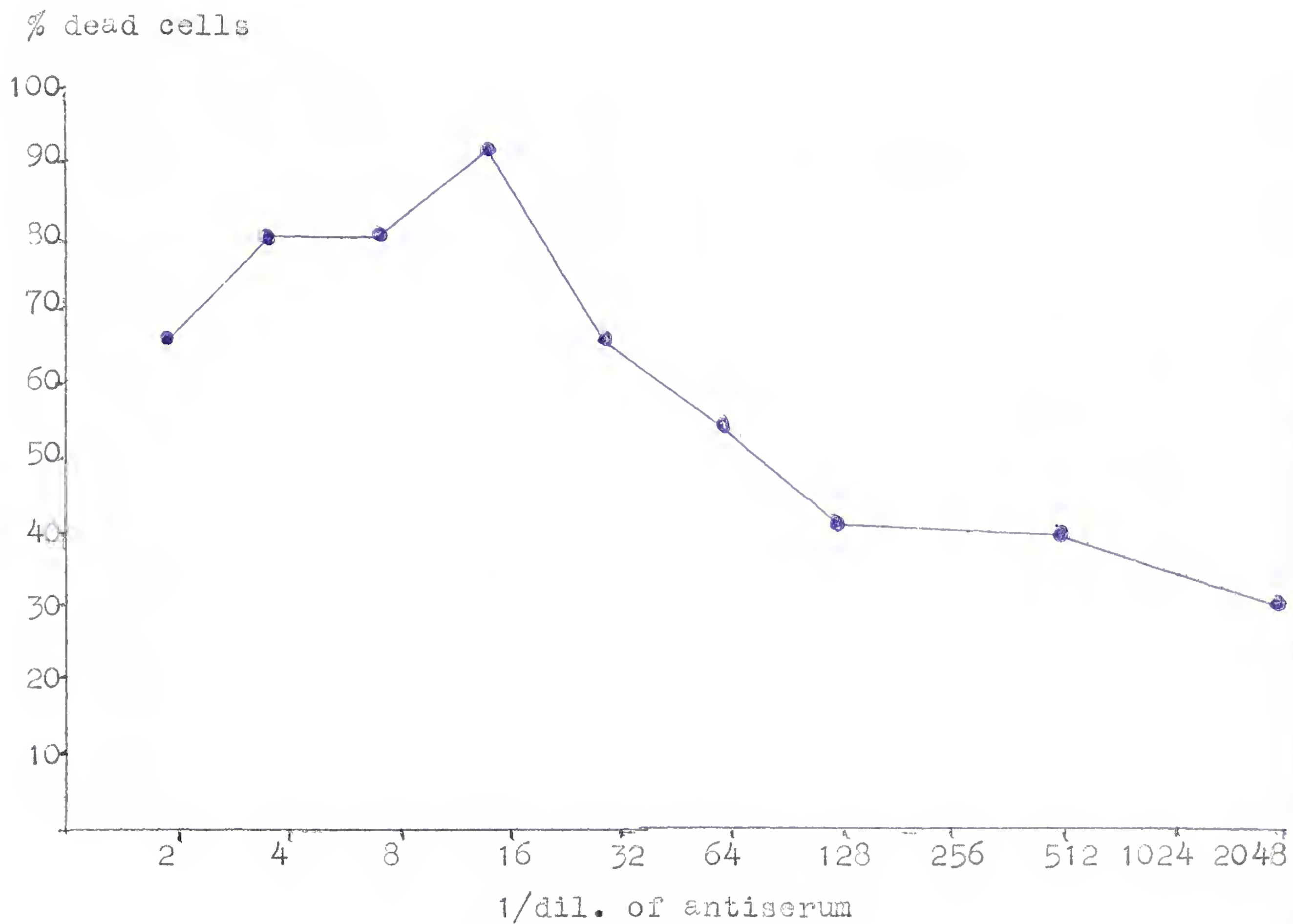
●——● AMS

○- - -○ ALS

▲- - -▲ NRS

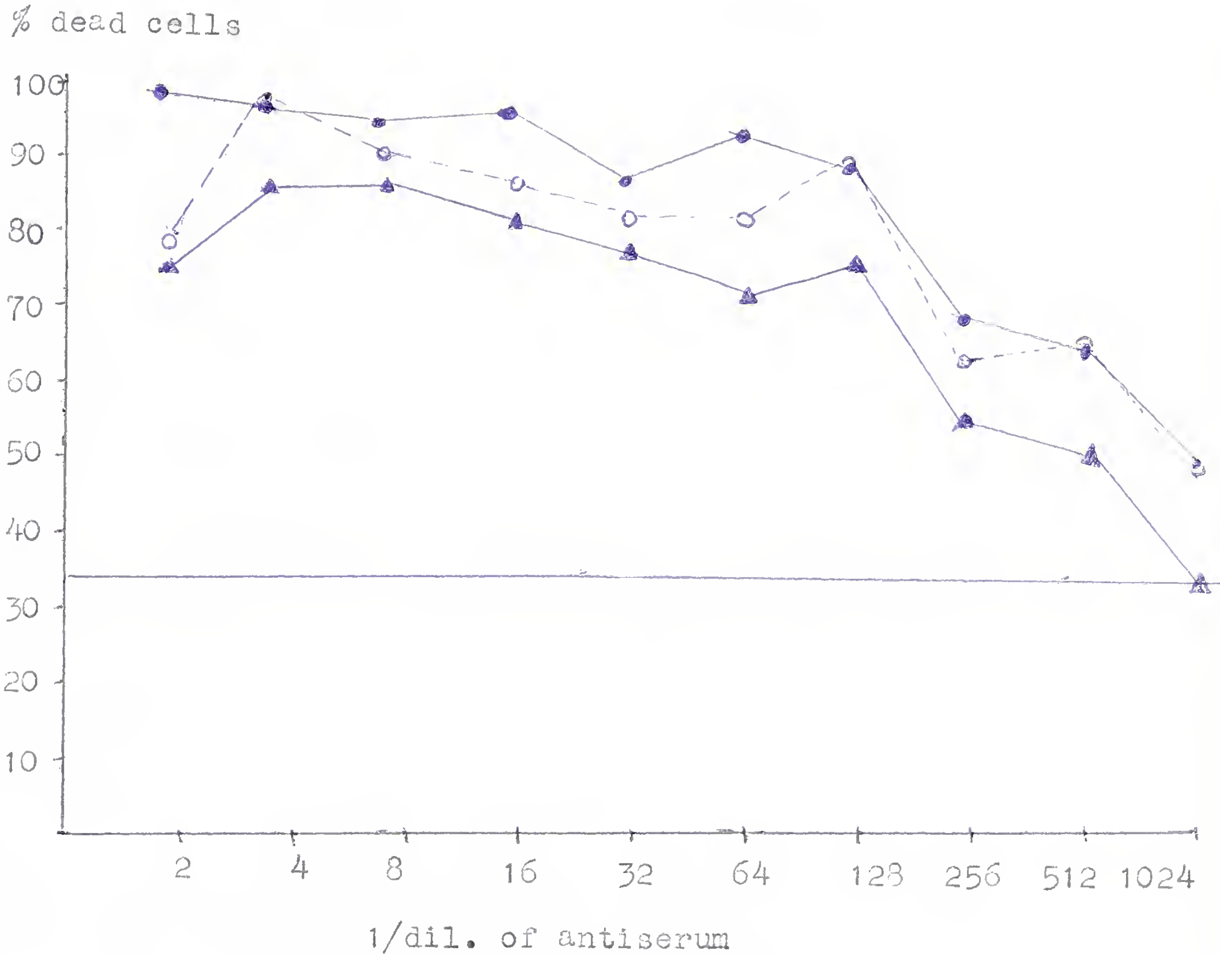
The three connected lines represent titrations done simultaneosly with the same cell sample. Separate points from AMS titrations done at two other times are drawn, but not connected.

FIGURE II - Cytotoxic activity of AMS against C3H lymph node cells



Complement control - 21%

FIGURE III - Titration of cytotoxic acivity of AMS, AMS absorbed with lymphocytes (AMS/L), and AMS sbsorbed with myeloma cells (AMS/M) against X5563 myeloma cells



Horizontal line represents 33% dead cells in initial cell sample
Initial complement control - 29% dead cells
Final Complement control - 31% dead cells

Legend

AMS

AMS/L

AMS/M

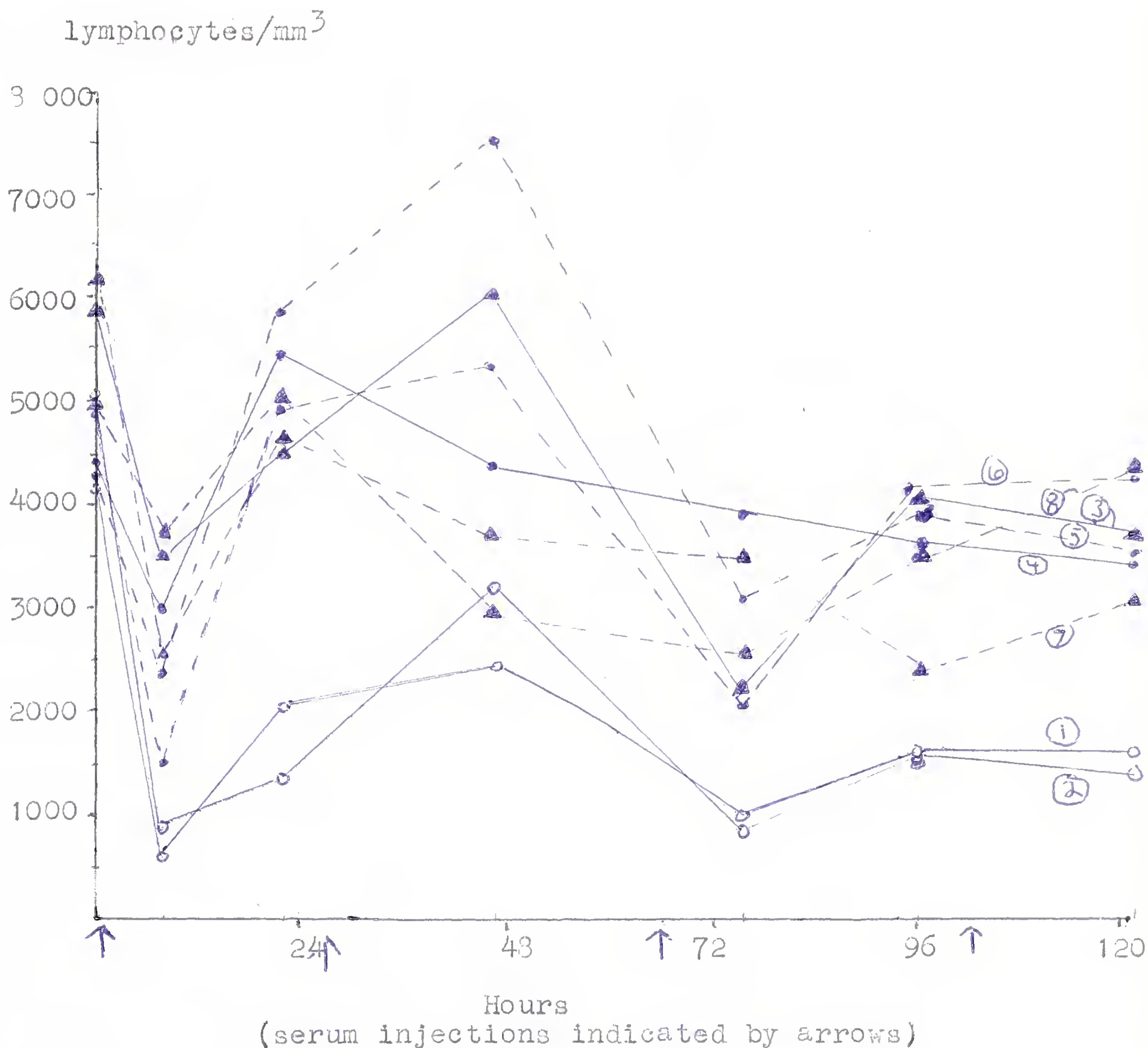
with myeloma cells should have lowered the curve to the baseline of % initial dead cells. It is tempting to conclude that, since the AMS/L curve is not lowered as far as the AMS/M curve, there are antigens on the myeloma cell foreign to the lymphocyte. This cannot be concluded from the experiment, however, until we are able to absorb the serum fully with myeloma cells, and perform equivalent lymphocyte absorptions.

It is clear, however, that our AMS has a high titer against X5563 myeloma cells, much higher than that of Dr. Monaco's very potent ALS, which was prepared identically. Our AMS also has a very low titer against lymphocytes. These data demonstrate that lymphocytes and myeloma share some antigenic components. One might argue also that they show that each cell type has specific antigens not shared by the other. Other factors may be involved in determining the cytotoxic titer of a serum versus a cell different from that against which it was raised, than the degree of antigenic identity of the two cell types one is dealing with. We hesitate to conclude with certainty, then, that lymphocytes and myeloma cells both have antigens not shared by the other, from this experiment alone. To be sure, we will have to perform more complete absorptions of each serum with both cell types, getting each serum completely absorbed with its "own" type of cell, absorb equivalently with the other cell, and see if any activity remains. Gel diffusion studies, not included in this protocol, are also planned.

Lymphopenia Experiment

Figure IV shows the results of an experiment performed over 120 hours on 8 C3H mice to determine the lymphopenic effects of ALS, AMS, and NRS. A total of 4 injections of serum was given to each animal. Two animals received injections each time of 0.25 c.c. ALS, 0.25 c.c. AMS, and 0.75 c.c. AMS. One animal got 0.25 c.c. of NRS each time, and another got 0.75 c.c. of the same. It is evident that all sera had a lymphopenic effect 8 hours after the first injection. Clearly, ALS had a far more profound effect at this time than the other sera, and this difference was maintained throughout the experiment. Animal #6, given injections of 0.75 c.c. AMS, had a particularly marked fall at 8 hours, but to a level twice as high as that in the animal with the lesser response to ALS. After 24 hours the lymphocyte count of animal # 6 returned to the range of those of NRS recipients, where it stayed. At 46 hours, the lymphocyte count of animal # 8, which got 0.25 c.c. AMS each time, fell within the range of the counts of ALS recipients (which had risen to this level and subsequently fell). However, animal # 8's lymphocyte count was within the normal range at all other times. Animals # 5 and 7, also recipients of AMS, had lymphocyte counts within the normal range at all times. It is quite clear from this experiment that Dr. Monaco's ALS, raised against A/Jax lymphocytes, exerted a profound and persistent depression over the course of the experiment on the peripheral lymphocyte counts of C3H mice; our AMS, however,did not exert

FIGURE IV - Effect of ALS, AMS, and NRS on peripheral lymphocyte counts of C3H mice



Legend of serum and doses given with each injection	animal no. :
o—o 0.25 c.c. ALS	(1,2)
▲---▲ 0.25 c.c. AMS	(7,8)
•---• 0.75 c.c. AMS	(5,6)
▲—▲ 0.25 c.c. NRS	(3)
•—• 0.75 c.c. NRS	(4)

an effect significantly different from that of NRS, even though the AMS has a high titer against a cell of C3H origin which shares antigens with lymphocytes.

Skin Grafting

Several regimens of AMS treatment were tested for their effect on the allograft response. Four groups of C3H mice received daily i.p. injections, for 7 days, of 0.25 c.c. AMS, and AMS absorbed with myeloma cells, lymphocytes, and C3H serum, respectively. The following day (day 0) al mice were grafted with Balb/c skin. A control group received similar injections of NRS. None of these mice received any serum after grafting. Another group received injections of 0.25 c.c. AMS for 7 days prior to grafting, as well as 0.5 c.c. on days +2 and +6. Still another group received daily injections of 0.75 c.c.AMS for 5 days prior to grafting; 0.5 c.c. on days 0, +1, +2, and +3; and 0.25 c.c. on days +6 and +7. A control group received similar injections of NRS. A small number of animals had grossly infected grafts when plaster casts were removed on day +6, and the recipients of these grafts have been excluded from the study. Figure V depicts,day by day, the number of surviving grafts observed in each group. The day on which a graft was observed to be rejected, and removed from the survival table, was chosen as the numerical determinant if the number of days the graft survived. The mean survival time of grafts in each group has been computed and included in Figure V; it was not felt necessary

to calculate standard deviations. Clearly none of the serum regimens prolonged allograft survival significantly. In fact, the lower doses of AMS were associated with a slightly accelerated rejection, if anything. The sera absorbed with cells, we have seen, were not effectively absorbed, and can be considered as AMS. Injections in the mice that received the very high serum doses were cut back, on day +3, because one of the animals in the NRS cage was obviously cachectic with weight loss, lethargy, and tachypnea.

FIGURE V - Effect of various regimens of AMS and NRS on the rejection of Balb/c skin grafts by C3H mice

serum treatment	no. of grafts surviving per day post grafting — Day: 6	7	8	9	10	11	12	13	27	MST (days)
no serum	11	11	6	4	0					9.0
0.25 c.c. i.p. daily X7 pre-graft with:										
NRS	12	12	9	4	2	2	1	0		9.5
AMS	12	12	6	4	3	1	0			9.1
AMS/L	7	4	4	1	0					8.3
AMS/M	9	9	3	0						8.3
AMS/S	6	4	0							7.6
0.25 c.c. daily X7 pre-graft, and 0.50 c.c. on days +2 and +6: AMS	4	2	2	0						8.0
0.75 c.c. daily X5 pre-graft; 0.50 c.c. days 0, +1, +2, & +3; 0.25 c.c. days +6 & +7:										
AMS	6	6	6	5	3	0				10.3
NRS	3	3	3	2	1	0				10.0
C3H isografts	8	8	8	8	8	8	8	8	8	∞

AMS/L, AMS/M, AMS/S = AMS absorbed with lymphocytes, myeloma cells, and C3H serum, respectively

MST = mean survival time of grafts in each series.

Survival time is defined as the number of the day a graft was found to be rejected (i.e. the day a graft was removed from the survival table).

DISCUSSION

We have presented clear evidence that a serum directed against a cell of C3H origin, other than the lymphocyte, has no significant effect on peripheral lymphocyte counts, and fails to depress the allograft response, when administered to C3H mice. In contrast, a serum prepared by the same protocol, against the lymphocytes of another strain of mice (A/Jax), has a profound lymphopenic effect on C3H mice. Our anti-myeloma serum (AMS) has a high cytotoxic antibody titer against myeloma cells, and a very low, but significant, titer against lymphocytes. In contrast, Dr. Monaco's ALS has a titer against myeloma cells two tubes lower than that of AMS. We did not perform a parallel cytotoxic titration of ALS against C3H lymphocytes. This titer can safely be presumed to be high, however, as Gray et al. (10) have shown that the same serum has a leukoagglutionation titer against C3H lymphocytes comparable to that achieved against A/Jax lymphocytes, which was high.

Jeejeebhoy (18) has recently objected that cytotoxic and leukoagglutinin titers, as well as induction of lymphopenia, cannot always be correlated with immunosuppressive effects of antilymphocyte sera, and that at present no satisfactory test is available for predicting the immuno-suppressive effects of batches of antilymphocyte serum. He raised serum against rat lymphocytes in both dogs and rabbits. He maintained that both sera had comparable cytotoxic leukoagglutinin activity against rat lymphocytes,

and seemed to imply that both sera produced initial lymphopenia in rats, although the rabbit serum did so more effectively; only the rabbit serum had any immunosuppressive effects, however. If these observations are accurate, they cast considerable doubt on the "cytotoxic" theory, as well as upon the validity of much of the in vitro work presented above. Jeejeebhoy's cytotoxic titers indeed seem to be similarly high with both sera, but close examination of his lymphopenia data reveals that, over the course of 4 hours, his dog anti-rat lymphocyte plasma, which had no immunosuppressive effects, had a lymphopenic effect not significantly different from that of normal dog plasma. He did not follow the lymphocyte counts beyond 4 hours. The point that lymphopenia (in the initial stages of serum treatment) cannot be correlated with immunosuppressive potency is not established by this study. Certainly his dog sera did have high in vitro titers against rat lymphocytes, however. It is quite conceivable that some dogs cannot recognize specific rodent lymphocyte antigens, however. A non-specific dog anti-lymphocyte preparation might then fix just as easily on many other tissues, or be eluted easily from lymphocytes onto other tissues. It has been shown in the studies of Gray et al. (10), and Monaco et al. (26) that the cytotoxic and lymphagglutinatin acitivity of their rabbit anti-mouse lymphocyte serum does correlate with immunosuppression and lymphopenia. We have used this animal system in our experiments, and feel we have achieved a good correlation.

Further absorption of both sera with both kinds of cells, followed by gel-diffusion studies or more cytotoxic titrations, are needed to quantitate fully how much antigenicity is common to lymphocytes and myeloma cells, as well as other tissues (which were not studied here), and whether the lymphocyte has specific antigens of its own. Nevertheless, our experiments suggest quite clearly, but probably in a qualitative way only, that lymphocytes and myeloma cells do share some antigens. Other factors may be involved, but it is tempting to conclude from our experiments that both the myeloma cell and the lymphocyte are capable of raising sera specific for themselves, at least having significantly higher titers against the immunizing cell than against other cells, and with, in the case of ALS, a "clinically" specific in vivo effect.

It is probably not reasonable to expect the lymphocyte to have its entire complement of anitgens cell-specific, simply because all cells come from the same fertilized ovum. Indeed, Russell and Monaco have observed (31) that the specificity of ALS may involve the whole array of individual specific antigens concerned with histocompatibility in allogenic combinations. It is certainly not unreasonable to hypothesize that individual cell groups, in particular lymphocytes, might have some antigenic specificities not present on other cells. The general physical availability of lymphocytes, a relative susceptibility to mechanical lysis (or transformation), or a high concentration of antigen (either cell or species specific),

may be contributing factors in the action of ALS. The failure of our high titer anti-myeloma serum, which had some activity against lymphocytes, to depress lymphocyte counts or prolong graft survival, seems to minimize the importance of the latter factors, however, as we might have expected AMS to be at least partially effective if these mechanisms were critical. It would seem highly likely that some degree of cellular antigenic specificity is involved. Levey (20) did achieve immunosuppression with an anti-epidermal cell serum, but less than that achieved with ALS. His results are not inconsistent with our hypothesis.

ALS has been proven to be a powerful immunosuppressive agent. The main practical question involved in the issue of specificity, it would seem, is whether absorption with other tissues might be of value in preparation of sera for use in vivo. If indeed lymphocytes possess specific antigens, one might conceivably expect such absorptions to lower the toxicity of the serum, and perhaps to increase its effectiveness per given dose - both effects by decreasing avidity for non-lymphoid tissues.

Our experiments have been directed at the specificity of anti-lymphocyte serum and other sera for the lymphocyte. We do not offer evidence in favor of either the "cytotoxic" or "sterile activation" theories of the manner of action of ALS. Indeed, both theories probably depend on the specificity of ALS for the lymphocyte.

The possibilities for the clinical use of antilymphocyte

serum in organ transplantation seem excellent. Hopefully our experiments will contribute to the quantitation of the degree of immunological specificity this serum has for the lymphocyte, and to understanding of the relevance of such specificity to the immunosuppressive action of the serum.

SUMMARY

The current literature pertaining to the specificity and mode of action of antilymphocyte serum as an immunosuppressive agent has been critically reviewed. Experiments are described in which a serum is raised against a mouse (C3H strain) transplantable myeloma. This serum has a high cytotoxic antibody titer against myeloma cells, and a lower, but significant, titer against C3H lymphocytes. In contrast, a potent known antilymphocyte serum, prepared by the same protocol, has a relatively lower cytotoxic titer against myeloma cells; this same serum has been shown by others to have high agglutination activity against C3H lymphocytes. Anti-myeloma serum did not have an effect on the lymphocyte counts of C3H mice significantly different from that of normal mouse serum, whereas antilymphocyte serum profoundly lowered peripheral lymphocyte counts. Finally, antimyeloma serum had no effect on the allograft response in any doses, both pre- and postgraft. Possible significance of these findings is discussed.

Acknowledgments

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I am very much indebted to Dr. Anthony P. Monaco for his kind donation of a sample of his anti-lymphocyte serum, which proved most valuable in this study.

Thanks are also due to Dr. Samuel Nelson for suggesting the use of myeloma cells, to Dr. Kikuo Nomoto for his help with the cytotoxic antibody titrations, and to Dr. Stanley Order for his advice and encouragement.

Last, but only as a fitting way to finish, I express my profound thanks to Dr. Byron H. Waksman, who served as faculty advisor for this thesis, for his unfailing advice, eminently constructive criticisms, and his sustaining good nature.

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