Studies on SaI-AT homograft rejection by A/jax and Balb/c inbred strains of mice
by Kathleen Erickson Jackson

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree 0f
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Abstract:
Investigations undertaken in this paper were able to develop for the first time a modification of the rosette test for use in a system employing homologous erythrocytes. Spleen cells from A/jax and Balb/c mice challenged with 20 x 10 6 SaI-AT cells were examined for their ability to form rosettes with A/jax target erythrocytes at intervals of 5, 10 and 20 days following tumor challenge. Results from these assays demonstrated a close relationship between numbers of rosetteforming cells and tumor destruction in immune Balb/c mice. That is, at the time tumor rejection appeared most intense in immune mice, rosette formation became maximal. A "cell associated" antibody believed to be bound to the small lymphocytes involved in homograft destruction was also detected for the first time in immune Balb/c mice, and was indicated by the appearance of small lymphocytes as the immune cell type chiefly involved in rosette formation with allogeneic target cells.

The exact nature of cytophilic antibody produded in response to anti-genation with allogeneic tumor cells was not determined. It appeared capable of passively sensitizing other lymphoid cell types and titres for cytophilic antibody, as demonstrated by the rosette test, bore a direct relationship to early rising hemagglutination titres in immune Balb/c mice. These results and the apparent absence of a complementfixing antibody suggest that cytophilic antibody involved in destruction of SaI-AT homografts may be of the 7Sγ class.

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STUDIES ON SAI-AT HOMOGRAFT REJECTION BY A/JAX AND BALB/C INBRED STRAINS OF MICE

by

KATHLEEN (ERICKSON) JACKSON

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Approved:

William G. Walters
Head, Major Department

Chairman, Examining Committee

Dean, Graduate Division

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ABSTRACT

Investigations undertaken in this paper were able to develop for the first time a modification of the rosette test for use in a system employing homologous erythrocytes. Spleen cells from A/jax and Balb/c mice challenged with 20 x 10^6 SaI-AT cells were examined for their ability to form rosettes with A/jax target erythrocytes at intervals of 5, 10 and 20 days following tumor challenge. Results from these assays demonstrated a close relationship between numbers of rosette-forming cells and tumor destruction in immune Balb/c mice. That is, at the time tumor rejection appeared most intense in immune mice, rosette formation became maximal. A "cell associated" antibody believed to be bound to the small lymphocytes involved in homograft destruction was also detected for the first time in immune Balb/c mice, and was indicated by the appearance of small lymphocytes as the immune cell type chiefly involved in rosette formation with allogeneic target cells. The exact nature of cytophilic antibody produced in response to antigenation with allogeneic tumor cells was not determined. It appeared capable of passively sensitizing other lymphoid cell types and titres for cytophilic antibody, as demonstrated by the rosette test, bore a direct relationship to early rising hemagglutination titres in immune Balb/c mice. These results and the apparent absence of a complement-fixing antibody suggest that cytophilic antibody involved in destruction of SaI-AT homografts may be of the 7S γ_1 class.

INTRODUCTION

Sarcoma-I ascites tumor (SaI-AT) is indigenous to and lethal for strain A inbred mice. Tumor development becomes grossly apparent typically, as distention of the abdomen 4 to 5 days following the injection of tumor cells into the peritoneal cavity. Death from massive tumor growth ensues approximately two weeks following tumor transfer. In marked contrast, the injection of SaI-AT into adult allogeneic recipient mice results in development of an immune response that destroys grafted tumor cells.

Mechanisms for rejection of SaI-AT by an allogeneic host, such as Balb/c or C57BL/Ks inbred mice have been a subject of controversy during the past several years. Although the role of the histo-compatability-II(H-2) locus in homograft rejection has been clearly established (Gorer, 1937; Snell et al., 1953 and Snell, 1957), the actual roles played by cell-associated antibodies, serum antibody and complement in response to transplantation antigen and rejection phenomena have not been clearly defined. The work of Kaliss (1954) and Gorer (1958) has shown SaI-AT to be highly insensitive to humoral antibody. Studies by Gorer and Kaliss (1959) demonstrated that the presence of circulating antibody may even produce an enhancing effect on SaI-AT tumor development. The more recent experiments of Chourlinkov et al. (1962) appear to resolve this. Results from these studies demonstrate that enhancement of SaI-AT homografts in specific tumor anti-serum represents a dose dependent phenomenon. Where hyper-immune sera from C57BL/6 mice was given intra-peritoneally (IP) to allogeneic mice prior

to tumor challenge, suppression of tumor growth resulted in three of five instances and enhancement was observed in the remaining two. Although potent anti-serum to SaI-AT does seem to suppress tumor development, the balance between tumor cell cytotoxicity and enhancement appears to be a delicate one. Studies of Phillips and Stetson (1962) also demonstrated cytotoxic activity of serum antibody and complement for SaI-AT when present in high titres.

Chambers and Weiser (1965) were able to demonstrate an apparent cytotoxic effect of humoral antibody for SaI-AT manifest as prominent blebbing of tumor cytoplasm. When viewed ultra-microscopically tumor cells showed increase cytoplasmic blebbing in C57BL/6K mice at onset of homograft rejection. This was apparent initially in A/jax strain mice bearing SaI-AT but subsided at the point it was seen to increase in the resistant strain of mice. Because this pronounced change in tumor cell morphology was evident where no contact with immune cells had occurred it is possible to attribute the apparent immuno-cytotoxic effect to antibody of a humoral nature. It is well established that at the time rejection begins and blebbing of tumor cell cytoplasm becomes more apparent in resistant animals, circulating antibody is present at its peak in the host.

The cytotoxic and protective effect of antibody to SaI-AT was further demonstrated by Tsoi, Weiser and Tokuda in 1965. Foster nursing experiments demonstrated that immune C57BL/6K mothers were able to passively immunize newborns against SaI-AT primarily via the lacteal

route. Tumor development from small challenge doses was also prevented when hyperimmune serum or ascitic fluid from immune animals was introduced directly into adult C57BL/6K treated with immunosuppressive doses of irradiation. This ability of immune serum to confer passive immunity to tumor susceptable hosts suggests that the antibody involved is humoral in nature.

The importance of complement to immune cytolysis of SaI-AT was suggested by Rief in 1963. Tsoi and Weiser noted in their studies (1968) that increasing doses of immune serum do not after a certain point afford additional protection. This was attributed to a lack of complement as a limiting factor in protection. The enhancing effect of complement on immune cytolysis was demonstrated in the work of Phillips et al. (1968). It is established that D10B2/old strain mice, lack the fifth component in complement (C'5) and display an impaired response to SaI-AT ascites homografts. When C'5 was restored to D10B2/old strain mice, tumor cell rejection occurred more rapidly than in the absence of this component.

That mouse complement may be inefficient for bringing a lytic system into full expression is suggested by the work of Holmes and Weiser (1966) who demonstrated enhancement of cytolytic effects upon addition of rabbit complement to tumor-bearing C57BL/Ks mice. Although the activity of complement in mice is limited by inherent factors, complement does appear, in the presence of serum antibody, to possess enhancing activities for immune cytolysis of SaI-AT.

The role of the lymphocyte in destruction of ascites tumor homografts is presently speculative and comprises several facets. The activity of lymphocytes as antibody producing cells has been established and implicates this cell type as responsible for the production of cytotoxic iso-antibody to SaI-AT homografts as previously discussed. Phillips and Stetson (1962) observed anti-tumor activity to SaI-AT following transfer of immune lymphocytes to immuno-incompetent hosts. Although lymphocyte activity as a factor in tumor suppression has been demonstrated both *in vitro* and *in vivo* by many investigators, the manner in which the lymphocyte exercises its anti-tumor properties is not known.

In vitro experiments of Rosenau and Moon (1964) and *in vivo* experiments of Wilson (1967) have demonstrated specific contactual agglutination of sensitized lymphocytes with target cell antigens prior to immune cytolysis. It is suggested (Wilson, 1967) that this may be an immunospecific step resulting from the presence of a preformed nontoxic substance on the surface of the lymphocyte. Similar observations made by Storb and Weiser (1967) on rosette forming cells *in vitro* suggest that specific agglutination can be attributed to the presence of cytophilic antibody on the surface of lymphocytes which have been sensitized following antigenation. Results from earlier studies of Amos (1960) also favored involvement of a cell-bound antibody where intimate immune cell contact with target homograft cells was seen to occur. The lymphocyte thus appears to function chiefly as a producer of iso-antibody and cytophilic antibody in response to an ascites tumor homograft.

The role of the lymphocyte as a specific agent in destruction of SaI-AT homografts has not been established. Although *in vitro* work of Rosenau and Moon (1964) suggests cytolytic activities for lymphocytes in contact with target cells, the following discussion shows the macrophage to dominate this phase of graft destruction in the SaI-AT homograft.

Accumulated evidence appears to favor destruction of SaI-AT grafts by the activities of immune peritoneal macrophages. The studies of Journey and Amos (1962) have demonstrated destruction of lymphoma cells following intimate contact with macrophages from a resistant host. The observation of Baker et al. (1962) that tumor cells remain viable in the presence of immune macrophages when implanted intraperitoneally in diffusion chambers suggests target-immune cell interaction as necessary to subsequent destruction of target cells. *In vitro* experiments by Granger and Weiser (1966) yielded similar results for immune macrophages introduced into tissue cultures of allogeneic target cells. Ultramicrographs show degenerative processes ensuing in target cells following intimate contact with membranes of immune peritoneal macrophages (Chambers and Weiser, 1964).

Tsoi and Weiser (1968) achieved suppression of SaI-AT growth following passage of immune macrophages into the peritoneum of allogeneic hosts. In a following study (Tsoi and Weiser, 1968) macrophages were shown to possess even greater capacity for homograft suppression when given simultaneously with immune serum; the effect appeared to be

synergistic. This would suggest that a serum factor may be responsible for sensitization of peritoneal macrophages.

Earlier work of Granger and Weiser (1966) indicated that events leading to cytoadherance demand biosynthetic activities of immune macrophages. It is possible that such activities may involve "exposing" of receptor sites necessary for attachment of a sensitizing factor such as cytophilic antibody from immune serum. Such sensitization can occur only as the result of active production of receptor sites by the macrophage (Boyden, 1960). That macrophages alone are capable of being passively sensitized by immune serum (Storb and Weiser, 1967) also suggests the involvement of specific receptor sites.

Enhancement of homograft destruction by macrophage-lymphocyte combinations has been demonstrated (Baker et al., 1962). That destruction of SaI-AT ascites homografts results from cytotoxic activities of the lymphocyte is regarded, however, unlikely (Baker et al., 1962; Granger and Weiser, 1964; Weiser et al., 1965). Accumulated evidence appears rather to implicate the lymphocyte as a producer of a sensitizing factor (cytophilic antibody) which imparts to the peritoneal macrophages the capacity for specific adherance to the target cell necessary to its subsequent destruction by the adherant macrophage.

This paper will investigate further the activities of the immune cells involved in the rejection of the SaI-AT homograft. The following experiments seek (1) to establish the presence of cytophilic antibody on lymphocytes and macrophages of SaI-AT immunized hosts using a

modification of the rosette technique; (2) to correlate activity of cytophilic antibody with serum titres and (3) to implicate cytophilic antibody production as a factor of tumor immunity.

MATERIALS AND METHODS

Experimental Animals

The animals used throughout this study were inbred Balb/c and A/jax adult females approximately two to four months of age.

The Balb/c mice were originally obtained from the National Institute of Health, Bethesda, Md. in 1966 and the A/jax mice from the Jackson Memorial Laboratories, Bar Harbor, Maine in 1964. Both strains have been maintained in this laboratory since by frequent brother-sister matings.

All stock animals were fed Purina Lab Chow whereas breeder mice were maintained on Purina Mouse Breeder Chow. Water was provided ad libitum.

Tumor Immunization

Experimental Balb/c animals were immunized intraperitoneally (IP) with 0.2 ml of Sarcoma-I (SaI-AT) ascites tumor cells. The tumor was maintained in this laboratory by transfer of 0.2 ml ascites fluid every ten days into the peritoneal cavities of normal adult A/jax mice.

Tumor cells used for challenge were drawn from the peritoneal cavity of A/jax mice five to ten days following tumor transfer using a one ml tuberculin syringe fitted with a 25 gauge needle. The tumor cells contained in two ml of harvested ascites were washed in 10 ml sterile phosphate buffered saline, pH 7.2, (PBS) at a speed not exceeding 100 x g for 12-15 minutes in an International table model centrifuge. Washings

were repeated until red blood cells made up no more than 3% of the total cell population. Other cell types generally comprised no more than 1 to 2% of the remaining cell count. Cells were counted after diluting 1:2 with 0.15% trypan blue using a hemacytometer under high power phase.

Washed tumor cells were resuspended in PBS to a concentration of 40×10^6 cells per ml. An immunizing dose (Granger and Weiser) of 20×10^6 tumor cells (0.5 ml) was then injected I.P. into Balb/c or A/jax mice. Control mice were injected with 0.5 ml of sterile PBS.

The Rosette Test

The rosette tests were conducted on cell suspensions made from individual spleens using a modification of the method of Biozzi, et al. (1966) and Storb and Weiser (1967). All work was done using silicon-washed glassware. Solutions were maintained on melting ice at $4^{\circ}C$ throughout the entire procedure. Spleens were removed from the mediastinal cavity of experimental animals at intervals of 5, 10 and 20 days following innoculation of SaI-AT tumor or PBS. Spleens were ground with a sterile glass plunger from a 10 ml syringe in Hank's balanced salt solution, pH 7.2 (HBSS). Cell suspensions were filtered through sterile gauze into 15 ml glass test tubes to remove coarse debris and washed three times for 12 minutes each at 800 x g in an International table model centrifuge. Red blood cells were harvested from adult A/jax female mice by bleeding from the tail vein into sterile PBS. The cells were washed as described above.

Following vital staining with a 1:2 dilution of 0.15% trypan blue, cells were counted under high power phase using a standard hemacytometer. A dose of 6×10^6 spleen cells contained in HBSS were pipetted into serology tubes with 24×10^6 A/jax red blood cells. Five hundredths ml bovine serum albumin (8 mg/ml) (BSA) was added to this and the final volume was adjusted to 1 ml with HBSS. The resulting cell suspension was incubated overnight (12 hrs) at $4^{\circ}C$ and examined for presence of rosettes.

Hematology

Following overnight incubation the clear supernate in each rosette preparation was carefully removed using a capillary pipette. The rosettes were resuspended in three to four drops of 30% normal A/jax serum. To avoid destruction of rosettes during resuspension care was taken to repeatedly invert the capped tubes containing the cell suspensions very gently in a melting ice bath for 10 minutes. The resulting suspensions were carefully smeared on clean glass slides and allowed to partially dry. The smear was then fixed and stained for 4 minutes in Wrights stain and buffered 6 minutes in glass distilled water using Wright's method. The stained preparation was examined for the presence of rosettes. A rosette was recorded as a white blood cell in adherance with 4 or more target red blood cells.

Antibody Titrations

Hemagglutination titers were determined according to the method of Stimpfling (1961).

Sera were incubated at 56°C for 30 minutes to inactivate endogenous complement. The sera were then diluted 1:1 in sterile PBS pH 7.2 containing 1.25% polyvinylpyrrolidone MW 160,000 (PVP) (General Analine and Film Corp.). Serum dilutions began at 1:10 and continued to 1:2560. One tenth ml of 1% thrice washed A/jax red blood cells in saline was added to each well and incubated at 37°C for one hour. Hemagglutination was read following incubation at 4°C for 12 hrs or overnight. A titre of zero was assigned to sera producing no detectable hemagglutination patterns at dilutions of 1:10. The final titre was expressed as the last serum dilution showing detectable hemagglutination.

Hemolysin titrations were carried out utilizing the hemagglutination reaction systems. PVP was however not employed in the reaction system. One drop of guinea pig or rabbit complement diluted 1:5 in saline was added to each well. The sera were incubated overnight at 4°C and examined for hemolysis the following morning. The titre was read as the last dilution to show complete hemolysis. Sera showing no hemolysis at dilutions of 1:5 or 1:10 were assigned a titre of zero.

Splenomegaly Assays

Following sacrifice by cervical dislocation experimental animals were weighed on a Mettler top loading balance and whole body weights recorded. Whole spleens were weighed on a Mettler balance immediately after excision, and the ratios of spleen:whole body weights determined.

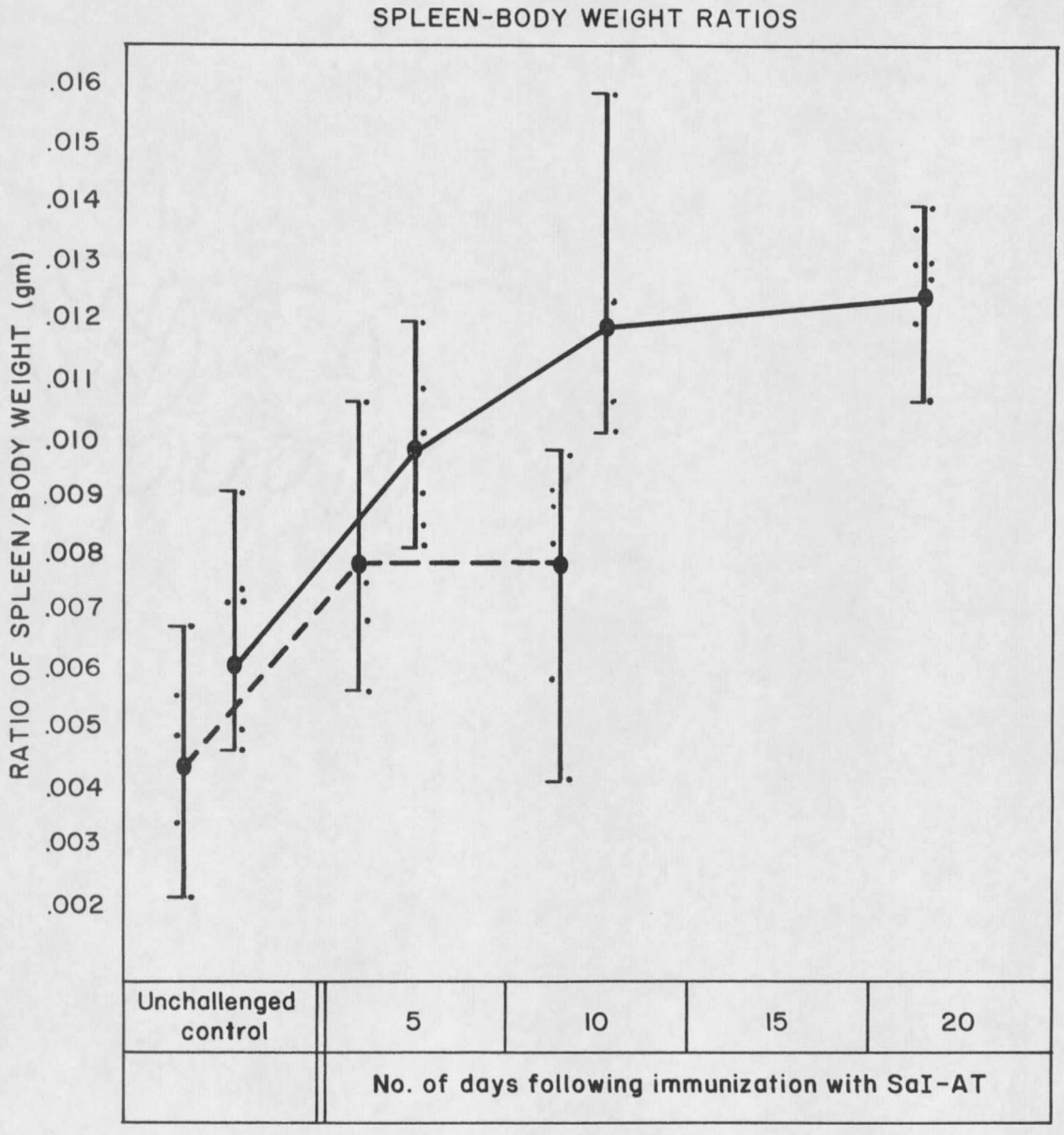
RESULTS

The natural history of Sarcoma I ascites tumor (SaI-AT) in adult Balb/c and A/jax mice. SaI-AT given intraperitoneally to Balb/c mice proliferated rapidly in the peritoneal cavity for at least 7 days following challenge. Total cell counts of peritoneal washings showed tumor cells to number 82 x 10^7 at day 5, increase to 95 x 10^7 at day 8 and became maximal (100 x 10^7) at day 10. From day 10 until two weeks, rapid decline in number of tumor cells was observed. Rejection was essentially complete by two weeks, although tumor cells have been observed in concentrated peritoneal washings of a few mice as late as day 20 post injection. The cytoplasm of these cells appeared heavily vacuolated and blebbed and nuclear changes typical of cells undergoing cytolysis (Baker et al.) were apparent. A/jax mice which received 10 x 10^6 SaI-AT succumbed, however, at days 10 to 12 to massive tumor growth.

Splenomegaly as an indicator of host response to SaI-AT. To establish a preliminary index of host responsiveness to challenge with SaI-AT, the spleen:body weight ratios of immunized Balb/c were determined at various time periods and compared to those ratios obtained from A/jax mice. The results of this study are presented in Table I. The spleen/body weight ratios for Balb/c and A/jax mice injected with SaI-AT showed a comparable increase (0.004 gms and 0.003 gms, respectively) at day 5 post-challenge. Splenomegaly became especially pronounced in Balb/c mice in the next 5 days whereas spleen/body weight ratios in A/jax mice failed to increase and, in fact, began to decline at day 10. These

Table I

Spleen-body weight ratios in A/jax and Balb/c inbred mice following challenge with SaI-AT.



– – – represents graph for A/jax mice.

——— represents graph for Balb/c mice.

|• represents ratio of spleen/body weight for one mouse.

● represents mean spleen/body weight ratio for one group of mice.

results suggest that the onset of specific immunologic responsiveness to SaI-AT cells in Balb/c mice occurred between 5 and 10 days, post-injection, and persisted for at least two weeks longer.

The ability of Balb/c mice to produce serum antibodies in response to SaI-AT challenge. Adult Balb/c mice injected with SaI-AT failed to produce antibodies capable of hemolyzing A/jax erythrocytes in the presence of rabbit or guinea pig complement. This was a surprising finding and suggested that macroglobulins of the 19S species were produced in insufficient titres or not at all. On the other hand, the data presented in Table II demonstrate the ability of Balb/c mice to produce hemagglutinating antibodies (HA), presumed to be of the 7S species, in response to immunization with SaI-AT. The production of HA antibody was clearly evident as early as day 5; maximum response was reached at day 10 and persisted through day 20. All Balb/c mice responded to SaI-AT immunization with high titres of HA antibodies whereas HA antibody titres were not detected in the sera of A/jax mice.

Rosette formation by spleen cells from Balb/c and A/jax mice challenged with SaI-AT. The spleen was selected as the source of cells to conduct rosette tests following challenge with SaI-AT because of its large number and varied population of lymphoid cells and its demonstrated role in tumor immunity. These studies employed the technique of rosette testing (see materials and methods) as a means of measuring cellular responsiveness to SaI-AT at various time periods after