STUDIES IN CELLULAR IMMUNOLOGY

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ABSTRACT

Antisera were prepared against pigeon thymus and bursa lymphocytes and against saline extracts of thymus and bursa. All unabsorbed sera were screened for differential reactivity against thymus and bursa-derived tissue by several techniques. Selected sera were absorbed with thymus and bursa tissue to demonstrate specificity. One serum with specificity against thymus extracts was identified by immunodiffusion. The thymus specificity was absent from extracts of pigeon bursa, brain, liver and breast muscle, but present in spleen extracts. This thymus specificity does not appear analogous to lymphocyte specificities identified in other species. Shared tissue specificities and a possible quantitative antigenic difference among the tissue extracts were also demonstrated by immunodiffusion and absorption analysis. In lymphocytotoxicity tests, fresh rabbit normal serum is highly toxic for pigeon thymus and bursa cells. This toxicity, in general, resembles the natural antibody present in rabbit and guinea pig sera against heterologous thymus cells. A rabbit anti-pigeon gamma globulin serum was rendered specific for bursa cells by absorption with thymus cells. Some standard anti-lymphocyte sera were shown to contain an antibody fraction

specific for thymus cells. Some or all of these reagents may be useful for distinguishing cooperating cell populations in a variety of immune responses.

An antigen was demonstrated on red cells from all pigeon squabs less than four days old. The antigen appears not to be secondarily adsorbed to the red cells from the fluids of the egg or the embryo. *In vitro*, masking of the antigen by components of adult serum does not occur under the conditions tested. Although the squab antigen behaves similarly to a known fetal red cell antigen in doves, it is probably qualitatively different from that antigen and from the known chick red cell antigen. The squab antigen is not detectable on lymphocytes from the bursa or the thymus.

Virgin female CBA/J mice were obtained after a variety of treatments and observed for primary tumors until either tumor onset or death. Included were mice which were: (1) immunosuppressed as adults by injection of anti-thymocyte serum (ATS); (2) injected with normal rabbit serum; (3) immunized with an irrevelant antigen or (4) untreated. Data were collected on tumor histology, incidence and time of onset for all groups. No tumors appeared during the period of ATS-immunosuppression or for several

months following treatment. The most frequently observed subsequent tumor was the typical mammary tumor. Although the first tumors appeared in ATS-treated mice, the mean age at tumor onset was not significantly affected by ATS-immunosuppression. No unusual tumors and no lymphomas were observed. Tumor incidences among groups of mice purchased at different times were different, but unrelated to ATS-immunosuppression. The failure of ATS-immunosuppression to affect growth is consistent with the fact that cellular immunity to mammary tumors is often specifically compromised.

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I. THYMUS AND BURSA SPECIFIC ANTIGENS OF THE PIGEON

A. Abstract

Lymphocytes of thymus-dependent and thymus-independent origins have separate roles in the immune response, but may cooperate in the antibody response to certain antigens. The lymphocytes look alike, but they can sometimes be distinguished from each other by serological means. The immune systems of birds are unique in that the functionally distinct populations of lymphocytes are anatomically separated in the central lymphoid organs — the thymus and the bursa of Fabricius. The avian immune system is used as a model for understanding mammalian immune systems. Cooperation between lymphocyte populations has been demonstrated in the avian antibody response although not with the two anatomically separate lymphocyte populations. Few serological markers are known for avian lymphocytes. Additional markers could more clearly identify distinct interacting cells and determine their roles in synergistic immune responses.

Antisera were prepared against pigeon thymus and bursa lymphocytes and against saline extracts of thymus and bursa. All antisera were prepared in rabbits — some as standard

anti-lymphocyte reagents and some in rabbits injected neonatally with large doses of pigeon cells. The neonatal injections were made to reduce the rabbits' antibody response to species-specific antigens upon challenge with pigeon lymphoid tissue.

All unabsorbed sera were screened for differential reactivity against thymus and bursa-derived tissue by passive hemagglutination, immunodiffusion, leukoagglutination and complement dependent lymphocytotoxicity. Sera identified in this manner were absorbed with thymus and bursa tissue to demonstrate specificity. One serum with specificity against thymus extracts was identified by immunodiffusion. The thymus specificity was absent from extracts of pigeon bursa, brain, liver and breast muscle, but present in spleen extracts. The thymus specificity was shared by extracts of thymus from two different breeds of pigeon but not extracts of chicken lymphoid tissue. This thymus specificity does not appear analogous to lymphocyte specificities identified in other species.

Shared tissue specificities and a possible quantitative antigenic difference among the tissue extracts were also demonstrated by immunodiffusion and absorption analysis.

In lymphocytotoxicity tests, fresh rabbit normal serum is highly toxic for pigeon thymus and bursa cells. This toxicity resembles the natural antibody present in rabbit and guinea pig serum against heterologous thymus cells. The activity against pigeon lymphocytes can be absorbed by pigeon thymus and bursa cells and is heat labile. Unlike the natural antibody against thymus cells of other species, the toxicity against pigeon lymphocytes could not be absorbed by agarose.

A rabbit anti-pigeon gamma globulin serum was rendered specific for bursa cells by absorption with thymus cells. Some standard anti-lymphocyte sera were shown to contain an antibody fraction specific for thymus cells.

Some or all of these reagents may be useful for distinguishing cooperating cell populations in a variety of immune responses. Avian species, in addition to the chicken, may provide additional data on the phylogeny of functionally distinct lymphocyte populations.

B. Introduction

Avian species are unique in the animal world for their structural compartmentalization of the immune system. The central lymphoid organs, the thymus (T) and the bursa of Fabricius (B), provide cells and maturation hormones for the immune system. They develop in the late embryo; and lymphocyte formation in these organs is maintained in the absence of antigen. The peripheral lymphoid organs — the spleen, lymph nodes, and circulating lymphocytes — require the presence of both central lymphoid organs for their subsequent development. Cells of the peripheral lymphoid organs participate directly in specific immune responses. Cells within the bursa or the thymus are not generally immunocompetent, although a small subpopulation may be immunocompetent. Both bursa and thymus are considered sites of maturation for stem cells of bone marrow origin. Once the cells become mature immunocompetent lymphocytes, they are seeded into the peripheral lymphoid structures where effective contact with antigen can occur.

The demonstration that bursa and thymus are functionally distinct was obtained by hormonal or surgical removal of each central lymphoid organ from neonatal chicks.

Bursectomy and thymectomy produce non-identical deficits in immune responsiveness. Immune dysfunction is most marked when surgical removal is combined with a general immunosuppressive or lymphocytotoxic treatment. This latter treatment kills immunocompetent lymphocytes which have already left the central lymphoid organs. Thymectomy or bursectomy of older chicks without such additional immunosuppression has almost no immunosuppressive effect. No significant deviation from normal may be evident until the animal reaches advanced age.

Neonatal thymectomy reduces all cell-mediated immune functions as measured by allograft survival and delayed hypersensitivity reactions. Circulating lymphocyte levels are severely reduced. Neonatal thymectomy depresses the primary antibody response to some antigens while response to other antigens remains intact. The secondary antibody responses are not significantly affected.

The complementary immune deficits are observed following neonatal bursectomy and x-irradiation of the chick. Specific antibody responses are severely depressed and serum immunoglubin levels are greatly reduced. Graft rejection and delayed hypersensitivity responses are virtually normal.

Surgical bursectomy of chick embryos at 19 days of incubation selectively prevents the later appearance of serum IgG immunoglobulins (16). If bursectomy is performed even earlier in ovo, at 17 days of incubation, neither IgG nor IgM immunoglobulins appear in the serum. Developmentally in the chick, the capacity to produce the distinct immunoglobulin classes procedes from IgM to IgG to IgA (35). An intact bursa is required at least until hatching for the complete developmental sequence although when the serum immunoglobulins are measured, they are produced by cells then outside the bursa.

The bursectomized and the thymectomized chicks are particularly useful models for understanding a dichotomy within the immune response which is partially observable in other species. The thymic role in cell-mediated immunity appears in many species to be equivalent to that in the chick. Neonatally thymectomized mice and cogenitally athymic mice and humans show severely impaired cell-mediated immunity. Antibody responses are generally normal although they may be secondarily affected following severe infection.

Congenitially agammaglobulinemic humans show the same impairment of humoral immunity as the immunoglobulin-

deficient, bursectomized chick. No experimental equivalent has yet been found in mice because mammals do not possess a bursa of Fabricius or similar anatomical representation of the bursa. A thymus-independent or bursal equivalent is indicated in mice, though, since humoral antibody responses are generally unaffected by neonatal thymectomy. Chromosomal and serological markers in reconstitution experiments indicate that bone marrow can provide the thymus-independent lymphocytes in this species.

A developmental progression of immunoglobulin classes as demonstrated by bursectomy in ovo, can be demonstrated in mice (38). In mice, neonatal injections of antisera specific for immunoglobulin class prevents the development of that immunoglobulin class and later developing classes of immunoglobulins. In humans an apparently congenital immunodeficiency restricted to the IgA class of immunoglobulins is known. In rats and mice, neonatal thymectomy decreases the IgA class of immunoglobulins. Congenitally thymusless mice also show depressed IgA levels (31, 40). This observation suggests that some functions of the avian thymus and bursa may be combined in the rat thymus and perhaps in other species as well.

No true equivalent of the bursa of Fabricius is known in mammals despite efforts to detect one. The properties of a potential bursa equivalent would be concentration of lymphocytes in close anatomical association with the gut and lymphocyte formation early in development of the lymphoid system. On this basis, tonsils, Peyer's patches and the appendix have been suggested as bursal equivalent structures. Attempts to demonstrate that these gut associated lymphoid tissues are functionally equivalent to the chick bursa of Fabricius have been unsuccessful. More likely, they are additional peripheral lymphoid organs.

Two Australian rodents reportedly have "dual thymuses". If it can be demonstrated that these two organs are not functionally identical to each other, these thymuses might be analogous to the central lymphoid organs of the chick or to subpopulations of lymphocytes within either of those organs; or one "thymus" might be a peripheral, rather than central, lymphoid organ.

Reptiles may have an anatomically closer representation of the avian immune system than do mammals. A few species of reptiles including the turtle have gut associated accumulations of lymphocytes (58). As yet, there is no evidence

that these lymphoid accumulations are functionally equivalent to the bursa of Fabricius in chicks.

Although the antibody response is generally considered a thymus-independent immune function, thymus-derived and bone marrow-derived lymphocytes of the mouse cooperate in the antibody response to sheep red cells and certain other antigens. The specific antibody response when both lymphocyte populations are present is greater than the sum of the antibody responses attributable to either cell population separately. Separate roles for these two populations are still evident in that thymus-derived cells are required although they, themselves, do not produce appreciable antibody. The specific antibody is produced almost exclusively by cells of bone marrow origin. An immunological role for the thymus-derived cells is indicated because these cells are probably antigen specific. Synergism between thymus-derived and bone marrow-derived lymphocytes has also been demonstrated in the antibody response to burro red cells and to heterologous serum proteins (13, 34, 63). These antigens have been called thymus-dependent antigens to distinguish them from antigens for which no thymus cell requirement could be demonstrated. Strong antigens, such

as polymerized flagella and bacterial endotoxin, are thymus-independent (4). Endotoxin is a mitogen specific for thymus-independent lymphocytes, but non-specific as to the types of antibody the stimulated cells can produce. Graft versus host disease, which also causes non-specific lymphocyte proliferation, may partially replace the thymus-derived lymphocytes in antibody responses. Interaction between thymus-dependent and thymus-independent lymphocytes has also been demonstrated in cell-mediated immune responses such as graft versus host and delayed hypersensitivity responses.

A variety of cell requirements have been demonstrated in both *in vivo* and *in vitro* immune systems. *In vitro* some cell requirements may be purely trephocytic. Cell cooperation has been most extensively studied in the antibody response to sheep red cells. In this system at least three distinct cell populations cooperate *in vivo* and *in vitro*. The antibody response to sheep red cells is therefore used as a general inclusive model for cellular interactions in immune responses (14). Adherent cells interact with two distinct populations of lymphocytes. Adherent cells are separable from lymphocytes because they adhere to glass or plastic surfaces; lymphocytes do not. The adherent cells

may be phagocytic or otherwise retain quantities of antigens. The term adherent cell includes several histologically distinct types of cells. Functional diversity within the population of adherent cells is known, but identification and purification of the effective cells has not been accomplished. The existence of large numbers of non-essential cells does not interfere with the immune response or the demonstration of an adherent cell requirement.

The amount of cell contact *in vivo* is difficult to estimate. The lymphocytes of the blood and lymphatic system recirculate constantly. The majority of these cells are thymus-derived, but a small population of recirculating thymus-independent cells exists. The structures of the lymphatic system including the peripheral lymphoid organs would appear to maximize contact between the recirculating cells, the stationary cells and antigen. Various degrees of cell contact have been observed between lymphocytes and adherent cells (29, 44, 56, 57). These observations provide evidence that several forms of cell contact can occur between adherent cells and lymphocytes. *In vitro* culture conditions which maximize cell contact also maximize the *in vitro* immune response.

The adherent cell-lymphocyte interaction is important in the induction phase of an antibody response (21, 22). Adherent cells may concentrate antigen, process antigen into a more potent immunogen or transmit informational RNA for the production of specific antibody. Adherent cells may even be found in the center of hemolytic plaques, indicating that they may secrete specific antibody. No evidence is yet available to determine whether the adherent cell synthesizes antibody or acquires antibody secreting properties after ingesting other cells. Adherent cells from nonimmunized donors or from tolerant donors are as effective in immune functions as adherent cells from immunized donors. The adherent cell has no inherent specificity for antigen. Phagocytosis and antigen processing are not identical functions. The two processes may be disconnected by x-irradiation; antigen processing is more radiation sensitive than is phagocytosis.

In order to demonstrate a requirement for a particular cell population, those cells must be the limiting factor for the immune response. All peripheral lymphoid organs are mixtures of cell types, cells of different origins and cells in different states of differentiation. The primary

lymphoid organs — the thymus and the bursa of Fabricius in chickens or the bursa equivalent in mammals — contain the immature precursors of immunocompetent cells and are not themselves effector cells in the immune response. For this reason, it is necessary to use immature cells and allow them to mature or to separate immunocompetent cells from complex mixtures of cells. In the former situation, the maturation steps and the interaction process are uncontrolled. In the latter, there may still be maturation steps which are antigen dependent.

Although the three cooperating cell populations are at least partially separable by their differential sensitivity to x-irradiation and different cell densities, these distinctions are not absolute (54). The adherent cell and the antibody secreting cell are generally of lighter densities than the lymphocyte and both are less sensitive to x-irradiation than are the lymphocytes. Lymphocytes of both thymus and bone marrow origins are presumed to divide in response to antigen. In the differentiation to antibody secreting cells, the precursor cell may undergo four to seven division cycles. All cells in division are sensitive to the prior effects of irradiation. The cell producing

antibody rarely divides and is resistant to 20,000R x-irradiation. Lymphocyte density is a function of the state of differentiation. Relatively immature or precurser cells may change density several times between antigen stimulation and demonstration of an immune response. There may be interconversion of histological cell types during this interval as well. Thymus and bone marrow derived lymphocytes are not histologically distinct from each other. Lymphocytes may convert to an adherent cell morphology, but it is unknown whether these lymohocytes are stem cells or are thymus-dependent or independent cells.

The cooperative effect between thymus-derived and thymus-independent lymphocytes has been demonstrated in mice, which species has no bursa of Fabricius. Although bone marrow cells of mice are often equated with bursal cell of chickens, synergism between cells of bursa and thymus origin in the chick has never been demonstrated. A recently published report indicates that, in the chick, synergism is demonstrable between bone marrow cells and thymus cells (43). The same experimental conditions failed to show significant synergism between bursa and thymus cells.

The failure to demonstrate synergism may be technical; for example, high background antibody production by bursal cells would obscure any synergistic effect of lesser magnitude. An alternative possibility is that chick bone marrow and bursa lymphocytes are not equivalent nor are the bone marrow cells of mammalian species necessarily equivalent to chick bursa cells. A cross species comparison of functional lymphocyte populations between avian and mammalian species may now have outlived its usefulness for the understanding of immune mechanisms at a cellular level.

There are limits to demonstrating a cell requirement — especially for cell populations which are capable of self replication. One is the number of cells within the mixed population which are able to interact and the second is the number of cells minimally needed to give a detectable response. Estimates of both have been determined for the adherent cell populations *in vitro* measures of immunity. Less than one in one thousand adherent cells effectively cooperates with lymphocytes in the antibody response. In the mixed leukocyte culture system in which cell-mediated immunity is assayed, less than one adherent cell per thousand lymphocytes will allow a positive response. In

migration inhibition tests, 2.5% lymphocytes in a population of adherent cells will determine the migration properties of the whole population. The effectiveness of small cell populations is increased by soluble mediators in the cell-mediated responses. Although these estimates do not directly pertain to the antibody response to sheep red cells *in vivo*, it is obvious that minimal contamination of a cell population with cells of another type of different function can grossly effect either the immune response or the behavior of the majority of cells. There is a need for techniques or serological reagents which can identify single cells or distinguish cells of different cell lineages.

Subpopulations of thymus-independent lymphocytes may be distinguished by the class of immunoglobulin which the cell produces. Bursectomy in ovo as well as the observation that a single cell may have one class of immunoglobulin on its cell surface and another in the cell cytoplasm suggest that the switch in class of immunoglobulin production observed in the serum during specific immune response also occurs at the single cell level.

Subpopulations of the thymus or of thymus-derived cells probably exist both as distinct states of differentiation and as functionally separate cell populations (12). Only

10-15% of thymus cells in the mouse are immunocompetent in assays of cell-mediated immunity. The immunocompetent thymus subpopulation is identified as cortisone-resistant medullary lymphocytes. Immunocompetent thymus-derived lymphocytes in the periphery are sensitive to anti-lymphocyte serum, cells in the thymus are unaffected. The difference in ALS-sensitivity is not a property of the cell, but is attributable to the blood thymus barrier. Adult thymectomy removes the primarily immature thymus cells, but not the thymus-derived cells in the peripheral lymphoid organs. Adult thymectomy and anti-lymphocyte serum treatment deplete thymus and thymus-derived lymphocytes, respectively.

Two functionally distinct thymus-dependent lymphocyte populations show synergism in the graft versus host response. The two populations appear thymus-dependent because both are sensitive to anti-theta serum and complement. Theta is present on both thymus cells and some immunocompetent cells in the periphery corresponding in location to the thymus-dependent areas of the peripheral lymphoid organs. Anti-theta is therefore used to distinguish thymus-derived immunocompetent cells. Theta is a thymus specificity

although many anti-theta sera contain antibodies of other specificities. Autoanti-thymus antibody, anti-Ly antibody and anti-allotype antibody are often present in the standard preparations of anti-theta sera. The autoantibody and anti-Ly are both directed against thymus cell specificities which are not detectable on lymphocytes in the peripheral lymphoid organs. Anti-allotype antibody, however, could kill thymus-independent cells in the presence of complement. The presence of this antibody in anti-theta reagents must temper the interpretation that the interacting cells are both of thymus origin when sensitivity to anti-theta is the only criterion of thymus origin.

Apparent thymus specificity may be indicative only of a state of lymphocyte differentiation rather than an independent line of lymphocytes (54). The so-called thymus specificities may be masked in cells of thymus-independent origin. Neuraminidase treatments of non-thymus-derived lymphocytes makes these cells sensitive to anti-theta serum and complement. The Lna specificity associated with the immune response locus, Ir-1, in mice is distinct from theta specificity, but is also present on peripheral immunocompetent cells (20). Anti-Lna reagents are not exclusively

thymus-dependent or thymus-independent reagents. They probably contain two or more specificities.

Thymus-independent cell markers include the anti-immunoglobulin, anti-MBLA and anti-PC-1. Anti-PC-1 apparently is present only on cells actively secreting antibody and not on antibody forming cell precursers. MBLA is apparently present on precurser cells as well as on secreting cells.

Of the two cellular interactions described for the antibody response, the interaction between the thymus-derived and bone marrow derived lymphocytes appears to be the more restrictive interaction. Both lymphocyte populations must possess the same major histocompatibility antigens for synergism in the antibody response to persits (36). If the cells are mismatched or only semi-allogeneic, as in parental and F1 hybrid combinations, synergism as measured by hemolysin response may be variable detectable in the first two weeks, but not thereafter. The interaction between adherent cells and lymphocytes on the other hand, does not require that the cell participants be histocompatible. Adherent cells and lymphocytes interact adequately even if they are of different histocompatibility types.

This requirement for histocompatibility may be a functional reflection of the close genetic linkage between some immune response genes and the major histocompatibility locus of the species. Such linkage is well established in the mouse and is indicated in other species as well. Exceptions to this association exist; they reflect genetic blockage at different levels within complexities of the immune response. Serological markers may help to identify interacting cell populations, but differences at some markers may in fact interfere with the interaction one wishes to study. It is therefore necessary to have at hand as many markers as possible both to identify the cell populations and possibly to determine the molecular basis of cellular interactions in the immune response.

The immune systems of birds other than the chicken have not been studied extensively, but the bursa of Fabricius appears to be the central lymphoid organ controlling the development of humoral immunity in these species as well. Because the Orders of birds diverged from each other between 60,000,000 and 120,000,000 years ago, differences may well exist among the immune systems of avian species. Subtle differences in the structure and function of the

avian immune systems would provide additional information on the phylogeny of immune mechanisms and perhaps provide additional insight into functionally distinct populations of lymphocytes.

Pigeons and chickens belong to separate Orders. At hatching, pigeons are considerably less mature than chicks. If the functional development of the immune system is also slower, the bursa-dependent and the thymus-dependent immunological functions may be more accessible to experimental manipulation. Pigeon eggs are laid in clutches of two — which hatch at approximately the same time. Paired squabs provide for an excellent control on age and environment for many experimental procedures.

In this thesis, serological markers were sought for the lymphoid cells and organs of the pigeon as a means of identifying functionally distinct cells in an avian immune system. In the synergistic response observed with avian thymus and bone marrow lymphocytes, the unique contribution of each population or of subpopulations can not be deciphered as long as the various lymphocytes are indistinguishable. The most useful serological markers would be the discriminating lymphocyte markers. Small antigenic differences may distinguish bursa and thymus lymphocytes or

their subpopulations or their immunocompetent descendants. Serological markers of less specificity, such as blood group antigens or histocompatibility antigens, could be useful in reconstitution experiments or in *in vitro* systems where cells from antigenically distinct individuals may interact.

Because bursa and thymus lymphocytes are so similar, they are expected to share many of the same antigens — from species-specific antigens to some possibly lymphocyte-specific antigens. In immunizations between individuals of the same species, species-specificities would not be antigenic, but individual differences could be. Smaller antigenic differences could be recognized by alloimmunization.

In cross species immunizations, species-specificities are often thought to be the most antigenic. Strong antigens may compete with and obscure the immune response to weaker, more subtle antigenic differences. Most species specificities would not discriminate among lymphocyte populations, although many organ specific antigens are detectable by heterologous antisera.

Numerous antisera were prepared in rabbits against pigeon bursa, thymus and spleen cells and against saline

extracts of these organs. Because species specificities may be a problem in heterologous antisera, a number of the antisera were prepared by a method which could reduce the species specificity. Fifteen newborn rabbits were injected with large doses of pigeon cells, in an attempt to induce tolerance to antigens common to all pigeon tissues and thereby reduce the antibody directed against these antigens. Challenge immunizations were done with pigeon lymphoid tissues or cells which were generally different from those used in the tolerizing phase of the inoculations. Three additional sera were prepared following a standard procedure for anti-lymphocyte sera . The specificity of standard anti-lymphocyte sera is usually demonstrated by *in vivo* immunosuppression of cell-mediated immunity. The lymphocyte antigen(s) recognized by ALS have not been identified, in any species. Antisera against the saline extracts of lymphoid organs were prepared in three adult rabbits in order to identify the large number of antigens which are certainly present in such extracts. Not all of these antigens would be expected to be recognized by the sera from neonatally tolerized rabbits. A summary of the sera prepared for this study is presented in Tables la and lb.

Rabbits Tolerized as Neonates[a]

Number of Rabbits	"Tolerogen"	"Immunogen"
1	B	T
2	B	Th
1	T	B
2	T	Bh
1	T	Sh
1	T	T
1	T	Th
1	rbc	B
1	rbc	Bh
1	rbc	T
1	rbc	Th
1	rbc	S
1	rbc (K30)[b]	wbc (K30)[b]

a. abbreviations used in this table:

B: bursa of Fabricius cells

T: thymus cells

S: spleen cells

h: Saline homogenate of lymphoid organ

rbc: red blood cells

wbc: peripheral white blood cells

b. single adult King used as cell donor

TABLE 1a. SUMMARY OF ANTISERA PREPARED AGAINST PIGEON LYMPHOID TISSUES

Rabbits Injected as Adults Only*

Number of Rabbits	Cells used in ALS Preparation
1	B
1	T
1	S

Number of Rabbits	Antigen
1	Bh
1	Th
1	Sh

* abbreviations used in this Table:

B: bursa of Fabricius cells

T: thymus cells

S: spleen cells

h: saline homogenate of lymphoid organ

TABLE 1b. SUMMARY OF ANTISERA PREPARED AGAINST PIGEON LYMPHOID TISSUES

Serum samples were taken from the rabbits before antigen challenge, after challenge and after reimmunization. The antisera were tested for specificity against both soluble and membrane antigens. Passive hemagglutination, immunodiffusion and immunoelectrophoresis were used for the detection of antibody against soluble antigens. Leukoagglutination and complement dependent lymphocytotoxicity tests were performed to assay specificity for membrane associated antigens. These tests were chosen to take advantage of the different properties of antibody populations including ability to precipitate or agglutinate the corresponding antigen and ability to fix complement.

C. Methods

Pigeons:

The pigeon colony at Caltech contains two distinct breeds of pigeons — Tumblers and Kings. The colony was started with three mated pairs of each breed. These mated pairs were housed separately in mating cages and fed grains, grit and water *ad libitum*. Progeny of these mating were banded and placed in fly pens after weaning. Nests were provided in each fly pen, but mated pairs were not separated. The two breeds were not housed in separate fly pens, but matings between the two were rare. Individual pigeons were identified by leg band numbers, preceded by T or K to identify the breed.

Bleeding Larger Squabs and Adults:

Blood was taken from the wing vein of larger squabs and adult birds into a syringe containing either an isotonic NaCl-sodium citrate solution (isocitrate) or Alsever's solution. Isocitrate was used if the cells were to be tested that day. If cells were kept longer, collection was done in Alsever's solution. All blood was stored at 4° C until use. Adult cells were used within a week. Squab cells were kept only overnight.

Preparation of Antisera in Neonatally Tolerized Rabbits:

Three pregnant New Zealand white rabbits were kindly supplied by Dr. Justine Garvey.

Newborn rabbits were injected with .5-1.0 ml of 10^8 - 2×10^8 pigeon cells in Hank's Balanced Salt Solution from the day of birth and every day thereafter for fourteen days (69). Cells were injected subcutaneously and intraperitoneally. Pigeon red cells, bursa cells or thymus cells were used in this phase of the injection schedule. Because there were not sufficient amounts of thymus and bursa cells, pigeon red cells from the same squabs providing the bursa and thymus cells were used. Rabbits receiving pigeon red cells were injected with 35% suspension of red cells on the first day and 20% suspension of red cells on the succeeding days. Squabs used as tissue donors were between a week and one month of age. Bursa follicles were broken up by passage through syringe needles of decreasing size to maximize the number of free bursa cells. One injection of bursa cells was substituted by an injection of a 15% bursa follicle suspension. All rabbits were bled at three months of age just prior to the challenge injections. For antigen challenge two ml of 1:1 saline extract of bursa or thymus were

injected subcutaneously once a week for three weeks. Ten days after the last injection all rabbits were bled for antisera.

Antisera Prepared in Adult Rabbits:

Antisera against bursa, thymus or spleen were prepared in adult female rabbits following a standard method for preparation of anti-lymphocyte serum (39). Rabbits were bled seven days after the last injection.

Antisera to lymphoid organ homogenates were prepared by thrice weekly injections of two ml of saline extracts of bursa, thymus or spleen. Injections were continued for three weeks. Rabbits were bled ten days after the last injection.

Rabbit Antiserum to Whole Pigeon Serum:

An adult female New Zealand rabbit received footpad injections of 1 ml of pooled pigeon serum in one ml of Complete Freund's Adjuvant. One month later, the rabbit was injected intraperitoneally with 1 ml of pigeon serum and intravenously with .5 ml two and five days later. The rabbit was bled ten days after the last injection.

Rabbit Antiserum to Pigeon Gamma Globulin:

This antiserum was purchased from Cappel Laboratories, Downington, Pennsylvania.

Reimmunizations:

Rabbits were reimmunized according to the availability of antigen. They were desensitized with an intraperitoneal injection of tissue extract or a subcutaneous injection of cells in a dose at least equivalent to the largest single dose of antigen the rabbit had recieved previously. Two and five days later, the cells or tissue homogenates were injected subcutaneously or intravenously. The rabbits were bled ten days after the last injection.

Rabbit Normal Serum:

Rabbit normal serum was obtained from an uninjected littermate of the "tolerized" rabbits or as preimmune serum from those rabbits subsequently immunized as adults.

Serum Handling:

Serum from individual bleedings was split into two aliquots. Aliquots for use in agglutination and complement dependent lytic tests were heat inactivated at 56° C for one hour. Sera for immunodiffusion and immunoelectrophoresis tests were not heated. All sera were stored at - 20° C.

Preparation of Cells Suspensions:

Red cells, bursa and thymus cell suspensions were prepared. Red cells were separated from whole blood in anticoagulant by light centrifugation and removal of the buffy

coat cells. Thymus and bursa were minced between scalpel blades to release the lymphocytes. When large quantities of cells were required the bursa tissue was forced through syringe needles of decreasing bore size to release additional cells from the bursa follicles. Cells were washed three times in medium and counted in a hemocytometer.

Tissue Extracts:

Fresh pooled tissue and an equal volume of saline were homogenized in a Waring blender for two minutes in thirty second intervals. The crude homogenates were centrifuged at 4° C at 17,000 rpm for 30 minutes and at 15,000 rpm for one hour, dialyzed overnight against at least twenty volumes of saline, centrifuged at 10,000 rpm for twenty minues. The supernatants were centrifuged at 105,000 x g for 2 hours at 4° C. The lipid layers at the tops of the supernatants were discarded and the fluid pipetted off the pellets. The extracts were stored at - 20° C.

Concentration of Antisera and Organ Homogenates by Ammonium Sulfate Precipitation:

Saturated ammonium sulphate was added to the solution to be concentrated up to a final concentration of either 33% or 50% (11). The precipitate was stirred for several hours

at room temperature, then pelleted at 1400 g for 30 minutes at room temperature. The precipitate was re-dissolved in borate-buffered saline (pH = 8.4) and dialysed against borate-buffered saline at 4° C until sulphate ions were no longer detectable in the dialysate. The material was clarified by centrifugation at 1400 x g for 30 minutes at 4° C and stored at - 20° C.

Concentration of Extracts by Pressure Dialysis:

The extracts were concentrated at room temperature by pressure dialysis using UM10 membranes (Amicon Corporation) at 20 psi. Concentrated extracts were stored in small aliquots at - 20° C.

Standardization of Extract Concentration:

Extracts were adjusted to 60 $O.D._{280}$ with saline.

Chickens:

White Leghorn chicks were obtained from Kimber Farms at one day of age and sacrificed for tissue when needed.

Micro-Hemagglutination:

Hemagglutination assays were performed in microtiter plates. 25 µl of normal rabbit serum diluent (1% normal rabbit serum in saline) was added to each well. Doubling dilutions of the antibody were made with a Takatsy micro-titrator. 25 µl of a 2% suspension of washed red cells was

added to each well. Plates were covered to prevent evaporation. Contents of the well were mixed by gently agitation of the entire plate. Agglutinations were read after three hours, at that time all the cells had settled to the bottom of the wells.

Reading Hemagglutination Assays:

Each individual well was observed and the degree of agglutination scored from - to ++++ (59).

The titer of the antiserum is the reciprocal of the last serum dilution which shows positive agglutination. For convenience the results were recorded as the titer $\log_2$ which is the same as the number of the last positive well.

Passive Hemagglutination:

Two techniques for the attachment of soluble extracts to tanned sheep red cells were used. A single sheep was used as the red cell donor in all cases. The procedures were the same as described by Kabat and Mayer (33) and by Nowonty (49). Identically prepared extracts of bursa, thymus and brain from pools of like organs from the same birds were used as antigen.

Immunoelectrophoresis:

A layer of 1% agar in Barbital buffer, ionic strength = 0.078, ph = 8.6 was allowed to solidify on a microscope slide. Antigen was placed in a well cut in the agar. The slide was electrophoresed at 6 ma per slide (about 60 volts) for two hours. Troughs parallel to the direction of electrophoresis were filled with antiserum and allowed to diffuse in a moist chamber for at least 24 hours.

Immunodiffusion:

Immunoelectrophoresis agar was allowed to solidify on clean microscope slides. The appropriate pattern of wells was cut with a gel punch and the agar plug removed by suction. Antibody and antigen were placed in the appropriate wells and the slides were stored in a moist chamber at least overnight.

Photographs:

Polaroid photographs were taken and kept as a record of the immunodiffusion or immunoelectrophoresis patterns.

Ficoll-Isopaque Separation of Lymphocytes:

Ten parts of 33.9% Isopaque (Nyegaard and Co.) and 24 parts of 9% ficoll (Pharmacia Fine Chemicals) were mixed at 4° C for several hours (64). Density of the mixture was

determined to be 1.073. The mixture was dispensed in 2 ml aliquots in clear plastic centrifuge tubes and stored at 4° C until use. Not more than six ml of cell suspension were layered on the gradient. Gradients were centrifuged for one half hour at 10° C. The gravitational force at the interface was 400 g. The white cells remained at the interface while red cells, dead cells, and cell aggregates pelleted. Cells were removed from the gradient immediately after separation.

Complement:

Complement from a number of sources was tested for toxicity and complement activity. Absorbed rabbit serum was used in most assays. Fresh rabbit serum was absorbed with pigeon thymus and bursa cells as described by Boyse et al. (10). One volume of .1 M EDTA was added to nine volumes of whole rabbit serum. For every seven volumes of treated rabbit serum one volume of packed cells was used for absorption. Cell suspension and washings were done in Hank's Balanced Salt Solution lacking Ca^{++} of Mg^{++}. Absorptions were done at 4° C for 20 minutes with frequent mixing. The absorbing cells were removed by centrifugation and the complement activity was restored with one volume of .1 M $CaCl_2$. The absorbed complement was stored in small aliquots at - 70° C until use.

Complement Titration:

Complement was titrated for residual toxicity against both bursa and thymus cells. Complement activity was determined with a dilution of unabsorbed rabbit antiserum which would kill 100% of bursa or thymus cells in the presence of adequate complement. The complement dilution which was twice the last doubling dilution to give 100% killing of both bursa and thymus cells was subsequently used in the lymphocytotoxicity tests.

Trypan Blue:

The trypan blue solution was made from 3 ml of a stock solution of 1% trypan blue in distilled water to which 7 ml of a 2% solution of EDTA in the culture medium (pH = 7.2) was added (3).

Lymphocytotoxicity:

Complement dependent lymphocytotoxicity was determined by dye exclusion using essentially the two stage micro-cytotoxicity method described by Amos (3). Modifications of medium and incubation times were necessary for pigeon cells. The general medium adopted for pigeon cells was Eagle's Minimum Essential Medium in autoclavable form (Grand Island Biological Company). The medium was prepared

according to package directions, one-tenth volume of 1.25% gelatin was added and the medium autoclaved. Before use for bursa and thymus cells, dextran (200,-300,000 MW, Clinical grade; Nutritional Biochemicals Corp.) was added to a final concentration of 2%. For peripherial blood leukocytes, 1% heat inactivated chick serum was added. One μl of an anti-serum dilution was incubated with one μl of a 2×10^6 cells/ml suspension at room temperature in microtiter plates. After 30 minutes the wells were filled with medium and the cells allowed to settle for ten minutes. The excess fluid was removed by quickly flicking the plate. One μl of absorbed rabbit complement was added to each well. The plates were then incubated at 37° C in a water saturated, 5% CO_2 atmosphere. After 20 minutes the wells were filled with a 0.3% solution of trypan blue. The cells were allowed to settle for ten minutes. Plates were read immediately under an inverted phase microscope. Each well was counted and the percentage of dead cells recorded. The serum titer is the reciprocal of the serum dilution which kills 50% of the cells. The percentage of dead cells in the absence of either antiserum or complement should be less than 15%.

Leukoagglutination:

A microagglutination test similar to the one described by Kissmeyer-Nielsen and van Rood was used (37). Two µl of antiserum dilutions and 2 µl of a suspension of 2 x 10^6 cell/ml of culture medium were added to the wells of a microtest plate. The plates were gently agitated to mix, and incubated for one and a half hours in a water saturated, 5% CO_2 atmosphere. At the end of the incubation, the wells were filled with trypan blue solution. Cells were allowed to settle for 10 minutes. Plates were read immediately with an inverted phase microscope. Plates were not scored if more than 10-15% of the cells were dead.

A second method of leukoagglutination was also used (53). For this assay cells had to be pipetted onto a microscope slide to determine the degree of agglutination.

Pigeon Normal Serum:

Serum samples from ten or more adult pigeons were pooled and heated at 56° C for one hour. The pooled serum was stored at - 20° C.

Lipid Extraction of Serum:

Pigeon serum was extracted with chloroform:methanol at 4° C (2, 23), centrifuged at 1000 x g for five minutes

and the aqueous layer retained. The extraction was repeated three times. The extracted serum was dialysed overnight against phosphate buffered saline pH = 7.2.

Solid Immunoabsorbants:

Solid immunoabsorbants of serum were prepared by cross-linking serum components with 2.5% glutaraldehyde (6). Serum was first dialysed overnight against saline at 4° C. Pigeon serum was also lipid extracted. The serum pH was adjusted to 5.0 with 1 M acetate buffer. Glutaraldehyde was added and the mixture allowed to stand at room temperature for several hours. The gel-like material was cut with a spatula and forced through a syringe to obtain smaller uniform pieces. The immunoabsorbant was washed with saline until the washes no longer absorbed at 280 mμ.

KCl Extractions of Pigeon Tissue:

Ten or more pigeons were exsanguinated by cardiac puncture and their thymuses, bursas and brains removed immediately. The tissues were washed in saline and like tissues pooled. Tissues were minced between scalpel blades into pieces about mm^3. Two hundred ml of 3M KCl in .01 M phosphate buffer at pH = 7.4 were used for each pool of

tissue (52). The tissue-KCl mixture was stirred overnight at 4° C, then centrifuged at 17,000 rpm for 30 minutes and at 15,000 rpm for an additional hour. The supernatant was dialysed against saline for 48 hours. The dialysed material was centrifuged at 10,000 rpm for 20 minutes and then stored at - 20° C.

D. RESULTS

Bursa and thymus cells should supply the most homogenous lymphocyte populations available from any lymphoid organs although there may still be minor subpopulations of cells derived from the other central lymphoid organ. The most easily quantitated cell subpopulation in bursa and thymus cell preparations consists of red cells. The oval-shaped avian red cells are nucleated and larger than lymphocytes. When the bursa and thymus lymphocytes are counted under the microscope, red cells are sometimes observed. Red cells are always less than two percent of the total cell population. Contamination of peripheral blood leukocytes preparations with red cells may be as high as 25% when cells are separated by centrifugation only. Separation on Ficoll-Isopaque gradients reduced the red cell content of the peripheral blood leukocytes preparations to about two percent. Spleen cells were injected without separation of the red and white cells.

Saline extracts of pigeon bursa, thymus and brain contain serum components which can be visualized in the concentrated extracts by immunoelectrophoresis. Inclusion of serum components is probably unavoidable for all tissue

extracts. Serum components are generally antigenic in heterologous species and immunization may pick up low level antigenic contaminants in the immunizing mixture. Antibody to serum components may be present in any antiserum prepared against crude tissue extracts.

Extracts used in injections and in the first screening of antisera by passive hemagglutination were not ultracentrifuged and therefore contained more membraneous material than the ultracentrifuged preparations. Ultracentrifuged preparations were used in all other passive hemagglutination experiments, in immunodiffusion, immunoelectrophoresis and in serum absorptions with soluble extracts.

All sera were screened by passive hemagglutination, immunodiffusion, leukoagglutination and complement-dependent, lymphocytotoxicity tests for differential reactivity against thymus or bursa tissue. The greatest difference in serum titers are denoted by boxes in the data tables.

In passive hemagglutination soluble antigens are attached to red cells. Upon reaction with the corresponding antibody directed against the soluble antigen, the red cells agglutinate and thereby make the antibody-antigen reaction visible. The extracts used in passive hemagglutination were

unconcentrated and were further diluted for attachment to tanned sheep red cells. Two slightly different methods of attachment of soluble antigen to tanned red cells were used (Tables 2, 3a-3d). The method described by Nowonty as the Boyden Method yielded moderate titers, but clear negatives. Agglutinations observed with the preimmune sera were similar to those obtained with normal serum from adult rabbits.

Serum from an uninjected littermate showed no agglutination with any of the extracts in either technique for antigen attachment. This serum was obtained at three months of age. Agglutination of red cells with attached pigeon extracts by adult rabbit serum at a 1:4 dilution or less suggest that adult rabbits may have low levels of natural antibody reactive with pigeon tissue extracts.

The method described by Kabat and Mayer yielded higher titers against thymus and bursa extracts while the titers observed with all rabbit normal sera remained low. Sheep cells with brain extract attached, however, spontaneously agglutinated even in the absence of serum. Serum concentrations higher than used in the diluent only slightly protected the cells from spontaneous agglutination. Comparisons between lymphoid tissue extracts and brain extracts

Rabbit Number	Tolerogen Cell Source	Bursa Exp. #1	Bursa #2	Bursa #3	Thymus #1	Thymus #2	Thymus #3	Brain #1	Brain #2	Chick Bursa #1	Chick Bursa #2
		Passive Hemagglutination Titers (log_2) with Extract of:									
W 1	B (bursa)	0	0	7	0	0	4-5	0	0	0	0
W 2	B	0	0	2	0	0	5	2	0	0	0
W 3	T (thymus)	2	0	—	2	0	3	2-3	2	0	0
W 4	T	2	5	5-6	2	5	6	2	5	0	0
W 5	rbc (red blood cells)	0	0	7	0	0	7	2	0	0	0
W 6	rbc	0	0	6	0	0	5	0	2	0	0
W 7	rbc	2	0	7	1	1	7	0	2	0-1	0-1
W 10	rbc (K30)	2-3	0	9	1	1	9	2	0-1	1	1
W 11	T	1	0	9	1	1	8	2-3	1	0	0
W 12	B	3	0	5	2	2	6	2	0	0	0
W 13	rbc	2	1	8	2	2	7	2	1	1-2	0
W 14	T	1	0	7	0	1	7	0	1	0	0
W 15	T	1	0	7	1	0	7	0	0	0	0
W 16	T	2	0	4	0	0	4	3	1	0	0
W 19	rbc	[0]	0	[7]	[6]	0	[>12]	0	0	0	0
W 21	None[a]	0	0	0	0	0	0	0-1	0	0	0
R 35	None[b]	2	1	1	2	1	0	1	1	1	1
R 36	None[b]	0	0	2	0	0	1-2	0	0	1	0
R 45	None[b]	1	0	0	0	2	0	0	0	0	0

a. untreated litter mate; b. pre-immune sera

TABLE 2. PASSIVE HEMAGGLUTINATION TITERS FOR PRE-IMMUNE SERA

(Experiment #1 was part of an immunology class experiment performed by John Brown, Jonathan Fuhrman, Ken Pischel and the author. Experiments 2 and 3 are described in the text. Figures placed in the boxes are for easier reference in the discussion.)

Rabbit Number*	Tolerogen Cell Source**	Immunogen Source**	Passive Hemagglutination Titers (log_2) with Extracts of: Bursa			Thymus			Brain		Chick Bursa	
		Exp.	#1,	#2,	#3	#1,	#2,	#3	#1,	#2	#1,	#2
W 1 a	B	Th	2	2	11	6	3	12	2	3	0-1	1
W 1 b			4	5	>12	3	6	>12	2	6	2-3	5
W 1 c			3-4	6	>12	3-4	8	>12	3	5	3	4
W 2 a	B	T	0	0	9	0	0	7	0	0	0	0
W 3 a	T	B	1	1	10	1-2	1	7	0-1	0	1-2	3
W 4 a	T	Bh	1	1	10	2	2	10	2-3	2	2	3
W 4 b			6	5	12	7	8	>12	5	6	3	4
W 5 a	rbc	Bh	3	2	5	2	3	9	2	3	2	3
W 5 b			4	4	10	1	5	>12	4	5	2	5
W 6 a	rbc	Th	2-3	5	11	2	5	9	1-2	5	1-2	5
W 6 b			4-5	8	>12	6-7	9	>12	4	9	1-2	6
W 6 c			8	6	>12	6-7	10	>12	5-6	9	3	7
W 7 a	rbc	S	2	4	11	2	4	10	2	4	2	4

* a, b, c are successive immunizations

** abbreviations used in this Table: B: bursa of Fabricius cells; T: thymus cells; S: spleen cells; h: saline homogenate of lymphoid organ; rbc: red blood cells; wbc: peripheral white blood cells

TABLE 3a. PASSIVE HEMAGGLUTINATION TITERS FOR IMMUNE SERA

Rabbit Number*	Tolerogen Cell Source**	Immunogen Source**	Passive Hemagglutination Titers (log_2) with Extracts of: Bursa			Thymus			Brain		Chick Bursa	
			Exp. #1,	#2,	#3	#1,	#2,	#3	#1,	#2	#1,	#2
W 10 a	rbc (K30)	wbc (K30)	1-2	0	6	0	0	6	1-2	0	0	0
W 11 a	T	T	4-5	7	[>12]	2	6	[8]	3-4	5	3	[3]
W 12 a	B	Th	3	4	9	2	6	8	5	6	5	3
W 12 b			4	7	11	6	7	10	5	8	3	7
W 12 c			5	8	>12	6	8	>12	6	9	7	8
W 13 a	rbc	B	2-3	5	[>12]	4-5	5	[9]	3-4	5	2-3	3
W 14 a	T	Bh	[4]	4	[>12]	[0]	3	[10]	4	5	3	4
W 14 b			4	7	>12	6	7	11	6	8	5	7
W 15 a	T	Sh	4	5	>12	5	6	>12	4	8	4	6
W 15 b			4	7	[>12]	6	8	[8]	5	9	5	8

* a, b, c are successive immunizations

** abbreviations used in this Table: B: bursa of Fabricius cells; T: thymus cells; S: spleen cells; h: saline homogenate of lymphoid organ; rbc: red blood cells; wbc: peripheral white blood cells

TABLE 3b. PASSIVE HEMAGGLUTINATION TITERS FOR IMMUNE SERA

Rabbit Number*	Tolerogen Cell Source**	Immunogen Source**	Bursa Exp. #1,	Bursa #2,	Bursa #3	Thymus #1,	Thymus #2,	Thymus #3	Brain #1,	Brain #2	Chick Bursa #1,	Chick Bursa #2
W 16 a	T	Th	3	3	11	4	4	11	3	3	3	4
W 16 b			5	5	11	4	6	>12	5	7	2-3	6
W 16 c			5	6	>12	4-5	6	>12	4-5	6	3-4	5
W 19 a	rbc	T	0	5	10	6	3	8	3	2	1	2
R 35 a	none	T	2	2	7	0-1	2	7	1	3	0	2
R 35 b			2	1	9	2	1	9-10	1-2	4	1	3
R 36 a	none	B	1	4	10	1	3	6	0-1	3	3-4	7
R 36 b			1	4	11	0-1	3	8	0-1	3	3-4	4
R 45 a	none	S	2	2	7	0	2	5	1	3	1	2
R 45b			3	3	9	3	4	10-11	2	7	4	7

Passive Hemagglutination Titers (log_2) with Extracts of: Bursa, Thymus, Brain, Chick Bursa

* a, b, c are successive immunizations

** abbreviations used in this Table: B: bursa of Fabricius cells; T: thymus cells; S: spleen cells; h: saline homogenate of lymphoid organ; rbc: red blood cells; wbc: peripheral white blood cells

TABLE 3c. PASSIVE HEMAGGLUTINATION TITERS FOR IMMUNE SERA

Rabbit Number*	Tolerogen Cell Source**	Immunogen Source**	Passive Hemagglutination Titers (log_2) with Extracts of: Bursa Exp. #1,	Bursa #2,	Bursa #3	Thymus #1,	Thymus #2,	Thymus #3	Brain #1,	Brain #2	Chick Bursa #1,	Chick Bursa #2
R 60 a	none	Bh	4	4	[11]	4	4	[7-8]	3	4	3	4
R 60 b			4	5	10	3	5	>12	4	4	3	4
R 61 a	none	Sh	4	6	10	2-3	6	9	2	6	1	4
R 62 a	none	Th	3	4	10	3	4	10	2-3	3	1	2
R 62 b			4	5	>12	4-5	5	11	4-5	5	2	4
rabbit anti-whole pigeon serum			n.d.	0	[>12]	n.d.	0	[8]	n.d.	8	n.d.	6

* a, b, c are successive immunizations

** abbreviations used in this Table: B: bursa of Fabricius cells; T: thymus cells; S: spleen cells; h: saline homogenate of lymphoid organ; rbc: red blood cells; wbc: peripheral white blood cells

*** n.d. not done

TABLE 3d. PASSIVE HEMAGGLUTINATION TITERS FOR IMMUNE SERA

were not possible when this technique was used for antigen attachment.

Rabbit antiserum to whole pigeon serum strongly agglutinated sheep red cells with bursa and thymus extracts attached by this latter method, but not by the former method. The agglutination patterns observed with this technique could be due in part to serum components in the extracts.

Passive hemagglutination titers for the preimmune sera using both techniques of antigen attachment are given in Table 2. Experiments 1 and 2 were done with red cells prepared as described by Nowonty (49) and experiment 3 with cells prepared as described in Kabat and Mayer (33). Differences in titer of two wells or less is within the error of the technique. The results of experiments 1 and 2 are consistent with each other except for W 19 serum. W 19 serum gave higher titer against thymus extract than against bursa extract in experiments 1 and 3, but equivalent titers in experiment 2. With this possible exception there are no other indications of specificity for bursa or thymus in the unabsorbed preimmune sera by passive hemagglutination. The reactivities of each individual serum sample against paired bursa and thymus extracts were nearly identical.

Passive hemagglutination by immune sera for the same three cell preparations is shown in Tables 3b, 3c and 3d. There were no sera with consistent differences in titer against thymus and bursa extracts for all three experiments. Serum W 14 (first immunization) shows a higher titer for bursa extract than thymus extract in two of the three experiments. The higher titer against bursa extract is consistent with the fact that rabbit W 14 was immunized with bursa extract. Sera from W 1, W 4, W 5, W 19, and R 36 show differences in only one of the three experiments. Differences in titer in a consistent direction were observed with later bleedings of W 5, W 19 and R 36, (for W 19 see pre-immune serum). Again the differences are consistent with a higher titer against the tissue used for immunization. In the case of W 19 one must assume that the peripheral white cells in the original red cell preparations used to "tolerize" W 19 acted to immunize that rabbit against thymus-derived cells or cell components.

In experiment 3 ten sera from seven rabbits show a difference in titer of three or more wells between bursa and thymus. Eight of the ten react more strongly with bursa extracts than with thymus extracts. A similarly high titer

against bursa extract was noted with the rabbit anti-whole pigeon serum antiserum. The fact that most of the differences are in one direction and do not necessarily correspond to the immunizing material suggest that the increase in bursa reactivity may not indicate anti-bursa specificity. This method may increase the attachment of bursa antigens (or possibly serum antigens shared by bursa cells) to red cells. Alternatively, red cells with sufficient attached bursa extract may have a greater tendency to agglutinate with low levels of antibody than cells with attached thymus antigens.

Bursas from two day old chicks were extracted for passive hemagglutination tests. Chick thymus tissue at this age did not provide enough material for testing. Chick bursa extract was used as an indication of the cross reactivity between avian species. Passive hemagglutination titers against pigeon brain and chick bursa extracts were generally of the same order as against lymphoid tissue extracts. Several exceptions in which the reactivity is either lower or higher are noted in Tables 3a through 3d.

Absorptions were performed on six of the sera to separate the possible specificities indicated in screening

of the unabsorbed preimmune and immune sera (Tables 4 and 5). Sera were absorbed with the pelleted material obtained in preparation of the extracts. This insoluble material was used because large quantities were available which were from the same pool of birds as the extracts, and absorptions could be performed without diluting the antibody. The pellets were stored at - 20° C and washed in saline before use as absorbants. The sera were multiply absorbed at room temperature and at 4° C. The pelleted materials were generally effective in absorbing out all reactivity against the corresponding soluble extracts. The pellets also absorbed reactivity against the other extracts tested. Those which only partially absorbed the corresponding specificity lowered the titers against other extracts as well. There was no indication of differential absorption of reactivity against the corresponding antigen. In none of the sera tested by absorption analysis and passive hemagglutination techniques (W 1, W 4, W 5, W 11, W 14, W 19) could tissue specificity be detected. If the possible specificity in some of these sera were against membrane components, as suggested by their titers in experiment 1, but not the following experiments, the membrane specificity was also

Rabbit Number**	Tolerogen Source *	Immunogen Source *	Absorbing Tissue*	Passive Hemagglutination Titer ($\log_2$) Against Bursa	Thymus
W 4 a	T	Bh	none	5	4
			B	0	0
			T	0	0
W 14 a	T	Bh	none	3	5
			B	0	0
			T	1±	0
			Br	0	0
W 5 b	rbc	Bh	none	5	5
			B	0	0
			T	1±	1±
			Br	0	0
W 19 a	rbc	T	none	5	3
			B	0	0
			T	0	0

* abbreviations used in this Table:

B: bursa of Fabricius

T: thymus

Bh: bursa homogenate

Br: brain

rbc: red blood cells

** a and b: successive immunizations

TABLE 4. PASSIVE HEMAGGLUTINATION BY ABSORBED SERA (BOYDEN METHOD)

Rabbit Number*	Tolerogen Source**	Immunogen Source**	Absorbing Tissue**	Passive Hemagglutination Titer log_2 Against	
				Bursa	Thymus
W 5 b	rbd	Bh	none	10	> 12
			B	0	0
			T	0	0
W 14 a	T	Bh	none	9	9
			B	0	0
			T	2-3	2
W 1 a	B	Th	none	11	12
			B	0	0
			T	0	0
W 11 a	T	T	none	11	> 12
			B	4	5
			T	0	0
W 19 a	rbc	T	none	8	9
			B	4	2±
			T	0	2±

* a and b: successive immunizations

** abbreviations used in this Table: B: bursa of Fabricius; T: thymus; Bh: bursa homogenate; Th: thymus homogenate; Br: brain; rbc: red blood cells

TABLE 5. PASSIVE HEMAGGLUTINATION BY ABSORBED SERA

(METHOD FROM KABAT AND MAYER)

absorbed out by the pelleted material. It is also possible that the pelleted material non-specifically absorbed out all antibodies. There was no control on this possibility except that the sera were also tested for specificity in immunodiffusion.

Immunodiffusion is less sensitive than passive hemagglutination for detecting low levels of antibody against soluble antigens. The technique has the advantage of separating the reactions to different antigens. The results are not seen as a single sum of the individual antibody-antigen reactions.

All sera were initially tested against the unconcentrated soluble extracts. Precipitin bands were not observed unless the antigen and antibody wells were refilled. Refilling wells may create artifactual precipitin bands. Both the antisera and the soluble extracts needed to be concentrated.

Saline extracts of bursa, brain and thymus were concentrated by two different methods — pressure dialysis and ammonium sulphate precipitation. The supernatants from ammonium sulphate precipitation were retained, dialysed against saline and concentrated by pressure dialysis. The

ammonium sulphate precipitable material was redissolved, but precipitated on dialysis against saline and was discarded. The concentrated nonprecipitable material formed a diffuse concentric halo around the antigen well in immunodiffusion tests. Precipitation in diffuse concentric patterns in the absence of antibody is generally indicative of aggregated or high molecular weight material. Such halos obscure possible precipitin bands which might be formed by antigen-antibody precipitation.

A method of antigen extraction other than making saline homogenates was attempted for preparing possibly simpler tissue extracts. Hypertonic KCl was used as a mild treatment for extraction of cell membrane antigens. Extraction of pigeon bursa, thymus, and brain with 3M KCl yielded material which, like the ammonium sulphate treated extracts, formed concentric halos when tested in immunodiffusion. Hypertonic KCl was not a useful extraction procedure.

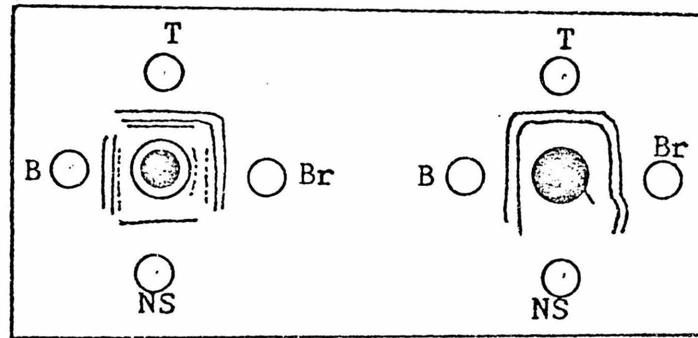
Crude saline extracts concentrated only by pressure dialysis precipitated only in the presence of antibody and were used in subsequent immunodiffusion assays. For screening the unabsorbed antisera, larger antibody wells and greater volumes of antisera were used as a substitute

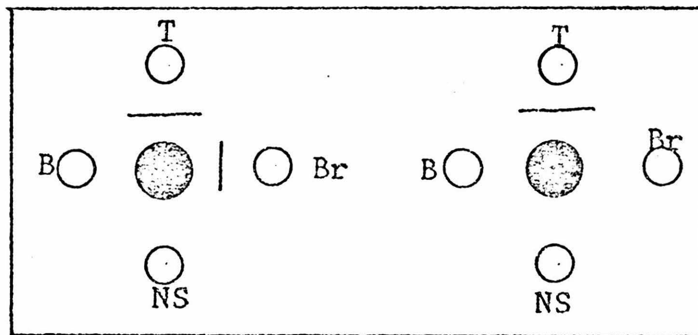
for concentrating each antiserum. All sera were tested against thymus, bursa and brain extracts, and normal pigeon serum. The concentration of the extracts was adjusted to the same OD_{280} level. Pigeon serum was more concentrated, but was used undiluted for the assays. No precipitin bands were observed with normal rabbit serum with the exception of R 36 preimmune serum. That serum formed a diffuse band against pigeon normal serum, but not against any of the tissue extracts. All preimmune sera and sera from rabbits immunized with whole cells produced no bands or faint precipitin bands. Immune sera prepared against the tissue extracts produced precipitin bands against all test fluids. Immunodiffusion patterns showing an additional band and possible specificity for thymus extracts were obtained from single bleedings of rabbits W 1, W 4, W 5, W 6, W 14, W 15, W 16, R 61 and R 62. Possible specificity was indicated in two bleedings from W 6 and from W 16. W 1, W 6, W 16 and R 62 had been immunized with thymus extract. W 4, W 14 and W 15 received thymus cells neonatally. W 5 was injected neonatally with pigeon red blood cells and R 61 was immunized with spleen extract. Thymus specificity would not be inconsistent with any of these injections. No serum

showed a pattern suggesting possible bursa specificity. Only R 61 and R 36 had been injected with bursa extract or bursa cells with no subsequent injection of thymus tissue or tissue containing a minor population of thymus components. Each serum showing possible specificity was concentrated by ammonium sulphate precipitation and absorbed with soluble extracts. An equal volume of the extract was added directly to the antiserum. Each serum was separately absorbed with pigeon normal serum, thymus, bursa and brain extracts.

The absorptions showed several common features. First, absorption with thymus removed activity against all of the tissue extracts. In almost every case an indistinct precipitin band remained against normal pigeon serum. Second, absorption with pigeon normal serum removed all activity against pigeon serum, but most sera showed two or three faint precipitin bands against the tissue extracts.

The bands remaining in W 1 serum were slightly stronger than with most of the sera. This serum produced two bands of identity for thymus, bursa and brain extracts (Figure 1). The first immune serum from W 16 was unusual in that it showed a distinct unique band against thymus extract. This specificity will be considered more extensively following this discussion of general patterns.





: Antiserum W 1

: Antigen Extract

( ): Absorption

B : Bursa

Br: Brain

NS: Pigeon Normal Serum

T : Thymus

FIGURE 1. IMMUNODIFFUSION PATTERNS WITH W 1 SERUM

Absorption with brain extract removed all activity against brain extract, but in most cases left in faint reactivity to normal serum, thymus and bursa extracts.

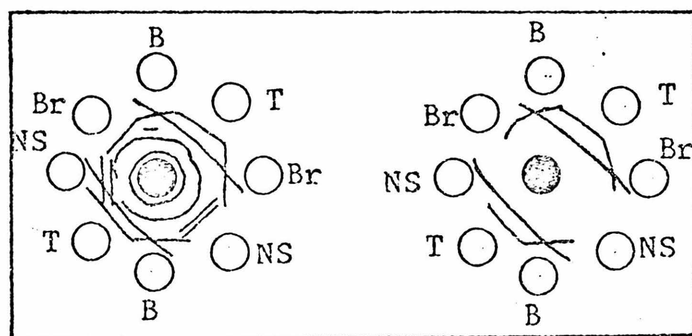
Absorption with bursa extract removed all activity against bursa extract, but left in a faint band against thymus extract. Both bleedings of W6 were exceptional in that reactivity against thymus extract was completely removed by absorption with bursa extract. Faint reactivity remained against brain extracts in serum from W 1, W 14, and the last bleeding of W 16.

In general, the precipitin bands remaining after absorption were too faint and did not extend far enough to determine reactions of identity or nonidentity in the several extracts and pigeon serum. Two exceptions to this generalization are W 1 and W 16 serum. Although W 1 identified two specificities shared by bursa, thymus and brain extracts, the two specificities behave differently in absorption analysis. One specificity can be completely absorbed by each of the three extracts. The second specificity is partially absorbed by the bursa extract, more so by the brain extract and completely absorbed by the thymus extract. The first specificity appears the same in all

extracts tested. The second specificity may be present in different quantities in the three extracts although quantitation by immunodiffusion is usually difficult.

Serum from W 16 shows a quite distinct pattern, both unabsorbed and absorbed, (Figures 2a through 2d). Absorption of W 16 with pigeon serum removes several specificities common to serum and tissue extracts. The same absorption leaves two precipitin bands — one fused band against thymus, bursa and brain extracts and a second which recognizes only thymus extract. Sequential absorptions were not performed on this serum because each absorption further dilutes the antiserum. The precipitin band for the common specificity is located closer to the antigen well than the band for thymus specificity. The common tissue specificity is also present in liver and muscle extracts (Figure 2b). The thymus specificity identified by this reagent is present in saline extracts of spleen, but not in saline extracts of liver or breast muscle, (Figure 2b). These results are indicated directly by immunodiffusion and confirmed by absorption analysis.

Because the thymus specific antigen appears to be a major antigenic component of the thymus extract, this



: Antiserum W 16

: Antigen Extract

( ): Absorption

B : Bursa

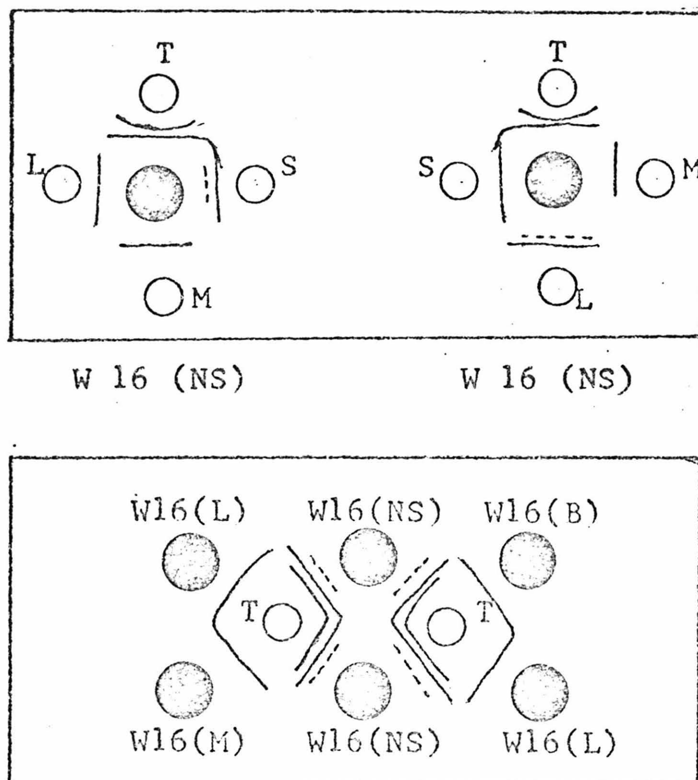
Br: Brain

NS: Pigeon Normal Serum

T : Thymus

FIGURE 2a. IMMUNODIFFUSION PATTERNS WITH W 16 SERUM

(Identification of Thymic Specificity)



◯ (shaded): Antiserum W 16

◯: Antigen Extract

( ): Absorption

B : Bursa

Br: Brain

L : Liver

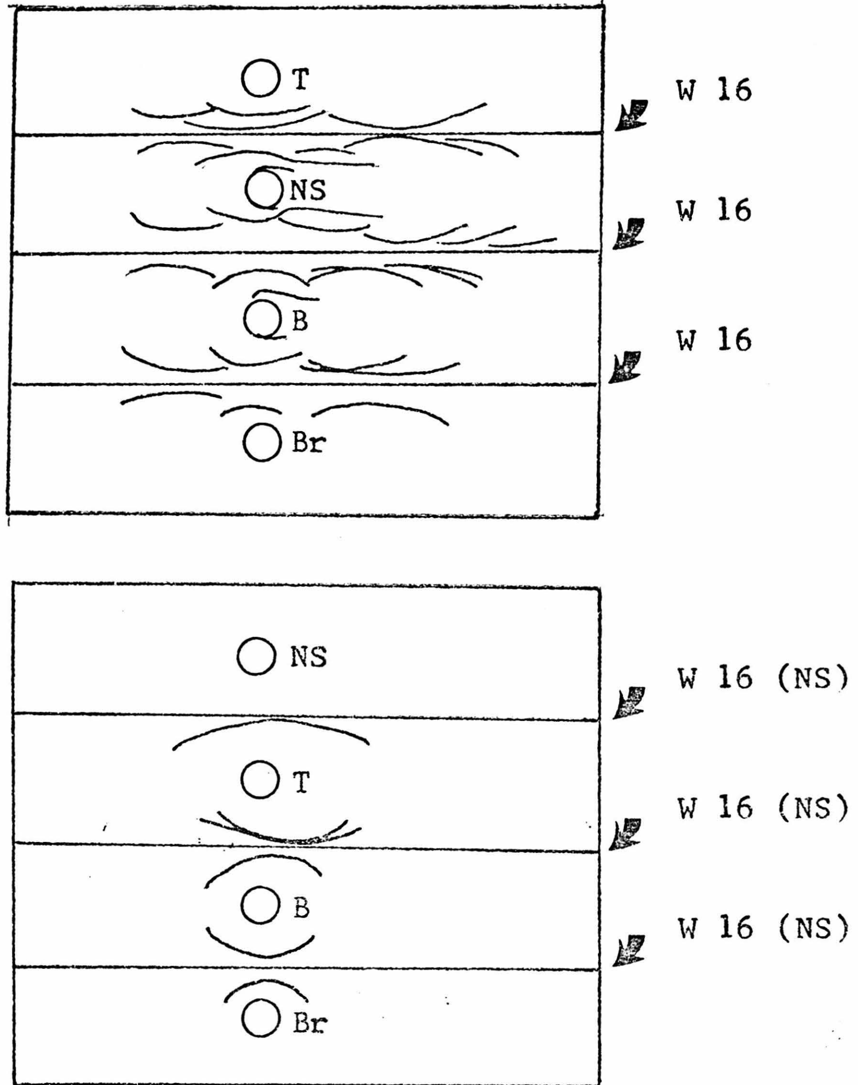
M : Muscle

NS : Pigeon Normal Serum

T : Thymus

FIGURE 2b. IMMUNODIFFUSION PATTERNS WITH W 16 SERUM

(Tissue Distribution of Thymic Specificity)



W 16: Antiserum W 16

○: Antigen Extract

( ): Absorption

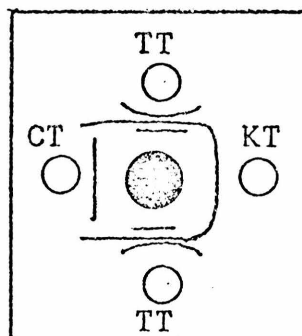
B : Bursa

Br: Brain

NS: Pigeon Normal Serum

T : Thymus

FIGURE 2c. IMMUNOELECTROPHORESIS PATTERNS WITH W 16 SERUM



W 16 (NS)

(shaded circle): Antiserum W 16

(open circle): Antigen Extract

( ): Absorption

CT: Chick Thymus

KT: King Thymus

TT: Tumbler Thymus

FIGURE 2d. BREED AND SPECIES DISTRIBUTION OF THYMIC SPECIFICITY

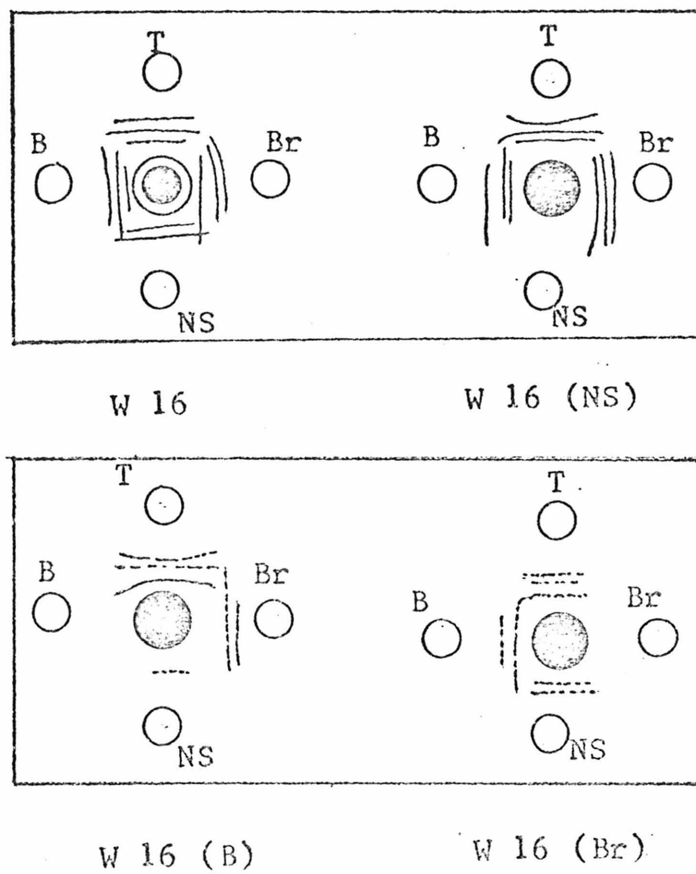
reagent was tested against the various extracts and pigeon serum in immunoelectrophoresis. The unabsorbed serum fails to reveal the thymus specificity as distinct from the several common specificities in the unabsorbed reagent (Figure 2c). Absorption with pigeon serum again indicates a thymus specific component (Figure 2c). Neither the thymus specificity nor the common tissue specificity migrates under the conditions of immunoelectrophoresis.

The absorbed reagent recognized the thymus specificity in extracts of King thymus (Figure 2d). It was non-reactive with bursa extracts from King pigeons and both thymus and bursa extracts from chickens. The shared tissue specificity appeared to be absent from the King thymus extract.

Later bleedings of W 16 failed to show an increased thymus specificity and recognized a more complex pattern of reactivity against the common tissue specificities (Figure 2e).

Direct agglutination of pigeon red cells was used as an indication of the depth of tolerance, the possible breed specificity and generalized membrane reactivity in the unabsorbed reagents.

Tumbler squabs had been used as the major sources of antigens — both cellular and soluble. A deliberate



: Antiserum W 16 late

: Antigen Extract

( ): Absorption

B : Bursa

Br: Brain

NS: Pigeon Normal Serum

T : Thymus

FIGURE 2e. IMMUNODIFFUSION PATTERNS WITH LATER BLEEDING OF W 16

exception was made for rabbit W 10. Rabbit W 10 was neonatally injected with red cells from a single adult King pigeon donor, K 30, and later challenged with K 30 peripheral white blood cells.

In the unabsorbed reagents, generally no difference in titer was observable between Tumbler and King red cells (Tables 6 and 7a through 7c). Preimmune sera from the neonatally injected rabbits indicated that tolerance if it existed at the time of bleeding was incomplete. Rabbits injected neonatally with red cells showed higher titers against red cells of both types than did rabbits which had been neonatally injected with thymus or bursa cells. The highest titer for a pre-immune serum, W 19 serum, was as high as might be expected from hyperimmune serum prepared against pigeon red cells. The high red cell titers are not necessarily indicative of specificity for the corresponding antigen, but could represent a dosage effect. The concentrations of red cells used in neonatal injections were higher than the concentrations of bursa and thymus cells.

The immune sera from these same rabbits showed a more restricted range of anti-red cell titers. The titers ranged from 4 to 256. The titer of immune serum from W 19

Rabbit Number	Tolerogen Cell Source	Hemagglutination Titers ($\log_2$) Against Tumbler (T10)[c] Red Cells	King (K30)[c] Red Cells
W 1	B (Bursa)	1	1
W 2	B	0	0
W 3	T (Thymus)	0	0
W 4	T	1	1
W 5	rbc (red blood cells)	5	4
W 6	rbc	7	8
W 7	rbc	5	5
W 10	rbc (K30)[c]	7	6
W 11	T	2	3
W 12	B	1	2
W 13	rbc	5	5
W 14	T	0	1
W 15	T	0	1
W 16	T	4	3
W 19	rbc	11	10
W 21	none[a]	0	0
R 35	none[b]	0	0
R 35	none[b]	0	0
R 45	none[b]	0	0

a. untreated litter mate

b. pre-immune sera

c. identification of pigeon cell donor

TABLE 6. DIRECT AGGLUTINATION OF PIGEON RED CELLS BY PRE-IMMUNE SERA

Rabbit Number*	Tolerogen Cell Source**	Immunogen Source**	Hemagglutination Titers (log_2) Against Tumbler Red Cells (T10)	King Red Cells (K30)
W 1 a	B	Th	5	5
W 1 b			3	4
W 1 c			5	5
W 2 a	B	T	3	4
W 3 a	T	B	3	2
W 4 a	T	Bh	2	4
W 4 b			6	6
W 5 a	rbc	Bh	7	6
W 5 b			6	5
W 6 a	rbc	Th	7	7
W 6 b			7	7
W 6 c			6	7
W 7 a	rbc	S	8	7
W 10 a	rbc (K30)	wbc (K30)	5	4
W 11 a	T	T	4	4

* a, b, c are successive immunizations

** abbreviations used in this Table: B: bursa of Fabricius cells; T: thymus cells; S: spleen cells; h: saline homogenate of lymphoid organ; rbc: red blood cells; wbc: peripheral white blood cells

TABLE 7a. DIRECT AGGLUTINATION OF PIGEON RED CELLS BY IMMUNE SERA

Rabbit Number*	Tolerogen Cell Source**	Immunogen Source**	Hemagglutination Titers (log_2) Against Tumbler Red Cells (T10)	Hemagglutination Titers (log_2) Against King Red Cells (K30)
W 12 a	B	Th	5	6
W 12 b			5	5
W 12 c			6	5
W 13 a	rbc	B	[3]	[6]
W 14 a	T	Bh	5	6
W 14 b			6	6
W 15 a	T	Sh	6	6
W 15 b			5	5
W 16 a	T	Th	8	6
W 16 b			7	6
W 16 c			7	8
W 19 a	rbc	T	7	8
R 35 a	none	T	7	6
R 35 b			8	7
R 36 a	none	B	10	8
R 36 b			5	7

* a, b, c are successive immunizations

** abbreviations used in this Table: B: bursa of Fabricius cells; T: thymus cells; S: spleen cells; h: saline homogenate of lymphoid organ; rbc: red blood cells; wbc: peripheral white blood cells

TABLE 7b. DIRECT AGGLUTINATION OF PIGEON RED CELLS BY IMMUNE SERA

Rabbit Number*	Tolerogen Cell Source	Immunogen Source**	Hemagglutination Titers (log_2) Against Tumbler Red Cells (T10)	Hemagglutination Titers (log_2) Against King Red Cells (K30)
R 45 a	none	S	11	9
R 45 b			[12]	[9]
R 60 a	none	Bh	7	6
R 60 b			6	6
R 61 a	none	Sh	7	6
R 62 a	none	Th	7	7
R 62 b			8	6

* a, b, c are successive immunizations

** abbreviations used in this Table: B: bursa of Fabricius cells; T: thymus cells; S: spleen cells; h: saline homogenate of lymphoid organ; rbc: red blood cells; wbc: peripheral white blood cells

TABLE 7c. DIRECT AGGLUTINATION OF PIGEON RED CELLS BY IMMUNE SERA

dropped to a level similar to that of other sera. The previously nonreactive or low titer rabbit sera showed increased serum titers, although some were still low.

The red cell titers of immune sera from some rabbits injected as neonates were comparable to titers obtained with sera from rabbits injected as adults with pigeon white cells. The highest titer obtained was from R 45 which had been injected as an adult with spleen cells. This was the only rabbit showing a higher titer against Tumbler red cells than against King red cells. Spleen cell preparations contain both red and white cells. Unabsorbed W 10 serum gave similar titers against both King and Tumbler red cells. Rabbits immunized with whole cells did not form a group distinct from rabbits immunized with tissue extracts, on the basis of the anti-red cell titers of their unabsorbed sera.

Leukoagglutination assays were performed by two different methods. In the method described by Schmid and Thein (55), the agglutination mixture must be pipetted onto a microscope slide to be scored. In this procedure the cell aggregates are disturbed and this could introduce errors in scoring agglutination. In the second technique described by

Kissmeyer-Nielsen and van Rood (37), it was not necessary to disturb cell aggregates to score agglutination. Cell aggregates in positive agglutination controls ranged from three to ten cells. Numerous free cells were also present. The small size of the cell aggregates and the large proportion of free cells made leukoagglutination assays difficult to score. All the unabsorbed reagents were screened by leukoagglutination using one or the other of these two techniques. The leukoagglutination titers were lower and showed fewer differences in titer than did complement dependent lymphocytotoxicity. The initial screening of sera in lymphocytotoxicity tests were done in medium which was less than optimal for cell survival. The background of dead cells was high and tests were scored only relative to the high background. These titers are relative and not directly comparable to titers obtained in later studies when the background was low. Investigations of selected sera were performed with supplemented medium, in which the background was reduced.

The conditions used in the lymphocytotoxicity tests had to be modified for testing pigeon lymphoid cells. Isolated pigeon bursa cells are generally less stable than isolated

pigeon thymus cells. The nonspecific bursa cell and thymus cell death was reduced by addition of 2% dextran to the medium. Cytotoxicity tests still had to be read soon after the final addition of medium or background levels of dead cells again rose to unacceptable levels. At 5% dextran levels both thymus and bursa cells were protected from non-specific death, but antibody mediated cell killing was also prevented. Dextran was found less toxic and more protective than fetal calf serum at serum dilutions from .5% to 20%. Peripheral blood white cells and red cells were more stable under the same conditions than bursa or thymus cells.

In preliminary experiments, a variety of complement sources were tested for toxicity and for complement activity. Fresh rabbit serum from five different rabbits, rabbit serum absorbed with agarose, rabbit serum absorbed with pigeon lymphoid tissues, lyophilyzed guinea pig serum and fresh pigeon serum were tested. Unabsorbed sera from all rabbits and the lyophilyzed guinea pig complement were highly toxic for both pigeon bursa and thymus cells even at dilutions of 1/20 or 1/40. There were no complement dilutions at which the toxicity was reduced below 10% and the complement was fully active with the rabbit antibody. Agarose absorption

of the rabbit serum did not reduce the natural toxicity of the rabbit serum to usable levels.

Undiluted fresh pigeon serum from the same pigeon whose cells were used for testing was nontoxic, but less than 90% of the cells were killed by the rabbit antibody and this complement. The antibody was unabsorbed rabbit anti-pigeon thymocyte serum from R 35. Complement dependent cytotoxicity dropped sharply with only a single doubling dilution of pigeon serum. The sharp fall in complement activity suggests that pigeon complement is not very active with rabbit antibody in this system rather than that the small volume of pigeon serum absorbs significant antibody activity.

Rabbit normal serum absorbed at 4° C with pigeon bursa and thymus tissue was nontoxic and was used as the complement source in all lymphocytotoxicity tests. The natural toxicity of fresh rabbit serum for pigeon thymus and bursa cells disappeared after the serum had been heated for one hour at 56° C. The serum remained nontoxic when rabbit serum absorbed with pigeon lymphoid tissue was added as the complement source.

Each individual rabbit reagent was tested for lymphocytotoxicity against thymus and bursa cells from the same

pigeon. Successive bleedings of the same rabbit were also tested against cells from the same pigeon. Serum samples from different rabbits were not necessarily tested against cells from the same pigeon donor. Preimmune serum samples from six rabbits injected as neonates showed similar titers against both thymus and bursa cells (Table 8). Preimmune sera from W 11, W 16, and W 19 showed higher titers for thymus cells than for bursa cells. Preimmune sera from rabbits W 2, W 3 and W 14 showed higher titers against bursa cells than against thymus cells. Higher bursa reactivity in W 3 and W 14 sera may result from the lower stability of bursa cells. These two rabbits had been injected only with thymus cells before the serum was taken.

The immune sera gave generally similar titers against thymus and bursa cells (Tables 9a and 9b). No immune serum showed a significantly higher titer against bursa cells than against thymus cells. Serum from rabbits W 12 and W 19 gave differences in titers against thymus and bursa cells of more than three wells. Both sera indicated possible thymus specificity and both had been immunized with thymic material. In some instances, the serum dilution series were not carried to the point of 50% toxicity for the test cells.

Rabbit Number	Tolerogen Cell Source	Lymphocytotoxicity Titers ($\log_2$) Against: Bursa Cells	Lymphocytotoxicity Titers ($\log_2$) Against: Thymus Cells
W 1	B (Bursa)	< 5	6
W 2	B	9	< 5
W 3	T (Thymus)	> 8	6
W 4	T	3[c]	< 5
W 5	rbc (red blood cells)	4[c]	7
W 6	rbc	8	8
W 7	rbc	< 5	5-6
W 10	rbc (K30)	9	9
W 11	T	5	> 8
W 12	B	4[c]	4
W 13	rbc	> 8	8
W 14	T	> 8	3
W 15	T	8	7
W 16	T	6	7-9
W 19	rbc	5	> 8
W 21	none[a]	< 5	< 5
R 35	none[b]	< 5	< 5
R 36	none[b]	< 3	< 3
R 45	none[b]	4	< 3

a. untreated litter mate

b. pre-immune sera

c. no clear end titers

TABLE 8. COMPLEMENT DEPENDENT LYMPHOCYTOTOXICITY TITERS FOR PRE-IMMUNE SERA

(This experiment was part of an immunology class experiment performed by Jonathan Fuhrman, Ken Pischel and the author.)

Rabbit Number*	Tolerogen Cell Source**	Immunogen Source**	Lymphocytotoxicity Titers ($\log_2$) (50% killing) Bursa Cells	Thymus Cells
W 1 a	B	Th	8	8
W 1 b			9	8
W 1 c			10	8
W 2 a	B	T	8	7-8
W 3 a	T	B	8	8
W 4 a	T	Bh	8	6-7
W 4 b			9	8
W 5 a	rbc	Bh	10	10
W 5 b			10	9
W 6 a	rbc	Th	> 8	> 8
W 6 b			> 8	> 8
W 6 c			> 8	> 8
W 7 a	rbc	S	8	8
W 10 a	rbc (K30)	wbc (K30)	7	< 5
W 11 a	T	T	> 8	8
W 12 a	B	Th	6	> 8
W 12 b			7	6
W 12 c			5	> 8

* a, b, c are successive immunizations

** abbreviations used in this Table: B: bursa of Fabricius cells; T: thymus cells; S: spleen cells; h: saline homogenate of lymphoid organ; rbc: red blood cells; wbc: peripheral white blood cells

TABLE 9a. COMPLEMENT DEPENDENT LYMPHOCYTOTOXICITY TITERS FOR IMMUNE SERA

Rabbit Number*	Tolerogen Cell Source**	Immunogen Source**	Lymphocytotoxicity Titers ($\log_2$) (50% killing)	
			Bursa Cells	Thymus Cells
W 13 a	rbc	B	> 8	> 8
W 14 a	T	Bh	7	> 8
W 14 b			6	> 8
W 15 a	T	Sh	7	8
W 15 b			8	> 8
W 16 a	T	Th	>10	>10
W 16 b			>10	>10
W 16 c			>10	10
W 19 a	rbc	T	5	> 8
R 35 a	none	T	>10	>10
R 35 b			>10	>10
R 36 a	none	B	> 8	> 8
R 36 b			7	> 8
R 45 a	none	S	6	6
R 45 b			8	7
R 60 a	none	Bh	7	10
R 60 b			8	>10
R 61 a	none	Sh	> 8	> 8
R 62 a	none	Th	< 5	< 5
R 62 b			9	9

* a, b, c are successive immunizations

** abbreviations used in this Table: B: brusa of Fabricius cells; T: thymus cells; S: spleen cells; h: saline homogenate of lymphoid organ; rbc: red blood cells; wbc: peripheral white blood cells

TABLE 9b. COMPLEMENT DEPENDENT LYMPHOCYTOTOXICITY TITERS FOR IMMUNE SERA

Although possible specificity was indicated for some of the preimmune sera in lymphocytotoxicity tests, immune sera did not appear as promising. Seven sera were selected for further study in lymphocytotoxicity assays. They were W 10, W 16, R 35, R 36, R 45, R 61 and rabbit anti-pigeon gamma globulin. W 16 was chosen because of its specificity for thymus in immunodiffusion tests. W 10 was selected because it was the only serum prepared against peripheral white cells and it could possibly show specificity for immunocompetent peripheral lymphocytes rather than lymphocytes of the central lymphoid organs. R 35, R 36 and R 45 sera were chosen as standard anti-lymphocyte reagents prepared against thymus, bursa and spleen cells, respectively. Conflicting reports in the literature indicate that such sera may have specificity for bursa or thymus lymphocytes (24, 32, 42, 50). R 61 was chosen as a strong reagent in which there might be many specificities. Rabbit anti-pigeon gamma globulin was selected mainly because the thymus independent cells of the mouse are recognized by their cell surface immunoglobulin determinants.

W 10 serum samples were absorbed with red cells from K 30, the same pigeon that had donated cells for injection

of W 10. Red cells used for absorption were centrifuged free of the buffy coat cells and then centrifuged on a Ficoll-Isopaque gradient and washed in medium three times to further free the red cells of contaminating white cells. Unabsorbed preimmune and immune W 10 sera gave titers of 10,240 and 320, respectively, in lymphocytotoxicity tests against K 30 peripheral white cells. Absorptions of the immune serum at 4°C and at room temperature with K 30 red cells reduced the toxicity against K 30 white cells to less than 10% above background at the lowest serum dilution tested (1:10). At the serum dilutions tested there appeared to be no specificity for peripheral white blood cells in the immune serum. Identical absorptions of the preimmune reduced its titer to 320 and still agglutinated K 30 red cells. Four further absorptions with K 30 red cells reduced the titer to 20. The absorbed serum at this point was similarly toxic for peripheral blood leukocytes from Tumbler 93 and for Tumbler bursa and thymus cells. Two additional absorptions with K 30 red cells decreased the complement dependent toxicity to 25% above background at 1:10 serum dilution. Higher serum dilutions were not cytotoxic. The absorbed serum alone at 1:10 dilution raised the background

to 17%. As for the preimmune serum, there is no indication of leukocyte specificity.

Anti-lymphocyte sera prepared in adult rabbits against pigeon thymus, bursa and spleen cells were tested in lymphocytotoxicity tests against thymus and bursa cells. The three unabsorbed sera were highly toxic for both types of cells. Absorptions of the three sera with thymus cells removed all the activity against both thymus and bursa cells. Absorptions with bursa cells removed all activity against bursa in the anti-thymocyte and the anti-bursa cell sera. Absorption of the anti-spleen cell serum was incomplete. In the anti-thymus cell and anti-bursa cell sera, absorption with bursa cells did not remove anti-thymus cell activity. In the anti-spleen cell serum there was no evidence to suggest differential absorption of the toxicity against either bursa or thymus. The results must be regarded as only indicative, because the background levels of dead cells for both bursa and thymus were high.

Rabbit anti-pigeon gamma globulin contained specificities for a number of pigeon serum components in addition to gamma globulin. Immunoelectrophoresis demonstrated specificity for gamma globulin as well as for two or three

serum components found in the region of the antigen well and a minor component in the β-globulin region. The unabsorbed anti-gamma globulin was toxic for both bursa and thymus cells at a titer of 320. The serum was absorbed once at 4° C with thymus cells. The absorbed serum was no longer toxic for thymus cells, but retained specificity for bursa cells. In this case, the cell donor for absorptions was not identical to the target cell donor. The immunoelectrophoretic pattern of the absorbed serum was indistinguishable from that obtained with the unabsorbed serum. If the bursa cell specificity of this serum is due to anti-gamma globulin or other serum specificity, many of the sera may show the same specificity after absorption. As noted in the immunodiffusion tests, all immune sera prepared against pigeon tissue extracts and one normal serum from an adult rabbit had precipitating activity against pigeon serum components. Distinct anti-gamma globulin activity was detectable by immunoelectrophoresis only in the final bleedings of R 61 and R 62.

A third bleeding of R 61 was absorbed at 4° C with K 30 red cells that had been separated on Ficoll-Isopaque gradient. The absorptions with red cells reduced the titer

against both bursa and thymus cells. The absorbed serum titers against bursa and thymus cells were very similar. The absorption with red cells was not meant to be complete, but only to reduce the amount of bursa and thymus cells needed for absorption. Subsequent absorption with thymus completely absorbed specificity for thymus cells; the titer against bursa cells remained high. Absorption with bursa cells was incomplete.

Aliquots of W 16 serum which showed thymus specificity in immunodiffusion were absorbed with thymus cells and with bursa cells. Complement dependent toxicity was reduced for both bursa and thymus target cells although neither absorption was complete. There was no indication of differential absorption by either population of lymphocytes. Absorptions with soluble extracts of thymus and bursa as used in immunodiffusion reduced the serum titer against both types of lymphocytes. This effect appeared to be an anti-complementary effect of the extracts. Additon of more complement increased the apparent titer of the absorbed sera without increasing the background of dead cells.

E. DISCUSSION

Antibody specificity against possibly weak antigens in a mixture of strong competing antigens may be difficult to obtain. The unwanted antibodies may be reduced by the induction of immunological tolerance to strong common antigens. This approach has been somewhat successful in preparing antisera to tumor specific antigens (8, 69), and was one method used in this study for the preparation of anti-pigeon lymphocyte sera in rabbits. The antigen preparations, tissue homogenates or cells, are complex mixtures. The immunogenicity and the concentration of the individual antigenic specificities within the mixture are unknown but important parameters. Tolerance is highly dependent upon the presence of antigen and antigen dose.

The levels of contaminating minor antigen populations within the antigen preparations are important because antigen dosage can determine the balance between immunity and tolerance. The form of the antigen, either cellular or soluble, is also important because tolerance is more difficult to induce against cellular antigens. For these reasons, some antigens in the preparation may immunize while others may induce tolerance. The soluble extracts of

pigeon bursa, thymus and brain contain serum components and probably some membrane specificities. These contaminants are difficult to quantitate. The importance of serum contaminants is indicated by the fact that a rabbit antiserum to pigeon gamma globulin was specifically cytotoxic for pigeon bursa cells after absorption with thymus cells. Serum contamination of antigen preparations could evoke anti-pigeon serum antibodies including some with anti-gamma globulin specificity. Serum from all rabbits that were immunized against pigeon tissue extracts contained precipitating antibodies against pigeon serum, but only three hyperimmune sera had demonstratable specificity for pigeon gamma globulin.

The inclusion of minor cell populations in the cell inocula can explain the anomalous reactivity of some unabsorbed antisera. Red cells included in bursa and thymus cell preparations represented less than 2% of the total cell population. Cross contamination of bursa and thymus cells in the pigeon cannot be assessed in the absence of distinguishing cell markers. For the chick, reciprocal bursa-thymus cell contaminations are estimated at approximately 5% (3, 42, 50). If some antigens present in the cell

inocula given neonatal rabbits immunized the rabbits, subsequent immunizations with antigenically different inocula could adversely affect the antibody specificity of the antiserum. The specificities and the titers of antisera from different bleedings of the same rabbit could be very different. Such differences were, in fact, observed in different bleedings of the same rabbit.

Antigen presence is required for tolerance maintenance. The timing of the immunizing series of injections coincided with the time the rabbits would be expected to have a spontaneous break in tolerance. The one serum showing antithymus specificity in immunodiffusion was prepared in a rabbit that received neonatal injections of thymus cells followed by injections of thymus extract. The specificity observed with this particular serum suggests that the tissue source of the tolerizing antigens need not be different from that used later for immunization.

All unabsorbed rabbit antisera reacted against pigeon serum, red blood cells, bursa and thymus cells, and saline extracts of many pigeon tissues. Tolerance to pigeon cells in the neonatally injected rabbits was incomplete. Titers of the unabsorbed sera against bursa or thymus or their extracts were often similar. Such sera may have contained

specificities which would not be apparent until specific absorptions were performed.

Titers obtained in passive hemagglutination tests measure the total reaction between all antibodies and their corresponding antigens. This technique is very sensitive; less than 50 antibody molecules per cell can cause agglutination. Only a very small percentage of the soluble antigen available is actually adsorbed to the tanned red cells. Because tissue extracts are highly complex antigenic mixtures, antigens within the mixture may compete for attachment to tanned red cells. All antigenic specificities would not necessarily be represented on these cells. This phenomenon could explain the high anti-bursa extract titers in one set of passive hemagglutination tests.

A totally different technique, immunodiffusion, was also used for detection of soluble antigens. Immunodiffusion screening of the unabsorbed antisera identified eleven sera with possible anti-thymus extract specificity. No anti-bursa extract specificity was indicated for any of the sera by immunodiffusion. The thymus extract was more effective in absorbing antibody than were the other tissue extracts. All sera prepared against soluble extracts showed

reactivity against all tissue extracts and against pigeon serum. One antiserum after absorption with pigeon serum demonstrated a shared tissue specificity and a possibly quantitative antigenic difference among the thymus, bursa and brain extracts.

Lymphocytes from mouse thymus show quantitative antigenic differences from peripheral, apparently thymus-derived lymphocytes for both theta and H-2 specificities (54). Theta is high on thymus cells, H-2 is low. The reverse quantitative relationship is true for the peripheral blood lymphocytes. These quantitative antigenic differences coincide with a difference in functional capacity between these lymphocyte populations. The murine TL antigen is present only on thymus cells in normal individuals. Normal lymphocytes outside the thymus do not express TL. The Ly and MSLA markers are present on peripheral lymphocytes and are lymphocyte specific (54). The H-2, theta, PC, H-14 and the natural specificity in normal guinea pig sera are not lymphocyte specific markers, although they can distinguish between thymus-dependent and thymus-independent lymphocytes (54).

A pigeon thymus specificity was detected by immunodiffusion in one rabbit antiserum. This reagent recognizes the same specificity in spleen extracts. The pigeon thymus specificity is not unique to the less differentiated state of lymphocytes in the thymus. This reagent did not react with pigeon brain extracts and therefore may not be the pigeon counterpart of the mouse antigens, H-2, theta, H-14 or the natural specificity recognized by normal guinea pig sera which are all present in brain. These mouse antigens are membrane antigens. The pigeon thymus reagent did not appear specific for intact thymus cells although the absorptions with soluble material were incomplete. Absorption with insoluble thymus tissue pellets removed all activity against thymus extract.

McArthur et al. (42) and Potworowski et al. (50) recently reported serological markers for both chicken thymus and bursa cells in complement dependent lymphocytotoxicity tests. The reagents of Potworowski et al. and of McArthur et al. were also cytotoxic for some lymphocytes in tissues other than the central lymphoid organs. Jankovic et al. (32) were unable to demonstrate bursa or thymus specificities by lymphocytotoxicity, leukoagglutination or passive hemagglutination techniques. Some *in vivo*

specificity was demonstratable in these latter sera by injection into chick embryos with guinea pig complement. Forget et al. (24) identified bursa and thymus specificities by immune adherence tests. The nature of these test systems suggests that the identified specificities are present on the cell membranes. No comparison of the various reagents has been reported so that the number of unique specificities may be no greater than two.

Malchow et al. (41) prepared antisera against crude membrane fractions of chick thymus and bursa cells. They identified two specificities shared by bursa and thymus and a unique specificity cells for each central lymphoid organ. The unique specificities were not detectable on lymphocytes from other tissues. The present study with pigeon lymphocytes was successful in detecting solublizable specificities for thymus and also two shared specificities.

The pigeon thymus antigen reagent could possibly be a useful typing reagent because the antiserum gives clear positive and negative reactions. It is not, however, a strong reagent and at present can be used only on pooled and concentrated extracts. A high titer reagent might be prepared because the antigen is separable from a number of serum specificities by its electrophoretic mobility.

Tissue extracts used for testing and for immunizations could also be freed of many pigeon serum specificities by absorption with insolublized antiserum to whole pigeon serum. Preliminary experiments indicated that rabbit antiserum to pigeon serum was easily rendered insoluble by glutaraldehyde treatment, the procedure used to insolublize pigeon serum. No prior extraction of lipids was necessary. Soluble antigens are particularly intriguing because they are, in general, more amenable to chemical analysis than are membrane antigens.

Strong reagents against pigeon lymphocytes may be obtained from rabbit antiserum to whole pigeon serum as well as by the procedures used to obtain standard anti-lymphocyte reagents. The former serum can be made bursa cell specific by absorption with pigeon thymus cells. This bursa specificity is consistent with the proposed role of antibody as the antigen specific receptor molecule on the cell membrane of immunocompetent cells, particularly of thymus-independent derivation. Some anti-lymphocyte sera can be made thymus-specific by absorption with pigeon bursa cells.

The molecular specificities of these sera are, in general, unknown, although heterologous anti-gamma globulin should be directed against the constant region of the immunoglobulin molecule. The natural antibody in heterologous serum against thymus cells of many species appears by inhibition studies to be directed against carbohydrate moieties.

Natural toxicity to heterologous thymocytes has been reported in normal serum from rabbits and from guinea pigs. Evidence that this toxicity is mediated by natural antibodies is that the toxicity can be absorbed out by thymocytes, and the toxicity can be isolated in the 7s and 19s serum fractions where IgG and IgM are found. Antibody to IgG removes the toxicity. Absorption with agar or agarose removes the toxicity without affecting the complement levels and is consistent with a carbohydrate specificity as indicated in inhibition studies. The toxicity is also heat labile.

The natural toxicity of rabbit serum for pigeon lymphocytes is similar to these previously reported systems in several ways: the toxicity is present in normal serum of rabbits and is more toxic for thymus and bursa cells than

for peripheral lymphocytes, is absorbed from the serum by thymus and bursa cells and is heat labile. A possibly significant difference from the preceding systems is that the toxicity for pigeon cells is not absorbed by agarose. If the natural toxicity depends on antibody in this case, the antibody specificity would be different than that for mouse and human thymus cells.

F. Summary

Antisera were prepared against pigeon thymus and bursa lymphocytes and against saline extracts of thymus and bursa. All antisera were prepared in rabbits — some as standard anti-lymphocyte reagents and some in rabbits injected neonatally with large doses of pigeon cells. The neonatal injections were made to reduce the rabbits' antibody response to species-specific antigens upon challenge with pigeon lymphoid tissue.

All unabsorbed sera were screened for differential reactivity against thymus and bursa-derived tissue by passive hemagglutination, immunodiffusion, leukoagglutination and complement dependent lymphocytotoxicity. Sera identified in this manner were absorbed with thymus and bursa tissue to demonstrate specificity. One serum with specificity against thymus extracts was identified by immunodiffusion. The thymus specificity was absent from extracts of pigeon bursa, brain, liver and breast muscle, but present in spleen extracts. The thymus specificity was shared by extracts of thymus from two different breeds of pigeon but not extracts of chicken lymphoid tissue. This

thymus specificity does not appear analogous to lymphocyte specificities identified in other species.

Shared tissue specificities and a possible quantitative antigenic difference among the tissue extracts were also demonstrated by immunodiffusion and absorption analysis.

In lymphocytotoxicity tests, fresh rabbit normal serum is highly toxic for pigeon thymus and bursa cells. This toxicity resembles the natural antibody present in rabbit and guinea pig sera against heterologous thymus cells. The activity against pigeon lymphocytes can be absorbed by pigeon thymus and bursa cells and is heat labile. Unlike the natural antibody against thymus cells of other species, the toxicity against pigeon lymphocytes could not be absorbed by agarose.

A rabbit anti-pigeon gamma globulin serum was rendered specific for bursa cells by absorption with thymus cells. Some standard anti-lymphocyte sera were shown to contain an antibody fraction specific for thymus cells.

Some or all of these reagents may be useful for distinguishing cooperating cell populations in a variety of immune responses. Avian species, in addition to the chicken, may provide additional data on the phylogeny of functionally distinct lymphocyte populations.

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II. DEVELOPMENTAL RED CELL ANTIGEN IN THE PIGEON

A. Abstract

Several fetal red cell antigens are described in the literature for species other than the pigeon. In this study, a fetal red cell antigen was sought as a possible serological marker for white cells as well. There are several reasons for thinking that such an antigen might be present on white cells. First, these two cell types are derived from a common stem cell. Second, a number of other antigens are known to be shared by white cells and red cells. In addition, the thymus and the bursa of Fabricius, which are the primary lymphoid organs in birds, are transient sites of erythropoiesis. Both lymphoid organs could be classified as developmental structures. At least one fetal red cell antigen in known to exist on cells other than mature red cells and to persist in these sites even after the antigen is no longer detectable on red cells.

The studies reported in this thesis did, in fact, reveal an antigen on red cells from all pigeon squabs less than four days old. The antigen appears not to be secondarily adsorbed to the red cells from the fluids of

the egg or the embryo. *In vitro*, masking of the antigen by components of adult serum does not occur under the conditions tested, nor can the antigen be made to reappear in the adult after frequent bleedings. Although the squab antigen behaves similarly to a known fetal red cell antigen in doves, it is probably qualitatively different from that antigen and from the known chick red cell antigen.

The squab antigen is not detectable on lymphocytes from the bursa or the thymus by either leukoagglutination or complement dependent lymphocytotoxicity. The antigen does not appear to be a lymphocyte marker. It is, however, an additional developmental antigen. If it is like other developmental antigens, it could be an interesting marker in both normal development and some disease states.

B. Introduction

An interesting subset of the developmental antigens are those present on red blood cells of the embryo and the neonate, but absent from normal adult red cells. Heterologous antisera have been used to demonstrate fetal red cell antigens in humans, chickens and doves (5, 6, 9, 10). The common fetal red cell antigen of humans, the i antigen, is detected by alloantisera. The relationship between this antigen and the one detected by rabbit antiserum is unknown (5, 11). Only in humans has an adult counterpart, I, of the fetal antigen been identified (11). The amounts of I and i present on human red cells are reciprocally related.

Avian blood is a particularly useful tissue for the study of developmental antigens because it is readily accessible and radical changes occur in both cellular and plasma factors between the time the blood first appears and the fully developed embryo hatches. The differences observed during embryonic development are both quantitative and qualitative (13, 14). The concentration of circulating red blood cells increases rapidly as hatching approaches, while the white cell levels rise more slowly. The first red cells in the avian embryo are a primitive

strain. Red cells in progressive stages of differentiation appear in the circulation of the chick embryo for the first six days of incubation. These cells are subsequently replaced by red cells of the definitive strain. These latter red cells quickly become the major type of red cells in the circulation, although primitive red cells may be found in the blood for as long as two weeks after hatching.

While changes in circulating red cells are occurring, the site of hematopoiesis is also changing (13). The progression is from yolk sac to fetal liver to spleen and bone marrow. The avian thymus and bursa of Fabricius are also minor sites of erythropoiesis. Erythropoiesis is observed in the bursa during the last days of incubation and ceases within several days after hatching. The avian thymus is even less involved in erythropoiesis. The circulating red cells and white cells of the blood appear to be derived from a common stem cell. The bursa and thymus which are minor sites of erythropoiesis are at the same time major and continuing site of lymphopoiesis. The thymus is the first lymphoid organ to develop and the first in which lymphopoiesis is observed. In the chick, the thymus develops at eight days of incubation. The bursa develops

2-4 days later and lymphocytes begin to appear in the bone marrow at about the same time. Small lymphocytes are found in the spleen 2-3 days later.

Red cells and white cells of the adult animal share many, but not all cellular antigens (23). The human blood group antigens A, B, M, N, S, Tj^a and probably the Rh complex are present on human leukocytes. These antigens are more difficult to detect on leukocytes than on red cells. Anti-leukocyte antibodies are more often found coincident with anti-erythrocyte antibodies than expected by chance in sera from multiparous or multiply transfused persons. In the mouse, the H-2 system, which was originally detected as a red cell antigen, not only is detectable on lymphocytes, but also is the major leukocyte antigen system in this species. In the chicken, the B blood group locus is that species' major leukocyte antigen system.

A chicken fetal red cell antigen, while absent from adult mature red cells, was detectable on other adult tissues (15). Spleen, liver, kidney and bone marrow cells of either adult or young chickens were able to absorb 100% of the hemolytic activity from the anti-chick cell reagent. In fluorescent antibody tests, adult red cells were negative, but blood leukocytes were fluorescent.

The I antigen of humans is absent from lymphocytes as indicated by the failure of anti-I sera to agglutinate or lyse lymphocytes in the presence of complement (21). One anti-I serum did lyse some lymphocyte samples, but lysis did not occur with all strong anti-I reagents. Lysis in the anomolous case was probably due to a contaminating antibody of different specificity. Absorption tests were not reported. The fetal antigen in chicks persists in adult tissues other than circulating red cells. If either the I or i antigens were present on human lymphocytes, the fetal antigen, rather than the adult counterpart, might be the more probable specificity. Anti-i was not tested against adult human lymphocytes.

The composition of the blood plasma may be important in determining the antigens on the red cells (19). There is a large positive association between blood group antigens and serum isoenzymes in several species (24). This association does not imply a mechanism of red cell antigen determination. The reason for the correlation remains to be explained. _In vitro_, red cells can adsorb bacterial and blood group specificities from the surrounding medium (19). Stormont demonstrated that the J antigen on cattle

red cells is, in fact, normally adsorbed to the cells from J+ plasma (20).

The composition of the plasma changes radically with development in the chick embryo (14). In the early embryo, the plasma solids are predominantly lipids. The concentration of serum protein increases especially in the week before hatching, and by hatching exceeds the concentration of serum lipids. The physical properties of the plasma also change. The pH changes from acidic to basic. The Cu and Fe levels and the Ca/Mg ratio increase.

All of these components — the physical properties of the serum, the composition of the plasma and the internal environment of the embryo (16) — may affect the expression of red cell antigens.

In the present study, because of the close developmental relationship between red cells and white cells, fetal red cell antigen(s) were looked for in lymphocytes of both thymus and bursal origin. The chicken fetal red cell antigen is present in tissues which at some time contain elements of both the white cell and red cell series. The bursa and thymus can both be regarded as developmental organs because of their early influence

on the development of the immune system, the transient erythropoiesis in both organs in the embryo, and the decrease in organ size and immune function in advanced age.

The possibility of polymorphism in fetal antigens is suggested from the I antigen system in humans (11). Clear polymorphisms exist for the i antigen and polymorphism in I may also exist. Even if no difference were observed between the bursa and thymus expression of a squab fetal antigen, there might be genetic differences among individuals within the breeds of pigeons or between breeds.

The relationship between the various red cell fetal antigens of different species is unknown. The chicken and pigeon systems are not expected to be necessarily identical or even equivalent. These two species are evolutionarily far removed and developmentally distinct. Pigeons belong to the altricial group of birds — those which hatch in a very immature state and develop slowly and require parental care. Pigeons, along with doves, are unique even among the altricial birds, in that parents produce crop milk to feed the very young bird.

The existence and nature of a fetal red cell antigen in pigeons is revealed in the following study. Its

relationship with the known chick and dove fetal red cell antigens is discussed.

C. Methods

Preparation of Antiserum:

An adult female New Zealand rabbit was injected in the toepads with .5 ml of a 20% suspension of washed squab red blood cells mixed with an equal volume of Freund's Complete Adjuvant. One month later an intraperitoneal injection of 1 ml of 20% squab cells in saline was given. Two and five days later the rabbit was injected intravenously with .5 ml of 20% squab cells in saline. Two reimmunizations, repeating the intraperitoneal and intravenous injections, were made at later times. The rabbit was bled ten days after the last injection in each series. Only the last serum sample was demonstrated to have specificity for squab cells after absorption with red cells from adult pigeons. Serum was heated for 1 hour at 56° C and stored at - 20° C.

Absorption with Adult Pigeon Cells:

The rabbit antiserum was absorbed at room temperature and at 4° C with washed, packed red cells from four adult pigeons. Three of these were the offspring of the three original Tumbler mating pairs and the fourth was

an adult King. Absorptions were repeated until the antiserum no longer agglutinated red cells from these same four pigeons.

Eggs:

Eggs are normally laid in a clutch of two, the second egg is laid somewhat less than two days after the first (1). Pigeons were checked three times a week or daily when more accurate ages and incubation times were necessary. Incubation times were determined from the time the second egg was laid because the first eggs are usually not adequately incubated until the female has finished egg laying. The two eggs often hatch the same day.

Bleeding Embryos:

The shell over the air sac was carefully removed to expose the internal fibrous membrane below (9). The membrane was flooded with a warm (37° C) isotonic NaCl-sodium citrate solution (isocitrate). A sharp scalpel was used to nick the visible surface blood vessels and the blood-citrate mixture was removed with a pipette.

Bleeding Squabs and One Day Old Chicks:

Small squabs and one day old chicks were bled terminally by cardiac puncture.

Chickens:

One day old (Kimber Farm) White Leghorn male chicks were obtained after use in short term learning trials.

A single adult rooster which had been immunized to the phage PM2 was used as an adult red cell donor.

Hemolytic Assays:

Hemolytic tests with pigeon red cells were performed as described for chicken red cells (15). Dilutions of antiserum were mixed with one-half volume of 2% washed red cells. Complement was added and the tubes were incubated at 37° C in a shaking water bath. The tubes were centrifuged and the degree of hemolysis estimated.

Complement:

Fresh normal rabbit serum was absorbed with agarose at 4° C for one hour (1). One rabbit was chosen as a complement source because its serum was less toxic for mouse thymocytes than were other rabbit sera. The absorbed serum was divided into small aliquots and stored at - 70° C until use. The complement was titrated in the hemolytic assay against both squab red cells and adult red cells.

Collection of Allantoic Fluid:

A fertile egg which had been incubated 8 days was candled. A pencil cross was made on the shell about one quarter inch below the air chamber and away from large blood vessels. A syringe needle was inserted through the shell at the pencil mark and allantoic fluid withdrawn.

Saline Extracts:

A one to two day old squab was sacrificed shortly after feeding, the crop was opened and the milk removed with a spatula. The egg yolk and egg white from an infertile egg were separated. Equal volumes of the egg yolk or egg white or crop milk were shaken with an equal volume of .001M MgCl in saline. The extract was spun down in a clinical centrifuge at top speed and the supernant collected and stored at - 20° C.

Incubation of Red Cells with Fluids or Extracts:

20 μl of washed adult pigeon red cells were incubated with 2 ml of appropriate fluid or extract at 37° C. Red cells for a two day old squab were similarly incubated in adult serum. After forty minutes the incubation mixtures were centrifuged and the fluid removed. The cells were not washed, but the fluid was replaced by enough saline to make a 2% suspension of red cells.

Serum Absorption with Chicken Cells:

The squab specific reagent was absorbed four times with red cells from one day old chickens. Absorptions were performed for five minutes at room temperature, followed by a five minute absorption at 4° C.

Additional Methods:

Additional methods may be found in the methods section of the preceding chapter.

D. Results

Absorption of the antiserum with red cells from a single adult pigeon removed most of the antibodies directed against adult red cells, but the serum still agglutinated red cells from some adult pigeons at low serum dilutions. No agglutination was observed when the absorbed serum was tested against red cells from the same pigeon as used for the absorptions. Absorption of the antiserum for detection of a possible squab antigen was not considered complete until the antiserum no longer agglutinated red cells from the four pigeons used to provide red cells for absorptions. The antiserum was also tested against red cells from the 12 birds used to start the pigeon colony. At no time did the absorbed antiserum agglutinate red cells from these additional adult birds. The parents of the positive squabs were also found to be negative, although the parents were not tested in every case.

The absorbed antiserum always agglutinated red cells from squabs between hatching and four days of age (Table 1). Because the Tumbler pigeons reproduce faster than the King pigeons, most of the squabs tested were Tumblers. Exceptions are noted in the table legend. Cells were

	embryo days of incubation			hatch → squab age													
Age	11	13	17	1	2	3	4	5	7	9	10	13	15	16	24	33	adults
Hemagglutination titer $\log_2$	5	6[c]	4	8	8	8	7-8	7[ad]	7	0	3-4	0	0	3	0	0	all 0
				7	4	8		6[a]	7	0			2-3				
				7	7-8	6[a]		0	0								
				6	7												
				8													
# Positive/# Total	3/3			13/13				4/6		4/7					0/2		0/20[b]
Average titer for positive squabs	5			7				7		3					—		—

a. weakly reactive; b. negative on subsequent bleedings also; c. King embryo;
d. King-Tumbler hybrid

TABLE 1. HEMAGGLUTINATION BY SQUAB SPECIFIC REAGENT AS A FUNCTION OF SQUAB AGE

generally positive at serum dilutions of 1:128. Cells from embryos between 11 and 17 days of incubation were agglutinated although the reactivity was somewhat less than that observed for the newly hatched squabs. Reactivity was heterogeneous for individual squabs between five and eight days after hatching. Some squabs reacted with the same serum dilutions as the very young squabs, and others were negative. Heterogeneity persisted until 16 days, but the degree of disparity between individuals decreased. Positive cells were agglutinated only with serum dilutions of 1:8 or less. There may be two prototype patterns of reactivity — which are indistinguishable until four days after hatching when the reactivity disappears in one and is diminished in the other. Heterogeneity in the persistence of reactivity was also noted in individual squabs which were bled every other day until their cells were no longer agglutinated by the antiserum (Table 2). Absorptions with red cells from individual squabs were limited because of the amount of material needed for the absorption and the volume of antiserum necessary to perform the tests. Complete absorptions were obtained with cells from two different squabs. Tests of the absorbed sera against

Squabs		Age of Squab 1	2	3	4	5	6	7	8	9	19
1	sibs	7	-[a]	0	-						
2		-	5	-	0						
3	sibs	4	-	6	-	7	-			7	
4		-	8	-	7	-	7				0

a. not done

TABLE 2. SQUAB SPECIFIC ANTIGEN IN INDIVIDUAL SQUABS

(Hemagglutination Titers $\log_2$)

red cells from other one to two day old squabs indicated that absorption with cells from a single donor did not remove reactivity to all other squab cells, but did reduce the serum titer significantly. Reciprocal absorptions were not performed.

Immunodiffusion tests were performed with the antiserum against saline extracts of egg yolk and of egg white and of crop milk and one to two day squab serum in Alsever's solution and allantoic fluid. Embryo red cells were mixed with some allantoic fluid during bleeding. The unabsorbed serum strongly precipitated a component in allantoic fluid, and weakly precipitated a component of squab serum and of milk extract. No precipitin bands against the egg white extract were observed. Egg yolk was highly opaque to an extent which made it impossible to distinguish any precipitin bands. Absorptions with adult red cells removed precipitating activity against all extracts and fluids except the allantoic fluid. The percipitin reaction with allantoic fluid was considerably reduced.

Because specific agglutination was observed in intact red cells, the antigen was assumed to be present on the cell surface. Attempts were made to attach or to reveal the

antigen on adult red cells. Incubation of adult cells in squab serum, allantoic fluid and saline extracts of egg white and egg yolk, failed to confer a positive reaction on adult cells.

Squab red cells are less stable when stored in Alsever's solution than are adult cells. They are more susceptible to lysis in distilled water and to complement mediated antibody lysis indicating that they are more fragile and perhaps that the additional red cell antigens of the adult confer stability upon the red cells and mask expression of the fetal antigen. Incubation of the squab red cells with serum from an adult bird did not prevent agglutination when the squab red cells were subsequently tested with the squab specific antiserum.

The adult serum did protect the squab red cells from spontaneous agglutination. Squab cells incubated with saline under identical conditions of incubation spontaneously agglutinated even in the absence of antibody, whereas those incubated with adult serum agglutinated only with the antiserum and only with the dilutions of antiserum to which they were normally reactive.

Red cells from a frequently bled adult bird were less stable than normal adult red cells upon storage at 4° C in Alsever's. These red cells formed less compact cell buttons in the absence of the antiserum, but still failed to agglutinate with the squab specific reagent. No evidence was obtained that the squab specific antigen was secondarily adsorbed to the squab red cells or that the antigen was present but masked in the adult red cell.

The unabsorbed antiserum reacts strongly in immunodiffusion with saline extracts of thymus and of bursa. These extracts also contained adult serum components. Passive hemagglutination which is more sensitive for the detection of soluble antigens than immunodiffusion showed some low level reactivity for extracts of thymus and bursa (Table 3). No reproducible difference in reactivity between extracts from thymus or bursa was found. The nature of these reactions or the antigens responsible are unknown. The antigens may not be membrane antigens. Specificity for intracellular soluble antigen, either common to both organs or unique, would not have been absorbed by intact adult red cells.

A. Passive Hemagglutination Results

experiment number	antiserum	bursa extract	thymus extract
1.[a]	unabsorbed	8[c]	6
	squab reagent	4	4
2.[a]	unabsorbed	5	3
	squab reagent	4	0
3.[b]	unabsorbed	>12	>12
	squab reagent	6	7

B. Leukoagglutination Results

antiserum	bursa cells	thymus cells
unabsorbed	5[c]	6
squab reagent	0	0

C. Lymphocytotoxicity Results

antiserum	bursa cells	thymus cells
unabsorbed	<10[c]	9-10
squab specific	<5	<5

a. Boyden method

b. method from Kabat and Meyer

c. titer $\log_2$

TABLE 3. SPECIFICITY OF RABBIT ANTISERUM FOR BURSA OR THYMUS EXTRACTS AND CELLS

A single absorption of the squab reagent with bursa cells failed to reduce the titer of the serum when tested against red cells from the same newly hatched squab. Bursa and thymus cells tested in either leukoagglutination or lymphocytotoxicity tests for comparative purposes were always from the same individual. Individual differences would therefore not be detected, but organ specific differences could be detected, if present. Bursa and thymus cells from squabs over a week old failed to react with the squab specific reagent in leukoagglutination or in complement dependent lymphocytotoxicity tests (Table 3). The unabsorbed rabbit antiserum tested against the same cells was used as a positive control.

The squab reagent agglutinated red cells from one day only chicks, but not from adult chickens (Table 4). The titer against chick cells was slightly lower than against red cells from one to four day squabs. Absorption of the reagent with chick red cells removed all reactivity against chick cells without significantly affecting the titer against squab cells. This cross reactive specificity could account for the red cell heterogeneity in squabs between five and sixteen days of age, but this possibility was not pursued.

Red Cell Donor	Agglutination (titer $\log_2$) by	
	Squab Reagent	Squab Reagent absorbed with chick red cells
adult chicken	0	n.d.[a]
one day old chicken	5	0
one day old squab	7	6

a. not done

TABLE 4. AGGLUTINATION OF RED CELLS FROM ADULT AND ONE DAY OLD CHICKENS BY SQUAB SPECIFIC REAGENT

E. Discussion

Absorption of the rabbit antiserum with red cells from a single adult pigeon failed to remove reactivity against red cells from all adult pigeons, but substantially reduced the titer against adult red cells. Because the antiserum was prepared against red cells from newly hatched squabs, those red cells must have already expressed some adult red cell alloantigens. These observations are in agreement with more extensive developmental studies of adult pigeon red cell antigens (2, 3). Of the five identified pigeon red cell antigens, three are already present at hatching and two are detected at one week after hatching. Adult antigen levels are not reached until one to three weeks after hatching.

During embryonic life and before adult antigen levels are reached, the red cells may express fetal antigen(s). Rabbit anti-squab red cell antiserum absorbed with adult red cells specifically agglutinates squab red cells. The strongest agglutination reactions are observed in red cells of embryos and squabs less than four days old. The transient appearance of the squab antigen as detected by hemagglutination is very similar to the time interval during which the known dove embryo antigen was detectable on dove

red cells. Ousterhoudt and Irwin reported that a dove embryo antigen was present on red cells from all embryos and disappeared four days after hatching (9). They tested thirteen pigeon embryos between six and thirteen days of incubation for the presence of the dove antigen, but no antigen was detectable.

The obvious developmental structures coincident with squab antigen expression are the egg yolk, allantoic fluid, egg white and primitive red cells. Incubation of adult red cells with extracts of egg structures and embryo fluids were performed to determine if the antigen could be adsorbed onto the red cells from the embryonic environment. The squab reagent weakly precipitates a component of allantoic fluid. Red cells of many species adsorb lipoproteins and serological specificities both _in vitro_ and _in vivo_. The present experiments indicate that the squab specificity could not be adsorbed by adult red cells from fluids and extracts, at least under the conditions of the tests.

The primitive red cells are only a very small percentage of the circulating red cells in the newly hatched chick. If the same is true in pigeons, there are not enough primitive red cells to account for the strong agglutination pattern observed with this reagent.

Because the squab cells are more fragile than adult pigeon red cells to hypotonicity and complement dependent lysis, the squab antigen could be present in the adult red cells but masked from reaction with specific antibody. Incubation of squab red cells in adult serum does not support this possibility. Frequent bleeding of an adult bird also did not change the non-agglutination of adult red cells with this reagent.

Both adsorption of serological specificity and masking of fetal specificity have been observed in other species and other red cell antigen systems (19). Sanders and Wright observed that papain treatment of red cells from adult brown trout revealed a specificity characteristic of red cells from yearling trout (18). Similar papain treatment of adult chicken red cells failed to reveal the fetal red cell specificity (15). Sanders demonstrated that the expression of the chick fetal antigen was not changed by in vitro or in vivo experiments in which the antigen might become covered and inaccessable to antibody. The antigen expression was apparently dependent upon a neonatal internal environment. The time of appearance of the chick fetal antigen did not correlate with observed quantitative differences in macroglobulin or lipid in the young chick and the adult chicken.

Levi and Schechtman reported that fluids and tissues from chick embryos failed to inhibit agglutination (6).

Ousterhoudt and Irwin found that the dove fetal red cell antigen was not removed from positive cells after washing, or treatment of the cells with either hyaluronidase or trypsin (9). The three avian fetal red cell antigen systems are similar in that simple direct mechanisms of antigen loss cannot be demonstrated. Sanders suggested that the antigen expression in the chick system might be determined by differential gene expression (15).

Although the chicken fetal antigen was detected on bursa and thymus cells of one day old chicks, the squab antigen could not be detected on squab bursa or thymus cells by either leukoagglutination or lymphocytotoxicity. Sanders et al. found that not all cells of the neonatal chick bursa and thymus express the chick fetal antigen (17). If the positive cells were stem cells (17), the squab antigen might also be present in thymus and bursa but not present on lymphocytes. The squab antigen does not appear to be a useful lymphocyte marker.

The squab specific reagent reacts with red cells from one day old chicks but not from an adult chicken. Agglutination of chick red cells is due to cross reactive

antibodies in the rabbit antiserum. Absorption with chick cells removes all reactivity to chick cells but has no significant effect on the agglutination of squab cells. It is not known if the specificity detected by this cross reactive antibody is the same as the reported chick fetal red cell antigen.

Because reactivity against chick cells was not necessary for the specific agglutination of squab red cells, the chick specific antigen is probably different from the major antigen detected on squab cells in this study. The squab antigen appears to be different from that reported in dove red cells because the dove embryo reagent did not agglutinate red cells from any of the pigeon embryos tested (9). Because the dove antigen cannot be detected on pigeon embryo red cells and the squab reagent can be rendered nonreactive to chick cells without affecting its squab specificity, the squab antigen appears distinct from the other avian red cell developmental antigens.

F. Blood Group Antigens in Ontogeny, Phylogeny and Disease States

The embryo specific red cell antigens which have been described in the literature are all present, at least neonatally, on the red cells of all individuals of the species. These are cell surface antigens, but their role, if any, in ontogeny is unknown. Since red cells are freely circulating, the antigens are not likely to function in cell interactions or in morphogenetic movements. They are not required for the formation or function of fetal hemoglobin. In humans, the i antigen and fetal hemoglobin are present concurrently in normal development. The rate of synthesis of the β chain of fetal hemoglobin does not correlate with the rate of synthesis of the I antigen (21). The two events are presumed to be under different regulatory control. In the rare adults whose red cells express the i antigen, adult hemoglobin — not fetal hemoglobin — is found (11). In normal adults whose red cells contain fetal hemoglobin the I antigen is present on the cell surface. In chickens, the fetal red cell antigen and the fetal hemoglobin are not present at the same times even in normal development (15). The presence of a normal fetal component in the

adult apparently is not necessarily indicative of a return to the fetal state in a genetic sense.

The significance of the fetal expression of some antigens is clouded by the fact that so-called fetal antigens may be fetal antigens only for a defined species. In one species of doves, for example, an antigen has been found on red cells for only a short period of development. In a second species the same specificity continued to be present in the adult (9).

The species distribution of fetal red cell antigens, or indeed of red cell antigens in general, are not necessarily indicative of evolutionary relationships among the species. Tha AB human blood group specificities are found throughout the plant and microbial species as well as in animal species other than humans (11). The relationships among the various fetal red cell antigens is unknown and only the Ii system has been looked for extensively in other species (25). Both I and i are widely distributed. The fetal i specificity is detectable in adults of some infrahuman species. The distinction between adult and fetal red cell antigens may have no significance outside the reference species.

In humans, the I antigen, unlike the other human blood group antigens is absent from fetal blood and from cord blood (11). It is, in that respect, late in developing, but it reaches adult levels by 18 months of age. The A and B, P and Lu^a human blood group antigens while present in human fetal blood may not reach steady adult levels until three, seven or fifteen years, respectively, after birth (11). The level of a particular antigen is not necessarily less than full adult level during that time. It may exceed adult levels and subsequently return back to adult norms. The A and B specificities in human fetuses are cross reactive with the A and B specificities of adults, but not identical.

The common ABO blood group polymorphisms in humans have been studied by many investigators in hopes of finding an association between antigen and disease susceptibility or resistance. Because individuals are tolerant of their own antigens and because a number of micro-organisms carry antigens highly cross reactive with the A and B blood group substances, it was thought that difference in ABO type might be responsible for a difference in fitness between individuals. Many small but significant associations between

ABO type and noninfectious and infectious disease have been reported (22), including diseases of older adults. Selective pressure for maintenance of polymorphism would have to occur prior to or coincident with reproductive capability. A significant influence of ABO type has been reported in very young children with regard to hospital admittance for gram negative infections (12), in spite of the fact that the AB antigens do not reach adult levels until three years of age and the fetal A and B substances are quantitatively and qualitatively different from the adult A and B substances.

ABO incompatibilities between mother and fetus have been suggested in association with early fetal death. A recent study by Hiraizumi et al. failed to demonstrate any significant correlation between ABO maternal-fetal incompatibilities and fetal death (4). They did report a significant effect of fetal genotype on fetal survival. Type A fetuses seemed to be favored. The advantage of blood group A individuals was less significant for infants in the first month after birth.

A different and more direct influence of blood type on disease is indicated in those diseases with recognizable antibodies directed against blood group substances.

Antibodies against the Rh factors are directly responsible for erythroblastosis fetalis. Rh is already expressed on the red cells in the human fetus. Autoimmune anti-I antibodies are responsible for the cold type autoimmune hemolytic anemia and a reexpression of the i antigen in the affected individuals (11). The complementary antibody, anti-i, was identified in several patients with reticulosis. Most individuals with either anti-I or anti-i in their serum have been detected because of attendant hematological disorders. Natural antibodies to these specificities are rare. These autoantibodies represent an apparent break in tolerance to either normal adult or normal fetal antigens. Expression of fetal cellular antigens at least in some disease states may be modified by environmental influences directly, without modification of gene action.

Malignancies directly affecting blood cells may change antigen expression on red cells. The reactivity of human red cells to a number of typing sera, including those having A, B or I specificity, is altered by leukemia (11). The natural antibodies to the A and B antigens are also altered. The antibody change is possibly a secondary effect of altered expression of the autologous antigens.

In chickens, the fetal red cell antigen reappears in leukosis (17).

The occurrence of fetal antigens in normal ontogeny and in some disease states has suggested to many investigators that these antigens may be useful in elucidating the relationship between environmental influences and cell surface specificities, the mechanism of differential gene expression or identifying the target cell for particular disease processes. The identification of new developmental antigens may provide additional markers for elucidating these processes.

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III. EFFECT OF IMMUNOSUPPRESSION ON PRIMARY TUMORS

A. Abstract

If immune surveillance suppresses spontaneous tumors which result from somatic mutation, environmental carcinogens, and oncogenic viruses, anti-thymocyte serum treatment should increase the tumor incidence and shorten the latent period. It might also allow the expression of tumors not normally seen in unsuppressed populations.

Virgin female mice of a single inbred strain, CBA/J, were obtained after a variety of treatments and observed for primary tumors until either tumor onset or death. Included were mice which were: (1) immunosuppressed as adults by injection of rabbit anti-mouse thymocyte serum (RAMTS), (2) injected with normal rabbit serum, (3) immunized with an irrelevant antigen (mouse serum β-lipoprotein) or (4) untreated. Data were collected on tumor histology, incidence and time of onset for all groups.

No tumors appeared during the period of ATS-immunosuppression or for several months following treatment. There was no evidence from this study or from the literature that anti-thymocyte serum has any carcinogenic effect in the absence of oncogenic viruses or carcinogens.

This strain carries mammary tumor virus as a result of neonatal infection via milk from infected mothers. The most frequently observed tumor, the mammary tumor, appeared at an average age of slightly less than two years. Although the first tumors appeared in ATS-treated mice, the mean age at tumor onset was not significantly affected by ATS-immunosuppression. No unusual tumors and no lymphomas were observed.

Tumor incidences among groups of mice purchased at different times were different, but unrelated to ATS-immunosuppression. Tumor incidence was not depressed by failure of mice to reach tumor age; the mean life span of non-tumor bearing mice was as long as or longer than that of tumor-bearing mice. Cause of the fluctuation in tumor incidence is not known.

Although tumor immunity reportedly exists at a time when palpable mammary tumors are present, immune surveillance does not appear to be the determining factor in the long tumor latent period. This conclusion is supported by published data from other laboratories which indicate that cellular immunity to mammary tumors is often compromised by immunological tolerance and/or the presence of

blocking factors in the serum. The failure of ATS-immunosuppression to affect mammary tumor growth is consistent with these observations.

B. Introduction

1. Histocompatibility - Necessity for Immunosuppression

Major histocompatibility loci have been identified in a number of vertebrate species and homology between these systems is often assumed (59, 66). The H-2 system in mice is the best studied and has been used to elucidate the basic principles of transplantation biology. The histocompatibility antigens, like blood group antigens, are expressed condominantly and are present to some degree on most tissues. If a graft donor has histocompatibility antigens different from those of the recipient, the graft will be rejected. Graft rejection is normally an immune phenomenon triggered by antigen-specific, thymus-dependent lymphocytes. It is associated with leukocyte infiltration and can be passively transferred by lymphoid cells, but not by serum. Damage appears to result from contact between "killer" lymphocytes and the target graft. For these reasons graft rejection is termed a cell-mediated immune response.

In humans, HL-A — a leukocyte antigen system, and ABO — the blood group antigens, function as histocompatibility antigens (65). Graft donor and recipient are cross-matched for ABO as they would be for a blood transfusion.

Significantly longer skin graft survival is observed when related donor-recipient pairs are HL-A matched. In unrelated individuals there is no correlation between HL-A compatibility and skin graft survival. If individuals are presensitized to a particular HL-A antigen, they will reject grafts bearing that antigen more quickly than they will grafts lacking the antigen. The effect of preimmunization demonstrates the potent antigenicity of HL-A and its importance when grafts are exchanged between unrelated persons. The difference in rejection times and the rationale for tissue matching prior to transplantation is that an individual usually reacts immunologically against only those antigens which he/she does not possess.

Sensitization to HL-A antigens is sometimes noted in multiply transfused or multiparous persons. Serum from such persons is the source of most HL-A typing reagents. Most potential graft recipients have been multiply transfused and therefore may be presensitized to some HL-A specificities. Circulating antibody to HL-A antigens has resulted in hyperacute rejection of kidney grafts carrying the corresponding HL-A antigens. Experiments in rabbits have confirmed the role of antibody in this unusually severe type of graft rejection.

Kidney grafts to immunosuppressed recipients who are matched to donor HL-A type function better and longer in proportion to the degree of HL-A matching. This result holds for unrelated as well as related donor-recipient pairs and has been observed both prospectively and retrospectively. The ability to match recipient and donor at HL-A is greatly impaired by the high degree of polymorphism associated with this locus.

An estimate of the number of alleles at HL-A was made from mixed leukocyte culture (MLC) data (7). In vitro as well as in vivo leukocytes respond to antigenic challenge by clonal expansion of the antigen-specific lymphocytes and of non-specifically activated leukocytes. Histocompatibility antigens are unique in that 40-60% of unsensitized lymphocytes respond in this way to allogeneic lymphoid cells. In the mixed leukocyte reaction, the mitotic activity of co-cultured allogeneic leukocytes is compared with that of the separately cultured leukocytes. Significantly increased incorporation of ^{3}H-thymidine is associated with HL-A differences between the leukocytes. ABO differences do not stimulate label incorporation under these conditions. Bach and Bach observed that 28% of mixed

leukocyte cultures from sibling pairs failed to stimulate. No instance of non-stimulation was observed in the 700 MLC between pairs of unrelated individuals.

The percentage of nonstimulation for sibling pairs is consistent with genetic data indicating that HL-A is a single genetic locus. From the frequency of non-stimulation in both cases, the effective number of alleles present in the test population is a minimum of twenty and more probably greater than thirty.

There is no simple relationship between the number of histocompatibility alleles and number of histocompatibility antigens (66). In H-2 there are at least 19 alleles and 36 H-2 associated antigenic specificities. The $H\text{-}2^a$ allele includes ninteen specificities and other alleles include varying, but lesser numbers of specificities in various combinations. These data are based on experiments with inbred strains of mice, most of which are related in origin. Preliminary results from Klein's laboratory indicate that wild mouse populations, presumably more representative of outbred populations, carry the known H-2 alleles and at least twenty additional H-2 alleles.

So far, HL-A is antigenically less complex than H-2 (65, 66). There are 11 internationally recognized HL-A

antigens. These antigens fall into two segregant series on the basis of apparent antigen exclusion. The present two subloci model does not account for all the data on HL-A, but is the prevailing model. Errors in the model are expected to be on the simplistic side. Refinement of HL-A serology will probably reveal hidden specificities in the present typing reagents — adding to the number and diversity of HL-A antigens.

If hidden specificities are present in typing sera, then individuals typed as identical for a given antigen could be antigenically identical for only a subset of the antigenic specificities. The serology would indicate more identity than really exists and would emphasize sameness.

In transplantation it is the differences which are important and which result in graft rejection. Serologically defined differences do not indicate the strength of the antigenic differences as recognized by graft rejection *in vivo*. There are no data in humans to indicate that certain serologically defined differences may be responsible for more vigorous cell-mediated responses. In mice, H-2 differences within the K region have been reported to be stronger stimulators of cellular immunity than differences

confined to the D region (112). The difference may be related to an additional locus within the H-2 region (5, 78).

Recognizing the deficiencies of the two sublocus model and its possibly oversimplistic representation of HL-A, Kissmeyer-Nielsen and Thorsby have used the model to make a minimum estimate of the number of possible HL-A phenotypes (65). Considering the 11 officially recognized HL-A antigens and 8 other antigens recognized by their own reagents and assuming a minimum of null alleles, they calculated that 4725 HL-A phenotypes are possible. In their population studies the most frequent HL-A phenotype was present in only 1-2% of the population. If a single antigenic difference is allowable, then an acceptable match for the most frequent phenotype would occur at random 11% of the time. The probability of a match declines rapidly when other phenotypes are considered. The chance of a perfect HL-A match between siblings remains one in four because recombination within HL-A is infrequent.

The fitness of a match between unrelated individuals is becoming an increasingly important medical problem. Prior to 1959 less than 10 transplants were performed

annually in the world. By 1971 that number was greater than 4000. The percentage of living donors, mostly relatives, has decreased from 54.5% to 32.8% over the same time interval. The corresponding increase in donors has been in unrelated, cadaver donors (1).

Polymorphism in minor histocompatibility loci affects the transplantability between related individuals as well as between unrelated individuals. In mice it has been demonstrated that differences at minor histocompatibility loci can act additively and resemble a major histocompatibility locus difference — causing rapid graft rejection (45). Humans, undoubtedly, have as yet unidentified minor histocompatibility loci.

With the difficulties in HL-A matching, the existence of minor histocompatibility loci and perhaps other antigenic stimuli, rejection is a virtual certainty for the graft recipient. Although matched related donors could alleviate the HL-A incompatibilities, rejection is also indicated. Skin graft survival time for an HL-A identical match is significantly prolonged compared to the survival time for a mismatched graft, but even then rejection occurs within twenty days — indicating far from infinite survivals.

Kidney may not be as antigenic as skin as illustrated by somewhat longer survival times for kidneys than for skin in both mouse and human; nonetheless, the antigenic disparity is sufficient to cause rejection.

Survival of a functioning transplanted organ requires suspension of the normal immune response. This occurs naturally in the case of corneal transplants or in the normal maternal-fetal relationship where the fetus may be considered comparable to an antigenically foreign transplant. The common feature of these two examples is that the circulating immunocompetent cell fails to recognize or be stimulated by the foreign antigens. For the cornea, this is a fortunate consequence of normally poor vascularization and small graft size (58). The fetus may be protected from effective contact with the immune system by the high sialomucin content of the trophoblast (58, 120). There is also some evidence that the pregnant female is specifically less reactive to histocompatibility antigens of paternal origin than to histocompatibility antigens not present on paternal cells (15). The specificity suggests an immunological reaction similar to immunological tolerance. Kidney, heart, lung, liver, bone marrow cells and

peripheral blood lymphocytes are subject to rejection and are not privileged transplants. Graft survival in these cases must be artificially prolonged by direct interference with the immune system rather than by masking or protecting the graft itself.

2. Methods of Immunosuppression

The ideal mode of immunosuppression would be specific immunological tolerance to donor histocompatibility antigens. In a state of tolerance an individual fails to respond to the tolerizing antigen but responds normally to all other antigens. Although there is evidence of partial tolerance or tissue adaptation in long term graft recipients, that population may be highly selected for reduced immunity.

Experiments with mice have demonstrated that tolerance to cellular antigens is much more easily established in neonates than in even slightly older individuals (69). Maintainence of tolerance requires that the individual be chimeric for lymphoid tissue of the two histocompatibility types. Since human transplants are performed on individuals past the neonatal stage, any scheme requiring identification

and treatment of the graft recipient as a neonate is impractical and unrealistic at this time.

In mice, induction of tolerance or immunity to soluble antigens depends upon the antigen dose. Tolerance can occur at dosages well below or above those which immunize. The amount of material per unit body weight necessary to induce high zone tolerance is equivalent in neonate and adults. The necessity and difficulties in identifying histocompatibility differences between donor and recipient are the same as previously discussed. The current preparations of soluble histocompatibility antigens are far from supplying the quantities and specificities required both for the induction and maintainence of tolerance.

In human transplantation, the risk and consequences of sensitizing the individual are much greater than the chance of establishing tolerance. Sensitization results in rapid second set or acute rejection which is more difficult to control than a first set rejection.

A generalized state of immunosuppression is more easily and reliably obtained. Immunosuppression may even facilitate tolerance induction. The immune response can be blocked at any point up to and including the reaction of

immune cells or antibody with antigen. Its effectiveness may depend upon the ability of the immune response to compensate for perturbations in other parts of the system. The target may be antigen-specific lymphocytes or non-specifically activated lymphocytes and macrophages. The block may occur in antigen recognition, antigen processing, lymphocyte activation by antigen, lymphocyte differentiation, cell recruitment and mitosis, release of soluble mediators, antigen expression or in any required synthetic process along the way.

There are five major groups of immunosuppressants which are therapeutically practical. They are antimetabolites, corticosteroids, x-irradiation, anti-lymphocyte serum (ALS) and surgical removal of lymphoid organs (26, 73, 131). Most antimetabolites are effective immunosuppressants at dosages which interfere with cell division although individual agents differ in their kinetics of inducing immunosuppression and their direct cytotoxic effects. The selective value of these agents is a result of the selective division of antigen reactive cells upon contact with antigen. Because of this division, immunocompetent cells become more sensitive to the toxic effects

of these drugs. The transplant patient is exposed to numerous environmental antigens as well as foreign histocompatibility antigens. At immunosuppressive doses the antimetabolites can also be toxic for other physiological systems. Because of the general toxicity of these drugs, it is likely that at least some antimetabolites interfere with normal immune processes at several levels simultaneously. The resultant immunosuppression is generalized and is not antigen specific.

The corticosteroids are lymphocytolytic and anti-inflammatory in rodents and therefore are potentially active both on central and peripheral elments of cellular immunity. In humans, steroids appear to act primarily by reducing inflammatory responses. Continued steroid treatment can cause a severe imbalance in a number of physiological systems.

X-irradiation causes chromosomal damage in both dividing and non-dividing cells, and results in cell lethality during cell division. It is therefore less specific in its action than are most other antimitotic agents. Sublethal doses of x-rays induce a persistent generalized immunosuppression. Because of their respective requirements for cell division

(clonal expansion), primary immune responses are more sensitive to x-irradiation than are secondary responses.

Thymectomy removes both a source of lymphocytes and hormone(s) — necessary for the maturation of precurser lymphocytes into immunocompetent cells. Cells within the thymus are not generally immunologically competent. They are considered to represent a precurser or immature form of the lymphocytes which are active in cell-mediated immunity in the periphery. The thymus-derived cell is equivalent to an immunocompetent cell active in cellular immunity.

The cells or precursers of cells which directly produce antibody are not affected by thymectomy and are therefore termed thymus-independent cells. Thymectomy obviously does not remove immunocompetent cells which have already seeded into the peripheral lymphoid organs. For that reason adult thymectomy alone is an ineffective immunosuppressant, whereas neonatal thymectomy is severely immunosuppressive. Thymectomy of the neonate or the adult has no confirmed direct effect on systems other than the immune system.

Splenectomy removes a mixture of antibody producing cells, cells active in cell-mediated immunity and a source of red blood cells. Thoracic duct drainage removes

circulating lymphocytes, most of which are thymus-derived, but also severely affects fluid and electrolyte balance.

Anti-lymphocyte serum (ALS) can be a very potent and specific suppressor of cell-mediated immunity (72, 128, 139, 140). It is effective in an otherwise nonsuppressed host if administered before or shortly after graft placement. It is not very effective if given much later than the antigen. These antisera are usually prepared in a heterologous species against lymphoid tissues of the graft recipient species. Anti-lymphocyte sera prepared in the same species as the host have not generally prolonged graft survival in normal recipients (10, 98, 129). The lymphoid tissue used as antigen may be limited by tissue availability and quantity. Whole cells or crude membrane fractions are effective as antigens. ALS prepared in a single species and by the same method against lymphocytes from different organs or cultured lymphoblasts are of different graft prolonging potencies (46, 93). ALS prepared against thymocytes is most effective, that prepared against lymph node lymphocytes the second most effective and the rest are of lesser potency. That thymocytes are the most effective antigen(s) might be expected, because thymus-derived lymphocytes are the effective cells

in graft rejection and peripheral lymphoid tissues are mixtures of both thymus-derived and thymus-independent lymphocytes. _In vivo_, ALS does not appear to affect the thymus — possibly because of the purported blood-thymus barrier. There is, however, a significant but transient lymphopenia after administration of ALS. The ALS target cells _in vivo_ are assumed to be immunocompetent thymus-derived lymphocytes.

The evaluation of an ALS by comparison of graft survival times is time consuming, expensive and wasteful of ALS. ALS show a high degree of species specificity — restricting tests to the species against which the serum was prepared or closely related species. Testing anti-human lymphocyte serums would necessitate using mostly subhuman primates.

It is clear that most _in vitro_ assays of ALS do not correlate with _in vivo_ suppression of graft rejection. Hemagglutination, leukoagglutination, lymphocytotoxicity or blast transforming assays are of no value in predicting ALS activity _in vivo_ (26, 60, 61, 128). This lack of correlation suggests that the sera may contain irrevelant and toxic antibodies — including and possibly in addition to anti-red cell, anti-platelet and anti-basement membrane

antibodies. It is also possible that the effectiveness of ALS depends upon more than one antibody population.

Some correlation with *in vivo* results has been claimed for the indirect globulin test (86), inhibition of rosette formation (6) and depression of pertussis induced leukocytosis (103). These results need to be confirmed.

The method of immunization — i.e., schedule of injections and use of adjuvant, is important in the apparent balance between effective and toxic antibodies. Early antiserum prepared without the use of adjuvant is most effective (72). Reimmunization of the same animal does not stimulate a marked increase in the relevant antibody, but rather the appearance of highly cytotoxic antibodies and a loss of graft prolonging potency when compared to earlier bleedings. Adjuvant, like reimmunization, elicits apparently cytotoxic and undesirable antibodies (62, 67).

The effective antigen(s) is unknown, but is presumed to be a membrane component. Absorption of ALS with red blood cells, liver or other nonlymphoid tissues does not absorb out the graft prolonging capacity of the serum. Absorption with lymphocytes removes immunosuppressive activity from the serum. The effective antigen(s) appears to be lymphocyte

specific. It is possible, but not established, that this antigen(s) is indicative of a particular state of lymphocyte differentiation. Because the effective antigen(s) is not conserved in widely disparate species, it may not be integral to the membrane or even integral to the immune functions of lymphocytes.

Anti-lymphocyte sera prepared by current methods need not be considered as acting at only one level of the immune response — if only because they contain antibodies of more than one specificity.

Isolated immunoglobulins from ALS retain their *in vivo* activity, whereas Fab' fragments of these molecules are almost ineffective (30). Because the antibody combining site is in the Fab' fragment and the Fc portion of the molecule functions in amplifying the immune response, more than a simple masking of antigen sites is suggested in ALS immunosuppression *in vivo*. The same conclusion was reached by Levey and Medawar, who showed a central immune deficit after ALS treatment (72). Lymphocytes transferred from an ALS-treated animal to a syngeneic x-irradiated recipient remained specifically nonreactive although these cells would have undergone several division cycles in a neutral environment prior to testing.

Monaco showed that ALS treatment combined with thymectomy prevented recovery of immunity to allogeneic histocompatibility antigens (87). Thymectomy apparently prevented the generation of new reactive cells which otherwise would replace those depleted by ALS treatment. These results indicate that ALS produces immunosuppression which may, because of concurrent antigenic stimulation, show some antigen specificity. The duration of immunosuppression depends upon the loss of antigen reactive cells.

ALS preparations do induce a transient depletion of circulating lymphocytes. Those preparations which are not cytotoxic also do not prolong graft survival, but lymphocytotoxicity alone cannot account for suppression of graft rejection. Recovery from lymphopenia does not restore immunocompetence nor does graft prolongation correlate with either _in vivo_ or _in vitro_ cytotoxicity titers for ALS.

Levey and Medawar noted hyperplasia of lymphoid tissue in ALS-treated animals and postulated that ALS might work by sterile activation of the lymphocyte population (72). This suggestion, however, was controverted by later work in the field. Woodruff and colleagues observed atrophy of lymphoid tissue after ALS administration and suggested that

hypo- or hyperplasia of lymphoid tissues *in vivo* may depend upon the local concentration of ALS (139).

Turk and Willoughby observed that ALS preparations are anti-complementary — indicating that ALS can suppress the peripheral nonspecific inflammatory responses (131).

Since ALS is prepared against complex antigens and mixtures of antigens, its antibody specificities could be expected to be similarly complex — especially in cross-species immunization. The total effect must represent a balance between the immune system and ALS, a balance within the parts of the immune system and a sum of local concentration effects.

ALS-immunosuppression is qualitatively different from that of other immunosuppressants. After absorption of anti-rbc and anti-platelet antibodies, ALS is more specifically toxic for the immune system than are any of the other immunosuppressants (26, 128). Toxicity in humans seems to be related mostly to the recipient's immune response against heterologous serum proteins in the ALS preparation — i.e., an indirect rather than direct toxicity (126). Within the immune system it shows high specificity for cell-mediated immunity and is effective in controlling both first set and

second set graft rejection. Its inhibitory effect on antibody production seems limited to the primary antibody response. The antigens tested were ones for which there is an identified T-cell dependent step prior to antibody production. ALS is generally ineffective in inhibiting secondary antibody responses. None of the other immunosuppressants is as specifically immunosuppresive with so little toxicity.

ALS-treated graft recipients demonstrate immunity to ALS and become refractory to immunosuppression by ALS. Attempts to demonstrate a causal relationship by passive transfer of serum have been negative. Pre-immunization with gamma globulin fractions results in more rapid clearance of anti-lymphocyte globulin and less prolonged graft survival times.

ALS from two different species when used consecutively are more effective in prolonging graft survival than either ALS preparation alone (32). These data are consistent with the idea that refractoriness to ALS is caused by recipient immune response to ALS — in particular the species-specific portions of the immunoglobulin molecule. If other parts of

the immune system could compensate for deficiency in cellular immunity, then the second ALS would not have extended graft survival.

All five types of immunosuppressants have been used to maintain human organ transplant recipients (26, 99, 101). The particulars of treatment vary with the institution and the individual patient — depending upon the grade of antigenic match between recipient and donor, the occurrence of rejection crises and individual sensitivity to prescribed medication. In human transplant patients the commonly and almost universally prescribed immunosuppressants are azathioprine and prednisone. Azathioprine (Imuran) is a -S- substituted derivative of 6-mercaptopurine (6-mp). Both drugs are purine analogs. Normal liver function is required for azathioprine to be immunosuppressive. Azathioprine presumably is converted to 6-mercaptopurine by liver enzymes. Prednisone is a corticosteroid. Other antimetabolites, including cyclophosphamide, are being tested for their ability to control or prevent rejection. The azathioprine-prednisone combination is effective because the additive effect of these agents allows a

reduction in the dose of each individual agent. Thymectomy and splenectomy often but not always supplement this basic treatment regimen.

Whole body x-irradiation is not widely used as an immunosuppressive treatment in humans, although irradiation of the graft before implantation may be effective in delaying rejection. Local irradiation to the graft *in situ* may be useful in controlling a rejection episode.

Anti-human lymphocyte serum is commonly prepared in horses, although clinical trials are being performed with ALS preparations from rabbits. Globulin fractions of ALS (ALG) are used in human patients to reduce the amount of irrelevant heterologous protein and thereby reduce the host's immune response to those serum components. ALG is not purified for anti-lymphocyte antibodies and is still a nonspecific immunosuppressant.

All forms of immunosuppression which are not antigen specific have the direct side effect of reducing the organism's resistance to infection. The immediate cause of death in these patients has most often been infection and sometimes infections which are not life threatening in a normally immunocompetent individual (1). In evaluation

of side effects from long term immunosuppression it may be necessary to distinguish between a direct effect of immunosuppression and the effect of chronic but unidentified infection.

In those cases where infections have been successfully managed and graft survival is maintained with adequate function, a long term threat of immunosuppression, either direct or indirect, has become evident. The immunosuppressed transplant patient runs an 80-fold greater risk of neoplasia than age matched individuals in the general population (99, 101).

3. Immunosuppression and Neoplasia

Immune surveillance, the idea that cellular immunity evolved to combat transformed cells arising spontaneously by somatic mutation, implies that transformed cells and the immune system are in balanced opposition (24, 44, 130). For this theory to have validity, it is necessary that the transformed cell be antigenically distinct from normal host cells. With sensitive techniques it is possible to demonstrate many tumor-associated antigens. The clearest examples of tumor-associated antigens are those of viral

origin. Tumors from individuals infected with the same virus express the same viral antigens which are identical to structural components of the virion. There are also cellular antigens characteristic of the virally transformed state which are not structural components of the virus. An additional class of antigens includes the individually unique antigens which are not shared by tumors of the same histological type or tumors which result from the same oncogenic stimulus. It is anticipated that most tumors will prove to be immunogenic and that immunity to autochthonous tumors may be the rule rather than the exception (105). The efficacy of immune surveillance is invoked to explain the high incidence of histologically malignant foci in human autopsy material compared to the much lower incidence of clinically frank neoplasm, occasional spontaneous tumor regressions and the low immunogenicity of spontaneous mouse tumors.

There are indications that at least some tumors may represent exceptions to or adaptations to immune surveillance. *In vitro*, cells from many tumors show a greater resistance to lysis by immune antibody and/or immune cells than do normal cells from the same individual. In some

cases this heightened resistance is attributed to increased membrane sialomucins.

The murine virally induced mammary tumor is an example of a tumor expressing viral antigens and individually distinct antigens, but which is highly resistant to immune destruction (118). The viral antigens are also present in normal mammary cells of infected females prior to malignant transformation. These cells are both antigenic and sensitive to immune destruction. The possibility exists that cells in the early stages of malignant transformation may be antigenic and normally sensitive to immune destruction — i.e., immune surveillance could be effective early in tumorigenesis.

Additional data indicate that the mouse mammary tumor can be made highly antigenic and subject to immune destruction in the tumor host if the tumor is injected with neuraminidase (116). Neuraminidase presumably removes some of the protective coating and bares the antigens of the tumor cells to immune recognition and effective immune responses. In such treated hosts, however, new tumors arise at an accelerated rate; they are believed to be antigenically distinct from the original tumor.

Another adaptation of oncogenic viruses to the immune surveillance function of lymphoid cells may be infection of host lymphoid tissue. The murine leukemia and mammary tumor viral antigens are normally expressed in the lymphoid cells of the infected host (11, 97). Infection at a time or in a quantity which would mimic expression of self-antigens would effectively negate an immune response just as immunity against self-antigens is suppressed. The balance between the immune system and antigen in some way determines the expression of the immune response as immune inhibition, tolerance or immune enhancement. The latter two would favor viral propagation and malignant transformation.

Apparent exceptions to immune surveillance do not deny a prior existence of immune sensitive stages during transformation nor do they suggest that effective tumor immunity cannot be induced. If any of the apparent exceptions from immune surveillance are true exceptions, they still do not dismiss the participation of immune surveillance in other tumor systems.

The theory of immune surveillance predicts a high incidence of spontaneous tumors in immunologically impaired

individuals. A lapse in this defense system combined with the superior growth properties of tumors might allow the tumor to reach its full growth potential, even if immunosuppression were of short duration. High tumor incidences are reported for humans with severe immunodeficiencies and for immunosuppressed kidney transplant patients when compared to normal populations of similar ages.

The most severely affected congenitally immunodeficient individuals do not survive past infancy. Those who do reach childhood exhibit an unusually high frequency of spontaneous lymphomas (35). These tumors are common in Wiskott-Aldrich syndrome, ataxia telangiectasia and Chediak-Higashi syndrome. Patients with these diseases exhibit deficiencies in both cell-mediated immunity and humoral immunity. Primary involvement of the central nervous system (CNS) has been noted in many cases, in contrast to lymphoma patients in general where only about 1% show CNS involvement. The increased tumor frequency, lymphatic involvement and unique location are the unusual features of these diseases. Dysgammaglobulinemia, which occurs much later in life, is a deficiency of immunoglobulins with variable impairment of cellular immunity.

These patients also run a higher than average risk of lymphoma. Tumors of the lymphoid system are increased even in Bruton's agammaglobulinemia, in which disease all classes of immunoglobulins are absent, but the cellular immune system appears normal. Individuals with more restricted deficiencies of the humoral system have not been reported to show an increased tumor incidence.

Tumor data for immunosuppressed transplant recipients are available only in the case of kidney transplant recipients (99, 101). Excluded from these data were tumors which were transplanted with the grafted kidney and were therefore of donor origin. Tumors of the recipient which were present prior to transplantation were also excluded. The apparent <u>de novo</u> tumor incidence is 80-fold increased compared to overall tumor incidence for the general population of similar age. All these patients were subject to nonspecific immunosuppression by azathioprine and prednisone. Some were additionally splenectomized, thymectomized or treated with ALG. There had been 125 tumors reported in kidney transplant recipients to the date of reference (100).

Tumors appeared on an average of 28 months after transplantation. Six tumors appeared in less than four

months after transplant. 61% of the tumors were epithelial tumors; the remainder were meschenymal tumors. Three instances of multiple tumors were reported. Of the meschenymal tumors, 86% were lymphomas and of these, 61% were reticulum cell sarcomas. Involvement of the central nervous system occurred in half the lymphoma cases — mimicing lymphomas in congenital immunodeficiency diseases. Immunosuppression is strongly suggested in the development of these tumors.

There is one unusual report in the literature of an immunosuppressed kidney transplant patient who recieved ALG and subsequently developed a reticulum cell sarcoma near the site of ALG injection (36). The tumor has been interpreted as possibly resulting from severe, local immunosuppression in the immediate area of the injection site. The significance of a single such case is obscure. Although ALG supplements other immunosuppressant agents, there is no indication that tumor incidence in transplant patients has increased since the use of ALG (101).

In addition to the data for immunodeficient and immunocompromised individuals, there is a strong association between cancer and spontaneous immunodepression (35).

Cancer patients, especially in the advanced stages, show a general, nonspecific reduction in immune responsiveness. Prognosis after surgical removal of the major tumor mass is correlated with delayed hypersensitivity responses to skin sensitizing chemicals. Although the immune depression observed in malignancy appears to be a secondary effect of malignancy, there are no data in these cases regarding immunocompetence prior to clinical malignancy. The striking almost constant association of immunological defect with malignancy does not implicate immunodeficiency in a causal way with malignant transformation, but does suggest that the immune system may be important in tumor control, similar to its role in dealing with exogenous pathogens.

Because (1) immunological defects are common features in the preceding examples of malignancies, (2) the immunological defect preceded malignancy in the case of immunodeficient and immunosuppressed patients, (3) tumors are or can be antigenic, (4) immunity can have an inhibitory effect on tumor growth, and (5) immune surveillance predicts an increase in tumors in immunologically impaired

individuals, suspension of immune surveillance is a reasonable mechanism to explain the increased tumor incidence in immunosuppressed transplant patients.

There is, however, some question about the "control" population to which tumor incidences were compared. Uremic patients without externally induced immunosuppression show a reduced capacity to reject grafts and a reduced activity in mixed leukocyte cultures — i.e., they demonstrate an impaired cellular immune response in the absence of immunosuppressive treatment (33, 64).

It has been suggested that they may have an elevated tumor incidence over the general population (101). The reported tumor increase in transplant recipients does not necessarily indicate an increased tumor risk following induced immunosuppression. Tumor data on uremic patients on dialysis are not adequate as yet to make the necessary comparisons (101).

Assuming that tumor incidence for the transplanted patients is increased, the question arises whether the tumors were pre-existing or arose *de novo*. If they were pre-existing, an increase in tumor incidence would indicate facilitated tumor growth.

If the tumors arose *de novo* following immunosuppression, the mechanism of tumor induction is in question. One possibility is that somatic mutations normally occur with great frequency and some percentage of these are mutations to malignancy (24, 44, 130). A second possibility is that the immunosuppressive treatment may itself be carcinogenic. A third hypothesis is that the partially suppressed immune system actually promotes induction of lymphomas.

Although antimetabolites may be effective immunosuppressants, a subset are definitely carcinogenic in animals. All immunosuppressants are not necessarily carcinogenic, but all carcinogens are immunosuppressive (35). Azathioprine is used in conjunction with steroids in the treatment of human diseases of presumed autoimmune etiology. The reports of tumors in these patients are fragmentary. A tumor incidence of less than 3 in 4000 is suggested for azathioprine-steroid treated patients and is similar to that reported in a placebo control group (76). The tumor incidence for both immunosuppressed and placebo control groups may be higher than for the general population. None of the authors of the individual case reports or the single control group report are the same. The observed

tumor incidence could be strongly affected by diligence with which tumors are discovered and reported by different individuals or institutions.

Penn and Starzl point to the occurrence of tumors in 20 psoriatic patients as a caution against the use of immunosuppressants although the tumor incidence in non-immunosuppressed psoriatic patients is not indicated (100). The immunosuppressive agents used to treat psoriasis in that report were methotrexate or aminopterin — not azathioprine and steroids. It is certainly not clear that all immunosuppressants affect malignant development in the same way. The data are at present insufficient to define the carcinogenic potential of individual immunosuppressive agents, either separately or in combination in human populations.

In mice, azathioprine and 6-mercaptopurine can induce lymphomas (27, 28, 37). Two of the three strains of mice used were not normally subject to spontaneous lymphomas. The third strain was susceptible to x-ray induced leukemia. Similar experiments in rats failed to demonstrate a carcinogenic effect of azathioprine. Azathioprine, as a purine analogue, is potentially mutagenic, and may also act to facilitate viral transformation.

Because lymphomas can be induced in mice by a graft versus host reaction, Schwartz has postulated that an increase in lymphoma incidence in humans with immunological abnormalities may have a similar basis (115). He postulates that humans carry a latent oncogenic virus which may be activated in proliferating immunocompetent cells. Such proliferation would normally be controlled by a feedback inhibition mechanism. Any interference with these "immunoregulatory loops" would allow antigen-induced cell proliferation and virus activation to procede under less stringent control. The subsequent tumor is presumed to develop from cells in close proximity to the released virions.

The most provocative finding in support of this theory is the demonstration in mice that leukemia virus is produced in the spleens from mice subjected simultaneously to ALS-immunosuppression and antigenic stimulation (56). Mice which were either ALS-treated or grafted showed no virus production in the spleen. Schwartz's theory has particular merit in that it can explain the elevated lymphoma incidence in immunosuppressed transplant patients. One would have predicted from the immune surveillance

theory that all tumors would be elevated in proportion to their usual incidence in the general population. If the immunoregulatory theory proves true, immunosuppression induced by all methods would be carcinogenic for transplant recipients.

One comparison of lymphoma incidence in mice receiving azathioprine or ALS — either alone or in combination with antigenic stimulation — has been reported (68). No lymphomas were observed when the mice were treated with either agent alone. No lymphomas were observed in ALS treated mice receiving antigenic stimulation, but nonmalignant plasmocytosis was noted. Only those mice receiving both azathioprine and antigen developed lymphomas. These results indicate that the particular immunosuppressive agent and antigenic stimulation may be important in lymphoma induction. The depth or extent of immunosuppression induced by different agents may still be the basis of the differential effect on lymphoma incidence.

Reports of tumors in immunosuppressed transplant recipients have opened up many questions regarding the relationship between immunosuppression and malignancy,

and the specific mechanism of tumor induction in these individuals. The answers to these questions may come from more extensive experiments in animal models. All the common immunosuppressive agents, while not proven carcinogens, can facilitate tumor growth in animals (99).

6-mercaptopurine may affect the host reaction to a transplanted tumor, aside from its demonstrated carcinogenicity. It has been observed both to accelerate and to inhibit tumor growth. The former may be due to its immunosuppressive properties and the latter indicative of direct toxicity for tumor as well as normal tissue. Both mechanisms depend upon the chemical's toxicity and are probably concurrent events *in vivo*. The end effect observed as tumor growth is dose-dependent.

The corticosteroids are similarly inconsistent in their net effect on transplanted tumors. They have been demonstrated to facilitate or inhibit carcinogenesis in mice. They can induce or cause accelerated metastatic growth. They prolong survival of tumor grafts across allogeneic and xenogeneic histocompatibility barriers.

Neonatal thymectomy increases the susceptibility of many species of experimental animals to tumors induced by

polyoma or adenovirus. Splenectomy, like thymectomy, potentiates tumor induction and growth resulting from adenovirus infection. Leukemogenesis following neonatal infection with Gross or Moloney viruses is inhibited by neonatal thymectomy (71, 79). The effect of removing lymphocyte target cells overcompensates for the tumor promoting effect of thymectomy in these instances. These same mice show an increase in lymphoma incidence after neonatal thymectomy. This increase is interpreted as malignant transformation in reticulum cells which are secondary target cells for the leukemogenic viruses (114).

Humans are susceptible to radiation-induced leukemias. The immunosuppressed human lacks thymus-derived cells and these individuals are particularly susceptible to lymphomas. Although the etiology of these two diseases in humans is unknown, the parallel with the conditions of lymphoma appearance in the mouse system is striking. Both the radiation-induced leukemias and the lymphomas in immunosuppressed transplant recipients appear starting several months after exposure and are significantly increased by three years.

Neonatal thymectomy in the mammary tumor system in mice inhibits the expression of mammary tumors (71, 75, 113, 142), although the effect may be strain specific. Strain specificity may be a reflection of differences in host susceptibility or a property of variant forms of the virus carried by the different strains.

Even in tumor systems, where thymectomy inhibits either tumor onset or tumor incidence, the number of tumors per mouse may be elevated (25, 54) or the number of hormone independent tumors may be increased (122). As expected from immune surveillance, more strongly antigenic tumors may arise (9).

Neonatal thymectomy consistently renders the host less able to resist allogeneic or xenogeneic tumor transplants. The positive thymic contribution to mammary tumorigenesis has not been fully explained, although immunological interpretations are still possible (see section D. Immunity and Murine Mammary Tumors).
In general, the effects of thymectomy on tumor growth are consistent with the immunological functions of the thymus.

Animals receiving ALS are demonstrably less resistant to tumor induction by adenovirus 12, Rauscher leukemia

virus, Moloney leukemia virus and polyoma than are RNS-treated or untreated individuals (57). The magnitude of difference between treatment groups is affected by virus dose and route of inoculation. In the adenovirus system, a large dose of virus can obliterate any difference between the control and ALS-treated groups. ALS-immunosuppression is generally instituted shortly after virus inoculation although the tumor latent period may be several months.

The affected immune response may be directed either against the virus or the transformed cell. The relative importance of these two responses depends upon the mechanism of tumor growth — whether additional cells are added to the tumor mass after viral infection or the tumor is restricted to cells arising by division from the initial transformant. The former type of growth has been demonstrated in murine mammary tumors (121), the latter is clearly indicated in transplanted tumor systems.

Transplantation of living tumor tissue can be facilitated by ALS-immunosuppression. If the tumors are histoincompatible with the host, their survival, like that of non-transformed histoincompatible tissue, is favored by immunosuppression.

When spontaneous tumors are transplanted into syngeneic hosts, ALS treatment may favor, inhibit or have no effect on tumor growth (see section C.4 Discussion). Carcinogen induced tumors of demonstrated tumor associated antigenicity tend to grow better in the suppressed syngeneic host than in the non-suppressed syngeneic host. Such tumors may even be induced more readily in an ALS-treated animal than in control animals (29, 110) Different syngeneic tumors almost certainly differ in antigenic strength. Only those tumors which are highly antigenic grow better under conditions of immunosuppression.

The interplay between the immune system and tumors may be quite complex as a function both of the antigenicity of the tumor and the quality of the host's immune response. The fact that immunosuppression may in some instances inhibit tumor development has led several investigators to postulate that immunity may actually stimulate spontaneous tumor development (106, 107, 121).

C. Retrospective Study on Effect of Anti-Thymocyte Serum on Primary Mouse Tumors

1. Introduction

Tumor data from immunosuppressed kidney transplant recipients indicates that these patients are significantly more likely to develop tumors than individuals of similar age in the general population (99, 101). All patients developing tumors had been immunosuppressed with azathioprine and prednisone. The tumors appeared shortly after transplantation — as early as several months and averaging 28 months post-transplantation. The length of the follow-up period is limited by the recency of kidney transplantation itself. More tumors may appear as the follow-up period is extended. Immunosuppression is strongly suggested in the development of the observed tumors. Because of the short time interval between transplantation and tumor onset, it is difficult to determine whether some of the early tumors were pre-existing or arose *de novo*. There is an additional risk of transplanting tumors inadvertently with the grafted tissue.

Assuming that many of the tumors arise *de novo*, there are at least three plausable alternatives which can explain

de novo tumor induction. The first is somatic mutation combined with suppression of immune surveillance (24, 44, 130). The second is that azathioprine, a demonstrated carcinogen in mice, may also be carcinogenic in humans (27, 28, 37). The third is that an imbalance within the immune system may actively contribute to subsequent tumor development (115).

The uncertainties in the human data such as the tumor incidence in non-immunosuppressed uremic patients and the origin of the observed tumors can be avoided in experiments in animal models. Here, too, environmental and genetic heterogeneity can be controlled. All the common immunosuppressants are capable of facilitating tumor growth in animals. Most of these data are based on experiments with known oncogenic agents or with transplanted living tumor tissue. Oncogenic viruses and their resultant tumors are antigenic and immunity to both may be affected by immunosuppression. It is difficult to conclude that suppression of immune surveillance is more important than suppression of an anti-viral response. Experiments using transplants of tumor tissue also introduce some difficulties in interpretation. One is that the tumor

used may be rejected by the host because of histoin-compatabilities unrelated to the malignant properties of the tissue. Second, the immune response is greatly influenced by the amount and route of antigen presentation. Some sites of tumor formation may represent naturally privileged transplantation sites. Depending upon how it is stimulated, the immune system can respond to tumor challenge by immune inhibition, tolerance or immune enhancement. The best tumor systems for obtaining information relevant to immunity to autochthonous tumors are therefore the primary tumors, themselves.

Data have been collected in the present study on the histology, incidence and time of onset of spontaneous tumors in virgin, female CBA/J mice in this laboratory. These mice fall into four treatment categories — immunosuppressed with rabbit anti-mouse thymocyte serum (RAMTS), treated with normal rabbit serum (RNS), immunized with an irrevelant antigen (mouse serum β-lipoprotein) and untreated normal mice. The last two categories of mice were observed in order to obtain tumor data on mice subjected to other experimental procedures and on untreated mice. None of the mice were experimentally exposed to known carcinogens or oncogenic viruses.

RAMTS was the sole immunosuppressive agent used in this study. Anti-lymphocyte sera are extremely potent suppressors of cellular immunity, and have little effect on humoral immunity. They can double or triple the mean survival times of H-2 incompatible skin grafts, and mice tolerate high doses of anti-lymphocyte serum. The sera used in this study were prepared by a method which appears to maximize the immunosuppressive properties of the sera and minimize their toxicity. All rabbit sera were absorbed with mouse red blood cells to further minimize the amount of irrelevant and possibly toxic antibodies.

The schedule of ALS administration is important relative to the time of antigenic stimulation, but the time of antigen stimulation in spontaneous tumor systems is unknown. The duration of treatment is effectively self-limiting because the host's immune response to the ALS, itself, limits its effectiveness. In other tumor systems in the mouse (19, 29, 70, 74, 110) a similar schedule of injections of RAMTS significantly increased tumor growth. This fact combined with significantly delayed skin allograft rejection indicate that the

severity and duration of immunosuppression in this study should have been adequate for tumor promotion.

A total of 58 mice were observed weekly for the presence of tumors until tumors were detected or the mice died of other causes. Observations continued over a period of three and a half years. Mice from individual purchases were the same age and used in the same experiments. Each purchase is referred to as a group and each group may be subdivided for experimental and control purposes.

Groups I and II were grafted with skin from another inbred line or given isografts. The groups were divided into subgroups treated either with rabbit anti-mouse thymocyte serum (RAMTS) or rabbit normal serum (RNS). Group I mice received subcutaneous injections of 0.5 ml of rabbit serum on days 2 and 5 after grafting and 0.25 ml twice a week as long as the allografts were maintained. Mice bearing isografts were injected on the same twice weekly schedule. The maximum number of injections per mouse was 12.

Group II mice initially received two subcutaneous injections of rabbit serum as above. At the same time they

were injected with 0.5 ml of syngeneic mouse serum from normal mice or mice immune to whole rabbit serum. After the initial tumors in Group I appeared in the RAMTS-treated subgroup, Group II mice were injected twice weekly with their respective rabbit sera until they had received the maximum dose given Group I mice.

Group III mice were either immunized with mouse serum β-lipoprotein in complete Freund's adjuvant or untreated. All mice in this group received skin allografts.

Group IV mice were used only as normal serum donors.

2. Methods

Mice:

Inbred mice of the CBA/J, A/J, and Balb/c strains were purchased from a single supplier (Jackson Laboratory, Bar Harbor, Maine) over the limited time period of 14 months.

After grafting, the mice were housed individually in small metal cages. The untreated blood donors were not individually housed until they were 1-1½ years old. All mice were fed Purina Rat Chow and water *ad libitum*.

Mice were examined at weekly intervals for the presence of tumors. Mice with tumors were sacrificed for histological examination of the tumors. Dead mice were autopsied for gross pathology. Mice which could not be accounted for at any point in the observations and one mouse which died within 24 hours of injection have been excluded from the data.

Skin Grafting:

Each graft recipient was anesthesized with an intraperitoneal injection of Nembutal (65 micrograms/kilogram). Full thickness grafts of back skin were transplanted according to the procedure of Billingham and Medawar (16). Bandages were removed after seven days and graft survival was evaluated daily or every other day. End point graft survival was reached when the entire graft was necrotic and could be peeled off without bleeding.

Rabbit Anti-Mouse Thymocyte Sera (RAMTS):

Rabbit anti-mouse thymocyte sera were prepared following the standard procedure of Levey and Medawar (72). New Zealand white female rabbits were injected intravenously with 10^9 living thymocytes from 2 month old CBA/J female mice. Two injections were made, two weeks apart.

Rabbits were bled seven days after the last injection and exsanguinated the following day. Sera from the same rabbit were pooled and stored at - 20° C. All sera were heat inactivated at 56° C for 20 minutes and absorbed with 1/2 or 1/3 volume of washed, packed mouse red blood cells for 10 minutes at room temperature and at 4° C. Normal rabbit sera were treated identically.

Rabbit Normal Serum (RNS):

Sera from untreated rabbits or sera from ALS rabbits taken prior to immunization were used as normal rabbit serum controls.

Serum Injections:

All serum injections were subcutaneous. Group I mice received injections only on the same side as the grafted skin (left). Group II mice were injected on both sides. Injections were made on days 2 and 5 after grafting and twice a week thereafter if additional injections were called for in the protocol.

Normal Mouse Serum (NMS):

Untreated young adult CBA/J female mice were tail bled into siliconized tubes. The blood was allowed to clot and the serum was removed, pooled and stored at - 20° C until use.

Immune Mouse Serum (IMS):

Mice from Group I which had received RAMTS were "re-immunized" 7 months later with 0.5 ml of RAMTS intraperitoneally, followed by an injection of 0.2 ml of a 1:5 dilution of RAMTS subcutaneously six days later. Serum was collected and pooled every other day beginning two days after the last RAMTS injection.

β-lipoprotein Isolation (β-Lp):

Pooled sera from young adult A/J female mice were adjusted to a density of 1.063 gm/ml with a stock solution of NaCl and KBr and centrifuged at 38,000 rpm for 18 hours at 13° C (138). The β-lipoprotein fraction was removed from the top of the serum and stored at 4° C.

β-lipoprotein Immunization:

A fresh preparation of A/J β-lipoprotein was mixed with an equal volume of Freund's complete adjuvant (FCA). Some mice were injected with 1 mg protein in FCA one week before grafting. In a later experiment, injections were made 11 days and 4 days prior to grafting and 4 days after grafting.

Immunoelectrophoresis:

A layer of 1% agar in barbital buffer, ionic strength = 0.078, pH = 8.6 was allowed to solidify on a

microscope slide. Antigen was placed in a well cut in the agar. The slide was electrophoresed at 6 ma per slide (about 60 volts) for two hours. Troughs parallel to the direction of electrophoresis were filled with antiserum and allowed to diffuse in a moist chamber for at least 24 hours.

Thymic Extracts:

#1. Fresh thymuses from young adult CBA female mice were homogenized in a ground glass homogenizer with a minimal amount of distilled water and frozen and thawed three times. The supernatant was retained and stored at - 20° C.

#2. Minced fresh thymuses from young adult CBA female mice were sonicated in saline for 2 minutes (in 30 second intervals) at 20 Kc/sec. The supernatant was retained and stored at - 20° C.

Tumor Histology:

Histological preparations and identification of mouse tumors were done by Dr. Raymond L. Teplitz (City of Hope National Medical Center, Duarte, California). Tissues were fixed in buffered formalin, dehydrated and embedded in parafin by standard techniques. The sections were stained with hematoxylin and eosin.

3. Results

The rabbit anti-mouse thymocyte serum (RAMTS) used for Group I mice substantially inhibited cellular immunity as indicated by its ability to prolong skin allograft survival (Table 1). The RAMTS preparation used for Group II mice after only two injections was not as strongly immunosuppressive (Table 2). These mice subsequently received additional injections of RAMTS. The degree of immunosuppression following these latter injections was not tested. This group of mice did not contribute greatly to the tumor data because of a low tumor incidence (Table 4).

Passive transfer of syngeneic mouse serum, either immune or normal serum, to Group II mice did not abrogate or prolong the observed RAMTS immunosuppression (Table 2). The immune mouse serum contained antibodies to a large number of rabbit serum components including gamma globulin. The precipitating activity of this mouse serum was weaker against gamma globulin that against the other serum components, but nevertheless present.

Immunization with β-lipoprotein has no significant effect on allograft survival (Table 3), and therefore is

Graft Donor	Number of Mice	mls of serum RNS[a]	mls of serum RAMTS[b]	Graft Survival-days (MST $\pm$ SD)[c]
CBA/J	3	4.0	——	∞
A/J	3	1.0-2.0	——	12.0 $\pm$ 0.0
A/J	2	——	1.0	17.5 $\pm$ 1.5
A/J	3	0.0-1.0	2.0	24.0 $\pm$ 1.4
A/J	3	——	2.75-4.0	33.7 $\pm$ 7.1

a. rabbit normal serum

b. rabbit anti-mouse thymocyte serum

c. mean survival times $\pm$ standard deviation

TABLE 1. Skin Graft Survival in RNS and RAMTS Treated CBA/J Mice - Data from Group I Animals *

* The skin grafting experiment was performed by Jill Fabricant, Charles Novitski and Bob Sellent as part of the immunology course.

Graft Donor	Number of Mice	mls of Serum RNS[a]	RAMTS[b]	IMS[c]	NMS[d]	Graft Survival-days (MST ± SD)[e]
CBA/J	2	——	1.0	——	1.0	∞
BALB/c	2	——	——	——	——	10.5 ± 0.5
BALB/c	4	1.0	——	——	1.0	11.2 ± 0.4
BALB/c	4	——	1.0	1.0	——	14.0 ± 1.2
BALB/c	4	——	1.0	——	1.0	13.3 ± 1.4

a. rabbit normal serum

b. rabbit anti-mouse thymocyte serum

c. serum from CBA/J mice immune to rabbit serum

d. normal mouse serum

e. mean survival time ± standard deviation

TABLE 2. Skin Graft Survival in RNS and RAMTS Treated CBA/J Mice Given Immune or Normal Mouse Serum

Treatment	Number of Mice	Graft Survival-days (MST ± SD)[a]
immunized with β-Lp[b]	5	12 ± 0.4
untreated	4	11.75 ± 0.4

a. mean survival time ± standard deviation

b. serum β-Lipoprotein

TABLE 3. Survival of Allogeneic Skin Grafts in β-Lipoprotein Immunized and Untreated CBA/J Mice *

* Grafting experiment was performed by Jonathan Fuhrman and Jeffrey Frelinger.

Group	Treatment	Grafted	Tumor Incidence	Age at Tumor Onset (days)	Age at Death Non-Tumor (days)
I a	RAMTS[a]	yes	6/8	445 ± 45[e] (310 - 599)[f]	503 ± 33 (456 - 550)
I b	RNS[b]	yes	7/7	513 ± 28 (410 - 639)	———
II a	RAMTS + MS[c]	yes	3/10	528 ± 83 (337 - 635)	662 ± 28 (545 - 790)
II b	RNS + MS	yes	1/3	711 ———	756 ± 74 (651 - 861)
II c	none	yes	1/2	430 ———	834 ———
III a	β-Lp + FCA[d]	yes	4/5	422 ± 57 (316 - 605)	968 ———
III b	none	yes	3/5	667 ± 88 (476 - 816)	878 ± 22 (847 - 910)
IV	none	no	3/18	730 ± 146 (629 - 879)	> 718[g] (577 - 896^{+})

a. rabbit anti-mouse thymocyte serum; b. rabbit normal serum; c. mouse serum; d. β-Lipoprotein in Freund's Complete Adjuvant; e. mean + S.E.; f. range; g. terminated at 896 days with 3 mice alive and showing no evidence of tumors at autopsy

TABLE 4. Tumors in Virgin Female CBA/J Mice

not suppressive for cellular immunity nor cross reactive with histocompatibility antigens of the mouse.

Humoral immunocompetence as judged by the mouse's ability to form precipitating antibodies to rabbit serum proteins was not affected when the rabbit serum was immunosuppressive anti-thymocyte serum. Serum from mice injected with RAMTS gave immunoelectrophoresis patterns against rabbit normal serum components similar to the patterns observed with serum from mice injected with RNS. The reactivity in both cases was weak. A β-globulin component of rabbit serum was recognized in every case. The number of rabbit serum components and the strength with which they were recognized were similar for the two classes of mice. The anti-thymocyte sera suppressed the cellular immune response and had little or no effect on humoral immunity as has been often demonstrated (72, 139).

The unabsorbed rabbit anti-mouse thymocyte sera contain strong hemagglutinins as observed by red cell agglutination when absorbed with mouse red blood cells. The absorbed sera are also lymphocytotoxic in *in vitro*, complement dependent, cytotoxicity assays, (Tommy C. Douglas - personal communication). The absorbed RAMTS precipitates a component of mouse

thymus extracts, but that component appears identical by immunoelectrophoresis to a β-globulin region component of normal mouse plasma. The component is not present in extracts of thymus cells which have been extensively washed in saline prior to preparing the extract. RAMTS when tested against normal mouse serum shows as many as three precipitin bands. Immunization against mouse serum components probably occurred as a result of serum contamination of the original thymocyte inocula. No thymus-specific component was identified in immunodiffusion or immunoelectrophoresis tests of thymus extracts.

Although prolonged survival of H-2 incompatible skin grafts indicated severe suppression of cellular immunity, no mouse showed any evidence of tumors or other gross pathology during the six weeks of RAMTS treatment or up to six months after cessation of RAMTS treatment. Observations on tumors are summarized in Table 4.

Nontumor bearing mice lived at least as long as, or longer than, tumor bearing mice. Tumors developed in both immunosuppressed and non-immunosuppressed mice. Fluctuations in incidence were noted; but tumor incidence was the same in matched RAMTS and RNS-treated mice. Incidence

within any group (purchase) of mice appears independent of the type of treatment which the mice within the group received.

The following account of the histology of the tumors is included through the courtesy of Dr. Raymond L. Teplitz, who collaborated in this part of the investigation.

Of the tumors observed, the most frequent tumor type was the typical mammary tumor (Figures 1a, 1b, 2a and 2b). Mammary tumors of mice are fundamentally of adenomatous nature. They recapitulate more or less faithfully the glandular components of the breast. Thus, most tumors show groups of small round or ovoid acini, lined by cuboidal or columnar epithelial cells. Nuclei, normally central or basal depending upon estrus, are of variable location. Likewise, nuclear size varies, as does the chromatin staining. Proliferative activity as monitored by mitotic index differs from case to case.

Distortion of structures follow upon obstruction and ectasia of ducts, leads to micro- or macrocysts. Hemorrhage and necrosis may occur, usually the result of vascular occlusion. All of these mammary tumors exhibit some or all of these characteristics. However, they may vary from

FIGURE 1a

100x This tumor is from a Group I mouse that received only two injections of rabbit anti-thymocyte serum. The tumor was first observed when the mouse was 520 days of age. The predominant structures in this tumor are small round acini. They are lined by a single layer of cuboidal (or low columnar) epithelium. A band of fibrocollagenous tissue separates two aggregates of these acini, forming a pseudo-capsule. Acinar size varies somewhat, but at X, cystic dilatation of a duct occurs, coincident with the presence of precipitated proteinaceous material.

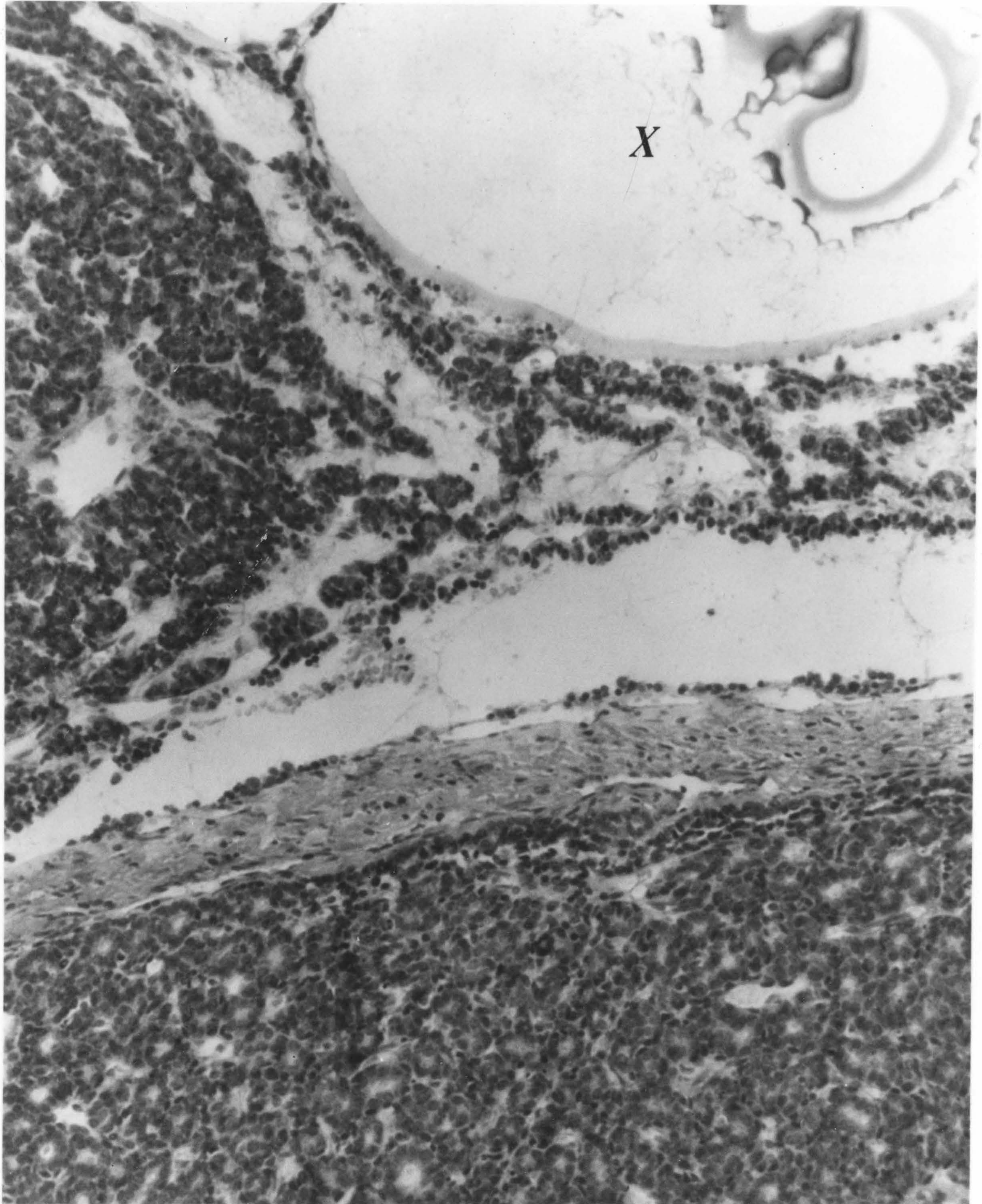

FIGURE 1a.

FIGURE 1b

400x Photograph adjacent to the pseudo-capsule. Variability in nuclear size, shape and chromatin distribution are hallmarks of the malignant character of these glands. No basement membrane is visible. Occasionally nucleoli are present, but mitoses are not present.

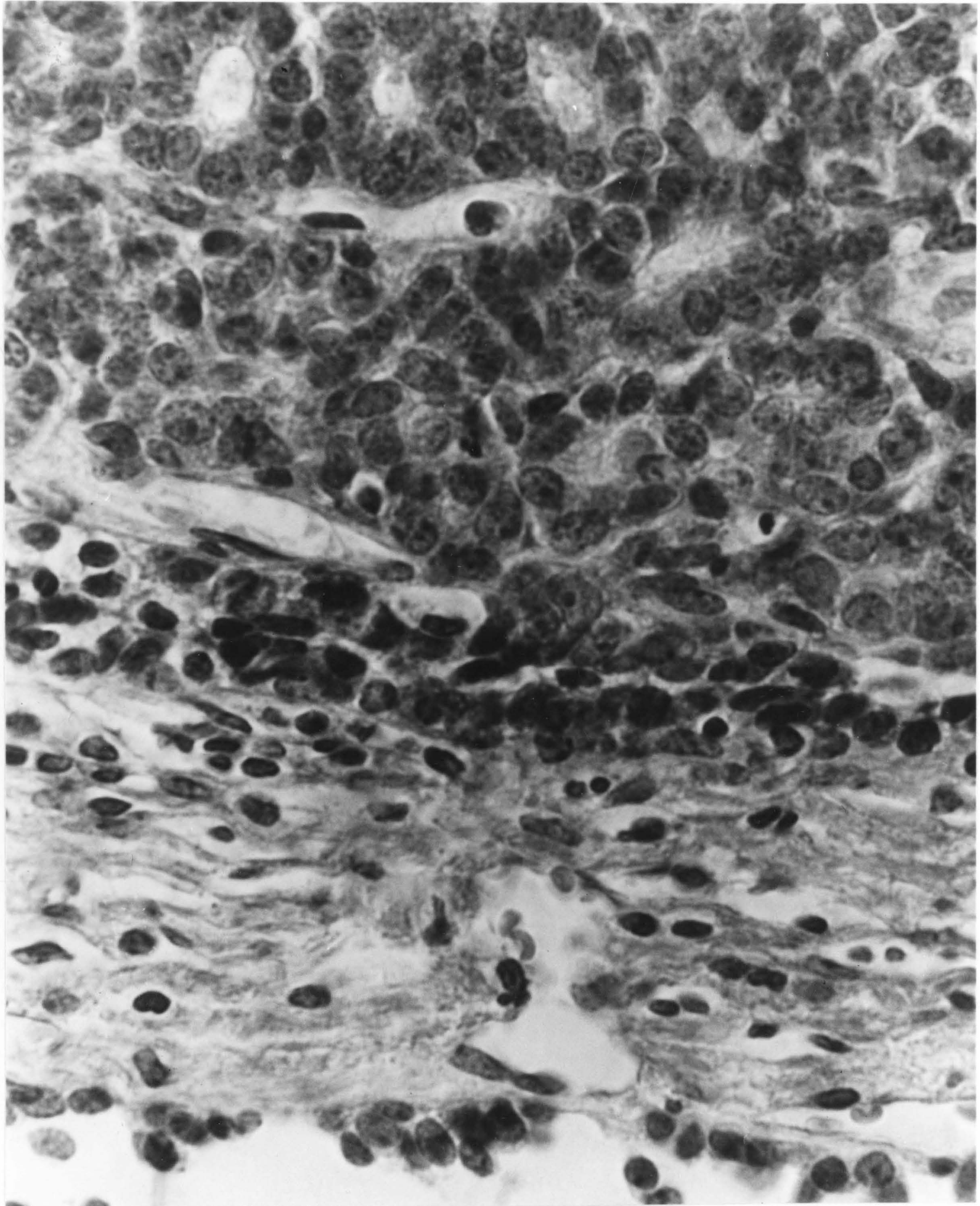

FIGURE 1b.

FIGURE 2a

100x This tumor is from a Group I mouse that received three injections of rabbit normal serum. The tumor was first noted when the mouse was 540 days old. The glands of this tumor are so compressed that a lobular pattern is produced. Acinar configuration is difficult to perceive, but cords of epithelium are more characteristic. Some duct ectasia is present (at Y), again associated with inspissated contents. Infiltration of connective tissue (Z) and fat (Z') testify to the invasiveness of the tumor.

FIGURE 2a

FIGURE 2b

400x At this magnification, the solid cord-like architecture is emphasized. Cells tend to pile upon themselves. No basement membranes are seen and at least one mitotic figure (M) is present. At V, destruction of a vessel is shown, with tumor cells forming the wall. Greater irregularity of nuclear structure and chromatin than in Figure 1 , together with more frequent mitoses, vascular involvement and poorer differentiation indicate that this tumor is more rapidly growing than the tumor in Figure 1.

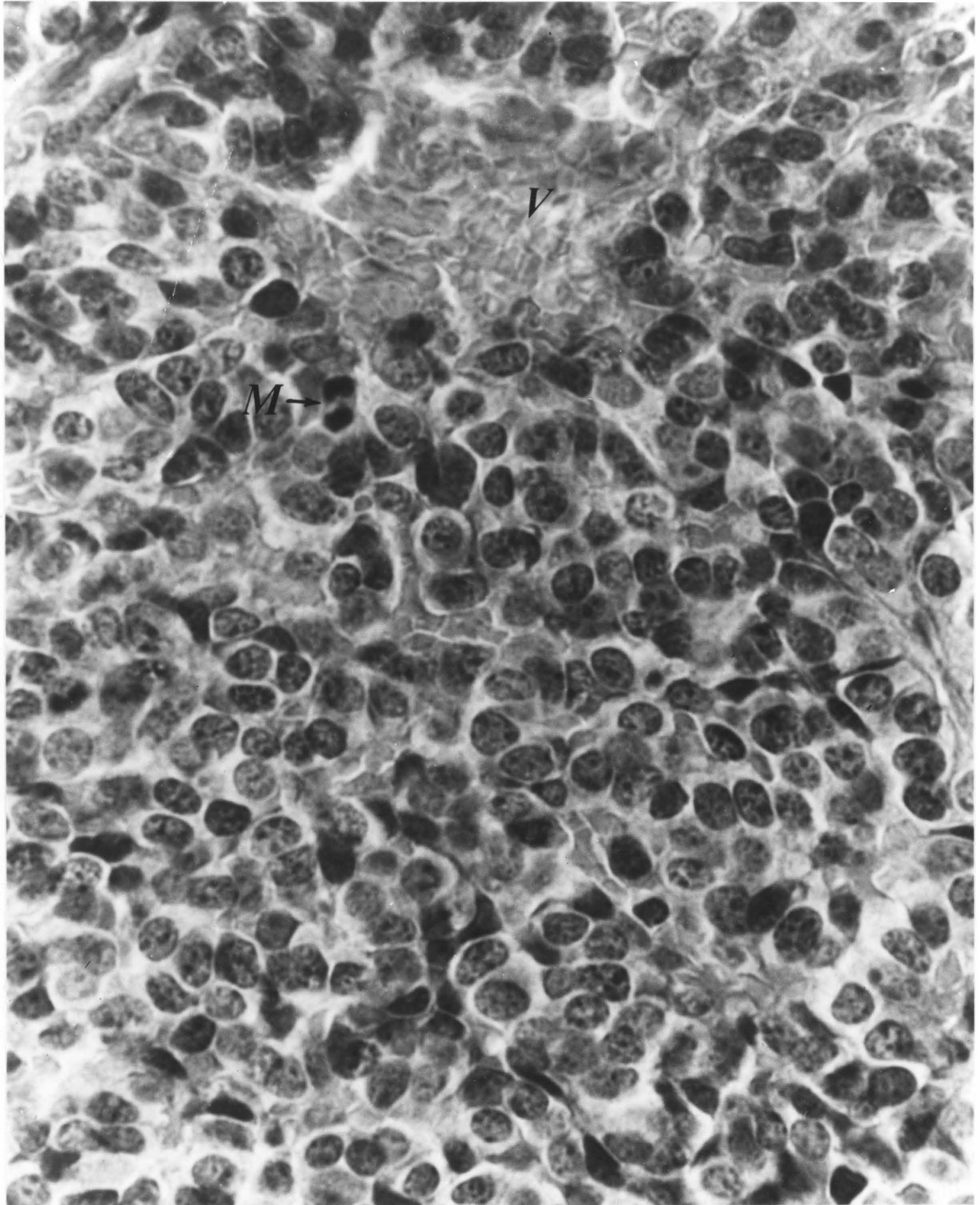

FIGURE 2b.

one portion of the tumor to another, a common feature of neoplasia.

Invasiveness is manifested first by perforation of the basement membrane juxtaposing the glandular epithelium. Vascular, lymphatic and adjacent structures are then involved, although not necessarily in the order presented. Metastasis to distant parts is generally slow in mice, showing a more benign course than in humans.

Of the three tumors of the series not of mammary type, two were classified as fibrosarcoma — one because of its location and the second was identified histologically as a low-grade fibrosarcoma. It displayed bundles or sheets of parallel, fusiform cells embedded in a deeply eosinophilic matrix. Bundles of cells intersect, but within each bundle the cells tend to be arranged parallel to one another. Nuclear size varies with cell size. Chromocenters tend to be distinct and range from 3-6/cell. Mitoses are rare, thus the characterization of "low-grade". The cell type and the fibrocollagenous matrix indicate the histogenesis: fibrosarcoma. The suspected fibrosarcoma was found in a Group I mouse that had received four injections of rabbit normal serum. The tumor first appeared at

507 days of age. The second fibrosarcoma occurred in an untreated mouse from Group IV at 682 days of age. The third tumor was a ventral tumor which did not display the characteristic adenomatous features of mammary tumors. Sheets of round or ovoid cells form the basic pattern with occasional cords of cells or adenomatous structures. The latter are lined with columnar or cuboidal cells, displaying small round nuclei. Nuclear chromatin is dense and distributed peripherally. Stromal connective tissue is loose. Mitotic figures are not encountered. Differential diagnosis: 1. Skin adnexal tumor with ~~meiosis~~ NECROSIS, and 2. Necrotizing (poorly preserved?) mammary carcinoma. This tumor occurred in a Group II mouse that had received RAMTS and normal mouse serum. The tumor appeared at 337 days of age.

Groups I and III have high tumor incidence equivalent to that reported for normal virgin females of this strain, whereas Groups II and IV have lower tumor incidences.

Tumor onset in immunosuppressed mice tends to be earlier than in the RNS-treated mice, but the mean time of onset does not differ significantly between the two treatment classes. The difference between the mean time of tumor onset in experimental and matched control mice was greater

within Group III than within the groups where immunosuppression was a factor, but the difference still was not significant.

The first tumor observed in any of these mice was in a RAMTS treated mouse and was located near the site of injection. No other mouse developed a tumor at or near the site of injection.

The anterior: posterior ratio of mammary tumors from the combined data for all these mice is 5.5:4, which corresponds to a random distribution of mammary tumors among the mammary glands rather than to the distribution of hyperplastic nodules (108).

Mammary tumors from Group I mice showed some preference for the left side over the right. The preference was not biased by the nature of the rabbit serum injected.

4. Discussion

For experiments evaluating the effectiveness of anti-lymphocyte serum (ALS) in prolonging graft survival, rabbit normal serum (RNS) treated animals are the appropriate controls. In these experiments one is interested in the antibody specific effects of ALS.

In experiments investigating the association of immunosuppression and tumor development, the appropriate controls should not be immunosuppressed. There is some evidence that RNS might be immunosuppressive. Unabsorbed RNS is cytotoxic for mouse thymocytes _in vitro_. _In vivo_, RNS causes a transient depletion of circulating lymphocytes (see also below). Some unabsorbed RNS may actually cause an immunosuppression which may be reflected by prolonged survival of histoincompatible skin grafts (41). Absorption of ALS or RNS with mouse tissue, like red blood cells (RNS [mrbc]) should remove many of the irrelevant antibodies such as species-specific antibody, anti-H-2 antibody and hemagglutinating antibodies. Absorptions should reduce the toxicity and non-specific effects of both sera. Immunosuppressive effects which are unrelated to natural antibody or irrelevant antibodies will not be removed.

Nehlson and Simpson have published observations on mice injected with RNS [mrbc] twice weekly beginning one day after birth and continuing for life (95). Prolonged administration of RNS [mrbc] did not depress immune responsiveness to allografts, xenografts, sheep rbc, bovine serum albumin or Keyhole limpet hemocyanin. RNS [mrbc] treatment did not affect graft versus host reactivity or response to oxazolone, but did induce lymphopenia. Although no specific deficit in immunity was demonstrable in either cell-mediated or antibody responses, a reduction in the number of lymphocytes suggests some small possibility of impaired immunity. RNS [mrbc] is assumed to have no effect on adult immunocompetence when given over a much shorter interval, as in this experiment.

In published experiments on a number of mouse tumor systems, tumor development was not significantly different in RNS-treated and untreated controls (39, 63).

Because there are a number of reports of apparent contamination of ALS with oncogenic virus, RNS treated animals may be preferrable to untreated animals as controls. The suggested source of viral contamination is the red cells used to absorb out the irrelevant antibodies (95). If ALS

and RNS used in the same experiment are identically absorbed, the contamination by viruses and other potential pathogens introduced by the absorption should be identical. This procedure may be the only valid control as it is not possible to identify all the oncogenic agents nor otherwise control for the secondary effects of unidentified pathogens.

The most common viral contaminant is polyoma (95). Polyoma causes a variety of tumors in mice including mammary tumors, salivary gland tumors and osteosarcomas (43, 95). Although mammary tumors are common in these mice, none of these other tumor types were found. Mammary tumor incidence and onset is similar for that reported in CBA/J mice as a function of their natural infection with mammary tumor virus (MTV). There is no evidence that the sera used in these experiments were contaminated with polyoma.

Antigens associated with mammary tumorigenesis reportedly exist in sera of infected animals (12-14). MTV as detected by infectivity of various tissues is relatively ubiquitous. MTV is present in whole blood, generally cell associated (94). Immunization with virus

positive mammary tumor tissue in Freund's complete adjuvant enhances growth of subsequent tumor inoculations. The adjuvant alone had no effect on tumor incidence, onset or survival time. The strain of mice from which the β-lipoprotein was isolated does carry a low tumorigenic variant of MTV. If the β-lipoprotein preparation were contaminated with virus or the tumor associated plasma antigen, it is possible that immunization with this material could affect tumor growth. Since β-lipoprotein is isolated by its special density and whole virions are much denser, whole virions are assumed not to be present in the isolated material. The tumor associated plasma antigen is an alpha-globulin, not a β-lipoprotein, although β-lipoproteins and alpha-globulins are immunologically cross reactive. The reported mammary tumor associated antigen is not a viral antigen, but may be indicative of a hormonal state which is important is mammary tumorigenesis. CBA/J mice apparently lack the antigen and therefore it could not play a role in mammary tumorigenesis in these animals.

There is no direct evidence that serum β-lipoprotein contains either infectious mammary tumor agents or tumor antigens. Blood associated tumorigenicity is reportedly

cell associated and is lost in cell free preparations (94). Mouse milk, which is the means of transmission of the standard mammary tumor virus (MTV-S), may contain infectious mammary tumor agent in a fraction of approximately the same density as β-lipoprotein (84). B-type virus particles, which are generally considered to include MTV-S, were not tumorigenic when isolated from the same milk samples. These findings are in contradiction to previous reports that it is the B-particle fraction from mouse milk which is tumorigenic (34, 49, 88).

Since there is no direct evidence that immunization with serum β-lipoprotein should have any effect on mammary tumorigenesis, β-lipoprotein is considered an irrelevant treatment in the context of this study. Data on skin graft survival indicate that β-lipoprotein immunization is not immunosuppressive. These injections should have no effect on tumor development either through immunosuppression or inadvertent immunization to tumor associated antigens.

There are marked variations in tumor incidence among the groups of mice. Mice in the same purchase, regardless of treatment, show the same tumor incidence. The murine

mammary tumor system is highly susceptible to environmental influences and to, as yet, undefined fluctuations There are numerous examples of unexplained variations in murine mammary tumor incidence in the literature (111, 119, 127). Because of the association by purchase, some of this variability may have been introduced before the mice were received in our laboratory.

While in our laboratory the mice, except for Group IV, were housed and fed identically. These factors should therefore not have contributed to the fluctuations in tumor incidence in the Groups I-III. Group IV mice were not housed separately. Andervont and Muhlbock showed that mammary tumor incidence was highest in female mice housed individually and decreased as the number of mice housed together increased (3, 91). These results were obtained with C3H and DBA strains of mice. No comparable studies have been carried out for the CBA strain, but it is presumed that CBA would show similar results. CBA mice are closely related to both C3H and DBA mice. They are assumed to carry the same mammary tumor virus (MTV-S) (20). They show a similarly high susceptibility to the

virus and their endogenous hormonal milieux are similar by several criteria (21, 42).

The numbers of tumors and the description of their locations are too few and incomplete to determine whether injections of anti-lymphocyte serum can influence the location of a subsequent tumor. Data are available from the literature on the site preference for mammary tumors in some strains of mice (108). CBA is not one of those strains and the observed age at tumor onset for this strain is in between the prototype patterns observed. Although CBA would not be the strain of choice, the observation that the first tumor to appear was located near the site of RAMTS injection suggests that the mammary tumor system is a possible model in which to examine the influence of serum injection on tumor location.

The results from this study differ from observations in immunosuppressed humans. No mouse tumors appear during or immediately following immunosuppression. The tumors which do appear later are predominantly mammary tumors and not lymphomas.

The absence of tumors during immunosuppression can be explained in several ways. If somatic mutation is the

mechanism of tumor induction, the frequency of malignant mutations may not be great enough to be observed in this study even though observations continued for the life of the animal. Since azathioprine was not used on any of these animals, the data only show that lymphomas do not appear in nontreated CBA/J mice. There is no positive control, i.e., that this strain would get lymphomas in the presence of azathioprine. Implicit in these arguments is the suggestion that a malignant tumor is the product of two processes — an inductive and a tumor promoting step. If either one of these steps is absent, then cancer is not manifested.

CBA mice are quite susceptible to lymphoma induction by estrogen (42), suggesting not only susceptibility but also the possibility of a latent lymphoma (leukemia) agent. Other investigators have reported a low incidence of spontaneous lymphomas in this strain of mice (119). There are many well documented examples of reciprocal viral interference between the mammary tumor agent and leukemogenic viruses (20, 48). Viral interference must be overcome or reversed in order to provide positive

evidence for the immunoregulation theory. Failure to do so does not negate the theory.

In the case of virally caused mouse mammary tumors where 100% of the individuals are presumed infected and the tumor frequency may be normally about 75% (1, 27), failure to observe an effect of immunosuppression requires additional explanation. Of the demonstrated naturally occurring tumors that have been well studied, virus infection occurs either prenatally or neonatally (11, 17). Infection is followed by a latent period of months or years during which tumor associated antigens are expressed on tissues accessible to the circulating immune cells. The tissues expressing the antigens may be normal untransformed target issue or tissues which are never known to be transformed by that particular virus. They may even be circulating cells of the immune system itself. In the face of this abundance of expressed and displayed foreign antigen, the immune system is not effectively stimulated. The developing mammary tumor _in situ_ may stimulate the immune system differently than does the mammary tumor transplanted to an extramammary site (22). The role of the immune system in these examples of naturally occurring

vertically transmitted oncogenic viruses may be quite different from that observed in adult infection by the same or another oncogenic virus or in challenge by transplanted antigenic tumors.

Young adult mice which were neonatally infected with the mammary tumor virus are reported to be tolerant when challenged with syngeneic virus positive tumors (89, 90).

By the time a primary tumor is detected, both humoral and cellular immunity exist coincident with the growing tumor (51, 92). Humoral factors, either free antibody, antigen or antigen-antibody complex, at this time are claimed to protect the tumor from immune destruction by sensitized lymphocytes (51, 53). As a result, the cellular immune system is specifically ineffective against the tumor and ALS-immunosuppression should not alter tumor progression.

Although the results of this study are consistent with both specific tolerance and enhancing serum factors, other investigators have found that short term ALS-immunosuppression can enhance or inhibit MTV tumorigenesis. Blair and co-workers reported that ALS treatment had no significant effect on the preneoplastic lesions in C3H

or BALB/cfC3H parous mice (19, 72). A definite inhibition of spontaneous mammary tumor onset was noted in the ALS-treated mice compared to RNS-treated mice. The role of immunosuppression in tumor inhibition is in doubt, however, because the ALS preparation used also inhibited the growth of transplanted normal mammary tissue. Inhibition of tumor development like the inhibition of normal tissue growth may have been a direct effect of the ALS on the mammary tissue itself. Since normal rabbit serum had no effect on spontaneous mammary tumor development, the ALS effect may have been due to an antibody population induced by lymphoid tissue, but not specific for lymphocytes. In that study, ALS was prepared by using Freund's incomplete adjuvant in the immunization followed by 3-4 challenge inoculations. The use of Freund's complete adjuvant and/or hyperimmunization have been noted previously to induce toxic antibodies, unrelated to those which induce immunosuppression (62, 67). Although the mechanism of mammary tumor inhibition is unknown, inhibition of tumor onset is also noted after neonatal thymectomy in C3H mice (75, 113).

An alternative immunological mechanism of ALS tumor inhibition is suggested by the fact that ALS-treated mice

developed circulating antibody to mammary tumor virus whereas control mice did not (18, 19). Syngeneic mammary tumor transplants to these two groups of mice failed to show any effective tumor immunity in the ALS-treated group. Humoral antibody would not be expected to be effective protection against the tumor challenge, but should be indicative of an altered state of immunity. If recovery from ALS alters the immune status of the animal, it could be as important in determining tumor development as the immunosuppressive phase of ALS treatment.

Fisher et al. reported that ALS treatment increases the growth of syngeneic transplanted mammary tumors in C3H mice (39). The ALS was prepared according to a method which used Freund's complete adjuvant.

Woodruff and Smith observed that ALS treatment inhibited the growth of intrastrain transplants of mammary carcinoma (141). Although their ALS preparation was prepared with Freund's complete adjuvant, it was absorbed with mouse red blood cells. The absorbed sera were still bound by and were cytotoxic for both lymphocytes and tumor cells. Absorption with spleen cells or thymocytes or tumor cells indicated the presence of

separate antibody populations directed against lymphocytes and tumor cells. Similar absorptions with methyl cholanthrene induce sarcoma indicated that enhanced growth of this tumor in ALS-treated mice was partially due to a direct enhancing effect of ALS on the tumor.

Inconsistencies have also been noted in the effects of different ALS preparations on murine leukemias (8, 63). The differences have been ascribed to the presence or absence of anti-viral antibodies in the anti-lymphocyte sera (8). Anti-viral antibodies which inhibited leukemia development were detected in ALS. When no activity was detected against the virus, the ALS had no effect on tumor growth or development. Since the lymphocytes are both the immunogens for ALS preparation and the presumptive tumor target tissue, and infected nontransformed normal lymphocytes express the antigen, the presence of anti-viral antibodies in the ALS should not be surprising. A comparable situation may exist in the mammary tumor system where viral antigens are expressed on lymphocytes, but not on the red blood cells used for absorptions.

It also seems reasonable that some of the differences can be accounted for by qualitatively different immune

responses elicited by the primary tumor *in situ* and by the tumor transplanted to an abnormal site. Evidence from transplantation studies in the mammary tumor system indicate that the mammary fat pad may be a privileged transplantation site (22). The presence of an intact basement membrane may confer this advantage on the spontaneous tumor (118). Other evidence suggests that normal mammary tissue components may be required for tumor growth and/or may sequester neoplastic cells from immune destruction (118). Although ALS is a relatively specific suppressor of cellular immunity, the facts that the relevant antigen is unidentified and that the sera are not rendered lymphocyte specific leave open many interpretations and may account for inconsistencies noted in the effect of ALS on tumor development.

D. Immunity and Murine Mammary Tumors

The murine mammary tumor must undergo a long developmental progression from the time of virus infection until a truly neoplastic, invasive tumor exists (20, 21, 118). Following infection, which occurs either in the neonate or earlier, viral antigens are present on otherwise normal tissues. The antigen is not restricted to presumptive target tissue, but appears to be ubiquitous. It is not until after puberty, when hormonal stimulation is adequate, that the mammary tissue may undergo preneoplastic changes. Hyperplastic alveolar nodules or plaque structures are indicative of this change. These tissues are higly antigenic and are sensitive to immune destruction (118). They are not themselves neoplastic and may persist in this condition for long periods of time. Frank neoplasia with its characteristic invasive growth properties and resistance to immune destruction may eventually occur. Since all forms of this developmental sequence are antigenically distinct from the host, the stages, whether they display viral or individually distinct antigens (132, 134) must have some special relationship to the immune system.

The immune system in the young adult female is specifically nonreactive to mammary tumor viral antigens as illustrated by the increased susceptibility of virus carrying females to mammary tumor transplants (89, 90). Susceptibility is ascribed to tolerance, not to the presence of enhancing antibodies. This observation has led to the suggestion that the distinction between susceptible strains and nonsusceptible strains is based on the relative ease in which immune tolerance can be induced in different mouse strains (21). Studies from Mintz's and Prehn's laboratories indicate that mammary tumor susceptibility is a characteristic of the genotype of the presumptive target tissue and not the internal environment of the mouse (81-83). Transplantation of preneoplastic lesions from allophenics or mosaics, in which the lesion is a mixture of cells from both high and low tumor strains, demonstrate that the neoplastic cells most generally originate from the high tumor strain. On the strength of these data, the immune response seems not to determine strain susceptibility to mammary tumors.

If classical immunological tolerance to mammary tumor virus and associated antigens exists in susceptible strains, then immunosuppression should not in any way alter tumor

development. Neonatally thymectomized mice neonatally infected with mammary tumor virus develop tumors less frequently than do sham thymectomized littermates. This observation has caused speculation that the thymus plays a positive and possibly a nonimmunological role in the development of the mammary tissue (113) or that thymus-dependent immune functions contribute to the expression of malignant potential (52, 77, 106, 107).

Some investigators have noted that neonatal thymectomy adversely affects subsequent development of the mammary glands (71, 75, 113, 142). The most direct mechanism suggested by these observations is that the thymus is a source of hormone which promotes normal mammary tissue development (75, 115). An inhibitory effect on the presumptive tumor target tissue would naturally have an adverse effect on tumor incidence and development. Other investigators have noted that neonatally thymectomized mice fail to develop normally in a great number of respects (79). Neonatally thymectomized germ free mice do not exhibit generalized growth defects, and the wasting syndrome of conventionally raised neonatally thymectomized mice has been attributed to infectious agents (79). In the absence

of data on the effect of neonatal thymectomy on tumor development in germ free mice, it may not be necessary to postulate the existence of a mammotrophic hormone.

Another mechanism to explain the decreased tumor incidence is that the thymus-derived lymphocytes may be requisite intermediate cell hosts for the virus. The mammary tumor virus is certainly present in these cells in a fully infectious state and the viral antigens are detectable on the surface of peripheral blood lymphocytes from infected mice (11, 94). Measurements of virus levels in neonatally thymectomized mice have failed to show a diminution from control values in sham treated mice (55).

Even if this virus infection and expression is not required for the eventual malignant transformation, it may be important in inducing immunological tolerance. Continued tolerance to cellular antigens requires that the host be chimeric in its lymphoid tissues for the specific cellular antigens. Host tolerance may be advantageous for the host in preventing an autoimmune reaction. Since the virus is expressed in numerous body tissues, in normal as well as in premalignant and malignant tissues and in tissues which have never been observed to be transformed by the oncogenic

virus, antiviral immunity could be extremely detrimental to the host in the absence of malignant transformation. Virus and host are symbionts at least during host reproduction and viral transmission to the progeny. For tolerance to explain the results of neonatal thymectomy, this type of tolerance must be an active process and require the presence of a thymus at least during tolerance induction. An example of required thymus presence during tolerance induction has been reported in mice made tolerant to bovine serum albumin (102). In both instances, spleen cells were unable to substitute for thymus (102, 142). Studies with milk-transmitted murine leukemia virus suggest that the virus carrier state may mimic, but is different from classical tolerance (109).

If MTV infection is delayed, neonatal thymectomy increases tumor incidence. That neonatal thymectomy has opposite effects depending upon the time of infection could be explained as an immunological phenomenon. Neonates are much more susceptible to tolerance induction than are older individuals or adults. Early infection could cause tolerance, whereas later infection could result in immunity. Heppner has evidence from *in vitro* studies that serum

blocking factors to mammary tumors are not produced in mice which were neonatally thymectomized (52). Such blocking factors are present in the serum of normal control mice. Leukocytes from thymectomized and control mice are equally capable of inhibiting tumor cell growth. These results suggest that the mammary tumor antigens are thymus dependent antigens. Thymus cells are required in a cooperative step with antibody forming cell precursors before formation of specific antibody against a number of heterologous serum proteins and red blood cells (31, 80, 85). Thymus dependence of enhancing antibody formation has not yet been established by other techniques or independent confirmation.

Immunosuppressive treatments, other than thymectomy, have profound effects on mammary tumor incidence or transplantability. Splenectomy decreases tumor incidence, but splenectomy combined with neonatal thymectomy restores a normally high tumor incidence (122).

Cortisone and x-irradiation both decrease the transplantability of virus-positive tumors (194, 107, 121). In most of these examples, immunosuppressive procedures also inhibit growth of virus positive tumors.

Virus-negative mammary tumors possess individually unique antigens (132-134). X-rayed recipients of virus

negative tumors show more tumor takes than do control mice. Splenectomy prior to transplantation of virus negative tumors decreases tumor incidence. In contrast to results with virus-positive tumors, immunosuppression generally facilitates the transplantation of virus-negative tumors (104, 107). Interpretation of the data may be tempered somewhat with the recognition that all immunosuppressive procedures may also be toxic for nonlymphoid tissues and that experiments with virus-positive and virus-negative tumors are rarely done in parallel.

Agents which nonspecifically stimulate the immune response may decrease mammary tumor development (20, 21). The result of specific immunization with mammary tumor tissue is, however, unpredictable in that either immune enhancement or inhibition of tumor growth may occur (20, 21).

Dissecting the immune response to mammary tumors is complicated by the different classes of antigens -- viral, tumor associated nonviral and individually unique antigens. The virion itself is not a single antigen, but a group of distinct antigens (96). The net immune response as measured by tumor development is the sum of the immune responses to

all the separate antigens and a product of the animal's past antigenic history with respect to these antigens or cross-reacting antigens. Weiss reports that the enhancing antibody in murine mammary tumor systems is directed against, not a viral or tumor associated antigen, but a normal constituent of mammary tissue (137). If this claim is confirmed, the antibody may have an important function in normal physiological responses by the mammary tissue to hormonal changes. Elimination of enhancing antibody may not be desirable prior to the occurence of frank neoplasia.

E. Summary

The immune surveillance theory postulates that neoplastic transformation continually occurs within an individual and that the immune system is the body's primary defense against tumors. A lapse in this defense system combined with the superior growth potential of tumors might allow the tumor to outgrow and overcome the immune system even if immunosuppression were of short duration.

In humans, generalized immunosuppression is used therapeutically to delay rejection of allografts of vital organs. Therapeutic immunosuppression is induced and maintained by a combination of antimetabolites and steroids. Other, more direct manipulation of the immune system may supplement this treatment. Sufficient numbers of long term survivors are available only in the case of kidney transplant recipients. Higher than normal cancer frequencies are observed in these patients — with lymphoma being the most frequent tumor type. Tumors appear relatively soon after the start of immunosuppressive therapy. Almost ten percent of the reported tumors appear within four months from the time of transplantation and the mean time of onset is twenty-eight months.

Although immunosuppression is assumed to be the major factor in the tumor increase, suppression of immune surveillance is not the only mechanism whereby these tumors might arise. There are at least three alternatives. One is that uremia may predispose humans to cancer. The second is a possible carcinogenic effect of the antimetabolites used in immunosuppressive therapy. Third, a direct toxic effect on the immune system coupled with constant antigenic stimulation might actively promote tumors of the lymphoid system. The contribution of immunosuppression to primary tumor induction and short tumor latency is therefore not completely clear.

The effect of immunosuppression in the absence of chemical toxicity or disease induced stresses can be evaluated in animal models where genetic and environmental heterogeneity are also controlled and where there is less chance of inadvertently transplanting a tumor with the graft.

Virgin female mice of the CBA/J strain were observed until tumor onset or death after short term (anti-thymocyte serum induced) immunosuppression or a variety of non-immunosuppressive treatments. The first few tumors were

observed in rabbit anti-mouse thymocyte serum (RAMTS) treated mice while control rabbit normal serum (RNS) treated mice were tumor free. Additional mice of the same strain were collected from other experiments, to obtain tumor data for untreated mice and mice subjected to other experimental procedures. None of the mice were experimentally exposed to known carcinogens or oncogenic viruses. RAMTS was the only immunosuppressive agent used in this study. Anti-lymphocyte serum is often used in human transplant patients to supplement other immunosuppressive treatments. Mice tolerate high doses of anti-lymphocyte serum (ALS) without toxic side effects. ALS shows greater *in vivo* inhibition of the cell-mediated immune reactions, such as graft rejection, than of humoral immunity. The preparations of RAMTS used significantly prolonged survival of H-2 incompatible skin grafts without grossly affecting humoral antibody.

In mice, ALS-immunosuppression similar to that used in this study can significantly affect tumor growth when tumors are primary tumors or transplanted tumors. These facts suggest that the severity and duration of immunosuppression in this study should have been adequate for tumor promotion.

No tumors appeared in any of the mice during or in a relatively long interval after immunosuppression. Tumors subsequently developed in both immunosuppressed and non-immunosuppressed mice. Neither tumor incidence nor onset were affected significantly by immunosuppression. Fluctuations in incidence were noted, but incidence was the same in matched RAMTS and RNS-treated mice. Cause of the variation in tumor incidence is not known.

The results of this study on mice differ in two major respects from the reports of tumors in immunosuppressed transplant patients. First, no tumors appeared during immunosuppression and second, the predominant tumors which appeared later were not tumors of the lymphoreticular system. During immunosuppression, the frequency of spontaneous tumors in CBA mice in the absence of a carcinogen must be low even under conditions favorable for tumor growth.

The most frequent tumor type was the typical mammary tumor. CBA/J mice carry the mammary tumor virus (MTV). 100 percent of the mice are assumed to be infected, and normally as high as 75 percent of the females will manifest tumors.

Failure to observe an effect of immunosuppression on mammary tumor development requires explanation. Infection with MTV occurs in the neonatal period when mice are most susceptible to induction of immunological tolerance. Young adult mice neonatally infected with the virus are reported to be tolerant when challenged with syngeneic, virus positive tumors. By the time a primary tumor is detected, both humoral and cellular immunity exist coincident with the growing tumor. Humoral factors, either as antibody, antigen or antigen-antibody complexes, at this time can protect tumor cells from immune destruction by sensitized lymphocytes. As a result, the cellular immune system may be specifically ineffective against the tumor and ALS-immunosuppression should not alter tumor progression.

Although the results of this study are consistent with the existence of specific tolerance and enhancing serum factors, other investigators have found that short term ALS-immunosuppression can enhance or inhibit MTV tumorigenesis. The reason for these differences is probably that ALS is a class of reagents, not a monospecific or even a lymphocyte specific reagent. ALS can affect tumor growth by three different mechanisms depending in part on the antibody specificities present in the ALS preparation. It can

facilitate tumor growth by suppressing the immune response against the tumor. It can inhibit (or enhance) tumor growth by a direct toxic (or enhancing) effect of ALS antibodies directed against presumptive tumor or target organ. It can alter tumor growth by the generation of new reactive lymphocytes during the recovery phase, providing that the host's anti-tumor capabilities are thereby changed.

ALS prepared using Freund's complete adjuvant or from hyperimmune animals are more broadly toxic than ALS prepared without adjuvant. The RAMTS in this study was prepared by a standard method without adjuvant in contrast to the ALS preparations shown to inhibit primary mammary tumor growth or facilitate growth of transplanted mammary tumors.

Since the thymus is the source of the peripheral lymphocytes involved in cell-mediated immunity, thymectomy should affect mammary tumor development in the same way as does ALS-immunosuppression. Under normal conditions of early vertical transmission of MTV, neonatal thymectomy decreases tumor incidence — quite the opposite from the effect expected from a suspension of immune surveillance. This observation has caused speculation that the role of the thymus in murine mammary tumor system may be more than just

immunological. There is some evidence that the thymus may provide a hormone which promotes mammary gland development directly. Another proposal is that the thymus-derived lymphocytes are necessary sites for MTV replication.

If MTV infection is delayed, neonatal thymectomy increases tumor incidence. That thymectomy increases or decreases tumor incidence depending upon the time of infection, suggests an immunological phenomenon. The murine mammary tumor system is a prime example of immunological enhancement of tumor growth. Neonatal thymectomy does not detectably affect viral (MTV) replication, but can prevent the development of enhancing serum factors. Thymus derived lymphocytes are necessary in a cooperative step with bone marrow derived lymphocytes for production of antibody directed against some antigens. The effect of neonatal thymectomy on murine mammary tumors could be explained if relevant tumor antigens were in this group of thymus dependent antigens. The proposed role of the thymus in the production of enhancing antibody does not preclude other mechanisms of "active" tolerance. Because MTV is a group of associated antigens, the immunological response to each individual antigen may be different and the eventual outcome

of infection depends upon superimposing the immunological responses to the individual antigens.

In the end, the effect of immunosuppression on primary tumor growth depends very much on the particular tumor system. There may be exceptions to immune surveillance, causing immunosuppression to be without effect or possibly to inhibit tumor growth. The effect of immunosuppression on spontaneous neoplasms would depend on four factors: (a) the presence of tumor associated antigens, (b) the past antigenic history of the tumor host, (c) the relative importance of nonimmune growth regulation, and (d) the mechanism of tumor induction. If there are no effective tumor-associated antigens or if the host is "tolerant" to those antigens or if other control mechanisms constitute the body's primary tumor defense, immunosuppression should have no effect on the various parameters of tumor growth. If immunostimulation of tumors exists, a lapse in immunity will retard tumor development. It is only when tumors are antigenic, when the host is capable of responding immunologically and in an inhibitory fashion, and when other control mechanisms are circumvented or secondary, that immunosuppression should significantly increase tumor expression.

The case for immune surveillance against tumors may be very limited when enough examples of primary tumors have been examined.

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