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Genetic analysis of transplantation immunity in the fowl

Louis William Schierman

Iowa State University

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GENETIC ANALYSIS OF TRANSPLANTATION IMMUNITY IN THE FOWL

by

Louis William Schierman

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of DOCTOR OF PHILOSOPHY

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INTRODUCTION

The transplantation of tissues and cells has significance with respect to a number of basic problems of special interest to the immunologist and the geneticist. In addition, tissue transplantation is an important research tool in experimental embryology, physiology, and pathology and has numerous applications in medicine and surgery.

Transplantation or histocompatibility antigens and red cell antigens show similar genetic characteristics. Both exhibit widespread polymorphism in animal populations and are controlled by a number of genetic loci. The question of whether transplantation antigens and red cell antigens are determined by the same loci and are polymorphic for the same unknown reasons has not as yet been completely answered.

The present study was undertaken to explore the possible relationship between blood type and skin graft acceptance or rejection in chickens. The relatively simple technique used in skin grafting of young chickens makes this species especially desirable for transplantation studies. The population chosen for this investigation was a moderately inbred Leghorn line (Line G) which had previously been blood typed at several loci (Schierman, 1961). Since a number of blood typing reagents had already been developed for use in Line G, this line was of particular value for histocompatibility studies.
Because the results obtained in the initial skin grafting experiment were considered worthy of further investigation, additional and related studies were also undertaken. One of these was to investigate the relationship between erythrocyte and leukocyte antigens. This is of particular interest in view of Medawar's (1959) contention that leukocytes but not erythrocytes contain all the antigens involved in transplantation immunity.

Another experiment undertaken involved the use of the graft-versus-host reaction. This reaction occurs when donor leukocytes (presumably lymphocytes) react against host antigens not present in the donor cells. One manifestation of this phenomenon is the production of nodular lesions on the chorioallantoic membrane of chick embryos previously inoculated with leukocytes from adult fowl. Results obtained from experiments of this type have been considered as possible evidence for an elective mechanism of antibody formation (Burnet, 1961). The genetic material available at Iowa State University again offered some unique advantages for research in this area.

The results obtained from the three related studies will be presented separately.
LITERATURE REVIEW

Blood Type and Histocompatibility

The possibility of selecting compatible donors for tissue transplantation by hemagglutination tests has been a subject of speculation and research dating back to the time the blood groups in humans were definitely established. Masson (1918), in a report from the Mayo Clinic, stated: "...blood grouping is just as important for good results in skin grafting as it is necessary in transfusions, and that it is governed by the same principles." Schawan (1919) drew similar conclusions on the basis of clinical experience. His results with 17 patients led him to believe that homografts obtained from donors of the same blood group (ABO system) as the recipient, or from Group 0 donors, would become permanent takes and grow almost, if not equally, as well as autografts. Since most of the patients in this particular study were released from the hospital within four to six weeks after grafting, the grafts were observed over a relatively short period of time. Nevertheless, Schawan's views were upheld by a number of workers including Dyke (1922) who reported three additional cases in support of this opinion.

In contrast to these results are those of Underwood (1914) who transplanted skin from 17 different individuals to a single patient. All grafts were rejected except that of the mother of the patient; that of the sister showed fraction-
al acceptance. Similar difficulties were encountered by Holman (1924). Of a total of 319 small, deep pinch grafts from a mother to a son, both belonging to the same ABO blood group, all were rejected. Loeb (1930, 1945) did not believe the blood group of donor and host was of probable significance in homograft results.

Gibson and Medawar (1943) noted that, with few exceptions, the early investigators made no critical distinction between the fate of native and foreign skin and that skin homografts were still occasionally used by surgeons without a clear recognition of their ultimate fate. They also pointed out the generally accepted fact that foreign skin does not form a permanent graft in human beings except, without doubt, between monozygotic twins.

Woodruff and Allan (1953), taking advantage of more recent developments in blood typing, interchanged small full-thickness skin grafts between two unrelated volunteers whose blood group combinations were both as follows: O; cde.cde; MN; S negative; Kell negative; Lewis (a) negative, (b) positive; Lutheran negative; Duffy positive. In addition, both gave a negative indirect Coombs' test with each other's red cells as well as with known Rh positive cells. Both homografts showed good initial takes but, in spite of the remarkable degree of red cell compatibility, were destroyed within three weeks. The authors suggested, however, that the two
volunteers almost certainly differed with respect to some red cell antigens not yet investigated. The possibility that these unknowns were identical with the skin antigens responsible for the breakdown of the grafts cannot be entirely excluded.

Rogers (1956) pointed out that the graft survival time found by Woodruff and Allan (1953) was somewhat greater than the average survival time of seven to nine days which he found for grafts from nonrelated donors. He stated, "When gross compatibility of all the major and minor blood groups and subgroups known to date exists between a donor and recipient, skin homografts transplanted under control conditions between this donor and recipient survive longer than skin homografts obtained from donors with dissimilar or incompatible blood groups and subgroups. In the light of these studies, it may also be assumed that extremely delicate and refined blood-typing techniques might possibly result in the identification of new or as yet poorly demonstrable blood subgroups, thus serving as a method of 'typing' other tissues, especially skin."

Evidence that human red cell antigens may also be present in skin has been obtained by Mourant (1955) who detected "immune" A antibodies in two patients who had received skin grafts. This type of antibody which was distinguished from the natural
antibody was considered to have been formed against the A antigens present in the donor's skin. In a larger study Griffiths and Crikelair (1962) found considerable increases in titer of A and B agglutinins after skin grafts were made between persons with incompatible blood types.

Ingebrigtsen (1912) made an early attempt to correlate homografting and hemagglutination in lower animals. On the basis of 14 carotid artery transplants in cats he concluded that transplantation results were independent of hemagglutination results.

Gorer (1937) first obtained evidence that isoantigenic factors determine the specificity of tumor transplants in mice. A sarcoma arising in an inbred albino mouse was transplanted to $F_2$ and backcross individuals from a cross with resistant black mice. He found that all susceptible individuals possessed an erythrocyte antigen (antigen II) derived from the albino ancestors and detected by immunized rabbit sera. He also found iso-agglutinins in the sera of mice where the tumor had recently regressed.

Gorer (1938) later showed that the agglutinins were a specific response to iso-antigenic factors in the tumor. The antibody was designated antibody II.

Gorer et al. (1948) demonstrated that an allele responsible for iso-antigen II was closely linked with genes for tail
defects on chromosome IX. The gene determining the iso-
antigen was labelled H2, and the locus is now referred to as
the histocompatibility-2 or "H-2" locus. Hocker et al. (1954)
reported that the H-2 locus controls a complex system of iso-
antigens.

In a recent review, Amos (1962) listed 18 known H-2 anti­
gens. Antigens of different strains may share one or more
factors. Cross reactions are thought to be due to a variety
of antibodies reacting with different degrees of intensity
against a few complex antigens, or alternatively, a larger
number of antibodies, each reacting against a specific antigen.
Two interpretations for the observed reactions, like the two
proposed for the inheritance of the Rh antigens in man, are:
(1) each gene of a multiple allelic series determines a highly
complex antigenic product; and (2) individual antigens are
side chains of a larger molecule and are controlled by closely
linked genes. Present data from recombinations would support
either hypothesis for the nature of the "gene" but would indi­
cate that at least three or four subdeterminants are involved.

The mouse has been used most extensively in transplanta­
tion studies due largely to the establishment of co-isogenic
lines. Breeding procedures for the development of such lines
have been described by Snell (1958).

The possibility that red cell antigens are related to
histocompatibility antigens in rats was suggested by Lumsden
(1938). He found that an iso-hemagglutinin was produced by rats resistant to implantations of a sarcoma. Recent studies by Bogden and Aptekman (1962) have established that the $\text{B}$ hemagglutinogen in rats functions as a major histocompatibility factor. They suggest that the $\text{R-1}$ locus in rats is a "strong" locus similar to the $\text{H-2}$ locus in the mouse. Kapitchnikov et al. (1962) and Hancock and Mullen (1962) reported finding hemagglutinins in the sera of rabbits which had rejected homografts.

### Transplantation Studies with Chickens

One of the first homografting experiments with chickens was that of Rous (1910) who successfully transplanted a sarcoma between birds. Pieces of tissue from a tumor found in a Barred Plymouth Rock hen were implanted into the breast muscle and peritoneal cavity of both related and unrelated stock. Successful transplantation occurred in a small percentage of related birds but in none of the unrelated adult birds. Partial success was observed in two out of 17 unrelated young chickens.

An early skin grafting study with chickens was that of Danforth and Foster (1927). The percentage of initial takes of homografts on newly hatched chicks was high, but many grafts, after becoming well established, were subsequently lost. This study further revealed that feathers produced on the graft have breed characteristics of the donor but sex
characteristics of the host.

Kozelka (1929) studied secondary sexual characters in the fowl by making both autografts and homografts of comb, spur and skin tissue. He found fewer cases of homograft rejection among closely related birds.

More recent studies involving genetic relationship and homograft reaction have been carried out by Craig and Hirsch (1957) using the procedure that Blumenthal (1939) had used with other species. They found that relative increases of lymphocytes in the circulating blood, following skin grafting, were correlated with donor-host relationship coefficients. Grafts exchanged between birds of different breeds gave greater response than grafts between individuals within a breed. Three and five week old chicks were used in this experiment. Berry and Craig (1959) were unable to show the same results with year-old birds.

Craig et al. (1960), using a system of scoring the macroscopic appearance of skin grafts, showed that in young chicks the rate of regressive change in transplanted skin is associated with the genetic diversity between donor and host. Different breed and crossbred donor tissue resulted in more extensive and statistically significant reactions than tissue from unrelated donors of the same heterogeneous strains.

Cannon and Longmire (1952) studied age effect of the recipient at grafting time on skin homograft survival. Chicks
from six different breeds or breed-crosses obtained from a commercial hatchery were used. From five to ten percent of the homografts were successful when the chicks were no older than three days at the time of grafting. Only one percent of the grafts remained intact in four-to-six-day old chicks observed for ten weeks post-grafting. No homografts lasted more than a few days in chicks grafted at 14 days of age.

In a similar experiment Cannon et al. (1954) investigated graft survival with respect to donor age as well as host age at grafting time. They concluded that: (1) the antigenic specificity of chick skin is developing at hatching and is complete by the 14th day; and (2) the ability to exhibit an immune response to homografted tissue is also developing at hatching and is complete by the seventh day post-hatching.

Weber et al. (1954) regrafted surviving homografts in chickens. In most cases grafts returned to the original donor were accepted and indicated permanent survival. However, second grafts from an original donor were rejected in four of five adult birds although they still retained grafts exchanged when donor and host were both less than three days old. They postulated that skin antigens may not be fully developed in young chicks. The repeat skin homograft from the original donor, when adult, was thought to contain antigens not present in the original graft. Therefore, either the original homograft was transplanted prior to their development, not develop-
ing these antigens, or the incompatible antigens were eliminated.

Billingham et al. (1956) found only one of 30 skin homografts from two-week-old Rhode Island Red donors to two-week-old White Leghorn hosts alive eight days after transplantation. In contrast, when adult Rhode Island Red donors were used, ten of 18 homografts showed some degree of survival at the eighth day. Older skin was thought to be, in effect, less antigenic than younger skin because grafts from older birds take longer to establish vascular and lymphatic connections with their hosts.

In the same study Billingham et al. (1956) demonstrated that animals become tolerant to foreign skin if inoculated with cells from the skin donor prior to or shortly after birth. This work followed Owen's (1945) demonstration that adult cattle twins are usually tolerant to each other's red cells, due to an anastomosis of their placental vessels.

Billingham et al. (1956) postulated that in the experiments of Cannon et al. (1954) the recipients of the older skin must have been a day or two more advanced in immunological development by the time the antigenic (and therefore tolerance conferring) stimulus took effect. This suggested that younger skin is antigenically more active in the sense of being quicker to exercise its antigenic power. They also postulated that rejection of a later homograft by a host apparently tolerant
to an earlier homograft comes about when, and only when, the state of tolerance is incomplete.

Terasaki et al. (1957) showed donor age and graft size effects in newly hatched chicks. On two-day-old chicks large grafts of two-day-old skin survived markedly longer than did smaller grafts. No large adult skin grafts, placed on two-day-old chicks, survived to the 14th week post-grafting while 32 percent of the large two-day grafts survived to this time. They interpreted the difference in survival to be due to some property of the graft and not to an effect on the host's immunological system.

Kozelka (1933) made one of the first studies concerning the relationship between transplantation antigens and red cell antigens in chickens. Integumental grafts were exchanged between chickens in which hemagglutination tests were made with absorbed rabbit anti-chicken sera. Transplants between individuals with practically identical or no additional agglutinogens, (demonstrable by the technique employed) were rejected about as frequently as transplants between individuals where donor agglutinogens were absent in the host. He concluded that factors responsible for homograft rejection are not identical or associated with factors causing hemagglutination with immune serum.

Haddow (1934) used absorbed normal ox serum to test the red cells of chickens and study the relationship of hemagglu-
tination to transplantation of an induced fowl sarcoma. Evidence obtained from three generations of birds indicated that the fate of transplants was not influenced by the antigen content of their erythrocytes.

Cock and Clough (1956) made skin homografts between chicks from three Reaseheath inbred lines. In one line, having a calculated coefficient of inbreeding of 98.75 percent, only six of a total of 86 homografts failed to survive to 50 days post-grafting. The authors noted that Dr. D. G. Gilmour of Cambridge University found the line to be segregating for at least one pair of alleles determining red cell antigens. In a personal communication, Gilmour stated that alleles at the A blood group locus were segregating in this line.

The question of a relationship between red cell antigens and transplantation antigens has been raised in connection with immunological tolerance. Billingham et al. (1956) found that whole blood or leukocytes injected into an embryo or newly hatched chick induced some degree of tolerance to subsequent skin grafts from the donor. The ability to induce tolerance for skin was found to reside only in the leukocytes, for the injection of red cells alone did not produce tolerance for subsequent grafts. These workers concluded that red cells lack antigens responsible for tissue transplantation immunity.

On the other hand, they inferred that every antigen in skin is also represented in the leukocyte.

In contradiction to the above finding were those of Cannon et al. (1958). By injecting the embryos at 14 or 15 days incubation and grafting at two days post-hatching, they found the red cells to be the most effective cell fraction in inducing tolerance to skin. Differences concerning procedure existed between the two experiments and may account for the conflicting results. In the latter study, the technique used for obtaining red cells free of leukocytes was apparently not entirely satisfactory.

Gilmour (1962) believed the findings of Billingham et al. (1956) did not settle the question of a possible common genetic determination of red cell and histocompatibility antigens. He postulated that the inability of red cells to elicit tolerance to other tissues may be due to red cells not carrying the full spectrum of histocompatibility antigens, or carrying them in a modified form.

That at least some correspondence exists between transplantation antigens and blood group antigens was indicated by Stark and Frenzl (1959) who found red cell agglutinins of low titer in some chicks which had rejected skin homografts.

Stark et al. (1961b) demonstrated that chicks tolerant to skin homografts were not tolerant to donor red cells. Ten of 13 chicks tolerating a skin graft produced hemagglutinins
after immunization with cells from the graft donor. Tolerance to the grafts had been induced in some chicks by grafting shortly after hatching and in others by grafting plus injection of donor blood shortly after hatching. After immunization with donor red cells the grafts remained intact on all except two chicks for at least six weeks, indicating that some erythrocyte antigens may be absent in tissue cells. However, in another study, Stark et al. (1961a) were unable to detect hemagglutinin formation in four of 20 chicks tolerating a skin homograft after repeated injections of donor red cells. In these cases tolerance to the graft had been induced by the graft alone. This led them to believe that skin grafts share all important antigens of red cells.

Gilmour (1962), in discussing the previously mentioned findings of Stark et al. (1961b), stated that it could again be assumed that antigens of red cells and tissue are the same in specificity but are carried in different ways such that physiological responses to them may differ. Stark (1962) found that in a larger series of similar experiments about 15 percent of chicks tolerating skin grafts showed red cell agglutinins of low titer before the stimulating series of blood injections. Gilmour (1962) noted the possibility that graft tissue may induce production of humoral antibodies, and that these have a self-enhancing action on survival of the graft. Such an interpretation was put forward by Cock (1962) to ex-
plain survival of tissue grafts in chickens despite production of humoral antibodies which reacted with donor red cells.

Leukocyte Agglutination Studies

That leukocytes share all the antigens involved in transplantation immunity has been suggested from a number of studies. Woodruff (1960) stated that an approach to selecting homograft donors as yet largely unexplored is that of developing methods of typing based on antigenic properties of leukocytes. Studies showing that human leukocytes have antigens not detected in red cells have been reviewed by Walford (1960). The inheritance of human leukocyte antigens has not been studied extensively although a recent investigation was made by Payne and Hackel (1961).

Amos (1953) reported the production of anti-leukocyte antibodies in mice following inoculation with either leukemic cells or normal tissue from different strains. Serum produced by one strain contained two antibodies not shown by red cell agglutination. Following rejection of skin homografts between different strains of mice, leukoagglutinins against cells of the skin donor strain were detected by Amos et al. (1954).

Terasaki et al. (1959a) developed a lymphocyte agglutination technique for detecting antibodies against blood lymphocytes in chickens. Lymphoagglutinins were produced in chickens which had been injected with spleen cells or received skin
grafts from birds of another breed. Whether the antibodies were tested with red cells was not reported.

In a similar study, Terasaki (1959a) showed that the lymphoagglutinating activity of serum from homografted chickens appeared two weeks after grafting and reached a maximum at about three weeks. The possibility that the lymphoagglutinins may play some role in the destruction of grafts was considered. Terasaki et al. (1960) investigated the appearance of lymphoagglutinins in partially tolerant chickens. A wide range of partial tolerance to homografts between four-day-old chicks of the same breed was found. In most cases lymphoagglutinins were demonstrable in the serum shortly after homograft rejection and appeared most markedly in chicks rejecting their grafts early.

Graft-Versus-Host Reaction

Embryonic and newly hatched chicks respond to the inoculation of immunologically competent cells with enlargement and histological changes in the spleen, and, to a lesser extent, in the liver and other organs. In the embryo, this reaction was first reported by Danchakoff (1916) and later by Ebert (1951, 1954) who found that small fragments of adult chicken organs, especially spleen, when transplanted to the chorioallantoic membrane of chick embryos greatly stimulated growth of the homologous embryonic organ.
Simonsen (1957) presented evidence to show that intact donor cells capable of proliferation and antibody production were necessary for host spleen enlargement. He observed host spleen enlargement following injection of adult spleen cells or whole blood into 18-day embryos or newly hatched chicks. By means of the Coombs' test, antibodies against blood group antigens of the recipient were found in the blood of the recipient. Transplantation of embryonic chicken spleen cells failed to cause splenic enlargement in the embryonic hosts, indicating that donor cells from immunologically mature animals were necessary to produce the phenomenon. Studies with inbred mice showed that splenomegaly resulted only if the transplanted adult spleen cells came from donors genetically different from the recipients. This observation was also reported later with chickens by Cock and Simonsen (1958). They postulated that the injected donor cells proliferate, colonize the host reticulo-endothelial system and, due to foreign host antigens, proceed to attack the host cells both by producing antibodies against the host erythrocytes, and by means of a homograft reaction.

Terasaki et al. (1959b) found that circulating leukocytes and the upper centrifugal layer of bone marrow cells from adult chickens caused a high percentage of deaths when injected into embryonic chicks. On examination of dead embryos the spleens were enlarged and often showed necrotic spots. Terasaki
(1959b) found that adult chicken blood lymphocytes produced splenomegaly when injected into chick embryos of a different breed or strain. Adult monocytes and thymocytes did not cause significant spleen enlargement. In another study, Biggs and Payne (1959) found that mitotic figures examined in spleens enlarged five- to twenty-fold were of both host and donor origin and were present approximately in the proportion of one-to-one.

The genetic basis for the graft-versus-host reaction was studied by Jaffe and Payne (1962). By injecting parental line blood into F2 and backcross embryos between two lines of inbred chickens, they estimated the number of loci involved. The proportions of embryos showing no splenic enlargement were compared with the theoretical expectations of (1/2)^n in the F2 and (1/2)^n in the backcross to the donating line, where n is the number of antigenic loci by which the lines differ. Their results suggested that the two lines differed with respect to one strong antigenic locus and possibly other weaker loci.

Boyer (1960) reported that cell suspensions prepared from adult fowl blood, spleen or thymus, when placed on the chorioallantoic membrane (CAM), produced nodular lesions or foci on the membrane in addition to splenomegaly and hepatomegaly. Again the leukocyte fraction of the blood was found to be responsible for the phenomenon; red cells and serum were incapable of producing the characteristic lesions. The number of lesions
which developed depended upon the number of cells inoculated. An inoculum containing $10^4$ to $10^5$ peripheral blood leukocytes was required to produce a single lesion.

The phenomenon of foci formation was thought to provide a useful tool in the study of certain problems of current immunological theory and seemed particularly relevant in the validation of the clonal selection hypothesis of Burnet (1959). This hypothesis, stated simply, is that every animal has a large population of immunologically competent cells; each cell has a predetermined pattern corresponding to only one, or at most, a few foreign antigens. An antigen introduced into the animal body would thus 'select' a particular antibody producing stem cell which would in some way cause it to proliferate and initiate production of the corresponding antibody.

In contrast to the clonal selection theory are the earlier hypotheses attributed to several workers including Pauling (1940) and Haurowitz (1952). These theories, usually referred to as the "template" theories, consider that antibody molecules have their specificity determined by being synthesized against a template of the antigen molecules themselves. Lederberg (1959) used the terms "elective" in describing Burnet's theory and "instructive" in referring to the template theories.

Burnet (1961) considered the ratio between the number of leukocytes inoculated on the CAM of the chick embryo and the number of lesions produced as possible evidence for an elective mechanism of antibody formation. One lesion was believed to represent the reaction of one donor cell. Also, the number of lesions produced was considered to indicate the proportion
of injected leukocytes with preformed patterns corresponding to, and reacting with, antigens in the chick embryo not present in the leukocyte donor.

Burnet and Burnet (1961) used the number of CAM foci produced to study the degree of genetic diversity between donors and hosts from two sublines of inbred White Leghorns. Their results were explained on the basis of three alleles at a single histocompatibility locus. The findings were considered to be in agreement with the clonal selection theory of immunity.

Szenberg and Warner (1961, 1962) made an investigation to determine the type of leukocyte responsible for the induction of CAM lesions. A highly significant correlation was found between the number of large lymphocytes present in the inoculated blood and the median lesion count. No significant correlation existed between the number of small lymphocytes inoculated and lesion count. The median values indicated that the number of large lymphocytes per lesion was between 1,000 and 2,000 with the values differing significantly between two inbred lines.

Szenberg et al. (1962) found that passage of cells from donors of known genotype through the spleens of outbred embryos gave cells capable of producing a full lesion count on the CAM of embryos having the same genotype as the original donor. This was clear evidence of a change in specificity of the donor cells. This finding was therefore considered to be incompati-
ble with any simple form of clonal selection theory as far as it applies to the primary reactivity of immunologically competent cells. The clonal selection theory was also thought to be untenable without modification in view of the findings of Lind and Szenberg (1961). In this study the adult donor fowl were first immunized with tissue from the embryonic host strain. The immunization did not increase the ability of the lymphocytes to produce lesions on the CAM.

Hilgard et al. (1962) recently studied tolerance phenomenon with respect to the CAM reaction. They found that by prenatal administration of embryonic spleen cells, chickens could be made tolerant so that their leukocytes were later incapable of producing lesions on the CAM of embryos whose genotypes corresponded to those of the spleen cell donors. The implication was noted, on the basis of these findings as well as the related studies previously mentioned, "that tolerance both to genotypically determined self-components and to artificially introduced components is a function of the internal environment not of the intrinsic genetic capacity of the cell."
PART I. RELATIONSHIP OF BLOOD TYPE TO HISTOCOMPATIBILITY
MATERIALS AND METHODS

Genetic Stock

Birds used in this investigation were from a White Leghorn line (Line G) in which inbreeding coefficients ranged from 0.40 to 0.54. Most of the birds in this line are descendants of a female from an unrelated White Leghorn line (Line H). This hen was mated to a Line G male in 1958 producing F\textsubscript{1} progeny. An F\textsubscript{1} male was later mated to Line G females and also to two full sibs so that backcross and F\textsubscript{2} progeny were obtained. In subsequent generations matings were designed to maintain segregation at several blood group loci.

All birds were blood typed prior to skin grafting. Grafts were exchanged between birds in a manner that provided a number of combinations of donor-host red cell incompatibilities. A study of four different blood group loci with respect to histocompatibility was possible. The number of alleles tested at the A, B, D and L loci was 2, 3, 1 and 1, respectively.

A total of 209 skin homografts was made with 58 chickens. This does not include nine grafts considered lost by accident shortly after grafting. Grafts were made between full sibs, half sibs and less closely related members of the same line. The coefficients of relationship between donor and host ranged from 0.57 to 0.85. An outline of the four skin grafting experiments performed is presented in Table 1.
Table 1. Details of four skin grafting experiments

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Loci studied</th>
<th>No. of birds</th>
<th>No. of grafts</th>
<th>Donor-host relationship</th>
<th>Coefficients of relationship</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A, B, D, L</td>
<td>19</td>
<td>68</td>
<td>Full sibs</td>
<td>0.83 and 0.85</td>
</tr>
<tr>
<td>2</td>
<td>A, B</td>
<td>9</td>
<td>34</td>
<td>Full sibs</td>
<td>0.84</td>
</tr>
<tr>
<td>3</td>
<td>A, B, D</td>
<td>18</td>
<td>66</td>
<td>Full sibs</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Half sibs</td>
<td>0.73</td>
</tr>
<tr>
<td>4</td>
<td>A, B, L</td>
<td>12</td>
<td>41</td>
<td>Full sibs</td>
<td>0.83 and 0.85</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Same strain</td>
<td>0.57, 0.60 and 0.74</td>
</tr>
</tbody>
</table>

Blood Group Determination

Segregation is now known to be occurring at the A-E, B, C, D and L blood group loci in Line G. Alleles determining red cell antigens of these five systems have been identified by the use of iso-immune reagents developed within Line G. Specificity of the reagents has been verified by testing a panel of birds with reference reagents made available by a commercial firm. The five systems investigated in this study were therefore presumed to correspond to the systems determined by Briles (1951, 1958), Briles et al. (1950a, 1950b) and Gilmour (1959). The blood typing procedure and immunization techniques have been described previously (Schierman, 1961).

1Hy-Line Research Laboratory, Johnston, Iowa.
Skin Grafting Procedure

Reciprocal skin grafts were made between chicks at 16 to 19 days post-hatching. Each chick received from two to four grafts, but not more than one graft from the same donor. Location of graft was randomly assigned to one of four positions on the back. In the initial experiment the size of the skin graft was approximately 8-mm.-square while in later studies the size was increased to about 8 x 10 mm. Preparation of the area for grafting consisted of first removing the down from the backs of donor and host. Flexible collodion was then applied a few minutes prior to grafting to stiffen the skin. This prevented the grafts from curling and also avoided gaping of the recipient area. Clipping was found preferable to plucking the down from the graft area because the remaining down stubble, becoming matted with the collodion, helped retain the rigidity of the skin until healing occurred.

After the collodion was applied, a small amount of physiological saline injected subcutaneously at the graft site separated the skin from the underlying tissues. A full thickness skin graft was then removed by cutting with a sharp pointed dissecting scissors. Grafts were turned 180° before being fitted into the prepared graft bed on the host. This permitted easy identification of grafts later by the feathers growing in a reverse direction.

After carefully blotting the graft area, a simple ready-
made "patch" dressing\(^1\) was placed over the graft. Thus, no suturing of the grafts was necessary.

The bandages were removed on the fifth or sixth post-operative day at which time the grafts were scored for degree of vigor. Observations were made daily or on alternate days for the next week and less frequently during later stages of the experiment. In scoring the grafts a series of arbitrary grades was used which provided a reasonably accurate determination of graft rejection time. The scoring system, presented in Table 2, is similar to that described by Polley et al. (1960). A score of three or less was considered a definite indication of an immunological reaction against the graft.

Table 2. Macroscopic scoring system used to estimate severity of homograft reaction

<table>
<thead>
<tr>
<th>Score</th>
<th>Description of graft</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Smooth and healthy appearing</td>
</tr>
<tr>
<td>4</td>
<td>Slight discoloration and/or inflammation, but smooth</td>
</tr>
<tr>
<td>3</td>
<td>Brown or red color and slightly shrunken</td>
</tr>
<tr>
<td>2</td>
<td>Brownish-black color and shrunken</td>
</tr>
<tr>
<td>1</td>
<td>Brownish-black or black color, shrunken and becoming detached at the edges</td>
</tr>
<tr>
<td>0</td>
<td>Graft sloughed off</td>
</tr>
<tr>
<td>X</td>
<td>Part of graft sloughed off but with some apparent rehealing</td>
</tr>
</tbody>
</table>

\(^1\)Plastic Band-Aid-Spots, manufactured by Johnson and Johnson Co., New Brunswick, N. J.
RESULTS

Experiment 1

Thirty-eight of the 68 homografts reciprocally transplanted in Experiment 1 were from donors possessing one or more red cell antigens not possessed by the host. Twenty such donor-host red cell incompatibilities involved the B system antigens. Thirteen of these 20 involved the B antigens only, while the remaining seven involved A, D or L antigens in addition to the B antigens.

Initial observations of the grafts were made on the sixth post-operative day at which time some grafts exhibited an edematous swelling and slight discoloration. On the seventh day, 13 of the grafts were dark red in color, indicating an immunological reaction on the part of the host. By the tenth day after grafting, 20 grafts had been rejected by ten birds. The remaining 48 appeared healthy and were healing in with the surrounding host tissue.

Notable was the fact that graft rejection occurred only in the 20 cases where donor and host were incompatible at the B blood group locus. That this may have occurred by chance is unlikely. The calculated chi square value was 67.94, with six degrees of freedom giving a probability of less than 0.001. This would indicate two co-dominant B alleles which control transplantation specificities as well as blood groups. The
two alleles were previously designated $B^1$ and $B^2$. Details of the results observed by the tenth post-operative day are presented in Table 3. The typical appearance of healthy and rejected grafts are shown in Figure 1 and Figure 2.

Table 3. Influence of $B$ locus genotype on homograft results

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
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<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(+)</td>
<td>(-)</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>3168</td>
<td>3</td>
<td>$B^1/B^1$</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>$B^1/B^2$</td>
<td>8</td>
<td>0</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>3159</td>
<td>4</td>
<td>$B^1/B^1$</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>$B^1/B^2$</td>
<td>6</td>
<td>0</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>19</td>
<td>2</td>
<td>$B^1/B^2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>$B^2/B^2$</td>
<td></td>
<td></td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
</tbody>
</table>

$^a$Tenth post-operative day

$^b$Number of grafts accepted (+) and rejected (-) from donor of genotype shown

The number of remaining donor-host incompatibilities involving the $A$, $D$ and $L$ blood group loci were 8, 4 and 13, respectively. These did not appear to influence histocompatibility in this experiment. Sex differences between donor and host did not appear to influence homograft rejection. Preliminary results of this experiment have been reported by Schierman and Noråskog (1961).
Figure 1. Line G host at eight days post-grafting displaying one healthy and one rejected homograft.

Figure 2. Line G host at ten days post-grafting displaying four healthy skin grafts. Grafts are circled for identification.
Periodic examination of the remaining grafts continued for 180 days post-grafting. Some of the grafts appeared to undergo a chronic mild reaction so that the survival end-point was not sharply defined. Although some grafts evidenced complete destruction, others exhibited a slow sloughing of the epidermis. Grafts were considered as having been rejected in those cases where shiny surfaced scars were eventually observed. Some grafts did not produce feathers even after a prolonged period of time in which no graft rejection had been observed. Since some reaction possibly had occurred between observations, the fate of these grafts was considered uncertain. Most of the B-locus compatible grafts produced feathers or short stubs of feathers. The production of feathers or feather stubs was the criterion used for judging graft acceptance. The typical appearance of a bird displaying grafts with reverse feather growth is presented in Figure 3. The status of the B-locus compatible grafts at 70 and 180 days post-grafting is given in Table 4. Of the 29 grafts intact at 180 days, six donor-

<table>
<thead>
<tr>
<th>No. days post-grafting</th>
<th>No. grafts intact</th>
<th>No. grafts uncertain</th>
<th>No. grafts rejected</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>31</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>180</td>
<td>29</td>
<td>13</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 4. Status of 48 B-locus compatible homografts
Figure 3. Line G host at 150 days post-grafting showing two grafts with reverse feather growth. Surrounding feathers have been removed for easier identification.
host incompatibilities were found for the A locus, two for the D locus and 11 for the L locus. That some of the grafts could be considered as permanent takes is indicated from these results as well as from observations made on four birds still available at 510 days after grafting. Three of the four birds had surviving grafts showing reverse feather growth. One bird retained all four grafts indicating acceptance from the four different full sib donors. Incompatibility at the A and L loci existed in two of the birds retaining grafts at 510 days post-grafting.

Experiment 2

The results of Experiment 2 were similar to the previous findings. Again, reciprocal grafts were made only between full sibs. In this experiment a third B locus allele, designated B³, was tested for histocompatibility along with the B¹ allele. Co-dominance of these two alleles was evident by the early rejection of the B locus incompatible grafts. Of 11 B incompatible grafts exchanged in this experiment, all were rejected by the ninth day post-grafting. The remaining 23 grafts appeared healthy and in good condition at this time. Only two showed signs of partial rejection prior to 30 days post-grafting. Final scoring of the grafts was made at 150 days. At this time the condition of the B locus compatible grafts on eight surviving birds were recorded as follows: 14 intact and healthy, three uncertain and four rejected.
Five of the 14 healthy grafts involved a donor-host incompatibility at the \( A \) blood group locus.

**Experiment 3**

Eighteen of the 66 homograft exchanges in Experiment 3 were \( B \) incompatible. Destruction of these 18 grafts was nearly complete by the eighth day after grafting. Although the remaining 48 grafts appeared healthy at this time, somewhat later, or commencing about the 14th post-operative day a partial breakdown was observed in some grafts. There were some in which only the center became necrotic and eventually sloughed. Three grafts of this type were found to reheel to the extent that feathers were eventually produced in the graft. However, most of the grafts did not produce feathers but apparently underwent a mild reaction such that surreptitious replacement of their epithelium by ingrowing host epithelium had occurred. That a lower percent of \( B \) compatible grafts was retained over the same period of time as in Experiments 1 and 2 was no doubt due to a lower degree of donor-host relationship in this experiment. The results of Experiment 3 involving the \( B \) compatible grafts exchanged between 12 full sibs and six half sibs are presented in Table 5. Of the grafts retained by the hosts at 150 days post-grafting, three were from \( A \) incompatible and four from \( D \) incompatible donors.
Table 5. Status of B locus compatible grafts at 150 days post-grafting

<table>
<thead>
<tr>
<th>Donor-host relationship</th>
<th>No. grafts intact</th>
<th>No. grafts uncertain</th>
<th>No. grafts rejected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full sibs</td>
<td>9</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>Half sibs</td>
<td>4</td>
<td>2</td>
<td>6</td>
</tr>
</tbody>
</table>

In Experiment 4, skin grafts were exchanged between full sibs and between chicks produced from two sire families of the same inbred line. Coefficients of relationship are shown in Table 1. Forty-one skin grafts were exchanged in this experiment of which 23 were $B$ locus incompatible. Three $B$ alleles ($B^1$, $B^2$ and $B^3$) were investigated. Again, all $B$ incompatible grafts were rejected within eight days post-grafting. However, two grafts considered to be $B$ compatible appeared to slough off one of the birds on the eighth post-operative day. Both grafts were from the host's full sibs. One explanation seems that such early loss of these two grafts may have been due to trauma. This would appear likely in view of the consistent results obtained from the other experiments. A bruised area surrounding one of the graft sites was noted. Half of the remaining 16 grafts were exchanges between full sibs, and half were exchanges between less related birds. Classification of
of the B compatible grafts at 150 days post-grafting is presented in Table 6.

Table 6. Status of B locus compatible grafts at 150 days post-grafting

<table>
<thead>
<tr>
<th>Donor-host relationship</th>
<th>No. grafts intact</th>
<th>No. grafts uncertain</th>
<th>No. grafts rejected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full sibs</td>
<td>4</td>
<td>4</td>
<td>2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Same strain</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Loss of these two grafts on the eighth post-operative day may have been due to trauma

Response to B Locus Incompatible Grafts

The results of the four skin grafting experiments clearly indicate that the B locus, in addition to being a red cell antigen determinant, is also a major histocompatibility locus. The intensity of the reaction which the B incompatible grafts elicited from their hosts was measured in terms of the median survival time (MST). The first day a graft scored three or less was the criterion for this evaluation. The MST's, 95 percent confidence limits and standard deviations were computed by Litchfield's (1949) nomograph method for the rapid solution of time-percent effect curves. The results of these computations are presented in Table 7.
Table 7. Median survival times of B-locus incompatible grafts

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>No. of birds</th>
<th>No. of grafts</th>
<th>MST (a) days</th>
<th>Standard deviation</th>
<th>Range of survival time days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>20</td>
<td>6.8 ± 0.5</td>
<td>1.16</td>
<td>6-10</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>11</td>
<td>6.6 ± 1.3</td>
<td>1.25</td>
<td>6-9</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>18</td>
<td>6.7 ± 0.6</td>
<td>1.22</td>
<td>6-8</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>23</td>
<td>5.9 ± 0.3</td>
<td>1.11</td>
<td>&lt;6 (b)-8</td>
</tr>
<tr>
<td>Pooled (grafts)</td>
<td>33</td>
<td>72</td>
<td>6.6 ± 0.2</td>
<td>1.14</td>
<td>6-10</td>
</tr>
<tr>
<td>Pooled (hosts)</td>
<td>33</td>
<td>--</td>
<td>6.9 ± 0.3</td>
<td>1.10</td>
<td>6-9</td>
</tr>
</tbody>
</table>

\(a\) Plus and minus 95 percent confidence limits

\(b\) More than half the grafts were rejected on sixth day post-grafting in Experiment 4

A total of 72 B-locus incompatible grafts was included in the four experiments although various combinations of three B alleles existed in each experiment. For this reason, MST's for each experiment were calculated as well as for the pooled data. Since the hosts frequently received more than one B incompatible graft, the possibility of a specific host effect would tend to nullify the independence of the observations. Although the data did not indicate such effects (15 out of 29 hosts receiving more than one B incompatible graft rejected
the grafts on more than one day), a separate MST was calculated using the average graft rejection time for those hosts receiving more than one graft.

The MST's computed from these studies suggest an expected survival time for B incompatible grafts. However, the investigation of B alleles not included in this study, as well as the effect of other donor and host ages are necessary before accurate predictions can be made.
PART II. RELATIONSHIP OF ERYTHROCYTE TO LEUKOCYTE ANTIGENS
MATERIALS AND METHODS

Lymphocyte Agglutination Tests

Specific lymphoagglutination tests to determine the relationship of leukocyte and red cell antigens in chickens were made in Experiment 5. Tests were carried out with lymphocytes from 18 adult Line G birds. The birds had been previously blood typed with reagents specific for antigens of the A, B, C, D and L systems. Red cells from some of the birds had reacted positively, and from others, negatively, to each of the reagents used.

Suspensions of essentially pure lymphocytes were obtained by a method similar to that used by Terasaki (1959b) and Terasaki et al. (1959a). Birds were bled by cardiac puncture into heparinized Hank's solution. Approximately 20 ml. of blood was taken from each bird. The blood was then incubated in 50 ml. centrifuge tubes for 30 minutes at 37°C to remove most of the polymorphonuclear leukocytes and monocytes which stuck to the surface of the glass tube. The blood was then centrifuged lightly at about 80 g. for seven minutes. The upper plasma layer, containing lymphocytes, was taken off with a pipette. Cells from the buffy layer were not removed as they frequently gave rise to spontaneous clumping. The lymphocytes were precipitated by centrifuging the plasma at 350 g. for ten minutes. The cells were then resuspended in Hank's balanced
salt solution to form a concentration of approximately 30,000 cells per cubic millimeter. Usually red cell contamination was slight, being less than five percent of lymphocytes. To make comparisons in titer and as a check on the accuracy of the results, separate red cell agglutination tests were repeated at the same time and used at the same concentration as the lymphocytes.

Fifteen reagents were used to test the cells. Of these, four were specific for the A system, seven for the B system, two for the C system and one each for the D and L systems. The number of antigens tested for these five systems was 2, 4, 1, 1 and 1, respectively. Fourfold dilutions of the reagents were made with Hank's solution. The lymphoagglutination and hemagglutination tests were made in small tubes using 0.05 ml. of cell suspension and 0.2 ml. of reagent. The tubes were incubated at room temperature for 90 minutes. Degree of agglutination was determined by examining a drop of the mixture with a light microscope.

 Attempts to Produce Lymphoagglutinins

The main objective of Experiment 6 was to produce agglutinins for lymphocytes which would be distinguishable from red cell agglutinins. In order to confer tolerance to the erythrocyte antigens, day-old chicks were injected with 0.5 ml. of 30 percent red blood cells from four adult donors. Red
cells were obtained by filtering the lower layer of centrifuged blood three times through glass wool, following a method described by Billingham et al. (1956). No leukocytes were found in any of several stained as well as unstained samples examined with a microscope. This did not insure that the preparation was entirely leukocyte free since the number of cells sampled was necessarily a small fraction of the number actually injected. Nevertheless, the method was considered satisfactory to obtain essentially pure red cells.

Twenty-three chicks were injected with the red cells. These chicks were known to lack a specific red cell antigen of the B system (designated B₃) which the donors had. The 23 test chicks and eight control chicks, not injected at hatching, later received two 8 x 10 mm. full-thickness skin grafts from the red cell donors or closely related birds having the B₃ red cell antigen. Further attempts at immunization were carried out with either two or three subcutaneous injections of peripheral leukocytes and spleen cells from the same or other birds having the B₃ antigen. Approximately 5 x 10⁸ leukocytes were administered per injection. Eleven test chicks and four control chicks were first injected with leukocytes and spleen cells at 17 days and grafted at 24 days after hatching; the remainder were grafted at 17 days and injected with leukocytes and spleen cells for the first time at 36 days after hatching. Injections were made at two-week intervals. In order to have
a control on the skin grafting results, 19 test chicks and all control chicks received an additional skin graft from a B-locus compatible donor at the same time the other grafts were made. Serum from each chick was tested for lymphoagglutinin and hemagglutinin titer two or three weeks after the last injection. These tests were carried out in the manner previously described.
RESULTS

Experiment 5

Remarkably clear-cut reactions were obtained in testing lymphocytes with specific iso-immune hemagglutinins. After gaining experience with the lymphocyte agglutination technique, consistent results were easily obtained.

Six of the 15 reagents used in this study did not agglutinate lymphocytes of birds whose red cells gave positive agglutination reactions. These reagents were considered specific for three independent blood group systems. Four of these were A system\(^1\) reagents used to test A\(_1\) and A\(_2\) red cell antigens. Similarly each of the D and L system reagents agglutinated only erythrocytes.

The possibility that the lymphocytes may contain A, D and L antigens in a state that excludes agglutination was investigated by absorption tests with lymphocytes. Unabsorbed reagents and reagents absorbed with an equivalent number of red cells served as controls. Confirmation of red cell specificity for the A, D and L antigens was obtained by showing that lymphocytes failed to remove antibodies of these three systems.

In contrast to the above findings, the B and C system

\(^{1}\)The A reagents possibly contained both A and E antibodies since the A and E blood group loci are believed to be closely linked. Reaction patterns obtained with A and E reference reagents were identical for Line G birds.
reagents agglutinated both lymphocytes and red cells. Tests for four B system antigens indicated their presence in lymphocytes as well as red cells. Two reagents specific for the C1 antigen gave similar results. Titrations with the B and C reagents revealed no differences in titer between the two types of cells.

Experiment 6

All attempts to produce iso-antibodies that would specifically agglutinate lymphocytes but not red cells were unsuccessful. Injection of red cells into day-old chicks markedly inhibited their capacity to produce agglutinins for both erythrocytes and lymphocytes. Detectable antibodies were produced by only four of the 23 test chicks, were demonstrable only with undiluted serum, agglutinated both lymphocytes and red cells and were specific for the B3 antigen. All eight control birds, not injected at hatching, displayed B3 agglutinins of higher titer (dilutions of 1:4 to 1:64).

Although the injection of red cells inhibited the production of humoral agglutinins, tolerance was not induced to the skin grafts. All grafts from birds having the B3 antigens were rejected within six to ten days after grafting. At the same time, B compatible grafts were accepted by all but two of 27 birds. Median survival time for the B incompatible grafts among test chicks grafted at 17 days post hatching was
8.8 ± 0.5 days and 7.3 ± 0.4 days for the chicks grafted at 24 days post hatching.

The fact that the MST's for grafts in this experiment were a day or two longer than those found for B incompatible grafts in the earlier experiments suggests that the injection of red cells at hatching influenced graft survival time. Some of the uninjected control birds appeared to reject their grafts earlier, but a statistical comparison would not be too valuable because the number of control birds in each group was small. However, the difference in MST's is more likely due to the use of adult birds as graft donors in this experiment. As previously noted, Billingham et al. (1956) reported that skin grafts from adult birds tend to survive a day or two longer than skin grafts from young chicks.

Thus, the results of Experiment 6 indicate that red cells injected at hatching do not induce any measurable degree of tolerance to subsequent skin grafts from the red cell donor. This finding is in agreement with the observations of Billingham et al. (1956) and in opposition to the results of Cannon et al. (1958). The fact that homograft tolerance was not obtained supports the contention that the red cells injected at hatching were free from leukocytes.

The results of Experiments 5 and 6 have been reported by Schierman and Nordskog (1962a).
PART III. INFLUENCE OF THE B LOCUS GENOTYPE ON
THE GRAFT-VERSUS-HOST REACTION
MATERIALS AND METHODS

Experiment 7 was designed to determine the influence of the B blood group locus on production of chorioallantoic membrane (CAM) lesions. Peripheral blood leukocytes from mature Line G birds were deposited on the CAM's of 14-day Line G embryos. Donor fowls were bled from a wing vein into sterile heparinized Hank's solution. The blood was centrifuged at slow speed and the buffy layer removed and resuspended in Hank's solution to give a concentration of approximately \(3.5 \times 10^4\) leukocytes per cu. mm. Although large numbers of red cells were usually present, they were of no particular disadvantage in this case. Each embryo was inoculated with 0.1 ml. of the cell suspension.

Preparation of the embryos for inoculation consisted of first candling and marking the side of the egg showing full development of the CAM. A 5 mm. square area of shell was removed from the center of the egg at a point 90° (clockwise, with the large end of the egg away from the observer) from the mark. Cutting of the shell was done with a small three-sided file with care taken to avoid damaging the shell membrane. An opening into the natural air cell was made with a sharp needle guarded 5 mm. from the end with a rubber stopper. Prior to opening the shell, the areas were painted with tincture of iodine. The inoculation procedure involved splitting the shell.
membrane with a needle point through a drop of sterile saline and applying a small amount of suction at the opening in the natural air cell. As soon as the artificial air space began to form, the egg was rotated slowly until the space had moved to a position under the originally marked area. The openings were then sealed with paraffin wax. After all the eggs to be inoculated had been prepared in this way, an opening through the shell over the artificial air space was made by a sharp jab with a triangular cutting edge needle. The inoculum was delivered with a finely calibrated one ml. size syringe using a 27-gauge needle from which the point had been removed. The eggs were again sealed and were incubated in the horizontal position at 38° C. until examined four days later.

The CAM's were removed for examination by inserting a scissors through the square hole in the side and cutting shell, shell membrane and CAM around the horizontal perimeter of the egg. The CAM was then peeled away from the shell membrane, washed in formol-saline and placed in a glass dish over a black background for observation. The lesions which were produced on the CAM were counted twice, and an average of the two counts was used in the analysis. For an additional measurement, the spleens of 20 embryos were weighed.

Three separate matings were made in order to produce embryos of known B-locus genotypes. Pedigree records were kept on all embryos. Embryos and adult leukocyte donors had
blood group genotypes designated B^1/B^1, B^2/B^2 and B^1/b^2 so that nine donor-host combinations were used. Seventy-seven embryos from 13 hens were tested with leukocytes from eight donors. From seven to 11 embryos were represented in each donor-host genotype combination.

In Experiment 8 host embryos had the same B genotypes as in the previous experiment. However, two adult B^3/B^3 birds were now used as the leukocyte donors. A total of 220 membranes was examined in five replicates. The number of embryos for each genotype were: 70 B^1/B^1, 45 B^1/B^2 and 105 B^1/b^2. Between replicates 1 and 2, males were shifted in the breeding pens so that B^1/B^1 and B^1/B^2 embryos were produced by the same hens but in different replicates. Sufficient time was allowed between matings to insure that the Replicate 2 embryos were sired by the new males.
RESULTS

Experiment 7

Four days after inoculation with adult fowl leukocytes, the membranes were removed and observed for the presence of lesions. The average number of CAM lesions or foci for each donor-host combination are presented in Table 8.

Table 8. Average number of CAM foci produced from nine donor-host genotype combinations\(^a\)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>B1/B1</td>
<td></td>
<td>0</td>
<td>32.3</td>
<td>40.3</td>
</tr>
<tr>
<td>B2/B2</td>
<td></td>
<td>95.7</td>
<td>0</td>
<td>79.0</td>
</tr>
<tr>
<td>B1/B2</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) These foci were observed as raised and opaque lesions. Some membranes contained very small and translucent lesions which are not included in these counts.

The results indicate that production of CAM lesions is due principally to \(B\) locus incompatibility, i.e., when the embryo has a \(B\) allele (thus antigen) not also represented in the leukocyte donor. Membranes from the four \(B\)-incompatible combinations yielded an average of 65 raised and opaque lesions. Counts on individual membranes ranged from 11 to 174. A membrane showing a large number of these primary lesions is pre-
sented in Figure 4.

No primary lesions were observed when donor and host were B-compatible. Upon close inspection, however, several membranes were found to contain a few small and relatively translucent lesions. These were presumed to be due to "weak" histocompatibility antigens controlled by genes at other loci. Distinction between the two types of lesions was generally possible, and only the clearly visible yellow or white nodular foci were included in the counts.

Further evidence for B locus control of the graft-versus-host reaction was shown by splenic enlargement in the B-incompatible embryos. Spleen weights from ten B compatible embryos ranged from 13.7 to 22.3 mgm. with an average weight of 17.6 mgm. For ten B incompatible embryos, the weights ranged from 22.7 to 49.0 mgm. and had an average weight of 33.8 mgm. Relative spleen size of the two classes is represented in Figure 5.

Experiment 8

All membranes exhibited the nodular foci previously described when B<sup>3</sup>/B<sup>3</sup> leukocyte donors were tested with B<sup>1</sup>/B<sup>1</sup>, B<sup>2</sup>/B<sup>2</sup> and B<sup>1</sup>/B<sup>2</sup> embryos. However, a number of relatively low foci counts were observed in two of the five replicates. In some cases, wide ranges in CAM foci counts existed between full sib embryos treated with leukocytes from the same donor.
Figure 4. Chorioallantoic membrane from 18-day Line G embryo showing nodular lesions resulting from the graft-versus-host reaction

Figure 5. Spleens from 18-day Line G embryos. The upper row of spleens was obtained from B incompatible embryos; the lower row from B compatible embryos
The average number of foci for \( B^{1}/B^{1}, B^{2}/B^{2} \) and \( B^{1}/B^{2} \) CAM's was 69, 66, and 90, respectively. An analysis of variance based on average foci counts obtained from each donor and host-genotype combination is presented in Table 9.

Table 9. Analysis of variance used in testing differences in CAM foci counts

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>Mean square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicates</td>
<td>4</td>
<td>6323.62**</td>
</tr>
<tr>
<td>Genotype of host</td>
<td>2</td>
<td>1669.71*</td>
</tr>
<tr>
<td>Donor</td>
<td>1</td>
<td>33.07</td>
</tr>
<tr>
<td>D x G</td>
<td>2</td>
<td>1097.19</td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
<td>458.93</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td></td>
</tr>
</tbody>
</table>

*Significant at .05 level
**Significant at .01 level

Differences in foci counts between replicates were highly significant (\( P < .01 \)). This is considered to be due to inconsistency in the number of leukocytes inoculated in different replicates. The two donors showed a considerable difference in leukocyte count. Although attempts were made to adjust for this difference, the number of leukocytes per donor
was not uniform for all replicates. That the number of leukocytes inoculated is linearly related to the average number of CAM lesions produced was shown by Burnet and Boyer (1961).

Differences due to the B genotype of the host embryos were significant at the .05 probability level. Comparisons of the average foci counts using Tukey's test\(^1\) revealed that the difference between \(B^2/B^2\) and \(B^1/B^2\) was significant at the .05 level while the difference between \(B^1/B^1\) and \(B^1/B^2\) failed to reach this level of significance.

An additional comparison of foci counts from 53 embryos produced by five hens was made. Data on these five hens was analyzed separately because they were considered to have a sufficient number of host embryos in the two replicates between which the sires had been changed. Other hens were not represented in both replicates. The reason for changing the males in the breeding pens was to test whether differences in foci counts could be reversed by changing the B genotype of embryos from the same hens.

The average number of CAM foci for embryos in this comparison is presented in Table 10. Mean foci counts for embryos of Dam Group a (two hens) and Dam Group b (three hens) appeared to be dependent upon the B genotype being produced.

---

Table 10. Average number of CAM foci for $B^1/B^1$ and $B^1/B^2$ host embryos of the same dams with $B^3/B^3$ leukocyte donors

<table>
<thead>
<tr>
<th>Host genotype $B^1/B^1$</th>
<th>Dam group</th>
<th>Donor 1</th>
<th>Donor 2</th>
<th>Host genotype $B^1/B^2$</th>
<th>Dam group</th>
<th>Donor 1</th>
<th>Donor 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rep. 1</td>
<td>a</td>
<td>65</td>
<td>113</td>
<td>b</td>
<td>149</td>
<td>175</td>
<td></td>
</tr>
<tr>
<td>Rep. 2</td>
<td>b</td>
<td>74</td>
<td>68</td>
<td>a</td>
<td>147</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Within each replicate the $B^1/B^2$ membranes yielded a considerably higher number of foci than the $B^1/B^1$ membranes. An analysis of variance using average foci counts on the CAM of embryos produced by the five hens is presented in Table 11.

Table 11. Analysis of variance used in testing differences in CAM foci counts of embryos produced by five hens

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>Mean square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor</td>
<td>1</td>
<td>67.3</td>
</tr>
<tr>
<td>Genotype/Donor 1</td>
<td>1</td>
<td>5806.4**</td>
</tr>
<tr>
<td>Dam Group/Donor 1</td>
<td>1</td>
<td>64.0</td>
</tr>
<tr>
<td>Gen. x D.G./Donor 1</td>
<td>1</td>
<td>33.6</td>
</tr>
<tr>
<td>Genotype/Donor 2</td>
<td>1</td>
<td>2265.7**</td>
</tr>
<tr>
<td>Dam Group/Donor 2</td>
<td>1</td>
<td>171.6</td>
</tr>
<tr>
<td>Gen. x D.G./Donor 2</td>
<td>1</td>
<td>3612.0**</td>
</tr>
<tr>
<td>Error</td>
<td>11</td>
<td>40.0</td>
</tr>
</tbody>
</table>

**Significant at .01 level
Differences due to host genotype, within each donor, were significant at the .01 probability level. This indicates that the number of CAM lesions produced was influenced by the B genotype of the host embryo and was not due to an effect of the host's sire or dam.

The effect of host genotype x dam group interaction within Donor 2 was also significant at the .01 probability level. This effect is confounded with replicates. The statistical significance of this effect is therefore considered to be due to non-uniformity between replicates in the number of Donor 2 leukocytes inoculated.

The results obtained in Experiments 7 and 8 have been reported by Schierman and Nordskog (1962b).
DISCUSSION

Blood Type and Histocompatibility

The most significant finding in this investigation was the effect of the B blood group locus on skin transplants between chickens. The results of four skin grafting experiments provided ample evidence that the B locus controls "strong" histocompatibility antigens in a manner analogous to the H-2 locus in mice. Yet the possibility remains that antigens responsible for graft rejection are controlled by separate genes, closely linked to the B locus. Additional transplantation studies using larger numbers coupled with serological studies on the distribution of the B antigens in various tissues would seem desirable. Of interest in this regard are the recent results reported by Basch and Stetson (1962) indicating that the H-2 iso-antigens of the mouse erythrocytes are identical to iso-antigens involved in homograft immunity.

Medawar (1959) favored the view that transplantation antigens and red cell antigens are different substances with the same genetic and immunological specificity and have the same antigenic determinants. He believed that the response elicited, whether cellular or humoral, may depend upon subsidiary molecular attachments. Results from the present experiments suggest that transplantation studies with chickens may provide some of the answers to these and other problems of fundamental
importance in the field of tissue transplantation.

The persistence of nearly all $B$ compatible grafts when the $B$ incompatible grafts were rejected indicates that no allelic segregation occurred in Line G at other "strong" histocompatibility loci. Further transplantation studies with $F_2$ or backcross progeny produced by crosses of unrelated inbred lines of chickens would help in determining the existence of other highly antigenic systems.

The $A$, $D$, and $L$ blood group loci did not influence skin graft acceptance or rejection; hence, these systems appear not to be associated with histocompatibility. Yet these systems may possibly determine relatively weak transplantation antigens. If so, a considerably larger number of birds would require testing in order to show this. Studies with other animals have indicated occasional prolonged or even lifetime tolerance for homografts containing relatively weak foreign histocompatibility antigens.

Although 14-day-old chicks are generally considered to be immunologically mature, this study raises some doubts as to the validity of this assumption. In some cases, grafts between birds with rather low coefficients of relationship still persisted after 150 days post-grafting. It would seem that these grafts would contain at least some antigens foreign to the host. Determination of the survival time of grafts exchanged between older birds of known relationship would be desirable. In this connection, Goulian et al. (1962) and Vrubel
and Vrubelova (1962) held that transplantation antigens are probably released for a relatively short time immediately after grafting depending on the healing process of the graft. Thus, if the birds in the present experiment had not reached complete immunological maturity at grafting time, some graft tolerance may have occurred. Therefore, other foreign histocompatibility antigens, less antigenic than the \( B \) antigens, may have been present in those grafts retained over a long period.

The present study raises at least two additional problems in which further research would be desirable. The first is the influence of the \( B \) blood group locus on histocompatibility in other lines and breeds of chickens. The second is to investigate possible linkage relationships between the \( B \) locus and other chromosome markers. This would help in defining the genetic nature of the \( B \) locus. Gilmour (1962) has suggested the possibility that the \( B \) antigens are controlled by a series of linked loci.

Chickens have some rather distinct advantages for tissue transplantation work. They are suitable in size, prolific, and have convenient embryonic characteristics. The development of co-isogenic lines of chickens would provide useful material for further tissue transplantation studies.

**Erythrocyte and Leukocyte Antigens**

Results of the lymphoagglutination tests clearly indicate that chicken erythrocytes have antigens not present in lympho-
cytes. Further experimentation is necessary before the significance of this finding can be evaluated. Why antigens of the A, D and L systems are erythrocyte specific, while antigens of the B and C systems are common to both lymphocytes and erythrocytes would be a question of fundamental importance. At the present time, practically nothing is known as to the physiological purpose of the antigens present in various tissues and organs of the animal body. Indications that antigens may play an essential role in growth and development were reported by Edds (1958) although work along this line has been inconclusive and difficult to evaluate. Studies with animals of known genotypes, with respect to antigenic make-up, very likely will prove valuable for further research in this area. In this connection, a study of the distribution of blood group antigens in other tissues and organs would seem desirable. Chickens may prove to be the most fruitful choice of species for such studies since several chicken blood group systems have already been identified.

Although only a small number of antigens were tested for erythrocyte and lymphocyte specificity in this study, other antigens belonging to the A, B, C, D, and L systems should have the same distribution among erythrocytes and lymphocytes. Amos (1962) believed that antigens belonging to each system should have a tissue distribution characteristic of that system.
The fact that \( C \) antigens, like \( B \) antigens, are present on lymphocytes, suggests that the \( C \) locus may also be associated with histocompatibility. Unfortunately, birds used in the skin grafting experiments had not been typed with \( C \) system reagents. Results from iso-immunizations have demonstrated that the \( C \) red cell antigens are less antigenic than the \( B \) antigens. Thus, the \( C \) locus may control "weak" transplantation antigens.

The failure to produce iso-antibodies specific for lymphocytes could have two explanations. The first is that the \( B \) antigens of the erythrocytes may contain all determinant groups in the \( B \) antigens of the lymphocytes. Thus, the injection of day-old chicks with erythrocytes containing \( B_3 \) antigens should induce tolerance to lymphocytes containing \( B_3 \) antigens. The second explanation is that the population does not have alleles segregating at other loci that determine antigens peculiar to lymphocytes. These inferences are subject to the possible limitations of the technique employed.

Graft-Versus-Host Reaction

The influence of the \( B \) genotype on the production of CAM lesions is clearly evident from this study. The macroscopic appearance of the lesions was similar to that described by Burnet and Boyer (1961). That these lesions or foci were not
found when donor and host were B compatible is evidence that the B locus is a major controller of the graft-versus-host phenomenon in chickens. The production of splenomegaly in the B incompatible embryos provides additional support for this contention.

The findings obtained in Experiment 8 are relevant to the postulations of Burnet and Burnet (1961). These workers considered each CAM lesion to be initiated by a single donor cell. They found one lesion per 20,000 to 40,000 donor leukocytes, i.e., approximately one lesion per $10^4$ lymphocytes and favored the view that only "preadapted" cells are able to react with the foreign antigens of the host. This hypothesis suggests that host embryos with two foreign antigens should have more CAM lesions than embryos with only one foreign antigen. The difference in the number of lesions produced should depend on the number of unlike antigenic determinants in the host antigens as well as the number of donor cells preadapted to each antigenic determinant.

In the present study, where $B^3/B^3$ donors were used, the $B^1/B^1$ and $B^2/B^2$ host embryos have one foreign B antigen while the $B^1/B^2$ host embryos have two. Since the average number of CAM lesions was higher for $B^1/B^2$ embryos, the results appear consistent with Burnet's "elective" theory of immunity. On the other hand, the higher lesion counts for $B^1/B^2$ embryos may be due to a qualitative effect of the host antigens. Thus,
if the antigens of the $B^1/B^2$ embryos, being more foreign, stimulate a larger number of analogous donor cells, the differences in lesion counts could be explained on the basis of an "instructive" or template theory of immunity.

Burnet and Burnet (1961) found at least as many foci using embryos with only one foreign antigen as compared with using random embryos. Since the random embryos are expected to have more foreign antigenic determinants, this was considered evidence against an "elective" theory of immunity.

In a personal communication, Burnet$^1$ has offered his interpretation why a defined one versus two antigen difference would show what is expected on the basis of an elective hypothesis while one versus a much larger random number of antigens would not. He stated: "The production of a lesion on the CAM requires (1) a close biochemical similarity -- chicken cells will not work on a duck CAM -- the greater the difference, particularly as regards genes not concerned with histocompatibility, the fewer the number of competent cells that can 'initiate' lesions; (2) 'recognizable' antigenic differences -- this will work in the opposite direction and conceivably the interaction between the two factors might account for the differences."

Burnet's interpretation (1) above may explain the rather

$^1$Burnet, F. M. Melbourne, Austalia. Results with the CAM reaction. Private communication. 1962.
wide differences in CAM lesion counts between full sib embryos in the present study. Since the birds assigned to this study were not highly inbred, genetic differences between donors and hosts at loci other than the B locus may have affected the number of lesions produced. In this regard Payne and Jaffe (1962) presented experimental evidence that splenomegaly is less severe when the donor and host are from less related avian species.
SUMMARY

Four experiments were conducted to determine the relationship between blood group genotypes and skin graft acceptance or rejection in a White Leghorn line (Line G). Two hundred and nine full thickness skin grafts were exchanged among 58 chicks at 16 to 19 days post-hatching. Prior to grafting, the birds were blood typed with iso-immune reagents specific for the A, B, D and L blood group systems. Grafts were exchanged between full sibs, half sibs and less closely related members of the same line. The coefficients of relationship (Wright, 1922) between donor and host ranged from 0.57 to 0.85. Inbreeding coefficients ranged from 0.40 to 0.54. Grafts were scored for degree of vigor beginning on the fifth or sixth postoperative day. A macroscopic scoring system provided a reasonably accurate determination of graft rejection time.

Of the 209 skin grafts 72 were exchanged between B incompatible birds, i.e., where the donor had B red cell antigens which the host lacked. These 72 grafts exhibited an edematous swelling and discoloration beginning usually on the sixth post-operative day. All B incompatible grafts were rejected by the tenth day after grafting. Only two of the remaining 137 B compatible grafts were rejected by the tenth day. Thus, the results clearly indicate that the B locus is
a major histocompatibility locus. Median survival time for the 72 B incompatible grafts was 6.6 ± 0.2 days.

Grafts exchanged between B compatible birds were often retained for the duration of the experiment (150 to 180 days post-grafting). Three of four birds observed at 510 days post-grafting had healthy grafts identifiable by feather growth. However, many B compatible grafts underwent a chronic mild reaction beginning about the 14th post-operative day making the survival end-point difficult to determine. The frequency of this type of graft rejection was greater among donor-host combinations having low coefficients of relationship.

The number of donor-host incompatibilities for the A, D and L blood group loci were 38, 21, and 15, respectively. No relationship between these three systems and histocompatibility was demonstrated.

A second objective of the study was to determine the selectivity of iso-antibodies for erythrocyte and leukocyte antigens in chickens. Agglutination tests with lymphocytes and erythrocytes of 18 adult Line G birds were made with 15 iso-immune reagents. Reagents of the A, D and L blood group systems discriminated between the two types of cells by agglutinating the erythrocytes only. Confirmation of red cell specificity for antigens of these three systems was obtained by absorption tests whereby lymphocytes were found not to re-
move A, D and L antibodies. In contrast, B and C system reagents agglutinated both lymphocytes and red cells. No titer differences were observed between the two types of cells.

Attempts to produce iso-antibodies specific for lymphocytes were unsuccessful. The injection of day-old chicks with erythrocytes inhibited the production of agglutinins for lymphocytes as well as erythrocytes but did not appear to affect homograft survival time.

As a third objective the influence of the B blood group locus on a graft-versus-host reaction was investigated. Peripheral blood leukocytes from mature Line G birds were deposited on the chorioallantoic membrane (CAM) of 14-day Line G embryos. After four days, counts were made of the number of CAM lesions produced. The lesions, which appear as raised yellow or white foci, are believed to arise from the interaction of donor lymphocytes with foreign antigens in the host tissue.

The results indicate that production of CAM lesions is due principally to B locus incompatibility, i.e., when the embryo has a B allele (thus antigen) not also represented in the leukocyte donor. Four B-incompatible combinations yielded an average of 65 nodular lesions. No lesions of this nature were observed when donor and host were B-compatible. Further evidence for B locus control of the graft-versus-host reaction was shown by marked splenic enlargement in the B-incompatible
When B^3/B^3 leukocyte donors were tested with B^1/B^1, B^2/B^2 and B^1/B^2 embryos, the average lesion counts were 69, 66, and 90, respectively. Differences due to host genotype were significant at the .05 level of probability. Following an "elective" theory of antibody formation, the greater number of lesions on the B^1/B^2 membranes are due to the leukocyte inoculum containing immunologically competent cells with preformed patterns, some corresponding to the B^1 and others to the B^2 antigenic determinants of the chick embryo. An "instructive" interpretation for the differences in lesion counts makes it necessary to assume that a qualitative difference in the antigens of the B^1/B^2 embryos causes a larger number of analogous donor cells to react.
CONCLUSIONS

The following conclusions seem warranted from the results obtained in this study:

1. The B blood group locus in chickens is a major histocompatibility locus or is closely linked to a histocompatibility locus.

2. The A, D, and L blood group loci are probably not associated with histocompatibility.

3. The A, D, and L blood group antigens are present in erythrocytes but not in lymphocytes.

4. Both erythrocytes and lymphocytes contain B and C blood group antigens.

5. Day-old chicks, injected with B incompatible erythrocytes, become inhibited in their power to produce antibodies for both lymphocytes and erythrocytes containing the foreign B antigen.

6. Day-old chicks, injected with B incompatible erythrocytes, do not become tolerant to subsequent skin grafts from the red cell donor or other birds with the foreign B antigen.

7. The B blood group locus is of primary importance in the graft-versus-host reaction.

8. A more pronounced graft-versus-host response seems to be elicited when host and donor differ by two B antigens compared with one B antigen.
GLOSSARY

**Autograft**—Tissue transplanted to a new site in the same individual.

**Co-isogenic**—A term used to describe inbred lines which are genetically identical except with respect to a single locus.

**Graft-versus-host reaction**—A syndrome occurring when inoculated immunologically competent donor cells react against "foreign" host antigens. This is also referred to as the graft-against-host reaction or Simonsen phenomenon.

**Heterograft**—A transplant between animals of different species.

**Histocompatibility antigens**—Antigens on or in the tissues of an animal which cause transplantation immunity. These are also referred to as T-antigens. In contrast, H-antigens more readily provoke formation of humoral antibodies. In the present study, antigens have been designated by a numerical subscript with the locus letter (e.g. B\(^2\)) and the corresponding allele by a superscript (e.g. B\(^2\)).

**Homograft**—A tissue exchange between animals of the same species except where donor and host are isogenic.

**Immunologically competent cells**—Adult reticulo-endothelial cells, such as spleen and lymph node cells, which are capable of reacting against foreign antigens in an immune or hypersensitive way. Peripheral blood lymphocytes
are considered to be immunologically competent.

**Incompatibility**—A condition in which the graft tissue contains one or more antigens not possessed by the host (except that the opposite condition exists in the case of the graft-versus-host reaction).

**Induced or acquired immune tolerance**—A state of reduced or absent immune reactivity to a particular antigen which would ordinarily evoke a specific immune reaction. This is brought about by previous exposure of an animal to the same, or a closely related antigen, usually at a stage of development at which the animal is incapable of reacting in an immunologically mature way.

**Iso-antigens**—Antigens peculiar to individual members of a species. Antibodies produced by the introduction of antigens into individuals of the same species who do not have the antigen are called **iso-antibodies**. The procedure is referred to as **iso-immunization**.

**Isograft**—A transplant between individuals isogenic with each other. In the case of animals, the donor and host may be either identical twins or members of a very highly inbred strain.

**Median survival time (MST)**—The time at which 50 percent of the grafts have undergone complete epithelial breakdown.
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