PRODUCTION OF IMMUNOLOGICAL TOLERANCE AS INFLUENCED BY THE AGE OF THE DONOR AND RECIPIENT IN CHICKENS

by

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INTRODUCTION

The state of acquired immunological tolerance to foreign histocompatibility antigens leading to prolonged or permanent retention of foreign tissue grafts may become of great practical importance in tissue transplantation. Acquired immunological tolerance (or tolerance) to foreign tissues may be defined as a specific central inhibition of the faculty of immunological response induced by cellular antigens (Gowland, 1965).

The tolerant state can usually be produced experimentally by injecting embryos or neonates with cells from genetically foreign donors. Grafts of skin and other tissue are subsequently accepted from the original donor when the animal is immunologically mature. Tolerance can also be developed in adults either after high doses of ionizing radiation or in normal adults exposed to high concentration of antigen.

Immunity and tolerance are alternative reactions to the same stimulus and the outcome depends on the time of administration and dosage of antigen. Tolerance to foreign antigen may be regarded as identical with the mechanism by which an organism becomes tolerant of its own body constituents.

The two chief characteristics of the tolerant state according to Jaffe (1966) are:

(1) Immunological unresponsiveness is restricted to the antigens with which the recipient initially comes into contact; the response to other antigens is normal.

(2) Tolerance is maintained only as long as the antigen is present.

Highly inbred animals are desirable for quantitative tolerance experiments. After mice, chickens are probably the best experimental animals for this purpose because of the availability of inbred lines, development of some knowledge about their histocompatibility antigens and easy accessibility of the chicken embryo for intravenous inoculation. Moreover, their immunological responses are as lively and versatile as those of mammals.

The chief objective of this study was to induce immunological tolerance in chickens by transplanting whole blood from hybrid donors into parental line recipients. Donors and recipients used were of different developmental stages from 14-day-old embryos to the adult. Ease of induction, duration and completeness of tolerance as related to developmental stage of donor and recipient and to genetic disparity were tested by the allograft and graft-against-host reactions.

LITERATURE REVIEW

Naturally Occurring Tolerance

Owen (1945) first reported the occurrence of erythrocyte chimerism in dizygotic twin cattle. He explained it as an interchange of immature hemopoietic cells through vascular anastomoses between the co-twins. Burnet and Fenner (1949) interpreted this phenomenon by advancing the hypothesis that the immunological system of the organism becomes non-reactive to foreign antigens with which it comes into contact during embryonic life. Anderson et al. (1951) and Billingham et al. (1952) have shown further evidence in dizygotic twin cattle, of the taking of

skin homografts exchanged between the partners, which they called tolerance.

Billingham et al. (1956), working with guinea pigs, reported that tolerance towards the antigenic spectrum of the mother could be induced under normal conditions by the leakage of the placental barrier between the mother and foetus, which they called maternally induced tolerance. Owen et al. (1959) and Ward et al. (1957) obtained indirect evidence for the possibility of spontaneous transplacental induction of tolerance only to the maternal Rh antigen. Lengarova (1957) and Nathan et al. (1960) reported induction of specific tolerance in rabbits to homografts of maternal origin in the progeny of pregnant females treated with various agents which are suppoed to increase the permeability of the placenta. Humphrey and Turk (1961) demonstrated experimentally the induction of transplacental tolerance to proteins in guinea pigs.

Variables in Tolerance Induction

Age of Recipient and Immunological Competence. Billingham et al. (1953) demonstrated acquired immunological tolerance in mice and chickens by exposing the embryos to cellular antigens. Hasek (1953), as reviewed by him (1956), first reported the accomplishment of experimental embryonic parabiosis in bird embryos, by the fusion of chorio-allantoic membranes of two embryos resulting in tolerance due to exchange of embryonic blood between the partners by vascular anastomoses. Lazzarini (1960) employed this technique for the induction of immunological tolerance among multiple bird parabionts.

Woodruff and Simpson (1955) induced tolerance in newborn rats by a single intravenous injection with homologous cells and reported survival of the grafts up to 84 days postgrafting. Billingham and Brent (1957) observed 95% tolerant and 80% highly tolerant mice when CBA strain spleen cells were injected intravenously into A strain newborn mice.

Martinovic and Pavlovic (1958) obtained success in conferring immunological tolerance by direct transplantation of the forebrain region in bird embryos before the establishment of circulation.

Medawar and Woodruff (1958) could induce tolerance in newborn rats by means of skin graft transfers. They observed the survival of the grafts for more than 50 days.

Puza and Gombos (1958) found injection of cells postpartum in dogs unsatisfactory in inducing tolerance. However by using a drastic technique of virtual exsanguination and transfusion of fresh blood in puppies they produced survival of skin grafts up to 90 days.

Billingham and Brent (1959) and Brent and Gowland (1962) produced tolerance in A-strain recipient mice of various ages by intravenous injection of (CBA x A)F_1 spleen cells. They found that when the dosage of antigen was constant, the percentage of highly tolerant mice declined rapidly with age between the range of ages 12 hours to 8 days.

Brent and Gowland (1961) observed that in the 13-day-old adult mouse repeated intravenous injections of antigen were required to induce tolerance and that tolerance in the young adult once induced had all the attributes of tolerance induced in the newborn.

Cannon and Longmire (1952) demonstrated that a small proportion of skin allografts from newly hatched donors transplanted orthotopically to newly hatched chicks induced a high degree of tolerance in respect to themselves. Billingham, Brent and Medawar (1956) demonstrated that two-week-old chicks were able to reject skin allografts.

Hasek (1956) reported induction of heterologous tolerance and interspecific blood chimeras after embryonic parabiosis between ducks and hens, hens and guinea-fowl, hens and pheasants and hens and turkeys.

Haskova (1957) found ducks to be very highly tolerance-responsive at hatching, since all the allografts transplanted to newly hatched ducklings induced tolerance in respect to themselves and survived for at least 80 days.

Howard and Michie (1962) and Brent and Gowland (1963) have shown that the neonatal mouse possesses a low degree of immunological competence. However they found that either tolerance or immunity could be induced at will in the newborn mouse, the operative variable being the antigen dosage employed. Solomon (1964) observed that in chicken embryos the maximum degree of tolerance could be induced with the smallest dose of cells injected at 13 days of incubation.

Cell Type, Route, Dosage, Thymectomy and Bursectomy. Billingham et al. (1953) found inoculation of mice and chicken embryos with viable cells to be more effective than skin grafting in inducing tolerance. They observed that of 7 chickens grafted with skin from the original donor after 14 days posthatching, 3 grafts survived to 50 days and 2 for more than 125 days. Prolonged survival of the grafts in 3 out of 5 mice was also observed.

Billingham and Brent (1956) reported that if newly hatched chicks were grafted with adult skin and at the same time injected intravenously with 0.5 ml of whole blood from the same adult donor, a high degree of tolerance could be induced in the majority of the birds due to synergetic effect.

Nakic and Silobricic (1958), Rubin (1959), Martinez et al. (1960) and Jensen and Simonsen (1962) observed tolerance in adult mice following parabiosis.

Albert et al. (1959), Fowler et al. (1960), and Maris (1961) reported induction of tolerance in human newborns treated with blood transfusion for hemolytic disease.

Billingham and Brent (1959) using CBA strain spleen cells and newborn A strain recipients demonstrated that the intravenous route was more effective than either the intraperitoneal or the subcutaneous routes.

Shapiro et al. (1961) and Martinez et al. (1962) could induce tolerance in adult mice by repeated doses of spleen cells injected intravenously.

Brent and Gowland (1961) observed that in the 13-day-old mouse repeated intravenous injections of antigen were required to induce tolerance and that tolerance in the young adult once induced had all the attributes of tolerance induced in the newborn.

Whole body irradiation with X-rays in sublethal doses will markedly suppress the immunological responses of an adult animal. Michie and Woodruff (1962) and Medawar (1963) demonstrated that sublethal X-ray irradiation greatly increased the efficiency of a given dose of antigen

in inducing specific transplantation tolerance in adult recipients.

Billingham and Silvers (1962) working with mice came to the conclusion that the greater the immunological competence of the inoculum the higher the tolerance inducing capacity. They observed that as a tolerance inducing stimulus, lymph node cells were superior to spleen cells, leucocytes, bone marrow cells and thymocytes in that order.

Miller (1962) and Good et al. (1962) reported that animals subjected to neonatal thymectomy showed in later life a reduction in the number of small lymphocytes normally present in the lymph nodes, spleen and blood, which may result in a severe impairment of the ability to reject homografts and heterografts. Dalmasso et al. (1963) and Miller (1963) observed that the restoration of immunological responsiveness of thymectomized animals could be brought about by the grafts of neonatal thymus and injections of spleen and lymph node cells from adult syngeneic donors. However, the experiments conducted by Osaba and Miller (1964) in which millipore chambers containing thymus tissue were implanted into neo-natally thymectomized mice showed that immunological unresponsiveness could be corrected without necessarily restoring the small lymphocyte content of lymphoid tissue to normal. Gowans (1965) reported that the mechanism by which the thymus restores responsiveness is not by increasing the cell numbers but by repairing a defect in individual lymphocytes. Goedblood and Vos (1965) working with thymectomized irradiated mice confirmed the findings of the earlier workers about the role of the thymus. They came to the conclusion that the presence of thymus is essential not only during the development of immunological responsiveness

in newborn mice, but also during the regeneration process in adult mice after lethal irradiation.

Silverstein and Kramer (1965) working with foetal lambs *in utero* came to the conclusion that the conventional circulating antibody did not play an obligatory role in the rejection of solid tissue homografts. They also suggested that the antibody or antibody-like activity responsible for the specificity of immunlogically active lymphoid cells resides not on the surface but rather within the cell.

Glick *et al.* (1956) reported the role of the Bursa of Fabricius in anti-body production. They bursectomized chickens at 2 weeks of age which produced a decreased response when challenged with *Salmonella typhimurium*. Mueller *et al.* (1962) observed that the reduction in antibody response to a variety of antigens was greater in birds bursectomized at one day of age and less pronounced in 5-week-old birds. Cooper *et al.* (1965) using surgical bursectomy followed by X-irradiation observed delayed rejection times as compared with controls receiving only X-rays.

Aspinall *et al.* (1963) reported that in the chicken neonatal thymectomy caused a striking reduction in the number of blood lymphocytes and lymphoid follicles of the spleen and no wasting disease was observed.

Jaffe (1966) while reviewing the role of Bursa and thymus in chickens observed that Bursa and thymus influenced two different populations of cells. The bursa controlled plasma cells and reactions concerned with these such as production of immuno-globulins, whereas the thymus affects the development of cells of the series which were concerned with cell bound antibody responses.

Genetic Variables. Schierman and Nordskog (1961) demonstrated that the B red cell antigen system in chickens was also an important histocompatability system. The conclusion that the B system was a histocompatability system in chickens was confirmed by Craig and McDermid (1963), Gleason and Fanguy (1964) and Crittenden et al. (1964). Shierman and Nordskog (1965) showed evidence that the C blood group locus was also a histocompatibility locus or was closely linked to such a locus.

Eichwald and Silmser (1955) demonstrated that females of many inbred strains of mice rejected isogenic male grafts. Hauschka (1955) and Snell (1956) interpreted this as due to the presence of an antigen associated with the Y chromosome in the male which was absent in the female.

Counce et al. (1956) reported that skin grafts exchanged between normal members of strains of mice differing at weak loci rejected relatively slowly compared with grafts between strains differing at strong loci.

Mariani et al. (1959) and McKhann (1962) showed that in the mature adult mouse, if the genetic barrier between the donor and recipient was weak, transplantation tolerance could be induced by a single large intravenous injection of spleen cells.

Hasek (1961a) working with ducks concluded that the percentage of acquired tolerance depends on individual variability in the immunological maturity of the newly hatched birds and the degree of antigenic differences between donor and recipient.

Hasek (1961b) demonstrated among chicken parabionts individual

differences in the quantitative manifestation of tolerance which might be interpreted as due to genetic differences.

Brent and Gowland (1962) working with mice stated that tolerance induction depended on dosage and strength of antigen. They demonstrated that a larger dose of antigen was required to overcome a strong H-2 locus than the relatively weak H-1 or H-3 genetic barriers. Billingham and Silvers (1962) reported similar results.

Gowland (1965) using the strong H-2 incompatible system could not induce tolerance in 13-day-old mice with one intravenous injection of a comparable number of cells.

Argyris (1965) working with mice observed that neonatal mice from different strains varied in their immunological response to transplantation antigens which he attributed to the genetic disparity between host and donor. His results also suggested that the rate of immunological maturation with respect to transplantation antigens varied among the different strains of mice.

Wilson and Talmage (1965) reported quantitative differences in the induction of tolerance among C57BL/6 and DBA/2 strains of mice. Newborn mice of parental strains were injected with adult F_1 spleen and bone marrow cells. Skin grafts were exchanged between parental strains of mice at the age of 6-8 weeks. It was observed that more F_1 cells are required in C57BL/6 mice to make them tolerant for the skin of DBA/2 mice whereas fewer cells are required in DBA/2 mice to make them tolerant for C57BL/6 skin.

Cross, Split and Partial Tolerance

Cannon et al. (1954) working with chickens observed that skin allografts transplanted to newborn chicks were more often tolerated when the donors were themselves newborn than when they were 2 weeks old. They concluded from this that the skin from the younger donors, being in some way more plastic was better able to adapt itself to its foreign host.

Weber et al. (1954) found that adult chickens carrying a skin allograft transplanted at hatching sometimes reject a second allograft from the original (now adult) donor, although the first allograft continued to survive. From this evidence they suggested that the first allograft had in some way adapted itself.

Woodruff and Simpson (1955) showed that an adult skin homograft transplanted to a rat to which cells of the prospective donor were administered just after birth survived indefinitely while a second graft from the same donor transplanted some months later to the same host was destroyed within a few weeks.

Terasaki et al. (1957) found in 2-day-old chicks, that large grafts of 2-day-old skin survived up to the 14th week in 32% of the cases, whereas none of the large adult skin grafts did survive. They attributed this great difference in survival to the fact that 2-day old skin being more pliable could adapt itself to the host, whereas the adult skin might not be able to do so.

Hasek (1961a) observed a similar phenomena in ducks when a second set skin graft grafted more than 6 months after the first was rejected,

though the first graft had taken permanently.

Hasek and Puza (1962) induced tolerance in adult ducks by exsanuination transfusion of homologous blood in large doses. Tolerance was tested by transplantation of a skin allograft from the blood donor and by survival of 51_{Cr}-labelled erythrocytes in the recipient's blood stream. The administration of a large dose of isoantigenic material induced a state of tolerance to erythrocytes whereas the skin grafts were rejected. They concluded that erythrocyte antigens could be different from "tissue antigens."

Craig (1963) working on genetic influences on completeness of tolerance and tolerance specificity in chickens observed cross-tolerance which he attributed to shared antigens between R and N line chickens.

Graft-Against-Host Immunological Responses

Simonsen (1957) reported in chickens the development of graft-versus-host reactions when adult spleen cells and peripheral blood leukocytes were injected into an embryo or newly-hatched chicken due to their reaction against host tissue. He also observed that if the donor and host differ widely genetically the reaction may take the form of a fatal wasting disease associated with lymphoid atrophy and hemolytic anemia.

Cock and Simonsen (1958), in chickens, and Billingham and Brent (1959), in mice, reported that spleen cells from hybrid donors when injected into parental recipients did not cause runt disease but induced

tolerance. Their results were in agreement with the graft-versus-host concept, as parental antigens were represented in hybrid cells. Burnet (1961) and Jaffe and McDermid (1962) showed in chickens that the graft-versus-host reaction was largely controlled by the B locus only. Simonsen (1957) showed that blood or spleen cells from 11-day-old chicks produced splenomegaly when injected into 18-day-old embryos. Solomon (1961a) found that splenic implants from 6-8 day old chicks could also produce splenomegaly. Using the spleen assay system, Solomon (1963b and 1964) demonstrated that the chick embryo possessed immunologically competent cells after about 15 days of incubation.

Gowans et al. (1963) reported in rats intermediate response of graft-against-host reaction when lymphocytes from partially tolerant parental donors injected into F_1 recipients. They also reported that lymphocytes from one such donor behaved as if they were from a normal animal although its unhealthy skin graft had not yet been shed.

Shierman and Nordskog (1964) reported occurrence of rejections of parental line grafts while hybrid grafts were surviving on tolerant chickens that were test grafted.

Solomon (1963a) obtained evidence that lymphocytes in chicken skin became immunologically competent at two days after hatching. He detected splenomegaly in embryos after grafting the skin of 2-day-old chicken onto the chorioallantoic membrane (CAM). The appearance of immunological competence of chick skin soon after hatching was further reported by Solomon (1964) performing an experiment originally suggested by Billingham and Silvers (1959). He found that the skin of

newly-hatched and one-day-old chicks became completely tolerant of their hosts whereas skin obtained from older chicks was rejected, apparently due to a graft-against-host reaction. Only a small proportion of skin homografts from 2-day-old chicks survived for more than 30 days, showing that immunological competence occurred by 2 days after hatching. Craig (unpublished data) was unable to confirm these results.

Terasaki (1959) showed in chickens that members of the lymphocyte family other than monocytes and thymocytes were involved in the production of splenomegaly. Szenberg and Warner (1962) found with the CAM technique a correlation between the number of pocks and the number of large lymphocytes, concluding that the large lymphocytes are the immunologically competent cells. On the contrary Solomon (1964) showed preliminary evidence that small lymphocytes isolated without contamination of large lymphocytes could cause splenomegaly in chicken embryonic hosts. Simons and Fowler (1966) confirmed Solomon's findings.

In rodents the cell-type responsible for the induction of graft-against-host reactions has been identified as the small lymphocyte by Gowans et al. (1962) and Hildemann et al. (1962). Gowans (1962) demonstrated that the small lymphocytes from the thoracic duct of parental strain rats caused a lethal wasting disease in normal adult F_1 hybrid rats.

Chimeral Status and Tolerance

Brent (1958) as reported by Mitchison (1959) reported that the persistence of antigen was not vital for the maintenance of tolerance.

On the contrary Billingham (1958) observed that tolerant mice, when carefully tested, invariably turned out to be chimeras in their lymph nodes and thereby concluded that persistence of antigen was required for maintenance of tolerance. Mitchison (1959) confirmed Billingham's observation by using heated blood for transfusions to induce tolerance in chickens. Balner (1964) feported in sublethally irradiated adult rats the possibility of persistence of tolerance towards the transplantation antigens across strong histocompatibility barrier, independent of chimeric state. He injected homologous bone marrow cells into sublethally irradiated young adult rats and tested for specific tolerance towards donor-type antigens by skin grafting at various intervals. About 50% of the animals though reverted to host type hemopoiesis when tested by serological typing, did not reject the skin grafts. They remained either partially or totally tolerant towards donor-type antigens. Zaalberg and Vandermuel (1966) working with allogeneic irradiation mouse chimeras came to the same conclusion.

Kelly et al. (1966) reported successful induction of tolerance among C57BL/1 female mice for male skin grafts with cell free extracts of antigenic material prepared from male liver, kidney, heart and lung, which had been stored from 2-6 months in a deep freeze prior to processing.

MATERIALS AND METHODS

Genetic Stocks

Two highly inbred lines and their reciprocal hybrids maintained at the Kansas State University Poultry Research Center were used as experimental genetic stocks. Line R is a Brown Leghorn with inbreeding coefficient of > 0.74 which was originally obtained from the Poultry Research Centre, Edinburgh, Scotland. It is believed to be homozygous at the major B-histocompatibility locus. RPL-6 is a White Leghorn originally obtained from the Regional Poultry Disease Laboratory, East Lansing, Michigan. It was reported to be under intensive inbreeding from 1939 (Crittenden, et al. 1964). A subline, $B^{13}B^{13}$, developed from RPL-6 on the basis of allelic differences at the major histocompatibility and erythrocyte antigen B locus, was used. Hence the B locus status of the parental lines and their reciprocal hybrids used in this experiment were: R-$B^{R}B^{R}$, RPL-6-$B^{13}B^{13}$, and reciprocal hybrids designated as $R6F_1$-$B^{R}B^{13}$.

Depending on space availability and fertility, natural mating or artificial insemination was used to obtain fertile eggs, which were held in an egg cooler until a sufficient quantity was accumulated for setting. However, the eggs were not held longer than one week. The eggs were set in the incubators maintained in the genetics laboratory of the Dairy and Poultry Science Department, Kansas State University. All chicks used for this experiment were pedigree hatched and wingbanded for identification.

Description of the Techniques Used in the Experiment

Embryonic Injections. Fourteen-day-old chick embryos were intravenously injected. The technique used was an adaptation of the procedure described by Billingham (1961). The eggs were candled and the position of a prominent chorioallantoic vein was marked on the surface of the shell with a pencil. A small rectangle, about 8 mm x 12 mm, was then cut in the shell overlying the selected vein by means of a fine toothed hack-saw blade. The cut rectangular piece of shell was carefully separated from the underlying shell membrane by means of a sharp lancet and discarded. A drop of sterile mineral oil was placed on the exposed shell membrane to render it transparent, revealing the underlying vessel with great clarity. The vein was entered with a number 30 gauge hypodermic needle fitted to a tuberculin syringe fixed to a micromanipulator, with bevel uppermost in the direction of blood flow and passed down the lumen of the vein for a short distance. The angle of insertion was kept as acute as possible. Then the required volume of whole blood was injected. The needle was withdrawn very slowly to minimize hemorrhage after injection. The window on the surface of the shell was sealed with transparent cellulose tape and the injected eggs were transferred back to the incubator.

Embryonic injections were given under a chemistry hood which was sterilized by ultra-violet light preceding use.

Intravenous Injections of Newly Hatched Chicks. Newly hatched chicks were injected with whole blood within 0-12 hours of hatching.

Injection was accomplished with a number 30 gauge hypodermic needle fitted to a tuberculin syringe inserted into the vein that runs superficially along the inner aspect of the lower part of the leg (Billingham, Brent and Medawar, 1956).

Intravenous Injection of Chicks Aged 24-48 Hours. Whole blood was injected into 24-48 hour chicks by the same method described in the case of newly hatched chicks.

Blood for injections was drawn from one-week-old male donors by cardiac puncture and from adult male donors from the brachial vein into a 3.5% solution of sodium citrate as anticoagulant fluid. The ratio of blood to anticoagulant was 1 to 4. A total volume of 0.25 ml was injected in all the experiments described, i.e., equivalent to 0.2 ml of whole blood.

Skin Grafting. The skin grafting technique used was essentially an adaptation of the Polley, Grosse, and Craig (1960) technique. Flexible colloidon was applied to the back of the chicks a few minutes prior to grafting to stiffen the skin of the donors and recipients. The chicks were anesthetized by injecting sodium pentobarbitol intraperitonially.

Two varieties of grafts were used in the experiment. Each parental line chick at the age of 16-17 days received two grafts, one from a hybrid donor and another from the other parental line chick of the same age. Each graft was rectangular and approximately 2 cm x 1 1/2 cm in size.

The entire skin on the back of hybrid male donor chicks was excised after killing the donors (for confirmation of sex). After removal, the

skin was placed raw side down on a filter paper moistened with physiological saline in a watch glass. An inkline was made at the anterior border of each graft prior to grafting.

In the case of skin from parental line donors, 2 rectangular full-thickness skin grafts were carefully cut out from the back of each chick. Removal of these grafts provided the necessary recipient graft beds. The anterior border of the skin was marked with ink. The excised skin tissue was placed flat on a filter paper moistened with physiological saline in a watch glass.

Position of hybrid and parental line donor tissues were assigned at random to the two graft beds made on the back of each chick. At the time of fitting the graft into the graft bed, the graft was reversed so as to bring the ink line to the posterior. Growth of feathers in reverse provided easy identification of most long-term viable grafts. After fitting the grafts to the sites, each graft was protected with a plastic bandage of 1" width. The bandaged chicks were transferred to electric brooders to provide warmth and early recovery from the anesthetic effect.

Bandages were removed on the fifth post-operative day and numerical scoring was done by adaptation of the scoring system suggested by Polley, Grosse and Craig (1960) as detailed below.

Score	Description
6	Smooth, bright and healthy appearing.
5	Slight discoloration but smooth appearing.
4	Some discoloration and/or inflammation apparent but smooth.
3	Discoloration and may be slightly shrunken.
2	Discolored, shrunken and crusty.
1	Graft sloughed.
X	Graft missing but not sloughed (faulty operative technique or accidental loss).

The grafts were scored every day till 30 days postgrafting and from then onwards once or twice every week depending on the graft situation. For the purpose of analysis of data, all grafts surviving up to 60 days with score of $>$ 4 were recorded as 60.

In the study of graft-against-host reactions, 14-day-old parental and hybrid embryos were intravenously injected with 0.2 ml equivalent of whole blood. On the 19th day of incubation the eggs were broken and viable embryos were killed. The spleens were taken out after cutting the abdomen and weighed in an electronic balance to determine the weight in milligrams.

Tolerance Induction as Measured by Allograft Reactions

Fourteen-day Embryo Treatment. Out of a total of 205 embryos, 162 embryos of lines RFL-6 and R were injected with whole blood from one-week-old and adult hybrid donors over a seven week period. The remainder, 43 embryos, acted as controls with some injected with Hank's solution

and some uninjected. During the first five weeks, equal numbers were included for each category in each treatment. Due to lower hatchability of line R embryos treated with one-week-old donor blood, two additional groups of line R embryos were injected with one-week-old donor blood to make up sufficient numbers.

Zero-twelve Hour Chick Treatment. Out of a total of 83 chicks, 71 chicks of lines RPL-6 and R were injected with whole blood from one-week-old and adult hybrid donors over a 4 week period. The rest, 12 chicks, acted as controls, receiving either Hank's solution or were uninjected.

Twenty-four-forty-eight Hour Chick Treatment. Out of a total of 30 chicks, 24 were injected with whole blood from one-week-old and adult hybrid donors. The rest, 6 chicks, acted as controls receiving either Hank's solution or were uninjected.

At the age of 16-17 days, all the chicks, both treated and controls received skin grafts as previously described. The number of chicks that survived and were skin grafted in each category is indicated in Tables 1 and 2.

Completeness of Tolerance as Tested by Graft-Against-Host Reactions

This experiment was designed to test for completeness of tolerance, among the birds which were tolerant of skin grafts, by means of a graft-against-host reaction.

A sample of birds believed to be highly tolerant to skin grafts of hybrid and parental donors on the basis of high scores and feather growth were selected from lines RPL-6 and R from different postgrafting

age groups. From the age group of 120-130 days, six tolerant birds, 3 each from lines R and RPL-6 were selected and 0.2 ml of their whole blood was injected into 14-day-old embryos of line RPL-6, line R and $R6F_1$ hybrids. Seventy-two embryos were injected on a single day. An equal number of embryos of each genotype was injected with blood from each presumed tolerant bird. R line embryos were treated with blood of R line birds tolerant of RPL-6 and $R6F_1$ skin grafts and RPL-6 embryos were treated with blood of RPL-6 birds tolerant to line R and $R6F_1$ grafts to serve as injected controls, in addition to a few uninjected controls. Treated eggs were returned to the incubator and on the 19th day of incubation the embryos were killed and their spleen weights were recorded.

Similar procedures were followed for presumed tolerant birds 80-90 days, 35 days and 8-14 days after skin grafting to test the completeness of tolerance.

RESULTS AND DISCUSSION

Intravenous inoculation of $R6F_1$ cells into R line and RPL-6 embryos and newly hatched chicks did not cause runt disease, since the parental antigens were represented in the hybrid cells. This was in accordance with the graft-against-host concept (Simonsen, 1957).

Skin was chosen as allograft tissue for testing the induction and persistence of tolerance since it was believed to be the test of greatest sensitivity and the results of this test are open to outward inspection from day to day (Billingham et al. 1956). Initial take of almost all

grafts and perfect survival of all autografts in chickens of lines RPL-6 and R indicated the surgical success of the technique used in skin grafting.

Induction of tolerance and its duration was measured in terms of mean skin graft survival time for an observation period of 60 days. Results are presented in Tables 1B and 2B for both lines of chickens. The relative graft survival rates for observation periods of 30 and 60 days are presented in Tables 4 and 5. Mean squares from analyses of variance of average skin graft survival times on treated groups only are presented in Table 3. The mean survival time of test grafts on untreated controls was 13-14 days in both lines (Tables 1B and 2B). No differences were observed in survival of the hybrid and parental grafts on the uninjected controls. The induction of at least some degree of tolerance was inferred if the survival of test grafts on treated subjects was prolonged beyond the mean survival time of similar grafts on untreated hosts. Prolonged survival of grafts (Tables 1B and 2B) was noticed in the case of subjects treated as 14-day-old embryos (14d E) and chicks within 12 hours of hatching (0-12h C) in both lines. In the case of 24-48-hour chicks, the treated chicks of line RPL-6 rejected both hybrid and line R grafts 16-17 days after grafting whereas line R chicks rejected RPL-6 grafts in about the same time but retained the hybrid grafts (5 out of 6) which had prolonged survival (Table 5). Analyses of variance (Table 3) indicated that the differences in mean skin graft survival times between embryonic and 0-12h C treatment combined versus 24-48h C treatment were highly significant in both lines.

Table 1. Effect of donor and recipient age on tolerance induction by injection of $R6F_1$ blood into RPL-6 recipients.

A. Number injected and subsequently skin grafted.

Age of recipient	Age of $R6F_1$ blood donors: Adult	Age of $R6F_1$ blood donors: 1-week	No. treated by recipient age	Uninjected controls
14d E	17	12	29	17
0-12h C	25	15	40	6
24-48h C	6	6	12	3

B. Survival period (days) of $R6F_1$ and R skin grafts.[1]

Age of recipient	Skin graft donor	Age of $R6F_1$ blood donors: Adult	Age of $R6F_1$ blood donors: 1-week	Means by recipient age	Uninjected controls
14d E	$R6F_1$	42.5	43.2	42.8	13.6
	R	40.8	40.2	40.5	13.8
0-12h C	$R6F_1$	37.6	39.1	38.4	14.0
	R	30.8	28.5	29.6	14.2
24-48h C	$R6F_1$	15.8	17.1	16.4	13.5
	R	15.8	17.5	16.6	13.5
Means by donor age	$R6F_1$	32.0	33.1		
	R	29.1	28.7		

[1]All skin grafting was at 16-17 days after hatching. Grafts were observed for 60 days, those surviving the entire period were given the value of 60.

Table 2. Effect of donor and recipient age on tolerance induction by injection of $R6F_1$ blood into R recipients.

A. Number injected and subsequently skin grafted.

Age of recipient	Age of $R6F_1$ blood donors: Adult	Age of $R6F_1$ blood donors: 1-week	No. treated by recipient age	Uninjected controls
14d E	15	9	24	8
0-12h C	17	14	31	6
24-48h C	6	6	12	3

B. Survival period (days) of $R6F_1$ and RPL-6 skin grafts.[1]

Age of recipient	Skin graft donor	Age of $R6F_1$ blood donors: Adult	Age of $R6F_1$ blood donors: 1-week	Means by recipient age	Uninjected controls
14d E	$R6F_1$	56.8	55.0	55.9	14.0
	6	53.7	49.0	51.7	13.9
0-12h C	$R6F_1$	59.0	58.6	58.8	14.2
	6	59.0	50.1	54.6	13.9
24-48h C	$R6F_1$	52.5	52.8	52.6	13.5
	6	16.1	16.1	16.1	13.6
Means by donor age	$R6F_1$	56.1	66.6		
	6	42.9	38.7		

[1]All skin grafting was at 16-17 days after hatching. Grafts were observed for 60 days, those surviving the entire period were given the value of 60.

Table 3. Mean squares from analysis of variance of average skin graft survival times.

Source of variation	d.f.	RPL-6 recipients	R recipients
Age of recipient (A_R)[1]	2	663.11**	589.14**
I + II vs III	1	1208.42**	1162.04**
I vs II	1	105.20**	16.24
Age of donor (A_D)	1	0.44	18.01
Genotype of graft (G)	1	39.24*	673.50**
$A_R \times A_D$	2	0.98	5.88
$A_R \times G$	2	21.00(*)	348.84*
I + II vs III x G	1	21.85*	697.68**
I vs II x G	1	20.17(*)	0.01
$A_D \times G$	1	1.84	9.91
$A_R \times A_D \times G$	2	1.12	4.64

[1]14d E = I, 0-12h C = II, 24-48h C = III

(*) = $P < .06$

* = $P < .05$

** = $P < .01$

Table 4. Effect of donor and recipient age on tolerance induction as measured by percentage skin graft survival on RPL-6 recipients.

Age of RPL-6 recipients	Skin graft donor	Age of $R6F_1$ Blood donors: Adult Surviving/ Total	% Survival	1-week Surviving/ Total	% Survival
A. At 30 days after grafting.					
14d E	$R6F_1$	12/17	71	8/12	67
	R	11/17	65	7/12	58
0-12h C	$R6F_1$	14/25	56	9/15	60
	R	9/25	36	5/15	33
24-48h C	$R6F_1$	0/6	0	0/6	0
	R	0/6	0	0/6	0
Survival by donor age	$R6F_1$	26/48	54	17/33	52
	R	20/48	42	12/33	36
B. At 60 days after grafting.					
14d E	$R6F_1$	7/17	41	6/12	50
	R	7/17	41	5/12	42
0-12h C	$R6F_1$	8/25	32	4/15	27
	R	3/25	12	2/15	13
24-48h C	$R6F_1$	0/6	0	0/6	0
	R	0/6	0	0/6	0
Survival by donor age	$R6F_1$	15/48	31	10/33	31
	R	10/48	21	7/33	21

Table 5. Effect of donor and recipient age on tolerance induction as measured by percentage skin graft survival on R recipients.

Age of R recipients	Skin graft donor	Age of $R6F_1$ Blood donors: Adult Surviving/ Total	% Survival	1-week Surviving/ Total	% Survival
A. At 30 days after grafting.					
14d E	$R6F_1$	15/15	93	8/9	81
	6	13/15	87	7/9	78
0-12h C	$R6F_1$	17/17	100	14/14	100
	6	17/17	100	13/14	93
24-48h C	$R6F_1$	5/6	83	5/6	83
	6	0/6	0	0/6	0
Survival by donor age	$R6F_1$	36/38	95	27/29	93
	6	30/38	79	20/29	68
B. At 60 days after grafting.					
14d E	$R6F_1$	14/15	93	8/9	81
	6	13/15	87	7/9	78
0-12h C	$R6F_1$	16/17	94	12/14	86
	6	16/17	94	9/14	64
24-48h C	$R6F_1$	5/6	83	5/6	83
	6	0/6	0	0/6	0
Survival by donor age	$R6F_1$	35/38	92	25/29	86
	6	29/38	76	16/29	55

It is one of the predictions of the Burnet and Fenner (1949) theory of immunity that the exposure of animals to antigens before the development of the faculty of immunological response should lead to tolerance rather than to heightened resistance. In accordance with this theory tolerance was induced by injecting intravenously 0.2 ml of whole blood from hybrid one-week-old and adult donors into 14-day-old embryos and chicks within 12 hours of hatching. However in the case of 24-48 hour chicks, intravenous injection of the same dosage of whole blood induced only slight and transient tolerance in line RPL-6 whereas in line R what is interpreted as partial tolerance could be induced. With the dosage level used in this study the power of antigenic stimulus to confer tolerance decreased as the age of the injected subject increased.

Differences could not be detected in mean skin graft survival times between chicks treated as embryos and within 12 hours of hatching in R line (Table 3). Most of the R line birds of both treatments had prolonged survival of test grafts with fully grown feathers of donor origin indicating a high percentage of tolerant birds in this line (Table 5). In line RPL-6 highly significant differences existed in skin graft survival times between the embryonic and newly hatched treatment groups (Table 3). The mean survival of skin grafts during the observation period was 42 days in the case of birds treated as embryos and about 34 days for birds treated as 0-12h chicks (Table 1B). Differences in the duration of the tolerance responsive phase between RPL-6 and R lines are therefore clear cut. Reasons for the existence of such differences as found between RPL-6 and R have been suggested by Hasek (1961a) as: (1) Variability in the immunological maturity between lines. Thus R line chickens

may have a relatively longer duration of the adaptive period. (2) The strength of the antigenic difference may differ, e.g., the B^R allele may be a greater antigenic barrier than the B^{13} allele. A similar situation was suggested by Billingham and Brent (1956) for the H loci in mice.

As age increases, a higher dosage of antigen is required to induce tolerance in mice (Brent and Gowland, and Billingham and Silvers 1962). Similarly a higher dosage of antigen might serve in the treatment of newly hatched RPL-6 chicks to produce tolerance equal to that obtained with embryo treatment. In general, the percentage of tolerant birds obtained in all the treatments as judged by the allograft reaction during the observation period was higher in the case of R line (Table 5) chickens when compared to line RPL-6 (Table 4).

The age of the blood donor had no significant effect on induction and duration of tolerance (Table 3) in either line of chickens as judged by mean skin graft survival times (Tables 1B and 2B) or relative rates of long term survivals (Tables 4 and 5). This clearly indicates that the antigenic stimulus contained in one-week-old donor blood is as competent as that of adult blood in the induction and maintenance of tolerance.

The effect of the genotype of the graft on test graft survival was highly significant in R line chickens ($P < 0.01$, Table 3) and significant in RPL-6 ($P < 0.05$, Table 3). Also the interaction between age of the recipient, i.e., embryonic and 0-12h C treatment combined versus 24-48h C treatment and genotype of the graft was highly significant in R line and significant in RPL-6 chickens (Table 3). The interaction between embryonic treatment versus 0-12h C treatment and genotype of the

graft approached significant level ($P < 0.06$, Table 3), in line RPL-6 only.

There were instances in individual R line chickens where the $R6F_1$ graft survived successfully with fully grown feathers and the RPL-6 graft was rejected. Similar instances were noticed in RPL-6 chickens also, though to a lesser degree. However, there were quite a number of birds in both lines keeping both grafts in good condition for a long time. Instances of this nature were reported by Solomon (1963a) and Shierman and Nordskog (1964). Solomon (1963a) explained rejection of parental line grafts on F_1 hosts as due to a local graft-against-host reaction. The theoretical explanation put forth by Shierman and Nordskog (1964) was that the parental line graft has twice as many foreign B antigen sites as the hybrid graft and the difference in survival of the two types of grafts might be due to a quantitative difference in immune response determined by antigen dose. The latter explanation seems more reasonable in view of the evidence obtained in this experiment as parental line grafts survived with much greater success on chicks treated as embryos or soon after hatching. Age of the recipient at treatment should have had no effect on any graft-against-host reaction following later skin grafting.

Several birds tolerant to skin grafts as indicated by 2 grafts in healthy condition with feather growth were selected for the purpose of testing for completeness of tolerance by the graft-against-host reaction (GAH). GAH reactions in the form of splenomegaly can be produced experimentally by injecting immunologically competent foreign cells into

chicken embryos. Simonsen (1957) and Billingham and Brent (1959) established that GAH reactions result from an immunological attack by the lymphoid graft against histocompatibility antigens in the cells of the host. Jaffe and McDermid (1962) found that splenomegaly response was primarily to B-locus incompatibilities. When 0.2 ml of whole blood from the birds tolerant to skin grafts was injected intravenously into parental line and F_1 embryos, GAH reactions of varying degree in the form of splenic enlargement were noted. In all 4 age groups tested, from 8 to 130 days postgrafting, a considerable number of reactive embryos was noted (Table 6). For the purpose of this experiment an embryo was considered to be reactive if its spleen weighed 26 or more milligrams. Mean spleen weights ranged from 7.3 to 8.1 mg in the case of uninjected controls and 10.0 to 11.7 mg in the case of syngeneic donor-recipient combinations (Table 7). Mean spleen weights and number of reactive embryos in each observed group (by weight class) are tabulated in Table 6.

GAH reactions by the cells of tolerant birds are not expected. According to Gowans and McGregor (1965) the lymphoid cells from donors tolerant of the tissues of the prospective recipient should not cause a reaction. But Gowans *et al.* (1963) reported in rats intermediate response of GAH reaction and one instance of full fledged response, when lymphocytes from partially tolerant (as judged by skin graft survival) parental donors were injected into F_1 recipients. In the present experiment the reacting embryos showed up to 5-6 times mean enlargement of the spleen over syngeneic donor-recipient combinations in the 3 age

Table 6. Completeness of tolerance as tested by graft-against-host reactions. Spleen weights of parental and F_1 embryos injected with blood of treated chicks tolerant to skin grafts.

		Postgrafting age of donors (days)																			
		120-130					80-90					35					8-14				
		Weight classes (mg)					Weight classes (mg)					Weight classes (mg)					Weight classes (mg)				
Line of recipient	Blood donor[1]	Mean wt.	0-25	26-75	> 75	Reactive Total*	Mean wt.	0-25	26-75	> 75	Reactive Total*	Mean wt.	0-25	25-75	> 75	Reactive Total*	Mean wt.	0-25	25-75	> 75	Reactive Total*
$R6F_1$	$R(R6F_1)$	52.4	4	7	2	9/13	50.0	0	3	-	3/3	56.9	-	8	2	10/10	23.0	7	3	-	3/10
	$6(R6F_1)$	48.7	2	10	2	12/14	-	-	-	-	-	39.8	3	7	-	7/10	-	-	-	-	-
	Not injected	8.9	4	-	-	0/4	8.5	5	-	-	0/5	8.4	3	-	-	0/3	8.0	2	-	-	0/2
RPL-6	$R(R6F_1)$	36.8	8	3	2	5/13	73.3	-	2	1	3/3	67.3	-	8	2	10/10	25.7	4	4	-	4/8
	$6(R6F_1)$	12.5	11	-	-	0/11	-	-	-	-	-	11.3	9	-	-	0/9	-	-	-	-	-
	Not injected	8.8	3	-	-	0/3	8.0	3	-	-	0/3	8.2	3	-	-	0/3	8.0	3	-	-	0/3
R	$R(R6F_1)$	11.0	7	-	-	0/7	11.3	3	-	-	0/3	10.1	7	-	-	0/17	10.0	3	-	-	0/3
	$6(R6F_1)$	80.4	2	1	7	8/10	-	-	-	-	-	58.8	1	5	2	7/8	-	-	-	-	-
	Not injected	6.6	3	-	-	0/3	7.1	3	-	-	0/3	6.6	2	-	-	0/2	6.0	2	-	-	0/2

[1] $R(R6F_1)$ indicates R line chick treated with $R6F_1$ blood.
$6(R6F_1)$ indicates RPL-6 chick treated with $R6F_1$ blood.

*Reactive spleens considered to weight > 26 mg.

Table 7. Mean spleen weight comparisons.

Mean spleen weight of	Postgrafting age of donors (days)			
	120-130	80-90	35	8-14
Uninjected controls	8.1	7.9	7.4	7.3
Syngeneic donor-recipient combinations	11.7	11.3	10.4	10.0
Allogeneic combinations	54.6	61.7	55.7	24.4

[1]Spleen weights of R($R6F_1$) and 6($R6F_1$) donors were pooled as no significant differences were found between the two lines.

groups with higher percentages of reactive embryos (120-130, 8-90, and 35 days). In one group (8-14 days) the mean enlargement was about 2 1/2 times greater than controls and with a low percentage of reactive embryos (Table 6).

The quantitative differences in splenic enlargement observed between the 8-14 days (low age) group and other groups (high age) suggests quantitative differences in numbers of nontolerant donor cells capable of an immune response. Another result of interest was that in each group of birds tested there were some unreactive embryos (Table 6) and among reactive embryos there were marked differences in spleen weights. This might indicate that the number of cells capable of GAH response was quite limited and that unresponsive embryos received either none or an insufficient number to elicit a measurable response.

A skin allograft is capable of producing an antigenic stimulus by means of vascular and lymphatic connections with the host. Hence a test graft can play a notable role in reinforcing tolerance by

supplying the antigen to the host which was made tolerant for the same antigens during embryonic life. The differences observed in spleen weights between high age group and low age group donor cells suggest that as the age of the graft increases the antigen supply will be less. These results are in agreement with the suggestion of Gowans et al. (1963) that long lasting skin allografts contribute less of antigenic material or none at all to the afferent lymph than a freshly implanted skin graft.

Judged by the GAH reaction observed in this experiment all the birds selected for testing, including the low age group, were in a state of partial tolerance. According to Billingham et al. (1956) and Gowans et al. (1963) a state of complete tolerance is revealed in a bird if it is carrying a fully feathered and healthy skin graft. All birds selected for testing were in a state of complete tolerance as judged by the apparent absence of allograft reactions but were found to be in a state of partial tolerance by GAH reaction. Hence the results of this experiment indicate that a redefinition of criteria indicating a state of complete tolerance may be needed. If birds do carry good skin grafts while in a state of partial tolerance or following loss of tolerance, then the theory of adaptation of grafts as reported by Weber et al. (1954), Cannon et al. (1954), Woodruff (1955), Terasaki et al. (1957) and Hasek (1961a), although generally not accepted, may need to be reexamined.

An alternate explanation for these findings may be advanced, i.e., that tested birds were in fact fully tolerant of skin allografts, but that a GAH reaction in the embryos was elicited due to the presence of

an age specific antigen. This explanation is vitiated by the absence of GAH reactions in the syngeneic donor-recipient combinations (Table 7).

The possibility of split tolerance may be considered since the host can develop complete tolerance for some donor strain antigenic constituents of the tolerance inducing complex without necessarily becoming tolerant of all of them as suggested by Hasek et al. (1961). Accordingly the findings in this experiment may also suggest that the antigens which provoked the GAH reactions may not be the same antigens that induced tolerance on behalf of F_1 and parental skin.

SUMMARY AND CONCLUSIONS

Tolerance induction was studied by injecting intravenously 0.2 ml of whole blood of $R6F_1$ one-week-old and adult donors into 14-day-old embryos, 0-12h chicks and 24-48h chicks of lines RPL-6 and R. The duration and completeness of tolerance were tested by allograft reactions and GAH reactions. With the dosage level used in this experiment, allograft reactions indicated that tolerance could be readily induced in 14-day-old embryos and 0-12h chicks of both the lines of chickens, whereas in the case of 24-48h chicks only a slight and transient tolerance in line RPL-6 and what is interpreted as partial tolerance in line R could be induced. In line R no significant differences could be observed in duration of tolerance between embryonic treatment and 0-12h chick treatment. In line RPL-6 highly significant differences were observed between these two treatments in duration of tolerance. The duration of the tolerance-responsive phase was longer

and the percentage of tolerant birds was higher in R line than in line RPL-6. The age of the blood donor was found to have no significant effect on induction and duration of tolerance in either line. The genotype of the graft was found to have a significant effect on mean skin graft survival times in both lines of chickens since hybrid grafts had longer survival than parental line grafts. The birds of both lines which appeared to be completely tolerant to skin grafts were found to be in a state of partial tolerance when tested by GAH reaction. Therefore, the results of this experiment indicate a need for (1) a redefinition of criteria for indicating the state of complete tolerance; (2) re-examination of the theory of adaptation of grafts; (3) further consideration of the possibility of split tolerance to histocompatibility antigens in chickens.

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PRODUCTION OF IMMUNOLOGICAL TOLERANCE AS INFLUENCED BY THE AGE OF THE DONOR AND RECIPIENT IN CHICKENS

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Tolerance induction, its duration and completeness was studied by injecting intravenously 0.2 ml of whole blood of (RxRPL-6) one-week-old and adult donors into 14-day-old embryos, 0-12 hour chicks and 24-48 hour chicks of lines RPL-6 and R. The duration and completeness of tolerance were tested by allograft reactions and graft-against-host reactions, respectively. With the dosage level used in this experiment, allograft reactions indicated that tolerance could be readily induced in 14-day-old embryos and 0-12 hour chicks of both lines of chickens, whereas in the case of 24-48 hour chicks only a slight and transient tolerance could be induced in line RPL-6 and what is interpreted as partial tolerance in line R. In line R no significant differences could be observed in duration of tolerance between embryonic treatment and 0-12 hour chick treatment. In line RPL-6 highly significant differences were observed between these two treatments in duration of tolerance. The reasons for the existence of such differences might be (1) variability in immunological maturity between lines (2) differences in antigenic strength. In general, the percentage of tolerant birds obtained in all the treatments as judged by the allograft reaction during the observation period was higher in the case of R line chickens when compared to line RPL-6. The age of the blood donor had no significant effect on induction and duration of tolerance in either line of chickens. This clearly indicates that the antigenic stimulus contained in one-week-old donor blood is as competent as that of adult blood in the induction and maintenance of tolerance. The genotype of the graft was found to have a significant effect on mean skin graft survival times in both lines of chickens since

hybrid grafts had longer survival over parental line grafts. The birds of both lines which appeared to be completely tolerant to skin grafts were found to be in a state of partial tolerance when tested by the graft-against-host reaction. Therefore, the results of this experiment indicate a need for (1) a redefinition of criteria for indicating the state of complete tolerance, (2) re-examination of the theory of adaptation of grafts, (3) further consideration of the possibility of split tolerance to histocompatibility antigens in chickens.