might in some cases be controlled by mechanisms not involving suppressor T cells. Such mechanisms could play a role in protecting the integrity of organ structures from autoimmune attack, or from bystander damage during local inflammatory reactions, by curbing in situ the activation and clonal expansion of immune T lymphocytes.

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Tolerance Induced by Thymic Epithelial Grafts in Birds

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Grafts of the anterior limb bud introduced at embryonic day 4 between histoincompatible chick embryos were subject to chronic, mild rejection beginning from several weeks to several months after birth. In contrast, quail wing buds similarly grafted into chickens started to be rejected at the first or second week after birth and finally autoamputated. Embryonic thymus epithelium from donor quail (before it had been colonized by hemopoietic cells) was grafted into chicks. A chimeric thymic epithelial stroma was generated in which the lymphocytes of the chick acquired the capacity to recognize the grafted limb as self either permanently or for a protracted period of time. In such thymic chimeras the grafted wings were not rejected.

SELF AND NONSELF RECOGNITION IS acquired during development and is then maintained throughout life. The thymus plays a central role in immunological recognition of self and nonself. T lymphocytes that have differentiated in the thymus become able to recognize, through a surface receptor, foreign antigens in association with molecules encoded by the major histocompatibility complex (MHC) (*1–4*).

Although the role of intrathymic differentiation is important in MHC restriction of T cells, the selection process of the T cell repertoire in the thymus is not understood. In particular, the respective roles of the stable endodermal component of the thymic stroma and of the macrophages and dendritic cells of hemopoietic origin are not known. Both cell types express class I and class II MHC antigens from early embryonic stages onward in mammals (*5*) and birds (*6*).

Attempts have been made to induce allotolerance in the adult mouse by grafting fetal thymuses depleted of their lymphocytes by deoxyguanosine or by culture at low temperature; these experiments have yielded controversial results. The grafted thymus, although expressing donor MHC antigens, survives for a long time in an allogeneic environment; nevertheless, it fails to induce allotolerance as tested in mixed lymphocyte reactions (MLR) when grafted into a normal histoincompatible recipient (*7*). However, thymuses cultured at low temperature before grafting, introduced into an athymic nude mouse, induce tolerance, as judged by MLR with both intrathymic and spleen cells (*8*). Thymic epithelium grafts (after deoxyguanosine treatment) can tolerize precursors of cytolytic T cells, but only for the minor and not the major histocompatibility antigens carried by the thymic epithelial cells (*9*). In fact, none of these experiments resolves if the induction of tolerance to self-MHC products is mediated through the thymic epithelium itself or by remaining cells of the dendritic and macrophage lineages, because neither method completely eliminates the latter category of cells.

We have investigated the establishment of tissue tolerance during ontogeny to see to what extent early embryonic grafting of allogeneic tissues leads to tolerance when the host's immune system is exposed to foreign antigens during development (that is, in the embryo itself). We grafted limb buds at day 4 of embryonic development (E4) between chick embryos of different histoincompatible haplotypes, and the embryos were allowed to hatch.

The allogeneic wing grew normally and did not show signs of rejection for a period of time varying from 1 to 5 months. Such signs eventually always appeared, however, showing that partial, but not complete, tolerance of the graft could be induced by early embryonic grafting experiments.

We then devised a system in which rejection of the embryonic graft takes place acutely as soon as the young chicken's immune system becomes mature (that is, during the second week after hatching). This was achieved in 100% of cases by xenogeneic grafting of quail embryonic limb buds onto chick recipients under conditions similar to those for allogeneic transplantations. In this model, grafts of quail thymic epithelium introduced before seeding of the epithelium by hemopoietic cells induced tolerance of the foreign limb.

The grafts were carried out as in Fig. 1a. Some of the chickens used in these experiments were partly inbred strains of the B14 and B19 genotypes; in other experiments they were F1 embryos of the inbred strains B15 × B21 (*10*). Fourteen chimeras hatched out of 114 grafted embryos. In most cases, the grafted wing was well formed and mobile. In others, the wing was somewhat shortened. Further development

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of the implant was normal. For example, down was replaced by remiges at the same time in the control and grafted wings. The graft remained healthy for several weeks and even for months in certain cases (Fig. 2). However, in all animals symptoms of chronic rejection appeared (except ADC 653 and 649, which are about 3.5 months old). These symptoms were variable and ranged from edema and chronic inflammation with loss of the feathers to destruction of the terminal limb segments. In several cases, the signs of rejection were reversible. In the birds with severe signs of rejection, mobility of the limb was altered, sometimes resulting in immobility. Autoamputation of the wing did not occur in any of the animals of this series, some of which are now nearly 2 years old.

Skin grafts from adults of the B4 haplotype (third party graft) were performed on four chick chimeras (ADC 38, 86, 102, and 105) (*11*). In all cases rejection of the explant occurred after 10 to 12 days, as it did in control chickens. MLR were performed with the peripheral blood cells of such birds. Their peripheral T lymphocytes proliferated in response to MHC antigens of a third party donor (B4) (*12*). Thus, the operated birds were fully immunocompetent.

We carried out the same operation with outbred white Leghorn chick embryos as recipients and outbred quail embryos as donors. Out of 242 operated birds, 30 hatched in an apparently healthy state. The grafted wing was or was not pigmented depending on whether it had been colonized by melanoblasts when it was removed from the donor; melanoblasts originate from the neural crest and reach the limb bud at stage 15 to 18 of Zacchei (*13*), about the time of removal from the quail donor. At birth, the grafted wing was smaller than the contralateral chick limb, due to the difference in size of the two birds (at hatching chick and quail weigh about 40 g and 10 g, respectively). During the first postnatal week the growth of the grafted quail wing was comparable to that of a normal age-matched quail.

Signs of immune rejection started during either the first or the second postnatal week with edema, and the rejection immediately became acute, with suppuration followed by necrosis and finally autoamputation of the wing in all cases (Fig. 3). During the acute phase of the immune response, the animal stopped growing, usually without losing weight. Then growth began again and the chimeras' weight reached approximately the value of age-matched chickens.

During rejection, the host developed a humoral response involving antibodies directed against common quail antigens that could be detected by immunocytochemistry on cultured quail embryonic fibroblasts. The antibodies against quail cell surface antigens only became detectable in the serum of the chimeras 1 to 8 days after the appearance of the first signs of rejection. This suggests that the cellular and humoral responses to the graft occur independently, a conclusion that was further supported by early bursectomy of the recipient embryos: in three chick host embryos, the anlage of the bursa of Fabricius, the site of B cell differentiation in birds, was surgically removed, at E5 (*14*), 1 day after implantation of the quail wing bud. Such animals cannot develop antibody response to injected antigens (*15*). However, they acutely rejected the wing at P10, P11, and P19, respectively.

We next tried to induce tolerance of the grafted quail wing. The limb buds taken from E3.5 quails were transplanted into chick recipients of the same developmental stage as above. Both donor and recipient eggs were returned to the incubator for 18 to 20 hours, after which the thymic rudiments were partly removed from the chick host (at E5) according to a variant of the technique described (*16*). The thymic epitheliomesenchymal anlagen were dissected from the quail embryo that had provided the limb bud and were grafted isotopically into the chick (Fig. 1b). At that stage, neither the quail nor the chick thymic primordia had yet been colonized by hemopoietic cells, and when transplanted heterospecifically, the lymphocytes, dendritic cells, and macrophages that they contain later on are derived from the host (*17*). Therefore no precursor cells of the monocytic or lymphocytic lineages are included in the thymic epithelial transplant.

Of the 291 chick embryos subjected to this operation, 16 hatched and survived in a healthy state. In all these birds, the grafted wing remained healthy longer than in the control chimeras that had not been grafted with quail thymus (Fig. 4).

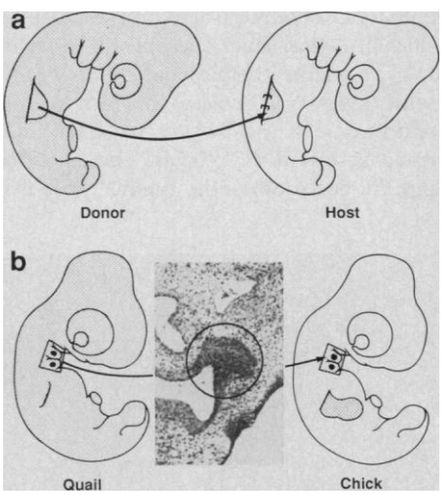

Fig. 1. (**a**) Limb bud transplantation. The right limb bud of either a chick embryo (allogeneic combination) or a quail embryo (xenogeneic combination) is substituted to its counterpart in a chick recipient. The recipients and donors are stage-matched (by reference to the developmental tables of Hamburger and Hamilton (*28*) for the chick and of Zacchei (*13*) for the quail. Quails are at stage 15 to 18 and chicks at stage 19 to 25). Platinous hooks maintain the graft. The extraembryonic membranes must be sutured after surgery to allow hatching (*14*). (**b**) Experimental design of the isotopic bilateral graft of a thymic rudiment between quail and chick into a recipient that has been transplanted 1 day earlier with a wing bud from the same donor [see (a)]. Circle, one of the four transplanted epithelial thymic buds shown in a histological section. Before grafting, the chick thymic rudiment is partly removed on both sides according to the method of Martin (*16*), slightly modified.

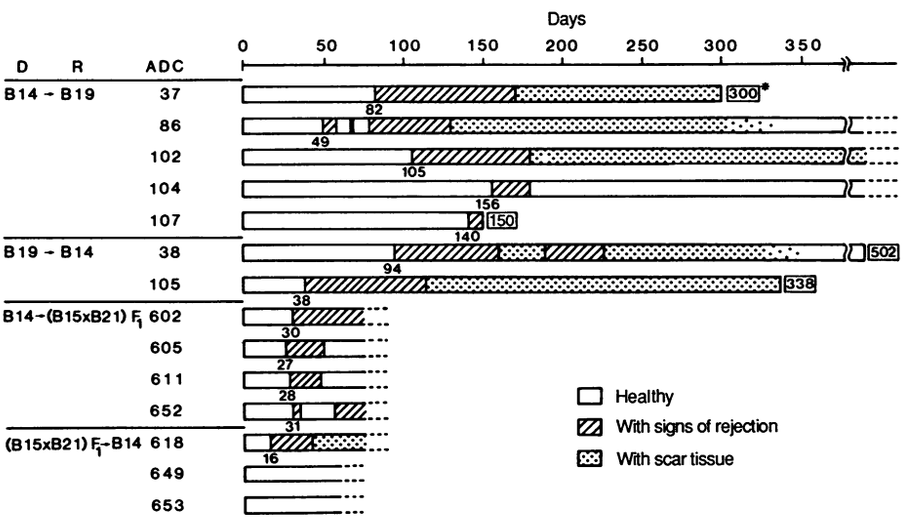

Fig. 2. Appearance after birth of chronic signs of rejection in allogeneic grafts of the wing bud performed at E4. The signs of rejection varied from local edema to fall of the feathers and suppuration. In some cases they were reversible. Autoamputation of the wing never occurred in these birds. *, Age at which the birds were killed; D, donor; R, recipient; ADC, wing chimeras.

One animal (ADC 60), which never exhibited any signs of rejection, was sacrificed at the age of 483 days (16 months). During the first 2 weeks after birth, the grafted quail wing had grown at a rate close to that of an age-matched quail. However, its growth subsequently decreased, leading, in the adult, to a smaller wing than in the adult quail control. Because, in this bird, the grafted wing was not pigmented, two biopsies were performed at 154 and 283 days. After sacrifice, the whole wing was also processed for histology. Skin, connective tissues, muscles, and bones of the quail type were identified on the basis of the structure of their nuclei. No sign of inflammation was present. A blood sample was taken from this animal before sacrifice. Concanavalin A stimulation of circulating T lymphocytes yielded a proliferative response comparable to that of age-matched chick controls (Table 1). No antibodies against common quail antigens could be detected by the immunocytochemical test on cultured fibroblasts (Fig. 4).

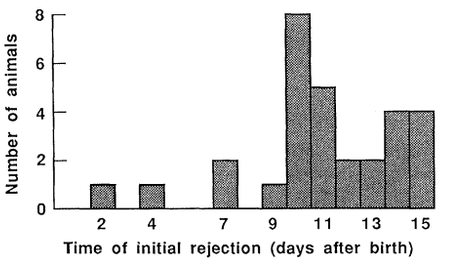

Fig. 3. Appearance after birth of acute rejection in xenogeneic grafts of the wing from E3.5 quail to E4 chick embryos. None of the 30 chimeric birds retained the grafted wing in a healthy state more than 15 days after birth. Most started to reject the wing during the second week after birth.

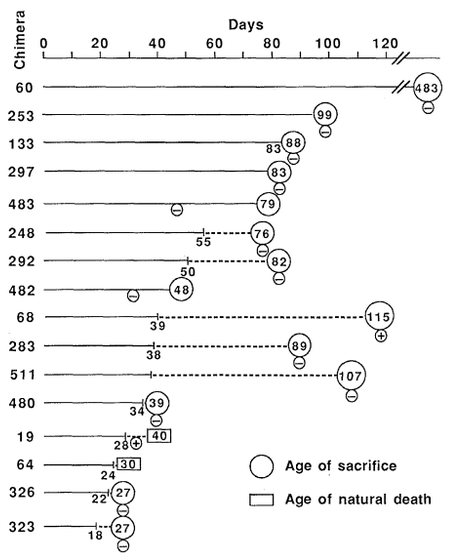

Fig. 4. Xenogeneic grafts of the wing bud (E4) and thymic epithelial primordia (E5) from quail to chick embryos. Solid line, maintenance of the wing in a healthy state after birth; dashed line, wing with signs of rejection. − and + indicate the result and timing of the test for antiquail humoral reaction detected by immunocytochemistry on cultured quail fibroblasts. Quail embryonic E8 skin was cut into small fragments, incubated 20 minutes in 0.1% trypsin (Difco) in Ca^{2+}- and Mg^{2+}-free phosphate buffer solution. Dissociated cells were suspended in minimal Eagle's medium supplemented with 10% newborn calf serum. Cells (10^5) were cultured for 1 day. Sera from the chimeras were applied to these cultured fibroblasts followed by a rabbit immunoglobulin antiserum to chicken immunoglobulin coupled with fluorescein isothiocyanate (FITC) (Nordic). Immunoreactivity was observed with a Leitz Orthoplan epifluorescence microscope. Sera from normal chicks did not exhibit a positive immunostaining with quail cultured embryonic fibroblasts.

A skin graft from a B19 male chick was performed at the age of 6.5 months and was rejected within the third week after grafting as in control chickens of the same flock.

Three injections of human gamma globulins were performed from 6 months of age at 7-day intervals. One week after each injection, the antibody response was measured (*15*) and was found to be similar to that of control chickens.

Similarly, no rejection of the wing was observed in four other birds (ADC 253, 297, 482, and 483) (Fig. 4). In ADC 133, a slight edema appeared on the wing at 83 days. In all the birds, as for ADC 60, wing growth slowed down around day 15 after hatching. None of these six birds produced antibodies directed against common quail antigens. ADC 19 and ADC 68, in which rejection of the wing started at P28 and P39, respectively, were the only chimeras of this group to produce antibodies to quail antigens. However, no antibodies were detected in other birds (ADC 323 and 326) in which rejection appeared earlier, showing once more that cell-mediated and humoral immune responses are dissociated.

Two skin grafts (one from B4 and one from B19 strains) were performed at 86 days in ADC 253 and at 73 days in ADC 297 and were promptly rejected. Concanavalin A stimulation on circulating T cells carried out on the birds that did not reject the wing led to normal proliferative responses (Table 1).

The birds showing tolerance were sacrificed at 1.5 to 3 months of age (Fig. 4), and their thymuses were examined histologically. Spontaneous regression had not occurred at this stage, and thymuses were always present. The number of thymic lobes, which generally varied in size, was smaller in operated than in control birds. Histological examination of the thymic tissue was performed either after Feulgen-Rossenbeck staining of DNA, which allows quail and chick cells to be distinguished (*18*), or after immunocytochemical detection of quail or chick B-L–antigenic determinants (that is, Ia-like) on the thymic epithelium (*6*). In some lobes, we detected a chick thymic structure (Fig. 5a) where no quail cells could be found. In contrast, the cortex of other lobes showed the typical quail B-L

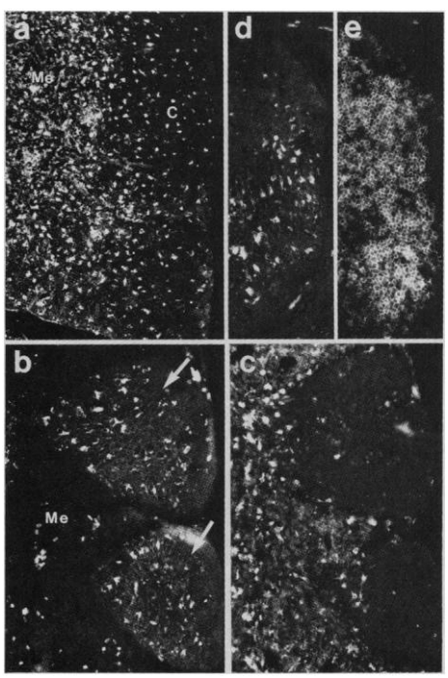

Fig. 5. Immunocytochemical analysis of cellular chimerism in thymuses from chickens engrafted at E5 with quail epithelial thymic buds following partial thymectomy of the recipient (see Fig. 1b). (**a**) Chick thymic lobe from ADC 297 immunostained with the monoclonal antibody against chick B-L antigen (TaP1) (*6*). In the cortex (C) only the epithelial cells are stained, while in the medulla (Me) both epithelial and dendritic cells of hemopoietic origin express the chick B-L antigen. This lobe is negative with the monoclonal antibody TaC1 specific for quail B-L antigen (*6*). ×60. Indirect immunofluorescence with FITC-conjugated antibody to mouse immunoglobulin (Capell). (**b**) Cortical regions of the thymus in ADC 253 (arrows) with epithelial cells of the quail type shown by immunostaining with the monoclonal antibody against quail B-L antigen (TAC1). In the medullary zone (Me) there are no quail B-L antigen expressing cells. Erythrocytes show autofluorescence. ×120. (**c**) Section consecutive to (b) immunostained with monoclonal antibody to chick B-L antigen (TAP1). All the cells, including epithelial cells, dendritic cells, and macrophages, are TAP1 positive. ×120. (**d**) Immunocytochemical analysis of another thymic lobe from ADC 253. Thymic lobe with epithelium of quail type as shown by immunostaining with the monoclonal antibody to quail B-L antigen (TAC1). ×100. (**e**) Section consecutive to (c) immunostained with the monoclonal antibody T10A6 against a T cell marker (*29*), confirming the functional nature of the chimeric thymic tissue. ×100.

immunoreactivity of the epithelial network (Fig. 5, b and d). Most lobes exhibiting the quail epithelial component were, in fact, chimeric, with zones formed by chick epithelium close to others formed by quail epithelial cells. By using both the stain for DNA and a monoclonal antibody that identifies a T cell surface antigen on quail thymic lobes, we demonstrated that chick host lymphocytes differentiate in an environment provided by the quail thymic epithelial graft (Fig. 5, d and e). Moreover, in all lobes (formed by quail or by chick epithelial cells), the dendritic, macrophage B-L–positive cells of the medulla were of chick host origin (Fig. 5, a and e). Therefore, these thymuses were chimeric in at least two respects—(i) in the epithelial network and (ii) in the quail epithelial lobes because of the chick nature of the hemopoietic cells that develop in these thymuses.

Our previous experiments involving neural tube grafts showed that the nervous tissue is completely tolerated in allogeneic combinations and rejected only after several weeks (up to 2 months) in xenografts (*19*). Our present work examines the immunological status, after birth, of transplanted embryonic tissues that (unlike neural tissues) do express class I antigens and for which there is no blood-tissue barrier. At E3.5 to E4, the limb bud is just becoming vascularized, so that, only a few endothelial cells of the blood vessel wall (but virtually no blood cells) are included in the graft.

In allogeneic combinations, a partial tolerance of the wing is apparently induced, since in all animals the grafted limb was maintained throughout life in a relatively healthy condition. Clearly, then, complete (nervous tissue) and partial (limb) tolerance can be brought about through early embryonic grafting in allogeneic combinations even in the total (neuroepithelial grafts) or virtual (limb bud grafts) absence of hemopoietic cells. It is known that immunological tolerance to histocompatibility antigens is inducible in allogeneic combinations provided that foreign blood cells are transferred in fetal or early neonatal life (*20–22*). This opinion was, however, challenged more recently by Flajnik *et al.* (*23*).

Whether the tolerance to the tissue grafted during early embryogenesis extends to adult skin grafts from animals syngeneic to the limb donors is unknown. Experiments to answer this question will require the use of highly inbred strains of chickens. Unfortunately, embryos of such birds have a lower resistance to surgery, and thus far, the experiment has not been feasible.

Our previous results showed that, in contrast to other grafted tissues [limb buds, neural primordia, and bursal rudiments (*15*)], the thymus did not undergo rejection. Tolerance of thymic grafts had been reported in allogeneic combinations (*7–9, 24*), but transfusion of blood from one species of bird to another was evidently not able to induce a stable state of tolerance (*25*). Therefore, given the central role of the thymus in self-nonself discrimination, our present experiments tested the effect of thymic graft on tolerance induction in this system. Because, at the stages we used, the quail and chick thymic epithelial rudiments were not yet invaded by hemopoietic cells (*17, 26*), the maintenance of the foreign limb induced by the thymic epithelium grafts demonstrated the role of this cell type in the process of self recognition. Recent experiments showed that the thymic epithelium also provided protection when other quail organs were grafted. Thus, the bursa of Fabricius, which can be exchanged between quails and chicks (*14, 15*), is tolerated after birth if the thymic epithelium from the donor is also implanted (*27*).

Table 1. Concanavalin A–induced proliferation of peripheral blood T cells from chimeras that tolerated the grafted quail limb thymic epithelial grafts. Peripheral blood cells (3×10^6) from chimeras (see Fig. 4) were cultured for 2 days in Iscove's modified Dulbecco's medium with 10% fetal calf serum, in presence or absence of concanavalin A (Con A) (Pharmacia).

Chimera	Age (days)	[^{3}H]Thymidine uptake (cpm)*	
		No Con A	Con A (10 μg/ml)
ADC 482†	48	962	34,585
ADC 297	68	815	14,108
ADC 253	72	220	6,028
ADC 483‡	79	174	25,043
ADC 60	161	593	117,027
Controls	48	206	26,541
	71	117	21,706
	161	242	52,693

*Arithmetic mean from duplicate cultures after 2 hours of [^{3}H]thymidine incorporation, 1 μCi per culture. †Frequency of CT-3–positive T cells (*30*), 53.3%; control, 69.3%. ‡Frequency of CT-3–positive T cells, 62.3%; control, 70.9%.

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