A Chick Embryo Model for the Study of Allograft Rejection[1] (36590)

ALICE P. WILLIAMSON, MARY ANN SOUTH, AND RUSSELL J. BLATTNER
Department of Pediatrics, Baylor College of Medicine, Houston, Texas 77025

For the study of components of the immune system, the chick embryo appears to offer important advantages for adaption as an experimental model. Since the chick embryo is immunologically immature during most of its development it will support allografts (1–3) and certain types of xenografts (4–6) on the chorioallantoic membrane (CAM). After hatching the chicken has acquired mechanisms which cause it to reject allografts and xenografts under all ordinary circumstances.

For these studies cardiac tissue was selected as the graft tissue since graft survival should be easily identified by continued contraction of the cardiac fibers. Whole heart transplants to chick embryos have been accomplished previously (7) in a small series of embryos (5 successful transplants out of 10 trials), but the technique used involved time-consuming surgical procedures. For the purposes of the present experiments it was necessary to devise techniques which would allow grafting of large numbers of embryos at a site where the grafts could be observed daily. Preliminary experiments demonstrated that this could be accomplished by using embryonic donors and placing a small piece of the cardiac tissue on the CAM of each of the recipient chick embryos (8). When the heart-grafted chick embryos also received spleen grafts a low percentage showed reactions which appeared to indicate some degree of rejection. In order to develop this model more fully studies were done to determine the earliest age at which the embryos were suitable for grafting and to determine conditions under which a high percentage of rejections would be obtained when lymphoid cells from adult chickens were administered to the graft.

Materials and Methods. Preparation of the recipient embryos. Eggs were obtained from outbred chickens, usually of the White Leghorn strain. They were incubated in a forced draft incubator at 99°F until the third or fourth day at which time a window was placed in the shells, using a method (9) in which the embryo is dropped away from the shell following one punch with a short blunt needle in the side of the shell and another over the air sac end. The embryo is observed with an egg candler while the entire embryo is pulled away from the shell membrane by suction with a rubber bulb on the air sac hole. The windows were made by grinding a square in the shell, then removing the shell and shell membrane to expose the embryo. The windows were sealed with Scotch tape and the eggs were returned to the incubator to be used at various ages.

Preparation of the grafts. Hearts were removed aseptically from chick embryos of various ages and, in some experiments, from hatched chickens. The hearts were cut into 1 mm fragments with small scissors in such a way that each piece contained both inner and outer surfaces of the heart. Each heart fragment was placed on the CAM of a recipient embryo and a 2 × 4 mm piece of Millipore filter (0.45 μ porosity) was placed over the edge of the graft in such a way that the graft was anchored firmly to the CAM. The eggs were returned to the incubator and thereafter were examined daily to observe the progress of the grafts. Those embryos in which the

[1] Supported by U.S. Public Health Service Grant AI-09380 from the Allergy and Immunology Branch of the National Institute of Allergy and Infectious Disease, and supported in part by research funds from Texas Children's Hospital, St. Luke's Episcopal Hospital and Texas Heart Institute.

graft showed no contractions on the third day were discarded from the experiment.

Preparation of lymphoid cells and administration to grafts. Plastic glassware was used for these procedures. Spleen cells were obtained by removing a spleen aseptically from a freshly sacrificed adult chicken. A portion of spleen was minced and mixed with 1 ml of Roswell Park Memorial Institute medium-1640 (RPMI) from GIBCO and ground gently in a Teflon tissue grinder. The tissue was then further diluted in the RPMI medium and drawn in and out of a 24 gauge needle to free the lymphoid cells from the stroma. Medium containing the suspended cells was removed and saved. Peripheral blood cells were obtained by drawing blood from the wing vein of an adult chicken into a heparin-rinsed syringe. The blood was mixed with RPMI in a ratio of 3 ml RPMI to 5 ml blood. The mixture was centrifuged at 1000 rpm for 10 min. The supernatant fluid was removed and the cells were resuspended in fresh RPMI and centrifuged again. Finally, 4 ml of fresh RPMI was added to the tube in such a manner that all cells remained undisturbed. The tube was then shaken gently until cells of the buffy coat were suspended in the medium. Cell counts were made using a B-D Unopette containing the red cell diluent in order to avoid lysing the few nucleated chicken erythrocytes which contaminated the suspension. The nuclei of such lysed cells could be mistaken for lymphoid cells. Otherwise, the cells were counted by standard techniques. The cells were diluted in RPMI to contain the appropriate number of cells per 0.05 ml. These were dropped on the CAM in the vicinity of the heart graft at a time interval of 3 to 4 hr after initial placement of the graft. Controls were treated with RPMI or the mixture of RPMI and plasma removed after the first centrifugation and passed through a 0.45 μ Millipore filter to remove all cells. The eggs were then returned to the incubator. In some experiments, grafts were removed at daily intervals and fixed for tissue sectioning for microscopic examination.

Results. Survival of heart grafts on the CAM. Heart grafts from donor embryos of ages varying from 5 to 14 days incubation were placed on recipient embryos of ages varying from 3 to 11 days incubation. In embryos younger than 5 days the CAM is not well developed and therefore does not present a suitable surface for grafting. In such embryos, grafts were placed in the coelomic cavity of the developing embryo. Few such grafts showed evidence of becoming vascularized, however, and it was also difficult to find the site of the graft when the embryos became older. The CAM embryos of 8 to 11 days of incubation accepted grafts of the heart fragments readily, yielding up to 90% of "takes." The CAM of embryos 7 days old or younger were found to be unsatisfactory for the survival of the heart grafts. Chick embryos of all the ages tested were suitable as donors but hearts from 10-day-old hatched donors became vascularized in only a low percentage of trials and none of these showed continued contractions of the cardiac fibers.

With the intent of using embryos in which development of the immune system was most immature, 8-day recipient embryos were routinely used for the experimental studies and, since it was practical to obtain donors and recipients from the same incubation setting, the donors were usually also of 8-days incubation. In the present studies the heart grafts survived without signs of rejection for periods ranging from 10 to 12 days, *i.e.*, until close to the time of hatching. Although some of the grafts showed superficial areas of necrosis with concomitant inflammatory response the major portion of the grafts remained pink and healthy. Considerable bleeding occurred in some grafts during the first few days. This was presumably due to the coalescence of vascular channels of graft and host tissue at a time when the wounded fragments of cardiac tissue had not healed. During the first 3 days vascularization took place. After this time the grafts began to increase in size, the tissues tending to adhere to and grow out over both upper and lower surfaces of the Millipore filter. In some instances the CAM tissue made a thin covering over the surface of the graft. In other instances both graft and filter became deeply embedded within the CAM. The grafts, how-

TABLE I. Effect of Lymphoid Cells from Chicken Blood on Heart Grafts to Chorioallantoic Membranes (CAM) of 8-Day Chick Embryos.

No. cells administered[a]	Total eggs	Embryos surviving 10 days after grafting: Ratio of grafts beating to total	Ratio of grafts rejected to total	Ratio showing graft-versus-host CAM lesions to total
1×10^6/egg	81	24/75 (32)[b]	71/75 (95)	35/75 (47)
0	114	94/108 (88)	0/108 (0)	0/108 (0)

[a] Cells were administered 3 to 4 hr following placement of the graft on the CAM.
[b] Percentage given in parentheses.

ever, remained in the form of easily recognizable, nodular masses, distinct from the tissues of the CAM proper. The contractions, characterized by a visible expulsion of blood during the peak phase of contraction and a refilling of the vascular channels as the muscle relaxed, continued throughout the period of observation in a high percentage of embryos.

Microscopic sections of the grafted CAM showed that, during the first few days after placement, the ectodermal layer of the CAM became eroded away directly beneath the graft. The graft then gradually became embedded in a matrix of mesodermal tissue and the ectoderm grew out to cover the wounded area. There was no evidence of epicardium surrounding the graft but the myocardial tissue remained discrete from the host mesoderm although mesenchymal tissue occasionally pentrated into the graft dividing it into sections. It was not possible to determine whether the endocardium lining the vascular channels was of host or donor origin. Mitotic figures were seen frequently among the cardiac fibers.

Graft rejection by lymphoid cells. When suspensions containing 1×10^6 or more washed spleen cells were administered to the CAM of heart-grafted embryos only 35 to 40% showed reactions which were interpreted as rejection reactions, *i.e.*, opaque or necrotic areas in the graft associated with cessation of contractions of the cardiac fibers. On the other hand, when suspensions containing 1×10^6 of peripheral blood lymphoid cells were administered to the grafted embryos, marked changes occurred in more than 90% of the grafts and these were associated with a significant reduction in the number of grafts which showed contractions of the cardiac fibers. Table I shows a comparison of the major effects observed in control embryos and those whose heart grafts were treated with lymphoid cells from the peripheral blood of chickens.

Through the fifth day after grafting there was little difference in the gross appearance of the cell-treated and control grafts. Between days 6 and 10 the cell-treated grafts enlarged markedly, became hard and pale and developed numerous white nodular areas (Fig. 1). Contractions lessened and gradually ceased by day 10 in most grafts. Contractions were often observed, however, even when evidence of rejection was far advanced. Histological studies indicated that the enlargement of the grafts was due in large part to massive lymphoid cell accumulations containing many mitotic figures. Cardiac fibers degenerated in areas where infiltration of such cells was dense. The microscopic sections confirmed that contractions persisted until the cardiac fibers had been virtually all destroyed. In some instances the entire graft turned black and appeared to be completely necrotic. Lesions which were characteristic of a graft-versus-host reaction were also observed on the CAM of some of these embryos. Autopsy of some of the experimental embryos showed that the cells occasionally caused enlargement of the spleen with white nodules appearing in the splenic tissue, a finding which is also characteristic of a graft-versus-host reaction in the chick embryo treated with allogeneic lymphoid cells. The grafted hearts alone did not cause any obvious change in the size or appearance of the

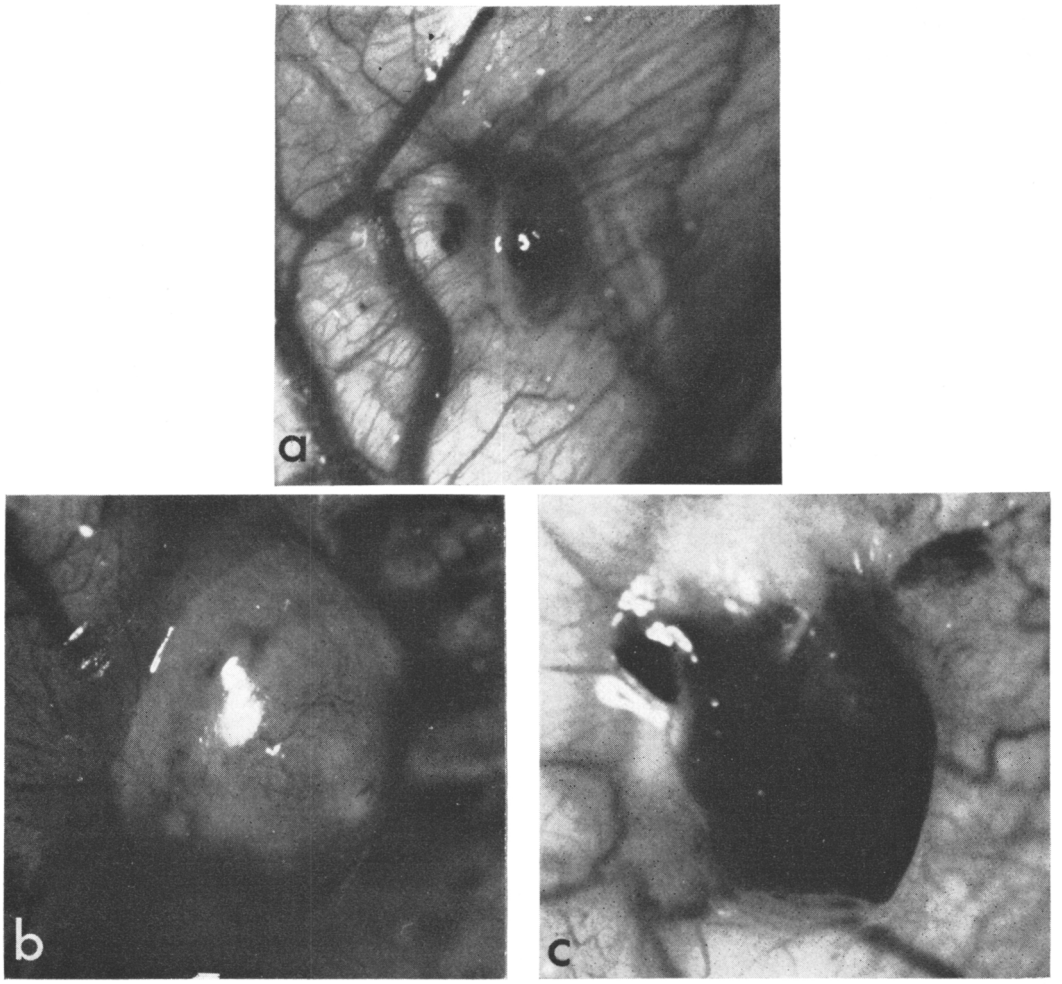

FIG. 1. Heart tissue from chick embryos grafted to the chorioallantoic membranes of other chick embryos 10 days previously. (a) Control shows well vascularized, healthy graft. (b) Graft treated with lymphoid cells from the blood of adult chickens shows marked enlargement, general opacity and white nodular areas within the graft tissue. (c) Complete necrosis of the heart graft following treatment with lymphoid cells from the blood of chickens. 18× magnification.

hearts of recipient embryos; nor were other organs of the embryo observed to be affected.

In order to compare the rejection reactions of lymphoid cells derived from peripheral blood with those derived from the spleen, cells were obtained from the blood and spleen of the same chicken. The results of several combined experiments (Table II) show that cells derived from the blood are consistently more effective in producing graft rejections than those of the spleen.

To determine the number of cells required to produce the heart graft rejections a dose–response curve was plotted. Lymphoid cells from chicken blood were prepared in 10-fold dilutions and suspensions containing the appropriate number of cells were administered to groups of heart-grafted chick embryos, each group containing 50 to 100 embryos. It is clear from Fig. 2 that the number of grafts showing rejection reaction is directly related to the number of lymphoid cells administered and that the number of grafts which survive and show contraction of cardiac fibers is inversely related to the number of cells administered. Few grafts are affected

TABLE II. Comparison of the Effects of Lymphoid Cells Derived from Chicken Spleen and Chicken Blood on Heart Grafts to the Chorioallantoic Membranes of 8-Day Chick Embryos.

No. cells administered[a]	Type cells	No. eggs	Embryos surviving 10 days after grafting: Ratio of grafts beating to total	Ratio of grafts rejected to total
5×10^5	WBC	45	17/42 (40)[b]	36/42 (86)
	Spleen	47	37/43 (86)	19/43 (44)
5×10^4	WBC	35	14/25 (56)	15/25 (60)
	Spleen	36	26/27 (96)	1/27 (04)
5×10^3	WBC	36	23/28 (82)	3/28 (11)
	Spleen	36	30/30 (100)	0/30 (0)

[a] Cells were administered 3 to 4 hr following placement of the graft on the CAM.
[b] Percentage given in parentheses.

when the cell dosage is 1×10^3 or less but the number affected rises sharply at dosages of 1×10^4 or more. It should be noted that white cell counts of chicken blood may include 50–60% of thrombocytes because the thrombocytes are nucleated and are sometimes difficult to differentiate from the white cells. However, dose–response curves plotted at increments of 0.5 $\log_{10}$ indicate that a difference of 1 to 5 times in the number of cells is of negligible significance.

Discussion and Summary. The data presented here indicate that fragments of heart tissue from outbred chick embryo donors of 5–14 days of incubation may be successfully grafted to the CAM of chick embryos which are 8 days of incubation or older. The cardiac tissue becomes vascularized, continues to grow and continues contractions which rhythmically expel blood from the vascular channels. The CAM of embryos younger than 8 days of incubation do not accept the grafts. Rejection of the grafts in more than 90% of the embryos can be effected by administration of peripheral blood lymphoid cells from outbred chickens. Washed cells obtained from the spleens of chickens can also bring about graft rejection but experiments comparing lymphoid cells from the spleen and the blood of the same chicken showed that the blood lymphoid cells are consistently more effective in producing rejection. The incidence of rejection reactions is directly related to the cell dosage. Conversely the number of surviving, rhythmically contracting grafts is indirectly related to the cell dosage. Histological studies showed that massive cellular accumulations occur in the cell-treated grafts. These are associated with destruction of most or all of the cardiac fibers by day 10 following administration of the cells.

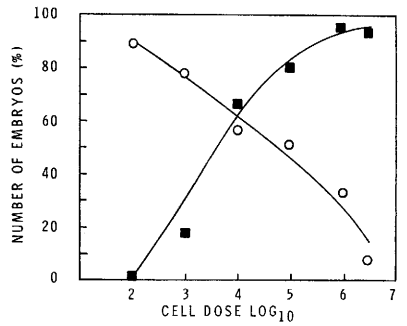

FIG. 2. Dose–response curve showing the effects of lymphoid cells from chicken blood on heart grafts to the chorioallantoic membranes of chick embryos. Observations were made on 10-day-old grafts. (○) grafts showing rhythmic contractions; (■) grafts showing rejection. Percentages are based on groups containing between 50 and 100 heart-grafted embryos.

1. Willier, B. H., Physiol. Zool. 3, 201 (1930).
2. Clark, L. F., Physiol. Zool. 9, 102 (1936).
3. Hancox, N. M., J. Physiol. (London) 107, 513 (1948).
4. Sandstrom, C. J., Anat. Rec. 52, 69 (1932).
5. Mirand, E. A., and Hoffman, J. G., Exp. Med. Sur. 11, 63 (1953).

6. Saccomanno, G., and Waldapfle, R., Arch. Pathol. **52**, 529 (1951).

7. Kalzberg, A. A., Plast. Reconstr. Surg. Transplant. Bull. **23**, 113 (1959).

8. South, M. A., Williamson, A. P., and Blattner, R. J., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **29**, 828 (1970).

9. Toth, T. E., Avian Dis. **13**, 895 (1969).

Received Mar. 9, 1972. P.S.E.B.M., 1972, Vol. 140.