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CELLULAR TRANSFER OF ANTIBODY PRODUCTION FROM ADULT TO EMBRYO IN DOMESTIC FOWLS

INTRODUCTION

Simonsen[8] demonstrated that the intravenous inoculation of adult fowl spleen cells or peripheral white blood cells into chick embryos gave rise to splenic enlargement and a Coomb's positive hemolytic anemia in the recipient chick. He found that this effect was species specific, required viable cells from donors over 11 days of age for its initiation, and could be passaged from chick to chick at least nine times without apparent diminution of the phenomenon. He concluded that the reaction represented colonization of the recipient spleen by the implanted adult cells and that the adult cells produced antibodies against the antigens of the recipient chick, as manifested by the Coomb's positive chick red blood cells and hemolytic anemia. This thesis was further supported by the subsequent finding[4] that only slight splenic enlargement occurred when cells were transferred between antigenically similar inbred strains.

These findings are particularly relevant to the "clonal selection" theory of antibody formation of Burnet[3] in which it is postulated that an antigen serves to create a selective environment for functional replication of previously existing clones of specific antibody-producing cells. This theory would imply that cells capable of producing specific antibodies such as viral antihemagglutinating antibodies could be transferred from an immune adult donor to an embryonic recipient and then serially passaged from embryo to embryo under the influence of an appropriate antigenic environment.

The work to be presented was prompted by a desire to test this possibility using influenza virus as an antigen and the method described by Simonsen as the test system.

MATERIALS AND METHODS

Fowl lines. The lines principally used in these experiments have been White Leghorns and Australorps obtained from a commercial hatchery. Eggs were placed in a 35° C. incubator until 24 hours after hatching and then transferred to an electric brooder. The

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inbred lines were obtained through the courtesy of the Animal Health Division of the Commonwealth Scientific and Industrial Research Organization. Each line has been propagated by successive matings of full and half sibs and has an inbreeding coefficient of approximately 80 per cent. Details of the method have been presented by Skaller.[9]

Antigen. The antigen used was Melbourne (MEL), an A influenza strain first isolated in Melbourne in 1935. The virus was grown in the allantoic cavity of chick embryos and freshly harvested suspensions were placed in a 56° C. water bath for one hour to destroy infectivity. Infectivity titres prior to heating ranged between $10^{6.8}$ and $10^{7.6}$ EID_{50} per ml. in the allantoic cavity of the chick embryo.

Preparation of cell inocula. Whole blood was obtained by cardiac puncture through a heparinized syringe. The cells were washed in saline and the buffy coat removed. Leukocyte counts were performed in a haemocytometer chamber and the cells diluted in normal saline to the desired concentration. Spleens from adult fowls or chicks were removed aseptically under chloroform anesthesia. The capsule was stripped off and cells teased apart with fine needles into ice cold saline. The suspension was then filtered through a loose wire mesh under light pressure, washed three times in normal saline, and resuspended to the desired cell concentration in normal saline after counting in the haemocytometer chamber. Penicillin and streptomycin were added to make a final concentration of 500 units of penicillin and 500 micrograms of streptomycin per ml. All cell suspensions were kept at 4° C. and inoculated within one hour of preparation. When mixtures of cell suspensions from different donors were inoculated, the suspensions were prepared separately and mixed together in equal volumes immediately prior to inoculation.

Inoculation of eggs. According to the method of Beveridge and Burnet.[2] The volume of the inoculum in all instances was 0.05 ml.

Antibody titrations. Embryos were bled by removing an area of the shell, clearing the membrane with sterile glycerine, and withdrawing blood by puncture from a chorio-allantoic artery. Hatched chickens were bled by cardiac puncture. The sera were separated and heated at 56° C. for 30 minutes. Although MEL virus is relatively insensitive to mucoid inhibitors, most adult fowl sera and occasional chick sera inhibit MEL in low dilutions in an antihemagglutinin (AHA) titration. To remove this non-specific inhibition, two volumes of M/100 potassium periodate were added to the sera, the mixture was allowed to stand for two hours at room temperature, and two volumes of 50 per cent glycerol were added to make a final serum dilution of 1 : 5. The AHA titrations were performed in the standard fashion with the exceptions that 2 to 2.5 agglutinating doses of virus and 0.5 per cent adult fowl red blood cells were used. A hyperimmune fowl serum of known potency was included in each test. All results presented in the same table or figure were performed on the same day.

Spleen weights. Spleens from embryos and young chicks were removed after exsanguination. They were dissected cleanly from adherent tissue, allowed to stand for 30 minutes on filter paper, and weighed to the nearest milligram.

RESULTS

COMPOSITION OF CELL INOCULA

The cell concentrations referred to in this article include all cell types excluding mature red blood cells found in the teased spleen cell suspensions

and all forms of leukocytes found in the peripheral blood. Since a vast body of evidence has incriminated cells of the lymphocyte and plasma cell series as antibody producers, it is of interest to know the relative proportion of these cell types in the inocula. Smears of spleen cell suspensions and peripheral blood were prepared, stained with Leishman's stain, and cell ratios determined after counting 2,000 to 5,000 cells. The morphologic criteria used for the identification of immature forms correspond to those of Makinodan, Ruth, and Wolfe.[6] Table 1 lists the percentage of these cells found in inocula

TABLE 1. CELL TYPES IN INOCULATING SUSPENSION

		Per cent cell type				
		Blast cell	*Prolympho-cyte*	*Lympho-cyte*	*Proplasma-cyte*	*Plasmacyte*
		Spleen cell suspension				
Normal	1	0.1	12.28	28.0	1.69	1.74
	2	0.8	20.1	29.0	0.9	1.2
	3	1.1	9.9	32.0	1.9	1.7
Immune	1	2.4	14.4	24.4	3.1	4.1
	2	1.9	11.5	20.0	1.5	2.5
		Peripheral leukocyte suspension				
Normal	1	0.0	10.0	43.5	0.0	0.5
	2	0.0	4.2	38.6	0.0	0.0
	3	0.0	6.8	60.6	0.08	0.8
Immune	1	0.0	4.0	52.4	0.0	0.0

prepared from normal adult nonimmunized fowls and also from adult fowls which had received two intravenous inoculations of heat-killed MEL virus at an interval of one month and were sacrificed 24 hours after the second inoculation. For the spleen cell suspensions, the figures represent per cents of all cell types found excluding mature red blood cells. For peripheral blood, the figures represent per cents of all leukocytes.

As would be expected, the results indicate that the spleen cell suspensions contained a variety of developmental stages in the lymphocyte and plasma cell series. Inoculation of 10^4 or more cells would contain a plentiful and representative supply of developing stages of these cells. No cells of an earlier stage than large lymphocytes were identified on a survey of 5,000 leukocytes of the peripheral blood. While this does not exclude the possibility that inocula of 10^4 cells may contain less mature forms, it makes the uniform appearance of immature forms in a series of inocula of this concentration unlikely.

GROSS AND MICROSCOPIC FINDINGS AFTER CELL TRANSFER

Spleen weights after inoculation. Inoculation of adult fowl spleen cells or peripheral leukocytes intravenously into chick embryos may give rise to massive enlargement of the recipient spleen. Figure 1 illustrates the magnitude of the difference obtained when 10^7 spleen cells from a normal adult fowl were inoculated into a 14-day-old embryo. The experimental spleen was removed five days after inoculation and is shown together with a spleen from a normal embryo of the same age.

The time course of enlargement after inoculation is shown in Figure 2.

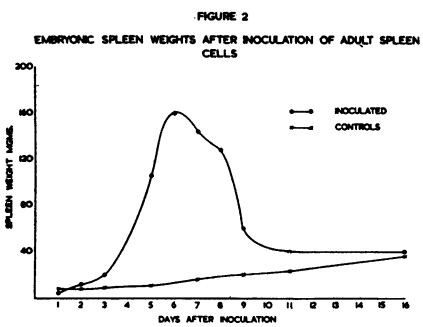


FIG. 2. Spleen weights from embryos inoculated with 2 x 10^7 adult fowl cells at 14 days of incubation. Controls represent spleen weight from embryos of the same age group given normal saline intravenously.

Embryos of 14 days of incubation were given 2 x 10^7 spleen cells intravenously, and controls of the same age were given equal volumes of normal saline. Each point on the graph represents an average of at least six spleen weights in milligrams. At one day after inoculation there was no apparent difference between the average spleen weights of the two groups. On the second post-inoculation day the experimental spleens were slightly heavier, but the numbers involved were too small to permit a valid comparison. By the third day the spleens of the experimental group were enlarged two times those of the control group. Progressive enlargement continued, reaching a maximum around the sixth day and thereafter declined. By the 16th day after inoculation the spleens of the two groups were comparable in size. It should be noted, however, that after the first week a high mortality, presumably from hemolytic anemia, was present in the inoculated group. Hemolytic anemia and spleen weights show a crude parallel, therefore a bias towards smaller spleens may have been present in the survivors after one week.

Factors influencing splenic enlargement. The species specificity of this phenomenon was tested by the inoculation of rabbit and mouse spleen cells, human peripheral blood and also by Hela cells of a strain being routinely used in this laboratory for adenovirus studies. The age specificity of the donor was also determined by the inoculation of spleen cell suspensions from fowl donors of varying ages. An indication of the viability requirements of the inoculated cells was obtained by subjecting spleen cells from an adult

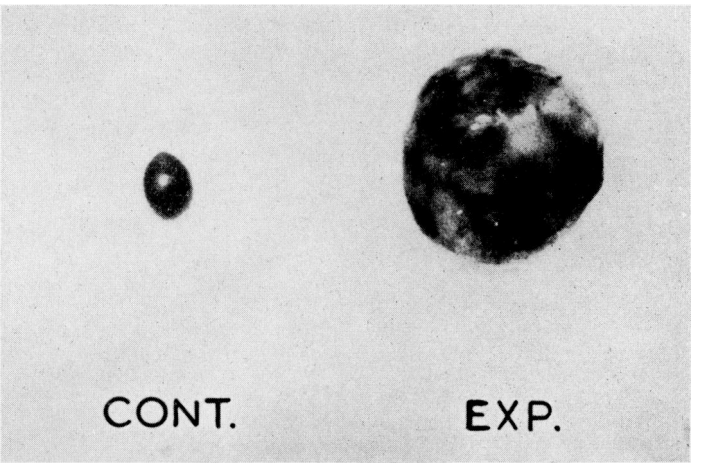


FIG. 1. Enlarged spleen from a 19-day-old chick embryo inoculated with 10^7 spleen cells five days previously. Shown together with a spleen from a normal 19-day-old embryo.

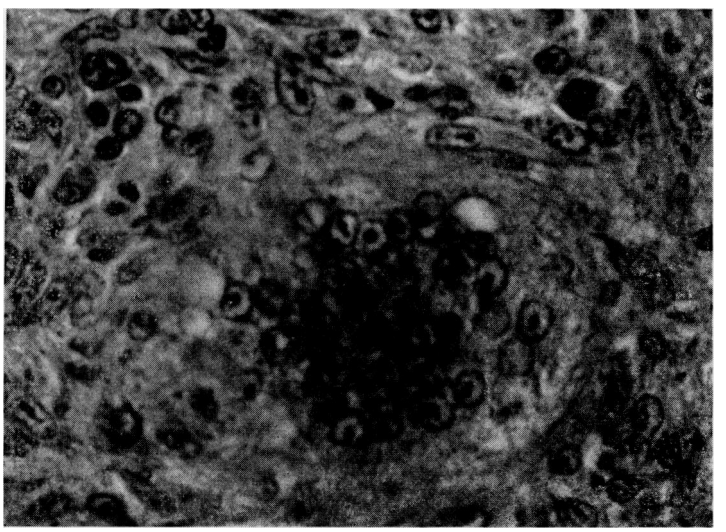


FIG. 3. Spleen from a 14-day-old embryo inoculated with 3×10^6 adult spleen cells two days previously. Hemotoxylin and eosin (x1,100).

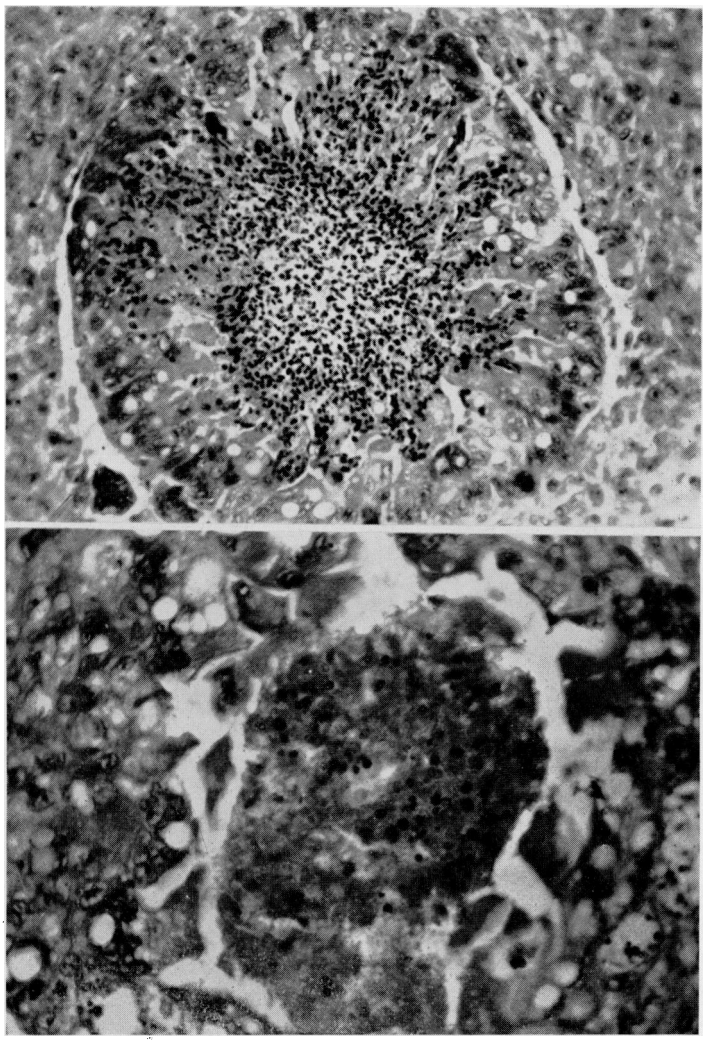


FIG. 4. Spleen from a 15-day-old embryo inoculated with 3 x 10^6 adult spleen cells three days previously. Unna Pappenheim (x500).

FIG. 5. Same spleen as Fig. 4. Hematoxylin and eosin (x600).

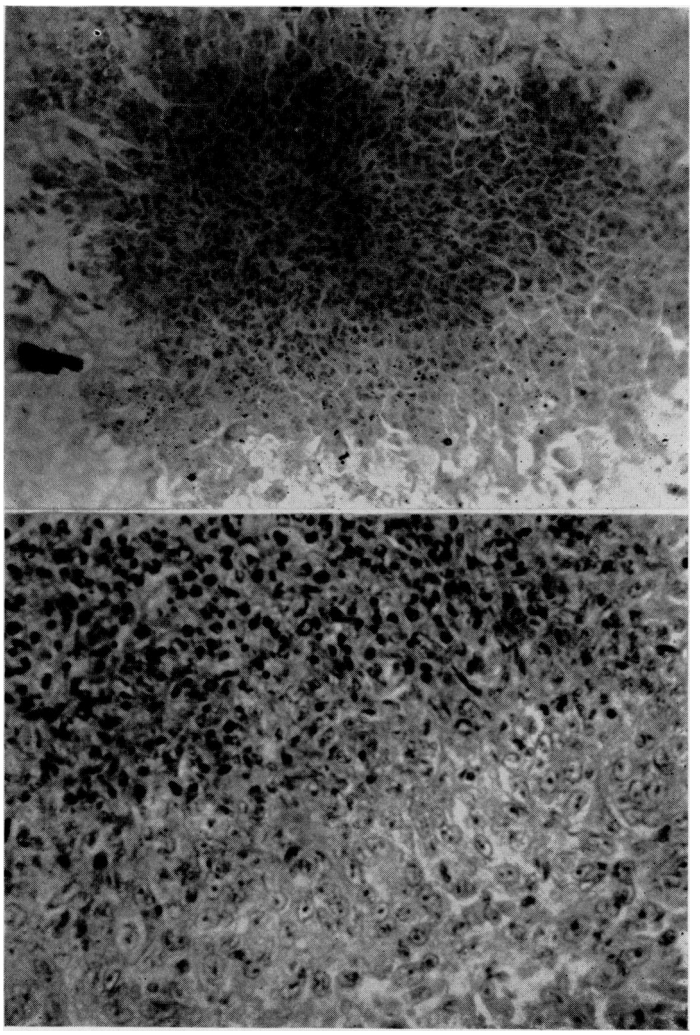


FIG. 6. Spleen from 17-day-old embryo inoculated with 4 x 10^6 adult spleen cells five days previously. Hematoxylin and eosin (x300).

FIG. 7. Spleen from a 19-day-old embryo inoculated with 4 x 10^6 adult spleen cells seven days previously. Hematoxylin and eosin (x525).

donor to freezing and thawing three times and also to heating at 56° C. for 20 minutes before inoculation. The results are summarized in Table 2. Of the inocula tested, only untreated cells from fowls 10 days of age or older were capable of initiating significant splenic enlargement. Cells from the 10-day-old donors gave rise to a lesser degree of splenic enlargement than

TABLE 2. FACTORS INFLUENCING SPLENIC ENLARGEMENT

Cell inoculum	*Donor*	*No. cells inoculated*	*Spleen weights in mgms.*		
			No. tested	*Range*	*Average*
None	—	—	50	5-15	8.0
Spleen	Adult rabbit	10^6	10	5-16	8.8
Spleen	Adult mouse	10^6	15	6-12	9.4
Peripheral leukocytes	Adult human	2×10^5	25	6-12	8.5
Hela cells	Human	3×10^5	4	10-14	11.0
Spleen frozen and thawed	10-wk. adult fowl	$*10^6$	20	6-15	9.8
Spleen heated 56° C.	10-wk. adult fowl	$*10^6$	20	7-15	10.0
Spleen	10-wk. adult fowl	10^6	30	45-320	182.0
Spleen	10-day chick	5×10^5	25	7-64	32.4
Spleen	5-day chick	10^6	15	4-14	8.5
Spleen	1-day chick	4×10^5	10	6-12	7.0
Spleen	19-day embryo chick	2×10^5	10	5-12	8.6

* Cell count prior to treatment.

a comparable cell concentration from more mature donors, indicating that there may be a gradual increase with age in the ability of the donor cells to initiate this phenomenon. These findings of species and age specificity and the necessity for viable cells are completely in accord with the data of Simonsen.[8]

The number of cells required to induce splenic enlargement is of interest, and attempts to determine this number were made by preparing serial dilutions of spleen and peripheral leukocyte suspensions from the same fowl and

inoculating these into 13-day-old embryos. Results are shown in Table 3. Totals of 3.75 x 10^4 peripheral leukocytes per inoculum were required to produce significant enlargement of the spleen in a majority of the recipients. Beyond this point, higher concentrations of cells tended to produce more rapid splenic enlargement and somewhat heavier spleens, but this relationship is not clear cut. In one instance an enlarged, nodular spleen was found in a recipient of only 4.7 x 10^3 spleen cells. Thus the enlargement of the

TABLE 3. NUMBER OF CELLS REQUIRED TO INDUCE SPLENIC ENLARGEMENT

Source	*No. cells inoculated*	*Spleen weights in mgms. 6 days after inoculation*
Spleen	3.0 x 10^5	200, 101, 74, 69
	7.5 x 10^4	120, 70, 61, 49
	3.75 x 10^4	106, 76, 64, 48, 17
	1.87 x 10^4	20, 16, 15, 14, 9
	9.3 x 10^3	14, 12, 12, 8
	4.7 x 10^3	38, 11, 11, 10, 9
Peripheral leukocytes	3.12 x 10^5	312, 171, 76, 49
	7.8 x 10^4	207, 193, 71, 68
	3.9 x 10^4	78, 58, 49
	1.95 x 10^4	49, 38, 34, 11, 8
	9.7 x 10^3	16, 13, 12, 10, 9, 8
Normals	—	12, 11, 11, 10, 7, 5

recipient spleen gives only a crude indication of the number of cells inoculated. The data suggest that peripheral leukocytes may be slightly more effective than spleen cell suspensions. If Table 1 is taken into consideration, however, and attention focused only upon cells of the lymphocyte and plasma cell series, no significant difference between the two types of suspensions can be found. The observation that splenic enlargement can be initiated by 1.95 x 10^4 peripheral blood leukocytes leads to the impression that mature cells can initiate the process, and this is strengthened by the finding that spleen cell suspensions, containing many forms of developing cells, are no more effective in causing splenic enlargement.

The age of the recipient embryo at the time of inoculation exerted a marked influence upon the type and degree of splenic enlargement obtained. Table 4 shows the resultant spleen weights when 5 x 10^6 from the same adult donor fowl were inoculated into embryos from 11 to 20 days of incubation. Splenic enlargement occurred in the recipients of all age groups tested but embryos from 11-15 days at the time of inoculation showed spleen

weights from two to three times greater than those of the older embryos. Aside from the greater weight in the younger group, their spleens also showed gross irregularity of the surface. When embryos in this group were sacrificed 3-4 days after inoculation, raised areas approximately 1 mm. in diameter were found scattered over the surface of the spleen. By the 5th to 6th day these areas appeared as yellowish white, glistening, well-circumscribed nodules quite distinct from the surrounding splenic tissue. On teasing the spleen with fine needles these nodules separated readily, and on further dissection of the nodules themselves a hard, white, central core could be shelled out.

TABLE 4. INFLUENCE OF RECIPIENT AGE ON SPLENIC ENLARGEMENT

Age of embryo at inoculation	*Spleen weights in mgms. 6 days after inoculation*		
Days of incubation	*No. tested*	*Range*	*Average*
11	21	36-195	92
13	19	54-312	178
15	16	46-270	162
17	25	31-84	67
20	18	49-91	74

The spleens of the older (17-20 days) recipients showed smooth surfaces and symmetrical enlargement throughout, with none of the gross features as described above for the younger embryos. The differences in total weight and morphological appearance between the younger and older age groups cannot be ascribed merely to dilution of the inoculated cells in the larger blood volume of the older recipients. Although there is a four-fold increase in whole embryonic weight between the 12th and 18th days, ten-fold differences in cell concentration between the inocula given to younger and older embryos gave rise to results identical to those just described.

Histological findings. For detailed description of the embryology of the normal chicken spleen with particular reference to the hematopoietic cells the reader is referred to Sandreuter.[7] In brief, granulopoiesis is present in the spleen by the 10th embryonic day, reaching a peak of activity at 14-18 days and thereafter gradually declining. Lymphocytic activity can be noted in 12-day-old spleens but does not become pronounced until 16-18 days of embryonic life and increases after hatching. Erythropoiesis is seen in the embryonic spleen but after hatching this function is taken over entirely by the bone marrow. These various hematopoietic elements are present diffusely

within the spleen, and well-circumscribed, focal or nodular collections of large numbers of hematopoietic cells are not present during embryonic life. It is noteworthy that plasma cells are found neither in liver, spleen, bone marrow, nor peripheral blood during embryonic life and do not make their appearance in any of these sites until 20 days after hatching.

The histological appearance of the spleens from embryos inoculated with adult cells was again dependent on the age of the recipient at inoculation with marked differences being observed between embryos inoculated at 11-15 and 17-20 days of incubation. When 12-day embryos were used as recipients, the following sequence of events was seen: one day after inoculation the normal architecture of the spleen was not disturbed but large, actively mitotic cells with pyrinophilic cytoplasm were found scattered throughout the sinusoids. Differentiation between this picture and that of a normal spleen of the same age was not easily provided, however. Two days after inoculation occasional small focal collections of these cells could be seen. Figure 3 is a microphotograph of such a focal collection. Mitotic figures are present and the cells are surrounded by an eosinophilic collar. The genesis of the surrounding material cannot be stated with certainty but the picture could be consistent with early proliferation of an embolic focus within a degenerating arteriole. By the third post-inoculation day extensive replication occurred and large focal collections of basophilic cells were present, often large enough to be discerned grossly when near the surface of the spleen. An Unna-Pappenheim stain of this stage is shown in Figure 4, illustrating the marked difference in the staining characteristics as compared to the surrounding cells. A higher magnification of the edge of the same cell focus is shown in Figure 5. At this point the cells show no mitoses and the nuclei are undergoing pyknosis. The lack of significant reaction in the surrounding tissue is also apparent. Figure 6 is illustrative of the situation at five days after inoculation. By this point frank necrosis of the cell mass has begun. The necrosis proceeds from the periphery inwards, leaving structurally intact cells only in the centre. Around the periphery are radially arranged, vacuolated epithelioid cells. The origin of the cells is obscure, but in structure and staining characteristics they resemble far more closely the surrounding embryonic spleen cells rather than the cells in the centre of the mass. By seven days necrosis is almost complete, leaving a cavity filled with eosinophilic debris and only a few structurally intact cells. This stage is shown in Figure 7. It represents the point at which spleen size no longer increased.

Thus the sequence of events was initially active proliferation from a small focus of cells leading to large nodular collections and later peripheral reaction and necrosis with final complete cell destruction. The histological

features of the later stages are entirely what would be expected if the embryonic spleen were considered to be confining, localizing, and finally rejecting an implant of foreign tissue.

The histology of spleen from older recipients was similar to that described by Simonsen[8] and consisted primarily in the appearance of numerous actively mitotic basophilic reticuloendothelial cells. Disintegrating polymorphonuclear leukocytes were also found as were occasionally syncytial giant cell formations. The large foci of mononuclear cells with subsequent necrosis as described in the younger embryos were not found in spleens from recipients 17-20 days old at the time of inoculation.

TABLE 5. INFLUENCE OF INBREEDING ON SPLENIC ENLARGEMENT

Donor adult	*Recipient embryo*	*Spleen weights in mgms. 5 days after inoculation*
Inbred	Inbred	24, 12, 13, 20, 18, 24, 34, 24
	Noninbred	278, 166, 360, 250, 144, 190, 205
Noninbred	Inbred	148, 76, 90, 89
	Noninbred	209, 49, 78, 29, 178, 91

Influence of genetic disparity. Cock and Simonsen[4] were able to demonstrate that when cell transfer was performed between donor and recipient of highly inbred fowl lines, splenic enlargement was significantly less than when the same cells were inoculated into embryos of crossed lines. These workers used newly hatched chicks as recipients, however, at which age the type and degree of splenic enlargement is less pronounced.

In order to determine whether the findings in younger embryos are also influenced by genetic similarity, 10^7 spleen cells from an adult female inbred donor were inoculated intravenously into her own eggs, eggs from full sisters inseminated by the same cockerel, and also into nonrelated eggs of the same age. Eggs of the inbred strain were also inoculated with spleen cells from an unrelated donor. Results are given in Table 5. Inbred embryos receiving cells from a donor of the same strain showed very little splenic enlargement as compared to uninoculated eggs, while the same cell concentration from the same donor caused massive splenic enlargement in the non-

inbred recipients. That the inbred embryos could respond is shown by the spleen weights of inbred recipients given spleen cells from an unrelated donor. The spleens became enlarged to the same degree as those of noninbred embryos receiving the same cell inoculum. These results therefore confirm the finding of Cock and Simonsen and also extend them to include the type of splenic enlargement found in young embryonic recipients.

Possibilities of infection. The gross appearance of the spleen resembled the findings in certain instances of infection of the embryo with microorganisms. Intensive search by histological and cultural methods failed to reveal any bacterial, fungal, or parasitic contaminant either in the inocula or the recipient spleens. Viral diseases are more difficult to rule out. Forms of avian lymphomatosis are prevalent in many fowl flocks and may resemble many of the features described after cell transfer. The accumulated evidences of age specificity of both donor and recipient, requirement for viable cells, effect of inbreeding, absence of demonstrable lesions in the donors, and the correlation with antibody production to be presented in the following section all serve to make the possibility of transmission of virual leukosis unlikely.

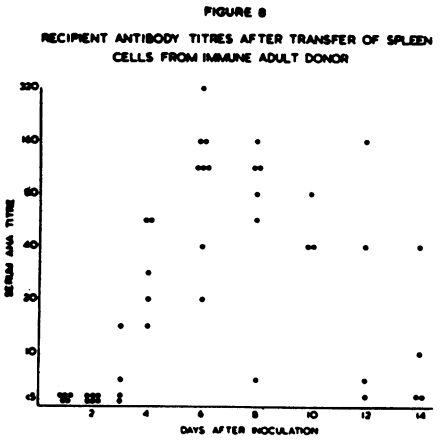
ANTIBODY FORMATION AFTER CELL TRANSFER

Transfer of spleen cells from immunized donors

Time course of antibody production. When the donor adult fowls were previously stimulated with heat-killed MEL virus, transfer of spleen cells to embryos resulted in the appearance of MEL antihemagglutinin (AHA) antibodies in the sera of the recipients. The time interval required for the appearance of these antibodies is shown in Figure 8. In this experiment a 10-week-old female donor was given two intravenous inoculations of 1.0 ml. of undiluted heated MEL virus with an interval of one month between inoculations. The fowl was sacrificed 24 hours after the second antigenic stimulus and inocula of 10^7 cells were transferred intravenously into 13-day-old embryos. Embryos and newly hatched chicks were sacrificed at the intervals shown and AHA titrations performed on the individual sera. As shown in the figure, no antibodies could be detected in the sera of recipients sacrificed on the first two days after inoculation. This complete absence of detectable antibodies for a 2-day period served effectively to rule out the possibility that any subsequent antibody was derived from passive transfer. Antibodies began to appear on the third day in low titres, reached the highest levels on the 6th day and thereafter showed a gradual decline, although levels could be detected as long as two weeks after inoculation. The curve

shows a general resemblance to the curve of spleen weights shown in Figure 2, but the antibody titres did not drop with the rapidity exhibited by the spleen weights.

Factors influencing antibody production after transfer. A procedure consisting of immunization of a donor, removal of spleen cells, and transfer to embryonic recipients obviously involves a number of complex steps, and the amount of antibody eventually found in the sera of the recipients is influenced by a wide variety of factors. The requirements of age and species



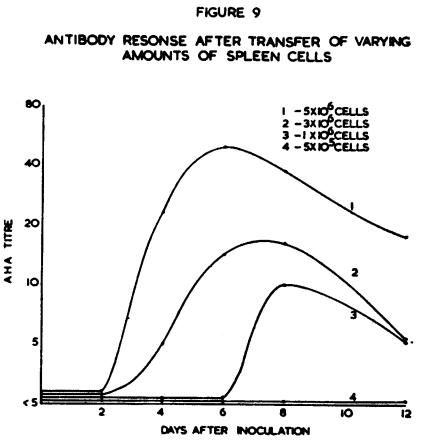


FIG. 8. Recipient MEL AHA titres after transfer of 10^7 spleen cells from an adult fowl immunized with heat killed MEL virus. Each dot represents a separate recipient.
FIG. 9. Recipient MEL AHA titres after transfer of varying amounts of spleen cells from an adult fowl immunized with heat killed MEL virus. Each dot represents an average of 5-8 determinations.

specificity and cell viability as shown in Table 2 for splenic enlargement also applied to the production of MEL AHA antibodies. Transfer of antibody production could not be obtained with heated or frozen and thawed cells, cells of different species, or cells from embryonic or neonatal donors.

The nature of the immunization of the donor, the presence or absence of antigen added to the spleen cells *in vitro* prior to transfer, and the age of the recipient embryo might also be expected to influence the degree of antibody response obtained. Table 6 summarizes the results from a series of transfer experiments designed to test these factors. The donor fowls were all females from 10 to 15 weeks old at the time of sacrifice. The concentrations of spleen cells were 5 to 6 x 10^6 per inoculum in all instances. In some cases heated MEL virus was added to the inocula to make a final concentration of 1:50.

When the donor fowl was given a primary intravenous or intraperitoneal stimulus and an intravenous booster after an interval of one month, transfer of spleen cells 24 hours after the second stimulus resulted in antibody formation in all recipients regardless of age or the presence or absence of antigen added *in vitro*. That the route of immunization was important was shown by the failure to transfer antibody formation when the second stimulus was given intraperitoneally. When the interval between stimuli of the donor was shortened to one week, the age of the recipient embryo assumed importance. Antibodies were found consistently and in a higher titre in embryos from 11-15 days at the time of inoculation as compared to recipients from 17-20 days of age. If no secondary stimulus was given *in vivo*, the addition of antigen *in vitro* was necessary and the influence of the age of the recipient became more important. In this instance only the younger recipients who had received cells plus antigen showed antibodies in their sera. When the donor fowl was sacrificed 12 hours after the primary stimulus, no antibodies could be detected in the sera of any recipients.

The results clearly indicate that younger embryos (11-15) served as more effective recipients for cellular transfer of antibody formation than did older (17-20 days) embryos. This division by age group follows closely the pattern of splenic enlargement by age group presented in the previous section. The data also show that antigen (MEL) could exert a marked stimulatory effect when added *in vitro* to previously stimulated cells although *in vivo* second stimuli were more effective.

The number of cells necessary to induce AHA antibody formation in recipients was determined by the inoculation of serial dilutions of spleen cells. The adult donor was given two intravenous stimuli of heat-killed MEL at a one-month interval, sacrificed 24 hours after the last stimulus, and spleen cells transferred to 13-day-old embryos. The results are shown in graphic form in Figure 9. Detectable antibodies were found in recipients receiving 10^6 or more spleen cells. The time of appearance of antibodies also showed a direct relationship to cell concentration. Antibodies appeared earlier and reached a more rapid peak in the recipients of the more concentrated cell suspensions.

A comparison of the relative effectiveness of spleen and peripheral leukocyte suspensions was also attempted by inoculating serial dilutions of these suspensions into 13-day embryos. While 10^6 spleen cells resulted in detectable antibody formation in the recipients, concentrations of 10^8 peripheral leukocytes per inoculum gave rise only to sporadic and low titred antibody levels in the recipients. This is in contrast to the results shown in Table 3 in which spleen and peripheral leukocyte suspensions appeared equally effective in initiating the phenomenon of splenic enlargement.

TABLE 6. FACTORS INFLUENCING ANTIBODY PRODUCTION IN RECIPIENTS AFTER CELL TRANSFER

Route of primary stimulus	*Interval between primary and secondary stimuli*	*Route of secondary stimulus*	*Interval between last stimulus and sacrifice*	*MEL* in vitro	*Number of recipients*	*Age of recipient embryo—days of incubation*	*Antibodies in recipients*
Intravenous or intraperitoneal	one month	intravenous	24 hours	present or absent	36	11-15	++
					49	17-20	++
Intravenous	one month	intraperitoneal	2-4 days	present or absent	14	17-20	0
Intravenous	one week	intravenous	24 hours	present or absent	28	11-15	++
					18	17-20	±
Intravenous	—	—	one month	present	27	11-15	++
					19	17-20	0
				absent	14	11-15	0
					12	17-20	0
Intravenous	—	—	12 hours	present or absent	16	11-15	0
					10	17-20	0

++ Antibodies detectable in all recipients. ± Antibodies detectable in less than half of recipients.

0 Antibodies not detectable in any recipients.

Transfer of cells from nonimmunized donors

Attempts at initiation of antibody formation were made by transfer of spleen cells from normal, nonimmunized adult donors with antigen added to the cell suspension *in vitro* immediately prior to inoculation. Cell concentrations were varied between 1 x 10^5 and 2 x 10^7 cells per inoculum. The concentration of antigen was varied between 10^{-1} and 10^{-5}, the dilutions representing heated allantoic fluid virus per unit of inoculating suspension. Dilution of the cell suspension in autologous and homologous sera and

TABLE 7. ATTEMPTED PASSAGE OF ANTIBODY-PRODUCING CAPACITY

Passage level	*Donor*	*Recipients*	*No. cells inoculated*	*AHA titres recipients at 5 days*	*Spleen weights recipients at 5 days*
1	immune adult fowl	13 d embryos	2 x 10^6	40, 120, 60 90, 20	148, 76, 52, 48, 60
2	1st passage recipients	13 d embryos	4 x 10^6	all less than 5	166, 22, 184, 46, 94, 36
3	2nd passage recipients	13 d embryos	4 x 10^6	all less than 5	101, 30, 89, 75, 59

concurrent administration of adult liver cells was also attempted. Both young and old age groups of embryos were used as recipients but the majority were in the 11-13-day age group since the previous data had indicated that this was the most sensitive age.

Of 328 recipient sera examined from experiments of this nature, only five showed detectable levels of MEL AHA antibody. The attempts were thus considered to be negative.

Attempts at serial passage of antibody formation

If functional replication of adult antibody-producing cells can occur within the spleen of a chick embryo, it would be reasonable to expect that these cells might then be serially passaged from embryo to embryo with demonstrable antibodies at each passage level. This possibility was tested by inoculating embryos with spleen cells from an immune donor, removing the recipient spleens after varying time periods, and inoculating those cells into further groups of embryos.

A representative example of these attempts is given in Table 7. Embryos of 13 days of incubation were inoculated with 4 x 10^6 spleen cells from an immune adult donor. After 4 days, serum AHA titres of the recipients

ranged from 30 to 240. At this point the recipient spleens were removed, a cell suspension containing 6 x 10^6 cells per 0.05 ml. prepared and inoculated into a further group of 13-day embryos. The spleens of this group of recipients showed comparable splenic enlargement, but no antibodies to MEL could be detected in their sera. One further passage again resulted in splenic enlargement of the same magnitude as the initial passage but antibodies remained undetectable.

A number of comparatively simple technical reasons could explain this failure to transfer antibody formation from embryo to embryo. The per cent of viable donor spleen cells which actually reach the recipient spleen after intravenous inoculation on first passage cannot be estimated with accuracy, but may be quite small. Thus an overwhelming dilution factor on first passage could be responsible for the failure of subsequent transfer. In an attempt to overcome this possible drawback, spleens were pooled from large numbers of first passage embryonic recipients and cell concentrations of up to 10^8 were inoculated into further embryonic recipients. Cell suspensions of this concentration are highly lethal to embryos, but of those surviving, none showed detectable levels of antibody.

Histological studies as shown in the previous section had indicated that some degree of cellular degeneration was apparent in recipient spleens as early as 3-4 days after inoculation of adult cells. Passage after too long an interval might therefore fail because of loss of cellular viability. For this reason second passage was attempted during 1-, 2-, and 3-day intervals after the initial cell transfer, again with negative results.

Results were also negative when passage was attempted in the presence of varying amounts of antigen and when normal adult fowl serum was added to the inoculum.

DISCUSSION

The postulated ability of adult reticulo-endothelial cells to colonize recipient and produce injurious antibodies against the antigens of the recipient has been termed the "donor vs. host" reaction. Phenomena of this general nature have been reported in various animal species using both embryos and irradiated adults as recipients of the implanted adult cells. Although the present study was not primarily designed to investigate this aspect, many of the findings confirm those of Simonsen[8] and lend further support to the thesis of immune response of donor to recipient. Firstly, enlargement of the recipient chick embryo spleen occurred only when cells potentially capable of producing antibodies were inoculated and it occurred only when cells were taken from a donor old enough to respond to antigenic

stimulation itself. Procedures which destroyed cells such as heating or freezing and thawing abolished the effect. When antigenic differences between donor and recipient were minimized by the use of inbred strains of chickens, transfer of spleen cells caused very little splenic enlargement in the recipient. Finally, this effect could be passaged from chick embryo to chick embryo indicating that the factor responsible for enlargement could undergo functional replication. These findings, together with Simonsen's demonstration that an antibody capable of coating the recipient red blood cells was found in the recipient, all suggest that the adult cells implanted in the recipient spleen were stimulated to reproduction and antibody formation by the antigenic differences of the recipient.

This cannot be considered a proven fact, however, for the crucial test of marking or tagging the implanted cells and subsequently identifying them as such in the recipient spleen has not yet been performed in this system. Thus the possibility remains open that the whole phenomenon represents inductive changes in the recipient spleen rather than multiplication of implanted adult cells. Such a mechanism has been postulated by Ebert,[5] and Andres[1] has presented more direct evidence of subcellular growth stimulating factors in the chick embryo. The histological features found in the present work after inoculation of younger age embryos speak against induction, however. At five days after inoculation the embryonic spleen gave the appearance of actively rejecting the cell focus. A reaction as marked and prompt as this would be difficult to imagine if the cell foci were considered to be originating as inductive response of the embryo's own spleen. Perhaps the simplest and most tenable way to avoid this apparent controversy between "induction" and "foreign cell proliferation" is to suggest that both mechanisms occur. Subcellular organ specific growth stimulating factors may be quite important in ontogeny, but may be relatively independent of the phenomena observed after intravenous inoculation of viable adult reticulo-endothelial cells.

Of particular interest in the present study is the marked influence of the age of the recipient embryo upon both splenic enlargement and cellular transfer of antibody production. Embryos of the younger age group (11-15 days) showed not only larger spleens after inoculation with adult spleen cells as compared to the older age group (17-20 days), but also showed the formation of foci of rapidly proliferating mononuclear cells large enough to cause gross nodularity of the spleen. There is nothing in the data to give conclusive reasons for this age difference, but the histological features found 5-7 days after transfer of adult cells to the younger embryos offer strong hints. As mentioned previously, at this point the histological picture is one of active rejection of the cell foci by the recipient spleen. This indicates that embryos as young as 16 days of incubation may possess at least one form of

protective response. It is not unreasonable to expect that this ability to reject foreign implants would show an increase in parallel with the age of the embryo. The reason for the differences in response according to age at inoculation could then be more readily explained. When embryos of the younger age group were inoculated with adult cells, the implanted cells were allowed to undergo almost unrestricted multiplication since the embryo at this age had very little ability to reject the cells. As the embryo aged, and the antigenic mass of the cell foci became large enough, a reaction occurred on the part of the host to the cell mass, and active destruction of the cell foci occurred. When embryos of an older age group were inoculated, multiplication of the implanted cells was more restricted because of the embryo's increasing ability to reject foreign cells and the large nodular foci of cells simply had no chance to form. Thus both the total weight and histological picture were different in recipients of the younger and older age groups.

The finding that MEL AHA antibodies could be transferred with greater ease to younger recipients is entirely consistent with this approach. When the donor fowl was sub-optimally stimulated (i.e. only one *in vivo* antigenic stimulus) and spleen cells plus MEL transferred to recipients of varying ages, antibodies appeared in only the younger age group recipients. It is very tempting to explain this on the same basis as given for the gross and histological differences by age group. It could be assumed that MEL AHA antibody-producing cells underwent functional replication in younger embryos, thereby producing enough antibody to be detectable in the sera. In the older embryos, antibodies did not appear because of the limited or absent functional replication of cells. While this explanation is consistent with the observed facts, it is also entirely possible that the observed differences in antibody production according to age are due to a longer life span of individual cells in the younger embryos rather than actual functional replication of the cells.

The number of cells required to induce splenic enlargement is of interest in view of Burnet's "clonal selection" hypothesis (1957). An essential feature of this theoretical approach is the requirement of clones of cells which are preadapted to produce only one specific antibody type. The presence of the corresponding antigen then allows for selective replication of these pre-existent antibody-producing cells in a clonal fashion. Since an adult fowl may respond to a wide variety of antigens, it would be necessary on the basis of this theory that a correspondingly wide variety of pre-adapted clones be represented in the fowl reticulo-endothelial system. Any single clone or cellular representative would then be present in very small proportion, and in order to insure the presence of any given cell type in an inoculum a large number of cells would have to be included.

In the present study, it was found that as few as 15,000 peripheral leucocytes could initiate this phenomenon. Of these 15,000, many are of a cell type (polymorphonuclear) not usually considered to be involved in antibody production. A certain proportion may have lost viability during manipulation and of the viable cells the number which actually reach the recipient spleen is undoubtedly further reduced because of filtration in lungs, bone marrow, and other chick organs. Therefore the number of cells required to initiate splenic enlargement is too small to be entirely consistent with all aspects of the clonal selection hypothesis.

When normal, nonimmunized adult fowl spleen cells were mixed with MEL *in vitro* and inoculated into young embryos, detectable levels of MEL AHA antibodies could not be consistently found in the sera of the recipients. A number of possible explanations could be advanced to account for this failure. It should be noted, however, that Trnka[10] has succeeded in initiating antibody production in newly hatched chicks after inoculation of adult fowl spleen cells exposed *in vitro* to bacterial antigens. The failure to initiate MEL antibody production *in vitro* in the present work may therefore be related to the type of antigen used and/or a lack of sensitivity of the test system, and a more elaborate explanation need not necessarily be invoked.

The failure to transmit MEL AHA antibody formation for more than one passage may be due either to inadequacies of the test system employed or to the fact that antibody-producing cells are "end cells" which either do not replicate at all or else lose functional ability on replication. The data presented offer no means of choosing between these alternatives. The fact that the phenomenon of splenic enlargement, which is considered to be an antigen-antibody reaction, can be serially passaged suggests that under certain conditions antibody-producing cells can retain immunological competence on replication in a foreign situation. A more definite answer will be obtained when serial passage is attempted with a wider variety of antigens and experimental recipients.

SUMMARY

When adult fowl spleen cells were inoculated intravenously into chick embryos from 11 to 20 days of incubation, the spleens of the recipient embryos became massively enlarged. The enlargement bore an inverse relationship to age with the younger recipients showing the largest spleens. Present in the spleens of only the younger embryos were large foci of mononuclear cells. These foci passed through several stages, initially showing active cellular proliferation, later peripheral reaction and cell destruction, and finally complete necrosis. This sequence of events could also be demon-

strated by the inoculation of leukocytes from the peripheral blood. When a highly inbred strain of chickens was used, transfer of spleen cells from the adult donor to embryos of the same strain resulted in very little splenic enlargement in comparison to inoculation of the same cells into noninbred embryos of the same age.

When the donor fowl had been previously immunized with heat-killed influenza virus (MEL), transfer of spleen cells to embryos resulted in the appearance of MEL antihemagglutinating antibodies in the sera of the recipients. The per cent of recipients showing antibody and the concentration of antibodies in their sera were dependent on the route of stimulation of the donor, the number of antigenic stimuli, the time interval between the last stimulus and removal of the donor spleen, and the presence or absence of antigen added *in vitro* to the cell suspension prior to inoculation. The age of the recipient embryo was also of importance with the younger (11-15 days) embryos serving as more effective recipients than the older (17-20 days) embryos. When MEL was added *in vitro* to normal, nonimmunized spleen cells prior to transfer, no antibodies were detected in the sera of recipients of any age group or genetic constitution.

Attempts at passage of antibody-forming capacity were made by removing the spleens from embryos showing high titres of MEL antibodies and inoculating the cells into further groups of embryos. Splenic enlargement and focal cellular proliferation were transferable without loss through several passages, but the ability to produce specific MEL antibodies was lost after the first passage.

Results are discussed with particular reference to their bearing on theories of antibody formation.

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