Immunology, 1975, **29**, 497.

Immunological Capacity of the Chicken Embryo

I. RELATIONSHIP BETWEEN THE MATURATION OF LYMPHOID TISSUES AND THE OCCURRENCE OF CELL-MEDIATED IMMUNITY IN THE DEVELOPING CHICKEN EMBRYO

B. D. Janković, Katarina Isaković, M. L. Lukić, N. L. Vujanović, Spomenka Petrović and B. M. Marković

Immunology Research Centre, Belgrade, Yugoslavia

(*Received 29th March* 1974; *accepted for publication 4th March* 1975)

Summary. In an investigation of the ontogeny of lymphoid tissue in chick embryos to relate maturation of lymphocytes with immunological competence, the numbers and sizes of lymphocytes were determined in the thymus, bursa of Fabricius, spleen, femoral marrow and peripheral blood of embryos from the 12th to 21st day of incubation, and in 6-day-old chicks. Results showed the thymus to be the first fully developed and most active lymphocytopoietic organ, followed by the bursa. The bone marrow was not lymphocytopoietic; the spleen and bone marrow were mainly granulocytopoietic and erythropoietic; some morphological differences between thymic and bursal lymphocytes were shown by light microscopy. It appears that in embryos and young chicks the lymphocytes are derived from the thymus and bursa, but not the bone marrow.

In tests of immunological competency, cells of the thymus, bursa, spleen, bone marrow and peripheral blood from 12–21-day-old embryos and 6-day-old chicks were transferred to chorioallantoic membranes of 12-day-old recipient embryos. There were distinct differences between the ability of various lymphoid tissues to induce formation of chorioallantoic pocks or splenic enlargement. The thymus, spleen and peripheral blood elicited both lymphocytic pocks and splenomegaly, the bursa elicited splenomegaly only, and the bone marrow was ineffective. The bone marrow, however, induced formation of nonlymphocytic pocks. It is concluded that the immunological activity of the chicken embryo is primarily effected by the thymus and bursa and that cell-mediated immunity appears in the 2nd week of incubation.

INTRODUCTION

Lymphocytes are considered essential for normal immunological function. However, in order to gain an understanding of the immune system it is necessary to study its development in relation to changes in morphological features and immunological activity. Among the developments and changes requiring study are possible quantitative and qualitative variations in lymphocytes and the exchange of primordial cells between lymphocytes and lymphoid tissues. It is also necessary to define the phenomena controlling the formation of

Correspondence: Dr Branislav D. Janković, Immunology Research Centre, P.O. Box 979, Vojvode Stepe 458, 11000 Belgrade, Yugoslavia.

even the smallest group of lymphoid cells capable of immunological activity. Despite some careful work (Solomon, 1971) there is still little information about the ontogeny of the immune response.

The chicken embryo is a rapid, experimental model for investigations of the appearance, distribution, maturation and functional expression of lymphocytes. Since the formation of chorioallantoic pocks (Boyer, 1960) and splenic enlargement (Howard, Michie and Simonsen, 1961) following treatment with allogeneic cells is generally thought to be unequivocal evidence of immunological competence of the donor cells, and reflect the number of effector cells (Seto and Allbright, 1965; Longenecker, Sheridan, Law and Ruth, 1970), it seemed appropriate to carry out parallel studies of lymphoid cell and lymphocyte development and of immunological competence. Thus, the first part of this paper reports the numbers of lymphocytes in lymphoid tissues of 12–21-day-old embryos and of young chicks. The second part reports the ability of thymus, bursa, spleen, bone marrow and peripheral blood cells of the various embryos and chicks to induce specific lesions in the chorioallantoic membrane and spleen. Special attention was paid to the relation between lymphocytes and the immunological activity of the embryonic thymus, bursa and bone marrow. In order to avoid possible confusion resulting from the source of the lymphoid cells (Janković, Isaković and Petrović, 1973) the following terminology was adopted: T (thymus-derived), Bu (bursa-derived), Bo (avian bone marrow-derived) and B (mammalian bone marrow-derived) cells.

MATERIALS AND METHODS

Embryos and chicks

White Rock eggs, obtained from 'Soko Salas' Poultry Station, Belgrade, were placed in incubators at 38–38·5° and at 65–70 per cent humidity within 5 hours of being laid. Embryos and chicks from these eggs were used in the tests.

Preparation of cells from thymus, bursa and spleen

Normal embryos were killed after 12, 14, 16, 19 and 21 days of incubation and chicks when 6 days old. Thymic lobes, bursa and spleen were carefully dissected, freed from surrounding tissue, weighed on a Mettler analytical balance, and minced separately over a stainless steel mesh. The cells were collected in Hanks's solution enriched with fresh normal chicken serum, pH 7·2, and the suspension of cells thus obtained was centrifuged at 700 rev/minute for 10 minutes. The supernatant fluid was discarded and cells resuspended in 2 ml of Hanks's solution. The number of cells in suspension was determined by using a Spencer haemocytometer, and the number of cells per milligram of original tissue sample was then calculated.

Preparation of cells from bone marrow

Marrow from both femurs was gently forced out with Hanks's solution under pressure into a previously weighed tube. The cells were dispersed by agitating the tube on a shaker for 2 minutes, and were counted as described previously (Burke and Harris, 1969; Janković *et al.*, 1973). This method satisfactorily revealed changes in the total cell number, and differential lymphocyte counts in 12–21-day-old embryos.

For morphological examination and differential cell counts, smears of thymus, bursa, spleen and bone marrow cells were prepared by brushing cell suspension on slides with a

Talens brush number 000, and stained with May–Grünwald and Giemsa. The same staining procedure was employed for smears of peripheral blood obtained from the chorio-allantoic vessels.

Differential lymphocyte counts

Differential cell counts were based on at least 5000 cells per tissue sample. The criteria of Lucas and Jamroz (1961) were used to distinguish lymphocytes from erythropoietic, granulocytopoietic, thrombocytopoietic, osteogenic and reticular cells and undifferentiated blast cells. The classification of lymphocytes was based on their diameters: small ($<6{\cdot}5$ μm), medium (6·5–9·0 μm) and large ($>9{\cdot}0$ μm). Smears were examined independently by three observers. Differential cell counts of smears from suspended cells provided more reproducible results than counts of cells in tissue sections. Results from some embryos and young chicks were omitted from the tests because the smears were unsatisfactory.

Host and donors

Fertile eggs obtained from a single flock of White Rock hens were used as recipients on the 12th day of incubation, whereas fertile eggs from another flock of White Rock hens served as donors of cells on days 14, 16, 19 and 21 of incubation. In addition, 6-day-old chicks were used as donors. No attempt was made to discriminate between female and male embryos. However, the antigenic disparity between two flocks of hens was demonstrated by a cross-skin grafting procedure. Birds of both flocks rejected skin grafts within 7 days.

Preparation of recipient embryos for inoculation

Twelve-day-old embryos were candled and the position of the chorioallantoic membrane (CAM) was marked. Separation of shell membrane from the chorioallantois and cutting of a 6×10 mm window were carried out as described by Ballantyne (1959). Embryos showing any abnormalities were not included in the experiment.

Preparation of cell inocula

Cell suspensions from the thymus, bursa, spleen and bone marrow of 12-, 14-, 16-, 19- and 21-day-old embryos, and from 6-day-old chicks were prepared as described above. Samples of chorioallantoic blood of embryos and venous blood from 6-day-old chicks were collected in sterile Hanks's solution containing 5 u/ml of heparin (1 volume of blood per 9 volumes of Hanks's solution). All cell suspensions were free of cell aggregates.

Thymus, bursa, spleen, bone marrow and peripheral blood cells were washed once in Hanks's medium containing 5 per cent inactivated normal chicken serum, 1 u/ml of heparin, and 100 u/ml of penicillin, then centrifuged at 700 rev/minute for 10 minutes and resuspended in enriched Hanks's solution. Viable cell counts were made with the aid of trypan blue. Each aliquot of cell suspension was smeared and stained with May–Grünwald and Giemsa for differential cell counts.

Induction of pock formation

Usually, the cell suspension from a single donor was inoculated into a single recipient embryo. Donor embryos younger than 14 days were not included in the experiment because insufficient spleen and bone marrow cells were obtained for inoculation. The method of inoculation was essentially that described by Longenecker *et al.* (1970). 2×10^5

viable cells were placed directly onto the epithelial surface of exposed CAM. The window in the shell was sealed off with sterile cellophane immediately after the inoculation, and the implanted eggs were reincubated for 4 days. The whole operation was performed on eggs transilluminated by means of a candling box and under sterile conditions. Sham-inoculated control eggs were treated in an identical manner except that they received 0·1 ml of sterile Hanks's solution on the CAM.

Counting of pocks and histology

For macroscopic inspection and histology, the inoculated eggs were opened on the 4th postoperative day, i.e. on the 16th day of incubation. The CAM was excised and washed, and nodular lesions were counted under a stereomicroscope. Then the CAM was placed in Carnoy's fixative and sections in the area of pocks were prepared by standard techniques, and stained with haematoxylin and eosin, or methyl green and pyronin. Differential cell analysis of CAM infiltrates included 85–90 per cent of pock cells. Those inoculated eggs which were not suitable for the counting of pocks because of the presence of confluent pocks were not included in the experiment.

Spleen index

The body and spleen weights of host embryos were recorded on the day of killing. The relative spleen weight (milligrams of spleen/10 g of body weight) was determined. The index of spleen enlargement for the group was calculated by dividing the mean relative spleen weight of each experimental group by the mean relative spleen weight of sham-inoculated controls. The presence of graft-versus-host reaction (GVH) was indicated by a spleen index greater than 1·30 (Howard *et al.*, 1961).

RESULTS

ANALYSIS OF CELLS OF THE DEVELOPING THYMUS, BURSA, SPLEEN, BONE MARROW AND BLOOD

An analysis of the results of the total number of cells and appearance of lymphocytes in various lymphoid tissues from the 12th day of incubation onwards (Table 1) showed that the thymus became a lymphocytic organ before the bursa. On the 12th day of incubation the thymus contained 86 per cent lymphocytes, whereas it was not until the end of the incubation period that the bursa contained a similar number. Lymphocytes were more numerous in the spleen immediately before and just after hatching, but they were never as numerous as in the thymus and bursa. Few lymphocytes were found in the bone marrow.

The principal feature of lymphoid tissue maturation, as revealed by micrometer cell measurements, was the increasing number of small lymphocytes, which were particularly numerous in the thymus, and then in the bursa. In contrast to these tissues, the number of small lymphocytes in the bone marrow remained small in both embryos and young chicks. Differential counts of embryonic white blood cells (Table 2) showed that there are relatively few lymphocytes in the peripheral blood, though there is a great increase in number in the 1st week after hatching.

Some morphological differences were seen between T and Bu cells. Usually all types of lymphocytes in the thymus were smaller than the corresponding bursa cells. Moreover, cytoplasmic basophilia was more obvious in T lymphocytes.

Granulocytes were only occasionally seen in the thymus, though they occurred regularly

in the spleen, bone marrow and bursa, and the spleen and bone marrow appeared to have similar granulocytopoietic activity at 12–16 days of incubation. The few liver preparations examined showed that this organ was not an important source of lymphocytes.

TABLE 1

TOTAL CELL AND LYMPHOCYTE COUNTS OF THYMUS, BURSA, SPLEEN AND BONE MARROW (CELLS/MG OF TISSUE × 1000 ± s.e.) OF EMBRYOS FROM 12–21 DAYS OF INCUBATION AND OF 6-DAY-OLD CHICKS

Age (days)	Total cells	Lymphocytes			
		Total	Large	Medium	Small
		Thymus			
Embryos					
12	1214 ± 173	1048·9 ± 166·4	170·5	360·5	517·9
14	1431 ± 158	1287·8 ± 195·0	139·5	344·9	803·4
16	1769 ± 243	1541·6 ± 295·9	85·7	271·3	1184·6
19	2646 ± 326	2516·6 ± 439·1	37·7	74·2	2404·7
21	3028 ± 395	2967·4 ± 504·8	18·2	84·5	2864·7
Chicks					
6	3160 ± 317	3065·2 ± 218·9	56·4	145·4	2863·4
		Bursa			
Embryos					
12	510 ± 62	52·8 ± 7·2	6·2	23·2	23·4
14	504 ± 69	143·7 ± 16·6	13·8	52·9	77·0
16	455 ± 35	322·5 ± 37·8	38·5	129·5	154·5
19	848 ± 56	652·9 ± 47·2	84·8	220·5	347·6
21	1310 ± 121	1048·0 ± 44·8	68·8	296·8	682·4
Chicks					
6	1504 ± 256	1273·6 ± 156·1	81·5	391·7	800·4
		Spleen			
Embryos					
12	682 ± 72	8·6 ± 0·5	0·7	2·3	5·6
14	841 ± 50	10·1 ± 1·3	1·1	2·5	6·5
16	732 ± 60	19·3 ± 1·6	2·4	5·6	11·3
19	738 ± 91	52·5 ± 6·2	3·2	7·8	41·5
21	1052 ± 66	177·0 ± 14·1	8·8	32·2	136·0
Chicks					
6	726 ± 21	448·9 ± 18·2	50·5	136·6	261·8
		Bone marrow			
Embryos					
12	n.d.	n.d.	n.d.	n.d.	n.d.
14	188 ± 24	3·4 ± 0·5	0·1	1·1	2·2
16	576 ± 42	11·7 ± 0·9	1·5	3·9	6·3
19	680 ± 52	14·1 ± 1·0	1·9	5·4	6·8
21	1476 ± 78	30·0 ± 1·7	5·0	9·7	15·3
Chicks					
6	1393 ± 223	34·1 ± 2·0	3·8	10·9	19·4

n.d. = Not determined.

LYMPHOCYTES IN THE INOCULUM

Cell preparations from the thymus and bursa contained many more lymphocytes than those from the spleen, bone marrow and peripheral blood (Table 3). The number of lymphocytes from the bone marrow of 6-day-old chicks was only 1/23rd and 1/20th of the number from the thymus and bursa respectively. The small lymphocytes were more numerous than other types of lymphocytes in the thymus, bursa and spleen.

INCIDENCE OF POCKS

Cells from thymus, bursa, spleen and bone marrow induced formation of pocks on the CAM of recipients, even when taken from 14-day-old donor embryos (Table 4). The number of pocks increased with the age and, therefore, maturation of the donors, the cells of 6-day-old chicks being the most active.

TYPES OF POCKS

It was considered necessary to examine the pocks histologically to relate the results with differences in lymphocytes in the lymphoid tissues (Tables 1 and 2) and differences in the number of lymphocytes in the inocula (Table 3) which may also contain haematopoietic colony-forming cells (Metcalf and Moore, 1971).

TABLE 2

DIFFERENTIAL COUNTS OF BLOOD LYMPHOCYTES OF EMBRYOS FROM 14–21 DAYS OF INCUBATION AND 6-DAY-OLD CHICKS

Age (days)	Number of lymphocytes/100 cells*			
	Total	Large	Medium	Small
Embryo				
14	2·3±0·49	0·3	0·7	1·3
16	3·4±0·45	0·4	1·5	1·5
19	5·6±0·68	1·0	2·5	2·1
21	8·9±1·13	2·7	2·4	3·8
Chick				
6	20·6±3·76	3·5	7·8	9·3

* Mature erythrocytes and thrombocytes are not included. Each age group represents average of ten to fourteen embryos or young chicks.

The pocks (Table 5) that contained 60–70 per cent of mature and immature lymphocytes were designated lymphocytic (L) pocks, and were thought to represent a typical graft-versus-host reaction. The second type of pock was composed of granulocytic cells (G pocks), the third of immature erythrocytes (E pocks), the fourth of thrombocytes in various stages of maturity (T pocks) and the fifth type of pock was of mixed cellular composition (Mix pocks).

Most of the L pocks were induced by inoculation of thymus, spleen and peripheral blood cells, the thymus being the most effective. Pocks of the G and Mix types were induced by bursal cells, and pocks of the G, E and Mix types by bone marrow cells. T pocks were induced by bursa, spleen, bone marrow and peripheral blood cells. Thus there was a great difference between the activity of the thymus, spleen and peripheral blood, and that of the bursa and bone marrow. An analysis of the data of the number of pocks and of the type of lymphocytes in the inoculum, indicates that medium and small lymphocytes probably induced formation of L pocks. The number of lymphocytes necessary to induce formation of one L pock depended upon the age of the donor and source of cells (Table 4).

SPLENOMEGALY

The splenomegaly-inducing ability appeared on the 14th day of incubation in the thymus and bursa, on the 16th day in the spleen, and about the 19th day in the peripheral

TABLE 3

MEAN NUMBER OF LYMPHOCYTES ($\times 10^3$) PER 2×10^5 OF INOCULATED CELLS

Age of donor (days)	Thymus				Bursa				Spleen				Bone marrow				Blood			
	Total	L	M	S	Total	L	M	S	Total	L	M	S	Total	L	M	S	Total	L	M	S
Embryo																				
14	161·2	17·5	43·1	100·6	57·1	5·5	21·0	30·6	2·5	0·2	0·7	1·6	3·6	0·1	1·2	2·3	4·6	0·4	1·8	2·4
16	191·4	10·6	33·5	147·3	152·4	18·4	56·2	77·8	5·6	0·7	1·6	3·3	4·6	0·6	1·5	2·5	6·6	0·6	2·8	3·2
19	193·3	2·9	5·7	184·7	162·4	21·3	54·7	86·4	14·2	0·9	2·1	11·7	4·7	0·7	1·8	2·2	10·4	1·6	4·2	4·6
21	194·0	1·2	5·6	187·2	165·1	11·4	45·2	108·5	34·3	1·7	6·4	26·2	4·7	0·8	1·5	2·4	18·6	5·0	5·6	8·0
Chick																				
6	195·3	3·6	9·2	182·5	163·2	9·8	52·3	101·1	123·5	13·9	37·6	72·0	7·8	0·8	2·6	4·4	42·2	7·2	16·2	18·8

L = large; M = medium; S = small lymphocytes.

blood (Table 4). The ability of the donor thymus, bursa, spleen and blood to evoke enlargement of the host's spleen increased with age. However, the bone marrow cells were quite inactive since significant splenic reactions were observed only in two out of thirty-nine recipients. An interesting observation was the particular ability of bursal cells to induce splenomegaly.

TABLE 4

POCK FORMATION AND SPLENOMEGALY IN EMBRYOS INOCULATED WITH THYMUS, BURSA, SPLEEN, BONE MARROW AND PERIPHERAL BLOOD CELLS FROM EMBRYOS AND YOUNG CHICKS

Donor		Host					
		Pocks				Splenomegaly	
Source of cells	Age (days)	Number of hosts	Number of hosts with pocks	Number of pocks/10^6 cells (mean ± s.e.)		Number of hosts with spleen index > 1·30	Spleen index for group
				Total	L pocks		
Thymus							
Embryo	14	7	3	8·33 ± 3·00	5·60	1	1·02
	16	10	5	6·25 ± 1·07	5·10	2	1·09
	19	10	8	18·20 ± 3·35	16·00	6	1·56
	21	8	8	26·42 ± 5·60	24·00	6	1·53
Chick	6	7	7	28·00 ± 5·50	25·80	5	1·32
Bursa							
Embryo	14	6	2	5·50 ± 2·02	0	1	1·05
	16	9	2	7·50 ± 1·77	0·15	2	1·23
	19	7	2	7·50 ± 0·50	0·75	2	1·79
	21	7	5	11·66 ± 4·00	1·63	4	1·46
Chick	6	6	6	15·83 ± 3·80	2·84	4	1·34
Spleen							
Embryo	14	9	5	8·33 ± 1·50	3·67	0	1·03
	16	8	6	10·83 ± 1·41	5·40	1	1·13
	19	6	4	11·00 ± 2·75	6·30	1	0·98
	21	7	7	25·71 ± 7·07	14·40	4	1·46
Chick	6	6	6	44·16 ± 9·87	32·20	4	1·38
Bone marrow							
Embryo	14	9	5	6·25 ± 1·07	0	0	0·95
	16	10	6	8·75 ± 4·77	0·70	0	1·06
	19	8	5	10·00 ± 1·79	0·70	0	0·98
	21	6	5	16·00 ± 2·19	1·40	1	1·07
Chick	6	6	5	21·00 ± 3·58	3·36	1	0·96
Blood							
Embryo	14	9	2	5·00 ± 0·50	0·70	0	0·83
	16	9	3	6·00 ± 0·50	0·90	0	1·08
	19	7	4	15·00 ± 0·75	5·70	2	1·41
	21	5	4	27·50 ± 4·15	10·70	4	1·50
Chick	6	8	8	60·00 ± 4·17	33·00	4	1·35

Histologically, enlarged spleens of 16-day-old recipient embryos contained circumscribed cellular changes of various size and intensity. These areas were composed of a large number of primitive reticulum cells, and of scattered deeply basophilic blasts, granulocytes (some necrotic), lymphocytes and macrophages. In some instances, the central portion of the lesion was a necrotic focus surrounded by multinuclear giant cells.

DISCUSSION

Two findings are of particular interest. The first is the difference in time of appearance and the number of lymphocytes in the embryonic thymus, bursa and bone marrow, and

TABLE 5

MORPHOLOGY OF POCKS PRODUCED BY THYMUS, BURSA, SPLEEN, BONE MARROW AND PERIPHERAL BLOOD CELLS

Age of donor	Thymus						Bursa						Spleen						Bone marrow						Blood					
	Number of pocks typed	Mean percentage of pocks					Number of pocks typed	Mean percentage of pocks					Number of pocks typed	Mean percentage of pocks					Number of pocks typed	Mean percentage of pocks					Number of pocks typed	Mean percentage of pocks				
		L	G	E	T	Mix		L	G	E	T	Mix		L	G	E	T	Mix		L	G	E	T	Mix		L	G	E	T	Mix
Embryo																														
14	14	67	26	0	0	7	7	0	38	0	18	44	32	44	22	26	3	5	14	0	14	36	21	29	7	14	15	14	14	43
16	16	82	9	5	0	4	13	2	37	10	19	32	32	50	19	24	3	4	37	8	27	29	9	27	14	15	25	15	15	30
19	74	88	4	4	0	4	15	10	31	9	14	36	28	57	28	7	7	1	29	7	25	26	15	27	21	38	6	14	2	40
21	90	91	4	2	0	3	48	14	32	12	10	32	83	56	33	4	6	1	60	9	13	45	14	19	39	39	18	8	17	18
Chick																														
6	96	92	6	0	0	2	47	18	30	10	0	42	107	73	15	6	4	2	62	16	9	53	9	13	57	55	23	10	5	7

L pocks mainly composed of lymphocytes; G pocks mainly composed of granulocytic cells; E pocks mainly composed of immature erythrocytes; T pocks mainly composed of thrombocytes; Mix pocks, mixed cellular composition.

the second is the appearance in these same organs of lymphocytes able to induce a graft-versus-host reaction. In previous work (Lukić, Vujanović and Janković, 1973; Vujanović, Lukić and Janković, 1973) we found large lymphocyte-like cells in thymic and bursal primordia of 6–7-day-old chicken embryos, and typical lymphocytes were always found in the thymus and bursa on the 8th day of incubation. The lymphocytes were first seen in the spleen and bone marrow on the 11th day. These findings are extended in the present work when differences in the number, size and type of lymphocytes were seen between the thymus, bursa, spleen, bone marrow and peripheral blood in embryos later than 11 days.

The most intriguing finding is the very active lymphocytopoietic activity in the thymus and bursa, and its absence from the bone marrow of embryos and young chicks, and since the bone marrow contains few lymphocytes (Table 1) it probably contributes little or nothing to the total lymphocyte pool, and those lymphocytes found in the marrow may be immigrant lymphocytes from other tissues. This contention may be questioned, because, though the marrow may contain few mature lymphocytes, it is the source of the lymphocyte stem cells. However, when considering the chick embryo bone marrow to be the source of stem cells, it should be remembered that the 'primitive bone marrow' of the 9th day embryo completely lacks haematopoietic activity (Dantschakoff, 1909). Consequently, at that stage it is very unlikely that stem cells migrate from the bone marrow to the thymic and bursal rudiments. Furthermore, though the morphology of the stem cells is uncertain, which hinders their identification (Metcalf and Moore, 1971) it seems that the first stem cells arise extravascularly from the immediate vicinity of the yolk sac blood vessels of 3–4-day embryos (Dantschakoff, 1908). These stem cells have been designated 'large lymphocytes' or haemocytoblasts (Maximow, 1924), or 'large basophilic cells' (Owen, 1970). They enter the thymic rudiments between 6th and 7th days of incubation (Owen, 1970), i.e. at the time when the bone marrow hardly exists. Despite this evidence, there is some suggestion that in the adult chicken the bone marrow, as an 'embryonic island', may become a source of stem cells which migrate to the thymus and bursa.

Chick embryo lymphoid tissues induced various graft-versus-host reactions. Although the B groups were unknown when pairing the donor and recipient embryos (Craig and McDermid, 1963), there was small variation of results within each group. Since the formation of pocks on the chorioallantoic membrane depends upon the interaction between donor lymphocytes and allogeneic embryonic cells (Coppleson and Michie, 1966; Lafferty and Jones, 1969) the formation of L pocks can be accepted to be a graft-versus-host reaction of 'allogeneic type'. One pock is probably derived from the proliferation of a single donor lymphocyte (Coppleson and Michie, 1966), and a relation has been established between the number of small lymphocytes in the inoculum and the number of pocks in the chorioallantoic membrane (Shortman and Szenberg, 1969). The data presented in this report are further evidence that small lymphocytes are more active than large lymphocytes in forming L pocks.

The thymus, bursa, spleen, bone marrow and blood elicited different pock responses, the bone marrow and bursa being almost incapable of forming L pocks. The almost total lack of lymphocytes in the embryonic bone marrow may account for this failure. It is pertinent to record that the role of B cells in cell-mediated immunity in mammals is controversial, in that some persons believe that a graft-versus-host reaction in irradiated recipients requires donor T and B cells, though the T cells effect the change (Hilgard, 1970); other persons do not accept that synergism occurs between T and B cells in this reaction (Cantor and Asofsky, 1970) or that participation of B cells in the thymus–bone marrow co-operation is

non-specific (Lonai and Feldman, 1970). Previous work had shown that thymus cells taken just before hatching are able to induce graft-versus-host reactions (Owen, Mawdsley and Harrison, 1964); it has now been shown that thymus and spleen cells can induce nodular foci as early as the 14th day of incubation. In contrast, the bursa, though containing numerous lymphocytes, induced very few L-type pocks. This may be due to a lack of formation of pock-forming lymphocytes in the bursa, or infiltration of active lymphocytes from the blood, and probably reflects a separation of immune responsiveness of the bursa from the thymus before the 14th day.

The lymphocytes of 14–16-day donors have the ability to induce splenomegaly in recipients. It has been shown that splenomegaly results from proliferation of donor-used recipient cells (Owen, Moore and Harrison, 1965) and the donor and recipient proliferations are interdependent (Biggs and Payne, 1959). The possibility that donor cells may proliferate at sites other than spleen and chorioallantoic membrane has not yet been tested. The splenomegaly-inducing activity of the bursa, and lack of it in bone marrow cells, may be due to a selective migration of circulation lymphocytes into the bursa, but not the bone marrow (Linna, Bäck and Hemmingsson, 1971), or due to different cell lines inducing formation of L pocks and splenomegaly (Tigelaar and Asofsky, 1973). In the mature bird, when Bo cells also participate, the various lymphoid tissues differ in immunological activity (Janković *et al.*, 1973). The thymus, containing many T cells, is primarily involved in cell-mediated immunity, the bursa and bone marrow containing Bu and Bo cells respectively are mainly active in humoral immunity, and the spleen, containing T, Bu and Bo cells (Linna *et al.*, 1971) participates in both cellular and humoral immune responses.

The stimuli to formation of the non-lymphocytic G, E, T and Mix pocks are not known. They could result from the colony-forming activity of haematopoietic stem cells (Metcalf and Moore, 1971). If the stem cells of embryonic bone marrow are multipotential, it would be anticipated that some would have differentiated into lymphocytic cells and induced formation of L pocks, but few L pocks were formed, thus confirming earlier evidence that avian embryo bone marrow is mainly committed to formation of granulocytes and erythrocytes (Lucas and Jamroz, 1961). If it is assumed that pocks were formed by proliferation of recipient cells (Weber, 1970), then the donor bone marrow cells were unable to induce the formation of lymphocytic pocks in recipients. Thus, formation of non-lymphocytic pocks by bone marrow cells can be accepted as further evidence of the non-lymphocytic function of the avian embryonic bone marrow and its non-participation in cell-mediated immunity of the graft-versus-host type.

ACKNOWLEDGMENTS

This work was supported by grants from the Republic Research Fund of SR Serbia, Belgrade.

REFERENCES

Ballantyne, D. L. (1959). 'Modification of technique used for tissue-grafting of the chorioallantoic membrane of the chick embryo.' *Transplant. Bull.*, **6,** 110.

Biggs, P. M. and Payne, L. N. (1959). 'Cytological identification of proliferating donor cells in chick embryos injected with adult chicken blood.' *Nature (Lond.)*, **184,** 1594.

Boyer, G. (1960). 'Chorioallantoic membrane lesions produced by inoculation of adult fowl leucocytes.' *Nature (Lond.)*, **185,** 327.

BURKE, W. T. and HARRIS, C. (1959). 'Total cell counts of the bone marrow of normal albino rats from 1 to 50 weeks of age.' *Blood*, **14,** 409.

CANTOR, H. and ASOFSKY, R. (1970). 'Synergy among lymphoid cells mediating the graft-versus-host response. II. Synergy in graft-versus-host reactions produced by Balb/c lymphoid cells of different origin.' *J. exp. Med.*, **131,** 235.

COPPLESON, L. W. and MICHIE, D. (1966). 'A quantitative study of the CAM reaction in the chick embryo.' *Proc. roy. Soc. B*, **163,** 555.

CRAIG, J. V. and MCDERMID, E. M. (1963). 'Prolonged skin homograft survival and erythrocyte (B locus) antigens in young chicks.' *Transplantation*, **1,** 191.

DANTSCHAKOFF, W. (1908). 'Über der Blutbildung im Dottersack des Hühnchens.' *Verhandl. anat. Ges. Ber.*, **22,** 72.

DANTSCHAKOFF, W. (1909). 'Über die Entwickelung des Kockenmarks bei den Vögeln und dessen Veranderungen bei Blutentziehungen und Ernährungstorungen.' *Arch. mikro. Anat.*, **74,** 855.

HILGARD, H. R. (1970). 'Synergism of thymus and bone marrow in the production of graft-versus-host splenomegaly in X-irradiated hosts.' *J. exp. Med.*, **132,** 317.

HOWARD, J. G., MICHIE, D. and SIMONSEN, M. (1961). 'Splenomegaly as host response in graft-versus-host disease.' *Brit. J. exp. Path.*, **42,** 478.

JANKOVIĆ, B. D., ISAKOVIĆ, K. and PETROVIĆ, S. (1973). 'Direct stimulation of lymphoid tissue of the chicken. III. Haemagglutinin production, haemolysin-forming cells and changes in lymphoid tissues following injection of guinea-pig erythrocytes into the bone marrow.' *Immunology*, **25,** 663.

LUCAS, A. M. and JAMROZ, C. (1961). *Atlas of Avian Hematology*. Agricultural Monograph 25, U.S. Department of Agriculture, Washington.

LUKIĆ, M. L., VUJANOVIĆ, N. L. and JANKOVIĆ, B. D. (1973). 'Lymphocyte differentiation in the chicken embryo. Relationship to the microenvironment.' *Microenvironmental Aspects of Immunity* (ed. by B. D. Janković and K. Isaković), p. 27. Plenum Press, New York.

LAFFERTY, K. J. and JONES, M. A. S. (1969). 'Reactions of the graft-versus-host (GVH) type.' *Aust. J. exp. Biol. med. Sci.*, **47,** 17.

LINNA, T. J., BÄCK, R. and HEMMINGSSON, E. (1971). 'Lymphoid cell migration.' *Morphological and Functional Aspects of Immunity* (ed. by K. Lindhal-Kiessling, G. Alm and M. G. Hanna, Jr), p. 149. Plenum Press, New York.

LONAI, P. and FELDMAN, M. (1970). 'Cooperation of lymphoid cells in an *in vitro* graft reaction system.' *Transplantation*, **10,** 372.

LONGENECKER, B. M., SHERIDAN, S., LAW, G. R. J. and RUTH, R. F. (1970). 'Measurement of graft-versus-host reactions on the chorioallantoic membrane of the chicken.' *Transplantation*, **9,** 544.

MAXIMOW, A. A. (1924). 'Relation of blood cells to connective tissue and endothelium.' *Physiol. Rev.*, **4,** 533.

METCALF, D. and MOORE, M. A. S. (1971). *Haematopoietic Cells*, p. 70, North-Holland, Amsterdam.

OWEN, J. J. T. (1970). 'Ontogenesis of immunological systems.' *Hanbuch der Allgemeine Pathologie. Immunreaktionen*, p. 129. Springer Verlag, Berlin.

OWEN, J. J. T., MAWDSLEY, A. R. and HARRISON, G. A. (1964). 'The role of the thymus in the development of immunological competence in the chick embryo.' *Transplantation*, **2,** 503.

OWEN, J, J, T., MOORE, M. A. S. and HARRISON, G. A. (1965). 'Chromosome marker studies in the graft-versus-host reaction in the chick embryo.' *Nature (Lond.)*, **207,** 313.

SETO, F. and ALLBRIGHT, J. F. (1965). 'An analysis of host and donor contributions to splenic enlargement in chick embryos inoculated with adult chicken spleen cells.' *Develop. Biol.*, **11,** 1.

SHORTMAN, K. and SZENBERG, A. (1969). 'The size and density distribution of fowl blood lymphocytes initiating a graft-versus-host reaction.' *Aust. J. exp. Biol. med. Sci.*, **47,** 1.

SOLOMON, J. B. (1971). *Foetal and Neonatal Immunology*. North-Holland, Amsterdam.

TIGELAAR, R. E. and ASOFSKY, R. (1973). 'Graft-vs-host reactivity of mouse thymocytes: effect of cortisone pretreatment of donors.' *J. Immunol.*, **110,** 567.

VUJANOVIĆ, N. L., LUKIĆ, M. L. and JANKOVIĆ, B. D. (1973). 'Appearance, morphology and distribution of lymphocytes during the early development of the chicken embryo.' *Phylogenic and Ontogenic Study of the Immune Response and its Contribution to the Immunological Theory*. INSERM, Paris.

WEBER, W. T. (1970). 'Analysis of host and donor cell population in chorioallantoic pocks.' *Transplantation*, **10,** 275.