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STUDIES ON THE ONTOGENY OF THE MOUSE IMMUNE SYSTEM

II. Immunoglobulin-Producing Cells

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Recent studies have shown that the precursors of cells which have the potential to participate in cell-bound immune reactions appear in the placenta and liver of the mouse embryo by the 9th or 10th day of gestation (1); they are not found in the yolk sac until 3 days later. Throughout pregnancy these cells are present in the liver. During the 11th to 15th days of gestation they are found in the upper trunk or in the thymus. On about the 15th day these lymphoid precursors appear in the lung and toward the end of pregnancy in the fetal bone marrow and spleen. Lymphoid precursors capable of maturation to cells which mediate *cell-bound* immune responses are not found in the gut until after birth. Following parturition these lymphoid stem cells are present in the liver, Peyer's patches, lung, bone marrow, lymph nodes, spleen, blood and thymus. However, by 6 weeks after birth the bone marrow appears to be the major or sole source of these cells.

In this report it will be shown that the precursors of cells which have the potential to produce immunoglobulins appear in the yolk sac, liver and the caudal half of the embryo by the 9th day of gestation, and that later they appear in the placenta, lung, gut, thymus, femur, spleen and blood. Certain of the observations strongly suggest that immunoglobulin-producing cells and those which mediate cell-bound immune responses arise as separate cell lines. In addition it will be shown that immunoglobulin production *per se* is not dependent upon the thymus (2).

MATERIALS AND METHODS

Pregnancies were surgically interrupted at various stages of gestation. The embryos and extra-embryonic tissues were dissected free of maternal tissue; they then were washed twice in cold TC 199 (Difco) in an effort to eliminate the possibility of contamination of the extra-embryonic tissues by maternal blood. Identical organs or tissues from a single pregnancy (5 to 11 embryos) were pooled in cold TC 199 and the cells were gently dissociated by mincing and aspiration through a 20-gauge needle. The age of the gestation was based on the following criteria: 7 to 8 days, a well developed ectoplacental cone and early membrane formation; 9 days, a well developed yolk sac, a conspicuous heart and no liver pigment; 10 days, early liver pigmentation; 11 to 12 days, a well pigmented liver and the absence of the thymus; 13 to 20 days, the relative size and development of the fetus. One host was the recipient of the pooled identical organ from a single pregnancy. The cell suspensions were injected intraperitoneally into the irradiated hosts.

Tissues from C3H.SW/Sn embryos and adult mice were transferred to thymectomized or non-operated adult congenic hosts (CWB/5Hz) (3) which had received 500 r whole body x-radiation. Tissues from CBA/J or C57Bl/6J embryos were transferred to lethally irradiated (870 r) thymectomized or nonoperated (C57Bl/6 × DBA/2)F_1 or (C57L × A)F_1 adult mice, respectively. Absence of the thymus was confirmed at autopsy. The above strain combinations were chosen because they have readily distinguishable γG_{2a}-immunoglobulin allotypes (4). (The results were similar with all strain combinations.) The lethally irradiated allogeneic hosts were bled from the retro-orbital plexus 45 days after irradiation, and the congenic hosts were bled 60 days after irradiation. The sera were assayed for donor γG_{2a}-globulins by a semiquantitative, inhibition of precipitation assay (5). The sera of uninjected irradiated mice and sera of known allotype were used as controls.

In addition, tissues were taken from CBA/J

embryos and adult mice as noted above and injected intraperitoneally into lethally x-irradiated (840 r) or sublethally irradiated (600 r) CBA-T6T6 mice. The lethally irradiated mice were protected against the effects of the radiation by two daily injections of urethan (0.2 ml of a 10% solution i.p.) (6). Approximately 30 days later these mice were killed and their tissues (thymus, spleen and bone marrow) were prepared for chromosome analysis. In most instances, at least 25 metaphase plates were scored for each tissue. The finding of donor cells in the thymus of the host was interpreted as indicating the presence of lymphoid precursors in the inoculum (7). The survival rate of the sublethally irradiated hosts was significantly greater than was that of the groups which received the higher dose of x-radiation; the results of the morphologic assays were identical.

RESULTS

As can be seen in Table I, donor-type immunoglobulins (γG_{2a}) were found in the sera of mice which had received yolk sac, liver or the caudal half of 9-day embryos. The following day precursors of cells capable of immunoglobulin production were present in the placenta. These stem cells persisted in the yolk sac until the 17th day of gestation and in the liver and placenta until parturition. Donor immunoglobulins were found in the sera of mice which had received gut and lung from 16- to 20-day embryos and in a few (3/11) of the mice which had received thymus cells from 16- to 18-day embryos. Late in pregnancy, precursors of immunoglobulin-producing cells were present in the femur, spleen and blood of the embryo. A few (2/6) mice which had received thymus cells from adult donors and all of the mice which had received adult bone marrow cells were found to have donor immunoglobulins in their sera. On the other hand none of the mice which had received 1.5 to 3 $\times$ 10^6 nucleated peripheral blood cells (0.25 to 0.5 ml of whole blood) had detectable donor-type γG_{2a}-globulins in their sera. These latter observations tend to exclude contamination of the extra-fetal tissues by maternal blood as a significant factor in the results obtained with yolk sac and placenta. However, when 20 to 30 $\times$ 10^6 nucleated peripheral blood cells (equivalent to approximately 5 ml of whole blood) are given, donor immunoglobulins can be found in the sera of the hosts (unpublished observations). All of the above observations correlated well with the results of the morphologic assays (Table II).

When tissues were taken from embryos prior to the appearance of the thymus, γG_{2a}-globulins of donor allotype were found in the sera of thy-

TABLE I

Production of γG_{2a}-globulins by various fetal and adult tissues when transferred to lethally irradiated allogeneic or sublethally irradiated congenic, thymectomized or nonoperated mice[a]

Tissue Injected	Age of Embryo (Days)								Adult
	7–8	9	10	11–12	13–14	15–16	17–18	19–20	
Liver			5/6	11/11	10/10	24/24	26/26	35/36	
Thymus			0/1[b]	0/1[b]	0/2	1/4	2/7	0/6	2/6
Gut			0/2	0/3	0/5	3/5	5/7	7/11	
Lung			0/1	0/3	0/4	1/5	2/7	6/10	
Femur			0/2	0/2	0/2	0/3	2/7	2/4	22/22
Spleen					0/1	0/2	1/2	3/6	
Blood					0/2	0/3	1/2	2/3	0/6
Skin				0/2	0/1	0/2	0/3		
Yolk sac	0/2	5/5	3/4	7/7	2/3	4/6	5/7	0/6	
Placenta	0/3	0/3	4/5	5/6	2/2	6/11	1/4	2/5	
Above diaphragm	0/3	0/2	0/2						
Below diaphragm	0/3	4/4	0/2[c]						
None	0/6	0/3	0/5	0/7	0/5	0/3	0/4	0/4	0/6

[a] The allogeneic hosts were bled 45 days and the congenic hosts 60 days after irradiation. The number of sera containing γG_{2a}-globulins of donor allotype are reported (no./total).

[b] Upper trunk without lung.

[c] Without liver or gut.

TABLE II

Per cent donor cells in the thymus, spleen and bone marrow of lethally or sublethally x-irradiated CBA-T6T6 mice 30 days after the intraperitoneal injection of various CBA embryonic or adult tissues[a]

Tissue Injected	Age of Embryo (Days)								Adult
	7–8	9	10	11–12	13–14	15	16–17	18–20	
Liver			*90*, 92, 90 *92*, 100, 80 *100*, 88, 100 *0*, 0, 0	*90*, 100, 100	*100*, 88, 92 *96*, 80, 80 *25*, 100, 84 *100*, 100, 100	*90*, 100, 100 *100*, 100, 100	*100*, 100, 84 *50*, 76, 44 *100*, 84, 92 *95*, 96, 93	*100*, 100, 100 (X5)[e] *90*, 90, 100	
Thymus				*0*, 33, 8 *0*, 0, 0	*4*, 0, 0 *0*, 0, 0	*0*, 0, 0 *0*, 4, 0	*12*, 8, 0 *0*, 0, 0	*4*, 0, 0 *0*, 0, 0 *0*, 0, 0 *0*, 0, 0	*0*, 4, 0 *0*, 0, 0 (X5)[e]
Gut			*0*, 28, 12 *0*, 0, 0	*0*, 0, 0 *0*, 0, 0	*0*, 0, 0 (X4)[e]	*0*, 0, 0 *0*, 0, 0 *0*, 12, 0	*100*, 100, 100 *96*, 33, 40 *24*, 50, 0	*90*, 70, 36 *90*, 94, 28 *30*, 40, 0	
Lung			*90*, 50, 12 *10*, 90, 30	*0*, 0, 0 (X4)[e]	*0*, 0, 0 *0*, 0, 0	*32*, 5, 0 *0*, 0, 0	*80*, 50, 40 *100*, 60, 80 *0*, 0, 0	*4*, 100, 0 *4*, 24, 0 *8*, 4, 0 *80*, 4, 0 *0*, 0, 0	
Femur			*0*, 0, 0	*0*, 8, 0 *0*, 0, 0	*0*, 0, 0		*80*, 50, 40 *90*, 100, 100 *0*, 24, 0 *0*, 25, 0	*100*, 96, 100 *100*, 84, 96	*25*, 8, 8 *100*, 70, 15
Spleen						*0*, 0, 0	*8*, 30, 4 *40*, 68, 72	*0*, 85, 25 *96*, 88, 68 *100*, 90, 100 *80*, 30, 82	
Blood							*0*, 80, 16	*100*, 24, 16 *100*, 72, 100 *8*, 68, 28 *0*, 0, 0	*0*, 0, 0 (X7)[e]
Skin			*0*, 0, 0		*0*, 0, 0 *0*, 0, 0		*0*, 0, 0	*0*, 0, 0 *0*, 0, 0 *0*, 0, 0	
Yolk sac	*0*, 15, 0 *0*, 0, 0	*0*, 4, 0 *90*, 40, 0	*84* 68, 16 *80*, 92, 16	*4*, 16, 0	*100*, 100, 96 *55*, 45, 24	*60*, 84, 80	*100*, 100, 100 *0*, 30, 12	*0*, 0, 0 *0*, 33, 45	
Placenta	*0*, 0, 4 *0*, 0, 0	*90*, 70, 4	*90*, 90, 100 *75*, 25, 24	*0*, 0, 0 *100*, 60, 44	*100*, 100, 100 *80*, 33, 100 *55*, 28, 0 *0*, 0, 0		*0*, 8, 4 *25*, 50, 4 *4*, 60, 40 *100*, 100, 64	*0*, 35, 30 *0*, 0, 0 (X4)[e] *80*, 40, 25 *0*, 60, 20	
Above diaphragm	*0*, 0, 0	*0*, 0, 0 *0*, 0, 0 *0*, 12, 0	*70*, 100, 100 *100*, 90, 80 *0*, 90, 100[b] *80*, 60, 100[b]	*96*, 92, 40 *0*, 30, 0[b]					
Below diaphragm	*0*, 0, 0	*90*, 80, 100 *100*, 100, 100[c]	*100*, 100, 100 *60*, 54, 32[c] *35*, 40, 24[d]						

[a] The lethally irradiated hosts were protected against the effects of the radiation by two daily injections of urethan prior to irradiation. The first and italicized number in each line represents the per cent donor cells in the host thymus. Some of these data have been reported previously (1).
[b] Without lung.
[c] Without liver.
[d] Without liver or gut.
[e] Number of mice giving above value.

TABLE III

Production of γG_{2a}-globulins by various fetal tissues when transferred to thymectomized, lethally irradiated allogeneic or sublethally irradiated congenic mice[a]

Fetal Tissue Given	Age of Embryo (Days)			
	7–8	9	10	11–12
Liver			3/3	6/6
Gut				0/3
Lung				0/3
Femur				0/2
Skin				0/2
Yolk sac	0/1	4/4	2/2	3/3
Placenta	0/2	0/2	2/2	2/2
Above diaphragm	0/2	0/2		
Below diaphragm	0/2	3/3		
None		0/3	0/1	0/7

[a] The allogeneic hosts were bled 45 days and the congenic hosts 60 days after irradiation. The number of sera containing γG_{2a}-globulins of donor allotype are reported (no./total). Some of these data have been reported previously (2).

mectomized hosts which had received yolk sac or the caudal half of 9-day embryos (Table III). Lymphoid precursors capable of immunoglobulin production in the absence of the thymus were present in liver and placenta of 10-day embryos and in liver, yolk sac and placenta of 11- and 12-day embryos.

DISCUSSION

It is apparent from these results that cells which have the potential to differentiate into immunoglobulin-producing cell lines appear in the yolk sac, liver and caudal half of the mouse embryo by the 9th day of gestation and on the following day in the placenta. The differentiation of these stem cells precedes the appearance of the thymus by 4 days. It is not clear from the data whether these cells have their origin in the yolk sac and then migrate to the embryo and placenta, or if they arise independently in these sites (cf. 8, ontogenesis of erythropoietic cells). Their late appearance in the thymus, gut, lung, spleen, femur and peripheral blood of the embryo suggests that they may be immigrants from other tissues, i.e., liver or yolk sac.

Certain observations in studies on the ontogenesis of cells which mediate cell-bound immune responses suggested that these cells may arise as a cell line(s) distinct from those which have the potential to produce immunoglobulins (1). Using chromosome marked cells (T6T6), donor cells were found in the thymuses of hosts given yolk sac from 9- to 12-day embryos or gut from 16- to 20-day embryos; these tissues gave no evidence of containing cells which had the potential to participate in graft-*vs.*-host or cell-bound immune reactions. The present studies clearly show that cells capable of immunoglobulin synthesis are present in these tissues at these times. That these observations are not merely the result of differences in the sensitivities of the two test systems is apparent when the findings with lung and thymus are examined. Lung obtained from 16- to 20-day embryos contains a significant number of cells which have the potential to participate in cell-bound immune reactions but the incidence of host sera which have detectable donor immunoglobulins is, if anything, less with lung than with gut of the same age (the immunoglobulin levels were quantitatively similar). Immunoglobulin-producing cells have been found in the embryonic thymus between the 16th and 18th days of gestation while cells which can participate in delayed-hypersensitivity reactions have been found in the upper trunk and in the thymus only between the 11th and 15th days of gestation. These observations strongly suggest that the cells which have the potential to participate in cell-bound immune reactions and those which are capable of immunoglobulin synthesis arise as distinct cell lines as early as the 9th day of gestation (cf. 9, 10).

As previously reported (2), cells which have the potential to differentiate into immunoglobulin-producing cell lines are present in the embryo at least 4 days prior to the appearance of the thymus and it has been shown here that they are capable of immunoglobulin synthesis in the absence of the thymus. Thus, while the thymus is essential for the production by these cells of detectable specific antibody in response to at least certain antigens (11, 12), these results demonstrate that the thymus is not essential for immunoglobulin synthesis *per se*. If one accepts the hypothesis that all immunoglobulins are preprogramed products of individual lymphoid cell clones, then the donor γG_{2a}-globulins found in these experiments may in fact be antibodies in the general sense that they are able to combine with certain substances but not with others. That is, in the absence of the thymus, the immunoglobulins produced in

response to an antigen may be of too low affinity to be serologically detectable. Thus one could postulate that the role of the thymus is to promote or to catalyze changes in the stem cell population or in the stem cell itself which result in the production of antibodies of increasing affinity in response to antigenic stimulation (13).

In vivo and *in vitro* studies have failed to detect immunoglobulin synthesis by the mouse embryo or by tissues known to contain cells capable of immunoglobulin synthesis (Tyan and Chin, unpublished data). In fact, antibody production, as judged by the detection of immunoglobulins of paternal allotype, does not normally begin in the mouse until about 3 weeks after birth (14). These findings suggest that immunoglobulin synthesis either is actively suppressed in the fetal and newborn mouse or that cells capable of immunoglobulin synthesis undergo a thymus-independent process of differentiation prior to coming under thymic influence.

SUMMARY

It has been shown that cells which have the potential to differentiate into immunoglobulin-producing cells appear in the yolk sac, liver and caudal half of the embryo by the 9th day of gestation. Late in pregnancy these cells are found in the thymus, gut, lung, spleen, femur and peripheral blood. Certain of the data suggest that immunoglobulin-producing cell lines and those cells which mediate cell-bound immune responses arise early in gestation as separate cell populations. It has been shown that immunoglobulin synthesis *per se* is independent of the thymus.

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