Role of the Thymus in the Development of Immunocompetence of Embryonic Liver Cells *in Vitro** (33378)

T. UMIEL, A. GLOBERSON,[1] AND R. AUERBACH

Department of Zoology, University of Wisconsin, Madison, Wisconsin 53706

The mechanism of development of immunocompetent cells has attracted much attention in recent years. Questions concerning the tissue origin of these cells and the conditions and factors necessary for obtaining their maturation have been studied in many systems, both *in vivo* and *in vitro*. The general picture that has emerged from these studies is that complex interactions between different lymphoid organs are involved in the differentiative events leading to the formation of immunocompetent cells (1).

It has been suggested that embryonic liver contains cells that are potentially immunocompetent (2); this suggestion was based on studies of adult irradiated mice injected with embryonic cells. In these studies, however, the various elements, host and donor, involved in the development of immunocompetence were hard to assess, since several cellular and humoral interacting factors could become involved in that development. For this reason it seemed appropriate to study the development of liver cells in an organ-culture environment in which the various cell components could be controlled and in which the immunological competence could subsequently be assayed. The system chosen was the *in vitro* graft-versus-host reaction as described by Auerbach and Globerson (3). In this system it had already been shown that it is possible to detect an immune reaction while at the same time one can follow the tissue origin of the reactive cells by appropriate immunogenetic controls (4).

Materials and Methods. Embryonic mice were obtained from strains C57BL6/JAu and F_1 (BALB/CAu $\times$ C57BL6/JAu) hybrids. Embryonic age was determined by observation of vaginal plugs. Dissections were performed in a mixture of horse serum and Tyrode's solution or in Tyrode's alone. Explants were grown for one day on top of a Millipore filter assembly to permit fusion of tissues when required. After this time they were cultured for an additional period of three to five days in the filter well of the assembly (3, 4). The nutrient medium consisted of Eagle's basal medium supplemented with 10% horse serum (Difco), 5% chick embryo extract (9-day embryos), and antibiotics. Cultures with 14-day embryonic liver were incubated in a water-saturated atmosphere of 57%oxygen, 5% carbon dioxide, and 38% nitrogen[2], while cultures of 16-day embryonic liver were incubated in a water-saturated atmosphere of 95% oxygen and 5% carbon dioxide. At the end of the culture period, tissue was removed and prepared as a cell suspension to be tested for competence to produce a graft-versus-host (gvh) reaction *in vitro*.

The assay for assessment of a gvh reaction was performed as described previously (3, 4). Paired explants of neonatal F_1-hybrid spleen fragments were placed in a double filter well assembly; appropriate test cells were then added. After one day, cultures were checked to be certain that matching had been adequate. Cultures were then scored again after three, four, and five days. Those cultures in which one fragment exceeded the other in size by more than 15% were scored as positive for splenomegaly. Representative cultures were subsequently fixed for histological verification of the occurrence of a gvh reaction.

Results. The first set of experiments was designed to determine directly whether the

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[1] Present address: Section of Cell Biology, Weizmann Institute of Science, Rehovoth, Israel.

[2] There have been many published variations in gas mixtures employed in culture systems. In the present instance this mixture was intended to be half air, half oxygen in 5% CO_2, and was arrived at empirically.

TABLE I. Splenomegaly Induction of Newborn F_1(BALB/C × C57BL) Spleen Explants by Embryonic Liver Cell Suspensions of C57BL and F_1(BALB/C × C57BL) Origin.

Origin of liver tissues		Number of experiments	Number of enlarged explants/total number of cultures
Age of embryos (days)	Strain		
14	C57BL	2	0/14
16	C57BL	5	3/27
14	F_1(BALB/C × C57BL)	2	0/13
16	F_1(BALB/C × C57BL)	3	2/23
Adult spleen cells	C57BL	27	62/84

embryonic liver contains cells that are able to evoke a gvh reaction. Cell suspensions prepared from liver obtained from parental or F_1-hybrid embryos 14 or 16 days old were added to neonatal F_1 spleen explants. Cell suspensions obtained from adult parental spleens served as positive controls. While splenomegaly was found to be readily induced by adult parental spleen cells, embryonic liver cells failed to do so (Table I). The occasional enlargement observed in response to liver cells did not represent a typical gvh reaction as judged by histological criteria but was suggestive of some proliferaton of liver cells within the spleen explant.

The second set of experiments was designed to determine whether the culturing environment might induce or encourage development of immunocompetent cells in the liver. Fragments from liver of parental and F_1 embryos were cultured for a period of two to six days and then tested for ability to evoke a gvh reaction. No detectable splenomegaly was elicited by any of the cells tested under these conditions (Table II).

Since tissue interaction with thymus was suggested as prerequisite to the development of immunocompetence (cf.1) experiments were then designed to test whether competent cells could be obtained by combination cultures of liver and thymus. Parental embryonic livers were combined with thymus of two-week-old F_1 animals for a period of from four to six days. Cells from such cultures were able to induce splenomegaly in 24 of 74 cultures (Table II).

These results raised the question whether the observed splenomegaly was in fact caused by an immunological reaction. If so, similar results should be obtained by cultures of allogeneic or semiallogeneic donor-host combinations but not in a syngeneic system. Following this principle, cultures of syngeneic combinations of thymus and embryonic liver,

TABLE II. Splenomegaly Induction of Newborn F_1(BALB/C × C57BL) Spleen Explants by Cell Suspensions Obtained from Embryonic Liver Cultures, Grown Alone or in Combination with Adult Thymus Tissue.

Origin of liver tissues				
Age of embryos (days)	Strain	Origin of thymus tissue	Number of experiments	Number of enlarged explants/total number of cultures
14	C57BL	—	4	0/15
16	C57BL	—	5	0/19
14	F_1(BALB/C × C57BL)	—	4	0/15
16	F_1(BALB/C × C57BL)	—	1	0/4
14	C57BL	F_1(BALB/C × C57BL)	4	10/28
16	C57BL	F_1(BALB/C × C57BL)	11	14/46
14	F_1(BALB/C × C57BL)	F_1(BALB/C × C57BL)	4	0/32
16	F_1(BALB/C × C57BL)	F_1(BALB/C × C57BL)	4	0/23

both obtained from F_1 animals, were tested against F_1 neonatal spleen explants. Under these conditions no spleen enlargement was observed in any of the 55 cultures tested (Table II).

Discussion. The results of the present experiments demonstrate that embryonic liver when grown in organ culture in the presence of thymus gains the ability to induce enlargement of a neonatal semiallogeneic spleen. This capacity was not detected in the case of embryonic liver grown alone in culture or of liver tested directly from embryos without prior culturing. This ability also was not evident when liver was cultured in the presence of adult spleen and lung (5).

One may raise the question whether the observed splenomegaly is really the result of a true graft-versus-host type of reaction, and whether it in fact represents an immune reaction at all. That it is essentially similar to the splenomegaly induced in a typical gvh reaction is suggested (a) by the fact that capability to evoke splenomegaly was manifested only in the semiallogeneic system but not in the syngeneic combination; and B) by the histological manifestations accompanying the induced splenomegaly (cf. also 3). Additional evidence concerning the immunological, gvh nature of the induced splenomegaly in culture was obtained in earlier studies testing the effect of spleen cells, and this evidence included radiation sensitivity, ontogenic pattern, and effects of preimmunization on radiation sensitivity (3, 4).

It appears from the present study that the ability to induce splenomegaly is not manifest in embryonic liver but can be induced by cultivation of liver in combination with thymus. The experiments suggest, further, that the competent cells are of liver origin, since F_1 thymus alone or in combination with F_1 liver did not evoke splenomegaly in F_1 neonatal spleen explants. While more complex explanations cannot be excluded without further studies involving, e.g., discriminant spleen assays, Millipore filter separation or specific antithymocyte sera, the similarity of the present system to others renders the simpler explanation most likely.

Whether the results of the present *in vitro* studies reflect accurately the ontogenic development *in situ* cannot be ascertained from the present experiments. One can only conclude that embryonic liver contains cells that are potentially competent to evoke the gvh reaction, and that these cells, at least *in vitro*, require a thymic stimulus for maturation.

Summary. The immunological competence of embryonic mouse liver cells was studied using the *in vitro* graft-versus-host system for assay. Embryonic liver cells did not have the capacity to induce splenomegaly when taken from embryos directly, nor did they acquire this capacity when cultivated as organ cultures for four to six days. The capacity to induce splenomegaly was acquired, however, when liver explants were cultivated for several days in combination with thymus tissue.

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