

## The role of endoderm in blood cell ontogeny in the newt *Pleurodeles waltl*

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### SUMMARY

In order to determine the origin of blood cells we performed embryonic grafts of different portions of mesoderm from various locations between diploid and tetraploid embryos at the tail-bud stage. The tetraploid animals were the hosts. The size differences between tetraploid and diploid cells made identification possible by direct microscopic examination of blood smears. In a previous report we showed the important role of truncal anterior mesoderm in the genesis of blood cells. We now establish that this effect is brought about by the inductive capacity of the hepatic endoderm or by the fact that the environmental conditions are more appropriate for blood stem cell development, whereas in the absence of the hepatic endoderm the blood stem cells fail to appear. Grafting of hepatic anlage containing endoderm and mesoderm gives rise to numerous graft-derived blood cells which last throughout the life span of the hosts. The same result is obtained by grafting truncal posterior ventral or lateral mesoderm onto hepatic endoderm. Heterotopic grafting of truncal anterior mesoderm isolated from its underlying hepatic endoderm leads to the formation of only a few blood cells which last only during larval life. This demonstrates that the whole lateral and ventral truncal mesoderm is able to differentiate into blood cells when associated with hepatic endoderm.

### RESUME

Afin de préciser l'origine des cellules souches sanguines nous avons pratiqué des greffes embryonnaires de différentes régions du mésoderme entre embryons 2n et 4n au stade du bourgeon caudal. Les individus 4n sont utilisés comme receveurs. La différence de taille entre cellules 4n et cellules 2n est telle qu'elle permet une identification de la ploïdie directement sur frottis sanguins. Dans un travail précédent nous avons montré le rôle important du mésoderme troncal antérieur dans le genèse des cellules sanguines. Nous établissons maintenant que l'endoderme hépatique joue un rôle inducteur responsable de la détermination des cellules souches sanguines ou qu'il crée les conditions de micro-environnement favorables à cette détermination. En son absence les cellules souches sanguines n'apparaissent pas. C'est ainsi que la greffe de l'ébauche hépatique comprenant l'endoderme et le mésoderme conduit à la formation de nombreuses cellules sanguines pendant toute la vie de l'hôte. Le même résultat est obtenu en greffant du mésoderme médio-ventral ou latéral sur l'ébauche hépatique endodermique. La greffe hétérotopique de mésoderme troncal antérieur isolé de l'endoderme se traduit par la présence de quelques cellules sanguines pendant la vie larvaire. Ceci démontre que l'ensemble du mésoderme ventral ou latéral est apte à donner des cellules sanguines lorsqu'il est associé à l'endoderme hépatique.

## INTRODUCTION

In vertebrates, the primary blood cells arise from mesodermal tissue associated with the ventral endoderm and yolk sac. Red blood cells in Amniotes arise in the yolk sac but leucocyte cell lines may also have their origin here. Yolk sacs taken from mice embryos between days 7 and 13 contain stem cells which can produce cells of the erythrocyte, granulocyte, megakaryocyte (Moore & Metcalf, 1970) and lymphocyte (Paige, Kincade, Moore & Lee, 1979) series, both *in vivo* and *in vitro*. However yolk sac blood stem cells may not be the only origin for all the embryonic and postembryonic blood cells. In birds, Moore & Owen (1965, 1966, 1967), Jotereau & Houssaint (1977), Jotereau, Houssaint & Le Douarin (1980), Beaupain, Martin & Dieterlen-Lievre (1979), Dieterlen-Lievre, Beaupain & Martin (1976) have indicated the role of the yolk sac in the development of blood and lymphoid cell systems.

However, other experiments have shown that the bird yolk sac is not the only source of blood cells which can also develop from intra-embryonic mesenchyme (Dieterlen-Lievre *et al.* 1980; Lassila, Eskola & Toivonen (1979).

In reptiles early haematopoiesis also occurs in the yolk sac (Vasse & Beaupain, 1981).

In amphibians, the first red blood cells come from the ventromedial mesoderm situated between the blastopore and the hepatic anlage and they make up the blood island. Removal of this anlage before blood circulation is established in both frogs (Federici, 1926) and newts (Goss, 1928) leads to a subsequent absence of red blood cells.

Cells from the blood island form a transitory population of cells which disappear during larval life. These results have been produced from embryonic grafts of blood islands between 2n and 3n animals in both *Rana pipiens* (Hollyfield, 1966; Turpen, Turpen & Flajnik 1979) and *Pleurodeles waltl* (Deparis, 1968).

During development the stem cells of the nascent haematopoietic organs do not come from the blood island. In *Rana pipiens* the lateral mesoderm of the kidney region contains blood stem cells which migrate during development to the haematopoietic organs (Turpen, Knudson & Hoeffen, 1981). In *Pleurodeles waltl* stem cells arise in the ventral mesoderm of the liver region (Deparis & Jaylet, 1975).

This study examined the role of the endoderm in blood cell development. In *Pleurodeles* embryos, the ventromedial mesoderm gives rise to blood stem cells in the anterior trunk region, which last the lifetime of the animal. However in the middle and posterior trunk regions it produces the blood island which gives rise to a transitory population of erythrocytes. There is therefore, either a real regional differentiation of the mesoderm, or a regional differentiation due to an inducing effect of an already regionalized endoderm.

## MATERIALS AND METHODS

Polyloid cells are bigger than diploid ones (Fankhauser, 1941). The large size difference between tetraploid and diploid cells (Fig. 1) make these two cell types easily recognisable (Deparis & Jaylet, 1975). In a previous report we showed that when  $2n/4n$  parabioses are made, blood mostly comprises diploid cells, even in the tetraploid individual (Deparis & Jaylet, 1976), so it is concluded that competition with diploid cells is unfavourable for tetraploid cells. This phenomenon seems to apply to all polyloid cells, since the triploid cells are often outnumbered when they are made to compete with diploid cells (Volpe, Gebhardt, Curtis & Earley, 1969). For this reason tetraploid embryos were chosen as hosts and diploid embryos as donors.

The tetraploid host animals were obtained according to the method described by Jaylet (1972). The diploid donor embryos at the tail-bud stage (stage 24 of the chronological table of Gallien & Durocher (1957) which corresponds approximately to stage 30 of *Ambystoma mexicanum* as defined by Schreckenberg & Jacobson, (1975) were cut into four pieces using a microscalpel (Fig. 2A). So, the anterior part with head (H) and the tail bud (T) were discarded isolating

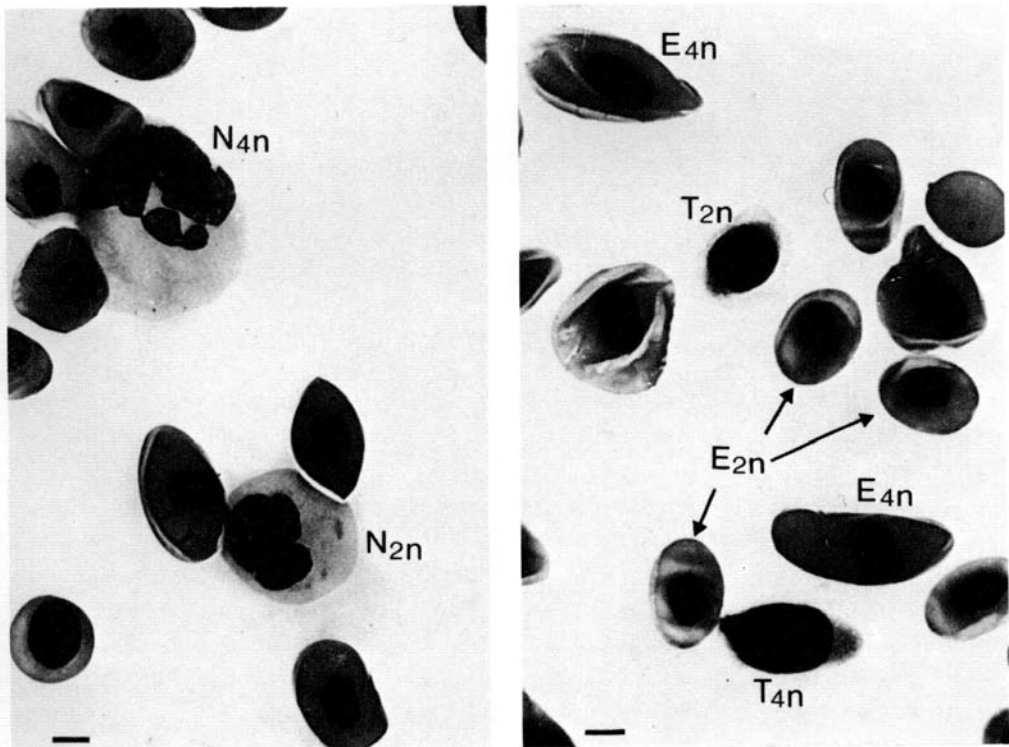


Fig. 1. Size difference between diploid and tetraploid cells. N: neutrophil; E: erythrocyte; T: thrombocyte. Scale bar =  $10\text{ }\mu\text{m}$ .

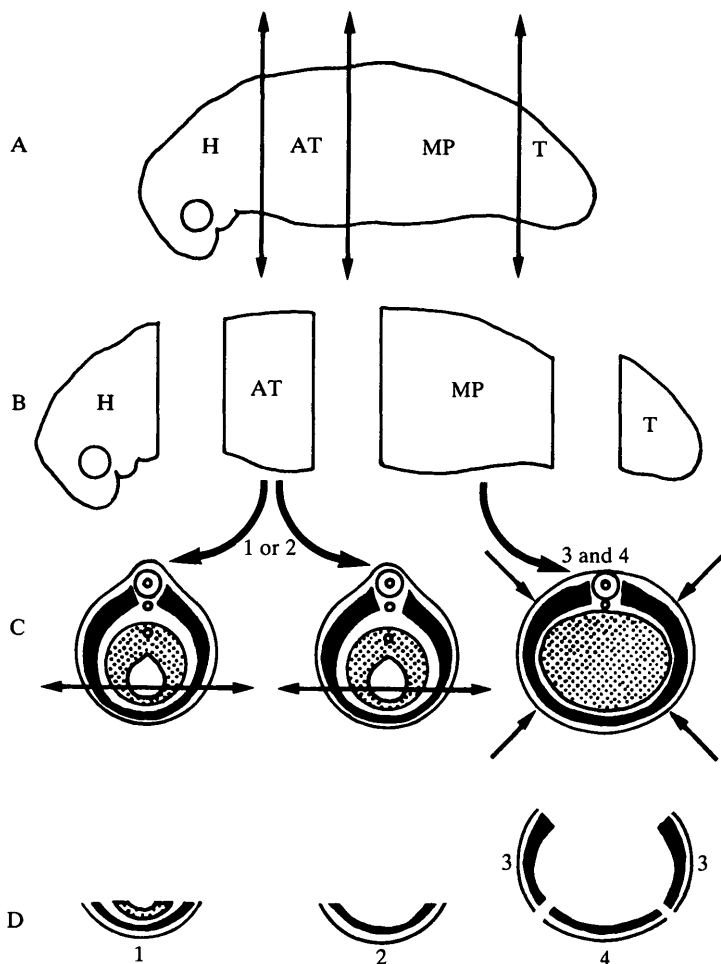


Fig. 2. Graft isolation. (A) Embryo at the tail bud stage, the arrows show the slice positions. (B) The two medial fragments are used for graft isolation. (C) The grafts were separated, the arrows indicate the slice positions. (D) Isolated grafts. Graft 1 includes the epiblast, the anterior mesoblast and the endodermal hepatic anlage; graft 2 is made up solely of the epiblast and anterior mesoblast; graft 3 represents the epiblast, and lateral median and posterior mesoblast; graft 4 is made up of the same tissue slices as graft 3 except that it is taken from the ventromedial region.

the anterior trunk region (AT) which includes the hepatic anlage and the median and posterior trunk region (MP) which contains some of the lateral mesoderm and the ventral mesoderm where the blood island originates (Fig. 2B). Both these two trunk samples (AT and MP) were placed in a twice concentrated operating Holtfreter solution for ten minutes, after which the endoderm was easily separated from the mesoderm/epiblast, enabling the various graft samples to be isolated (Fig. 2, C and D). The proportions of 2n erythrocytes, thrombocytes and neutrophil leucocytes in the hosts' blood were directly

determined by counting blood smears stained with May-Grünwald-Giemsa solution.

For thymic lymphocytes we used a microdensitometric determination of DNA after Feulgen staining. Large size variations in lymphocytes make the distinction between 2n and 4n cells in lymphocyte populations impossible.

Microdensitometric measurements on slides is made difficult by the fact that the nuclei are rarely whole. We used the following technique to surmount this problem. The thymus was removed and placed in a mixture of three parts ethanol and one part glacial acetic acid for two minutes. After this fixing period, it was rinsed for one minute in distilled water and then digested in 50 % acetic acid. One drop of the resulting cell suspension was placed on a slide coated with dry albumin and a coverslip placed on top. The preparation was then pressed under a wad of filter paper and placed in 50 % acetic acid to unstick the coverslip. The slides were stained using the Feulgen method. Using this technique we were able to obtain whole and well-separated nuclei which made DNA measurement relatively straightforward. We used a Leitz MPV2 scanning microdensitometer with monochromatic light at 542 nm. The values obtained for 4n controls were  $4240 \pm 93$  s.e. and  $2240 \pm 30$  s.e. for 2n controls.

## RESULTS

We have already shown that when the liver anlage is grafted onto an embryo at the tail bud stage it gives rise to a supernumerary liver and to numerous blood cells (Deparis & Jaylet, 1975). The graft is then composed of ectoblast, mesoblast and endoblast. This type of embryonic graft is the only one that leads to blood cells originating from the graft during the life span of the host. It is known that blood cells have a mesodermal origin so we can conclude, from the preceding experiments, that either the hepatic endoderm acts on mesodermal cells which are induced into blood stem cells or that mesodermal cells of the hepatic region can differentiate autonomously into blood stem cells. Five graft types were used in order to analyse the role of the hepatic endoderm.

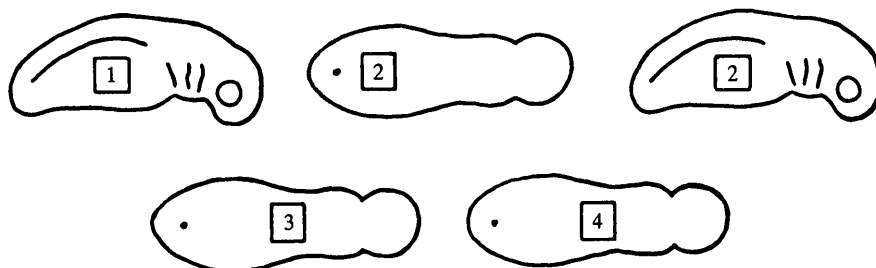


Fig. 3. Grafting sites. The type 1 grafts are placed on the flank of the host animals. The type 2 grafts are either placed in the posterior ventromedial region or on the flank. The type 3 and 4 grafts are placed on the endodermal hepatic anlage.

Table 1. *Percentages of 2n cells in the 4n hosts.*

Type of graft	Erythrocytes			Neutrophil leucocytes			Thrombocytes		
	Larval life	Metamorphosis	4 months and more after meta.	Larval life	Metamorphosis	4 months and more after meta.	Larval life	Metamorphosis	4 months and more after meta.
Type 1 graft (ectoblast/mesoblast/endoblast from anterior trunk region) grafted on the flank (11)	3-90	4-88	24-96	0-35	0-26	0-34	30-95	26-95	36-90
Type 2 graft (ectoblast/mesoblast from anterior trunk region) grafted on the belly (7)	1-12	0-1	0	0-3	0	0	2-61	3-28	0-2
Type 2 graft (ectoblast/mesoblast from anterior trunk region) grafted on the flank (7)	1-10	0	0	0	0	0	0-16	0	0
Type 3 graft (lateral ectoblast/mesoblast from median and posterior trunk region grafted onto hepatic anlage (4)	10-53	50-78	81-90	5-36	26-32	22-38	30-88	19-86	72-80
Type 4 graft (ventral ectoblast/mesoblast from median and posterior trunk region grafted onto hepatic anlage (11)	6-81	4-61	0-65	0-20	0-32	0-33	45-70	44-80	46-82

The figures represent the percentage range found for each series.

The figures in brackets indicate the number of animals examined for each type of graft.

Two graft samples were isolated from the anterior trunk region. One included the epiblast, the mesoblast and the endodermal hepatic anlage (type 1 graft, Fig. 2) and was grafted on the host's flank (Fig. 3). In this location it is very easy to observe the development of a supernumerary liver during larval life. There is no fusion of the graft with the host hepatic anlage, a situation which often occurs when the graft is placed onto the ventromedial region.

Throughout the life span of the hosts a high proportion of diploid blood cells from the graft were found in the blood (Table 1).

The thymus of these animals was found to contain numerous diploid lymphocytes. The graft thus produced cells which colonized the thymic epithelial anlage (Deparis & Jaylet, 1976; Jaylet & Deparis, 1979) (Table 2).

The other type of graft consisted solely of the epiblast and the mesoblast (type 2 graft, Fig. 2). It was grafted either on the flank or on the posterior ventromedial region in order to be well separated from the host's hepatic anlage in the two cases (Fig. 3). These locations were chosen to test the autonomous possibilities of differentiation into blood cells of these mesoblastic cells. In these last two cases the blood of the larvae from the host embryos were found to contain, for a transitory period, only a low proportion of diploid cells coming from the graft. The cells were seen in the larval stage but by metamorphosis they had practically disappeared (Table 1).

So, we examined the possibilities of blood cell formation from the mesoblast of the anterior trunk region. It appears that it can only do so when it is associated with hepatic endoblast.

In two other series of experiments we associated other kinds of mesoblast with hepatic endoblast in order to determine the role of this anlage in blood cell origin.

From the medial and posterior trunk region we obtained two new types of graft which were placed on the hepatic anlage after removal of the superficial region (Fig. 3). One was made up of ventromedial epiblast and mesoblast which gives rise to the blood island (type 4 graft, Fig. 2), while the other was of epiblast and lateral mesoblast which do not normally lead to blood cell lines (type 3 graft, Fig. 2).

Table 2. *Percentage of 2n thymocytes in the 4n hosts established by microdensitometric determination of DNA several months after metamorphosis.*

Type of graft	Number of animals examined	2n thymocyte percentage range
Type 1 graft (ectoblast) mesoblast/endoblast from anterior trunk region) grafted on the flank	5	17-89
Type 3 graft (lateral ectoblast/ mesoblast from median and posterior trunk region grafted onto hepatic anlage	4	48-92

In both cases the host animals blood was found to have significantly raised levels of diploid blood cells coming from the graft (Table 1). The thymus was also well colonized (Table 2).

#### DISCUSSION

Our results show that several regions of the ventral and lateral mesoderm in the tail-bud stage can give rise to blood cell lines in *Pleurodeles waltl*. However they have to be associated with endoderm from the anterior trunk region, more especially with the endoderm of the hepatic anlage. Under these conditions the mesodermal cells are influenced by the endoderm in what can be considered to be a form of induction. We can also invoke the micro-environmental conditions that lead to blood stem cell determination (Dexter, Allen & Lajtha, 1977; Dexter, 1982). This is particularly clear in the case of grafts of posterior or lateral ventral mesoderm onto the hepatic anlage. In normal development the ventromedial trunk mesoderm gives rise to the blood island. It produces a transitory population of blood cells which disappear during larval life and are completely absent at metamorphosis (Deparis, 1968). But this same mesoderm when associated with hepatic endoderm gives rise to blood cells which are found throughout the life span of the animal. This indicates the presence of stem cells. The lateral mesoderm of the posterior trunk region does not lead to blood cells during normal development, but when grafted onto the hepatic endoderm it receives an inducing influence and leads to the formation of blood stem cells. In these two cases there is therefore a radical modification in the determination of these mesodermal cells. It should also be pointed out that all types of blood cells are represented even though they do not all differentiate in the liver (Deparis, 1968). It is probable that the hepatic endoderm guides blood cell differentiation which is further defined in the other haematopoietic organs as they become colonized by stem cells (Deparis & Jaylet, 1975). This is especially the case for the spleen which is the haematopoietic organ in *Urodeles* where the stem cells come from the liver. The thymus is another example of a haematopoietic organ which is colonized by exogenous cells, which it transforms into highly specialized lymphocytes (Jotereau *et al.* 1980; Tompkins, Volpe & Reinschmidt, 1980; Deparis & Jaylet, 1976). With respect to *Pleurodeles* at least, our experiments show that the cells that colonize the thymus come, as do all blood stem cells, from the mesoderm that covers the hepatic endoderm.

Our results show the inducing role exerted by the endoderm. This role has been shown in amphibians where the ectoblastic origin of the mesoderm is under an endodermal influence (Nieuwkoop & Ubbels, 1972). Boterenbrood & Nieuwkoop (1973) have studied the regional differentiating influence of the endoderm and have shown that only the dorsal endoderm can induce cord formation while the lateral or ventral endoderm induce various mesodermal structures including the blood cells. In *Triturus alpestris* Asashima (1975) has also shown the inducing



action of the endoderm on the ectoderm with subsequent blood cell production. Capuron & Maufroid (1981) have observed erythrocyte formation after mesodermal grafts on *Pleurodeles* embryos. In the chicken, Miura & Wilt (1969) consider that erythrocyte determination results from endodermal-mesodermal interactions. On the other hand Okada (1960) demonstrated that endoderm differentiation is partly governed by mesoderm at the neurula stage in *Triturus pyrrhogaster*. This author has also observed blood cells in the anterior part of the neurula. In fact there are very strong interactions between the endoderm and the mesoderm during development, especially in the liver, this has been well demonstrated in all Vertebrate embryos (Le Douarin, 1975). Our experiments point to a regional differentiating effect of the inducing action of the endoderm. The ventromedial mesoderm, under the inducing influence of the endoderm from the same region, leads to a transitory population of red blood cells which, when inserted onto the hepatic endoderm, gives rise to the blood stem cells which produce blood cells throughout the life of the individual. This is also the case for lateral mesoderm from the trunk region when grafted onto the hepatic endoderm. Thus there are both anteroposterior and dorsoventral differences in the inducing capacities of the endoderm in the Urodeles amphibians.

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