Development 121, 407-416 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

An investigation into early placental ontogeny: allantoic attachment to the chorion is selective and developmentally regulated

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SUMMARY

Culture of postimplantation conceptuses was used in conjunction with microsurgery to investigate the timing, the mechanism and the developmental regulation of chorioallantoic fusion in the mouse. The timing of fusion was determined in both freshly recovered conceptuses and in those that had been cultured from as early as the mid-streak stage. Attachment of the allantois to the chorion was found to have occurred in most conceptuses by the 6-somite stage, irrespective of whether they had been cultured.

In investigating the mechanism of fusion, we wished to determine whether it depended on directed growth of the allantoic bud or on its differential adhesion to the chorion. Microsurgery was used to transplant allantoic tissue into the exocoelomic cavity of conceptuses from which the resident allantois had been removed. In synchronous grafting experiments, transplanted allantoises typically attached to the chorion despite loss of their connection with the hindgut region of the fetus. Hence selective attachment of the allantois to the chorion clearly cannot depend simply on its directed growth.

While the transplanted allantoic tissue attached to the chorion selectively, it did not attach to it precociously, despite being favourably positioned to do so. These findings argue that the initial attachment of the allantois to the chorion depends on a selective adhesive mechanism that is developmentally regulated. Further grafting experiments in which donor conceptuses were either more or less advanced than hosts revealed that attachment of the allantois to the chorion depends primarily on the stage of the allantois rather than on the stage of the chorion.

Collectively, these findings support the hypothesis that the initial stage of chorioallantoic fusion depends on selective adhesion between regionally differentiated mesodermal surfaces which is governed principally by the stage of development of the allantois.

Key words: allantois, chorion, placenta, adhesion, morphogenesis, mouse, gastrulation, embryos

INTRODUCTION

Eutherian mammals exhibit an extreme form of viviparity in which the embryo or fetus is wholly dependent on the mother for the supply of nutrients, respiratory exchange and the elimination of toxic metabolites. Crucial to the success of this mode of development is the establishment of an extensive, intimate relationship between the vascular systems of the mother and conceptus, which is achieved through the development of a placenta. In rodents and certain other mammals, the visceral yolk sac, an early site of haemopoiesis, may play a significant role as an organ of exchange during development of the fetus. Thus, in rodents, in situations where the visceral yolk sac constitutes the only functional placenta, as in normal conceptuses in culture or diploid parthenogenetic or gynogenetic conceptuses in vivo, development can reach mid-gestation (New, 1990; Surani et al., 1990). Nevertheless, the later developing chorioallantoic placenta unquestionably provides the most important surface of exchange between mother and fetus throughout eutheria.

Although much comparative work has been undertaken on the structure and function of the mature chorioallantoic placenta (Mossman, 1987), little effort has been devoted to investigating the development of this organ, particularly during the initial stages of its ontogeny. An intriguing feature of its development is that it is, as its name implies, the product of the fusion of two extraembryonic structures, the allantois and the chorion, that are initially well-separated from each other by a fluid-filled cavity. Both the allantoic bud and the chorion are present by the headfold stage in the rat and the mouse but, depending on the strain of mouse, may be present together as early as the neural plate stage (Ellington, 1985, 1987; Downs and Davies, 1993). Subsequent enlargement of the allantoic bud so that it extends across the exocoelomic cavity, is clearly instrumental in establishing contact between them. Once chorioallantoic fusion has begun, the allantois soon becomes overtly vascularized.

At present, the origin of the allantois and chorion has been documented by lineage analysis only in the mouse (reviewed

in Gardner, 1983; 1985). In this species, the chorion itself is clearly of dual origin, consisting mainly of extraembryonic ectoderm, a trophectoderm derivative that is bounded internally by part of the primitive ectoderm (epiblast)-derived mesoderm which lines the entire exocoelomic cavity. The allantois is wholly of primitive ectodermal (epiblast) origin, consisting of a core of mesoderm overlaid by part of the lining of the exocoelomic cavity into which it grows from the posterior end of the primitive streak by a combination of mitosis and distal cavitation (Ellington, 1985). Recently, clonal analysis has revealed that the precursor cells of the allantois lie in the proximal region of the primitive ectoderm at the onset of gastrulation. The more posterior cells within this region contribute preferentially to the apical core and enveloping mesothelium of the structure and the more anterior ones to its base (Lawson et al., 1991; Lawson and Pedersen, 1992; K. Lawson, personal communication).

How the allantois makes and maintains specific attachment with the chorion is not known. Extension to the chorion might be due to intrinsic directed growth properties of the allantois or, at the opposite extreme, depend entirely on extrinsic cues or constraints provided by the neighbouring visceral yolk sac or amnion. If the allantois reaches the chorion without making contact with any other part of the exocoelomic surface, there would be no need for adhesion between these two tissues to be selective. If not, spatial or temporal differences in mesodermal adhesiveness would be required to ensure the observed specificity. Firm adherence of the allantoic bud to the mesodermal surface of the exocoelom outside the chorionic region has only been described in conceptuses that are homozygous for deletion of *Brachyury* (Rashbass et al., 1991). Here, attachment of the bud to the amnion is commonly observed.

To investigate the mechanism of chorioallantoic fusion, we have developed a microsurgical technique for removing the allantois and reinserting its apical half in the exocoelomic cavity, which can be used in conjunction with whole embryo culture. In the first part of the present study, the validity of this approach was assessed by specifically addressing two issues. The first was whether the initial steps in chorioallantoic fusion were perturbed by the culture and manipulation of conceptuses. The second was whether there would be sufficient regeneration from the stump of tissue remaining after removal of the allantois to complicate interpretation of grafting experiments.

In the second part of the study, we report the results of experiments designed to test how specific fusion of the allantois with the chorion is achieved. Allantoic tissue was transplanted between conceptuses of the same or different developmental stage. In the synchronous grafting experiments, transplanted allantoises were found to resemble those developing in situ in both the specificity with which they attached to the chorion and the stage at which they did so. Finally, asynchronous grafting experiments revealed that the stage specificity of mutual adhesion depended primarily on the allantois which, unlike the chorion, only showed attachment if it had reached the stage when this normally occurs.

MATERIALS AND METHODS

Source, recovery and staging of conceptuses

A closed-bred colony of PO mice (Pathology, Oxford), a Swiss-derived albino strain (Kelly and Rossant, 1976), that were exposed to light daily between 07.00 and 19.00 hours, were used throughout this study, except for the 18 hour asynchronous experiments. In the latter series of experiments, conceptuses were from matings between the F_2 generation of (C57Bl/6 × CBA) mice. Oestrus females were selected by vaginal inspection (Champlin et al., 1973), pairs placed with single males overnight, and the females inspected for copulation plugs the following morning. Taking the time of mating as the mid-point of the dark period, pregnant females were killed at approximately 8.0-8.5 days postcoitum (dpc) and their uteri placed in Dulbecco's A phosphate-buffered saline (PBS, Oxoid) for isolation of decidua. Once isolated, the decidua were placed in Hepes-buffered DME medium with 7.5% fetal calf serum (K. Lawson, personal communication), the conceptuses isolated, and then divested of their parietal yolk sac.

Before microsurgery and culture, conceptuses were staged by visual inspection according to the scheme of Downs and Davies (1993). After culture, they were staged again, either in an intact condition before processing for histology, or after both the yolk sac and amnion had been torn open and the embryo stretched with forceps to aid the counting of somites. Where nascent somites were present, they were recorded as whole ones.

Culture, labelling and microsurgery

Staged conceptuses were cultured in 1 ml of medium in Falcon tubes (12×75 mm, Becton-Dickinson, NJ), either individually or in matched pairs, in a roller system of diameter 30 cm which rotated at 0.5 revs/minute (Beddington and Lawson, 1990). The culture medium contained equal parts of bicarbonate-buffered DME medium and rat serum (K. Lawson, personal communication). Storage of the rat serum used in this study was at −20˚C for up to 1.5 years. The medium was invariably made up shortly before use and pre-equilibrated for at least one hour at 37˚C in the gas phase of 6.2% CO_2 in air before conceptuses were incubated in it.

For [^{3}H]methyl thymidine labelling, conceptuses were pooled from several litters and incubated in groups for 2 hours in organ tissue culture dishes (60×15 mm with center well, Falcon, Becton-Dickinson, NJ). The dishes each contained 500 μl of medium to which both [^{3}H]methyl thymidine (specific activity: 40-49 Ci/mmol, Amersham, UK) and cold thymidine (Sigma) had been added to obtain a final specific activity of 10 Ci/mmol at a concentration of 10 μCi/ml (Cleaver, 1967; Beddington, 1981). Control conceptuses were cultured similarly in medium supplemented with cold thymidine alone (1×10^{-6} M). After incubation, conceptuses were pipetted through several changes of Hepes-buffered DMEM that contained 1×10^{-6} M cold thymidine, and then placed in culture medium containing 1×10^{-6} M cold thymidine and cultured for 20 hours.

In most grafting experiments, pairs of staged conceptuses were manipulated. As much of the allantois as possible was removed by suction from the host conceptus using a mouth-controlled pipette of approx. 70-90 μm internal tip diameter that was introduced into the exocoelom through the left side of the yolk sac. In all shorter-term grafting experiments, only the apical half of the allantois was used for transplantation so as to avoid inclusion of primitive streak tissue or primordial germ cells in the grafts. However, whole allantoises were transplanted in the 18 hour asynchronous experiments because of concern about recognizing smaller grafts after longer intervals. The visceral yolk sac of the donor conceptus was torn open so that either the entire allantois or its apical, slightly ballooned portion could be excised with a fine glass needle. The donor allantoic tissue was drawn inside a pipette which was inserted into the exocoelomic cavity of the recipient conceptus via the wound in the yolk sac through which the host allantois had been removed. In the remaining grafting experiments, apical halves of allantoises that had been isolated by suction were reinserted into the exocoelom of the same conceptuses from which they had been removed. In other conceptuses, the yolk sac was punctured with a pipette and exocoelomic fluid withdrawn, leaving

the allantois in situ. These sham-operated conceptuses were cultured separately, but concurrently, with operated ones.

Examination of conceptuses

The location and possible attachment sites of the allantois were scored initially by examining conceptuses with the yolk sac intact. Conceptuses carrying allantoic grafts from [^{3}H]methyl thymidine-labelled donors were fixed in Carnoy's fluid at 4˚C for 3-16 hours and then held in 70% ethanol for at least 3 hours to eliminate the chloroform present in the fixative. They were then processed for wax-embedding, sectioning and autoradiography. Most of the remaining conceptuses were dissected further so that the allantois could be moved gently with forceps in order to determine whether it was adherent to the chorion or elsewhere. The allantois was recorded as adhering if it resisted separation or showed obvious damage thereafter. Other conceptuses, including those used to assess the affect of [^{3}H]methyl thymidine-labelling on growth and development, were fixed intact for 24 hours in Bouin's fixative (Gurr, UK) for conventional histology. Following fixation in Bouin's, conceptuses were dehydrated, cleared in Histoclear (National Diagnostics, Atlanta, GA), embedded in fibrowax (Raymond Lamb, UK) at 56˚C, sectioned at 6 μm and then stained with haematoxylin and eosin.

Sections for autoradiography were immersed for 30 minutes in cold 5% trichloroacetic acid after they had been de-waxed and hydrated (Copp et al., 1986). They were then rinsed thoroughly in Milli-Q water and coated with a single layer of Kodak NTB-2 emulsion (5 parts: 7 parts 1% glycerol). Once dry, they were stored at 4˚C for 21 days before being developed, fixed and mounted unstained in DPX (Gurr) via Histoclear.

RESULTS

Early development and fusion of the allantoic bud in situ

Both the size and shape of the allantoic bud varied among freshly dissected conceptuses of the same stage (Fig. 1). The variation was particularly marked at the late headfold stage, when the bud ranged from a small unexpanded knob to an elongated, apically swollen rod (Fig. 1A). By the 3-somite stage, the base of the allantois had thickened considerably (Fig. 1C) and, by the 5- to 7-somite stage, the spaces between cells in its apical region had generally increased (Fig. 1E-G). At all stages lateral blebs were common (Fig. 1B,D). The bifurcated appearance that is evident in the 5-somite-stage allantoises illustrated in Fig. 1E was also not unusual in more advanced specimens.

Attachment of the allantois to the chorion was exceptional in conceptuses developing in vivo that had fewer than 5 somites (2/261, 0.07%), but was the norm among those with 6 or more (Fig. 2). It showed very similar stage specificity in vitro, irrespective of whether conceptuses had been cultured intact or following sham-operation for removal of the allantois (Fig. 2).

The allantois had formed focal adhesions with the mesodermal surface of the amnion or the yolk sac in approximately 38% of in vivo conceptuses that had not reached the stage of

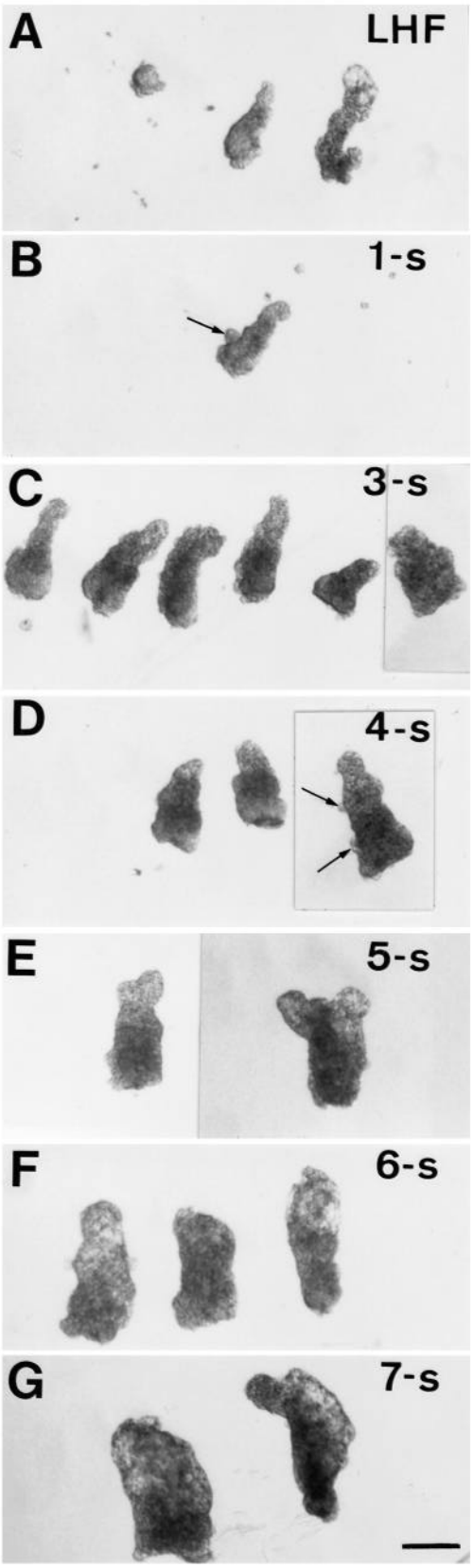

Fig. 1. Variability in the size and shape of the allantois between the headfold- and 7-somite stage. Allantoises displayed in these panels were cut from their basal insertion in the posterior primitive streak. The basal surface is consistently at the bottom, although the orientation of the long axis is variable. In A-E, the allantoises were not attached to the chorion, but in F-G, the allantoises were attached. (A) Late headfold (LHF). (B) 1-somite pair (1-s). (C) 3-somite pairs. (D) 4-somite pairs. (E) 5-somite pairs. (F) 6-somite pairs. (G) 7-somite pairs. The arrows in B and D point to examples of allantoic 'blebs'. Scale bar: 200 μm.

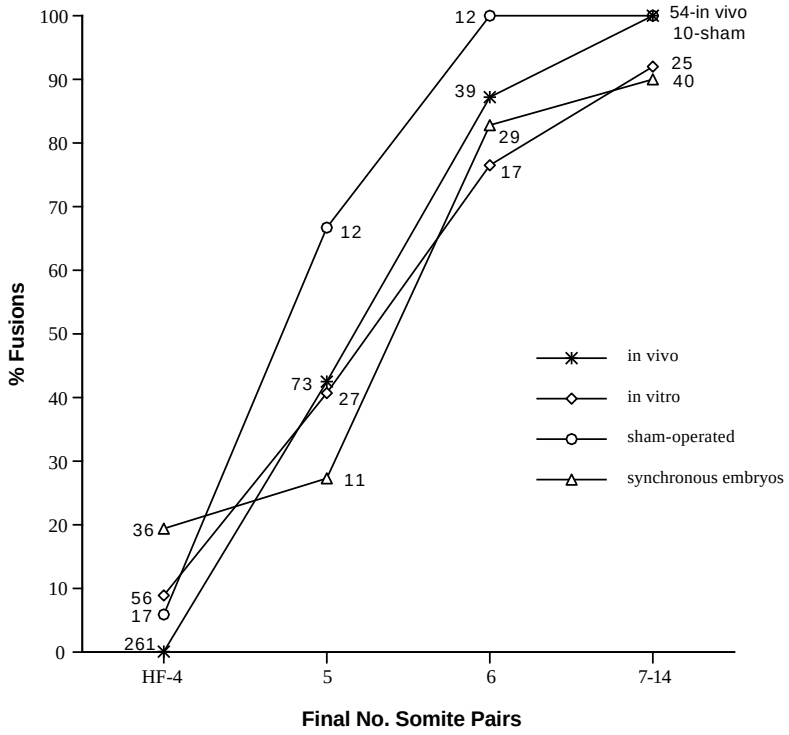

Fig. 2. Staging of attachment of the allantois to the chorion. These data are from the PO strain and demonstrate graphically the stage at which chorioallantoic fusion took place in vivo, in vitro, in sham-operated conceptuses and in conceptuses receiving synchronous allantoic grafts. Conceptuses in vitro were cultured from the mid-streak through the neural plate stages (Downs and Davies, 1993), and were scored after at least 8 hours in culture. Two conceptuses from these experiments (1: 8-s; 1: 9-s) showed abnormal somitic development, vesiculation of the surface ectoderm along the trunk and a clubbed allantois that touched, but did not adhere to, the chorion. These conceptuses were initially at the neural plate/early and late bud stages, respectively, and had been cultured for 29 hours. All other conceptuses in this study appeared normal. The numbers next to each data point are the numbers of conceptuses examined.

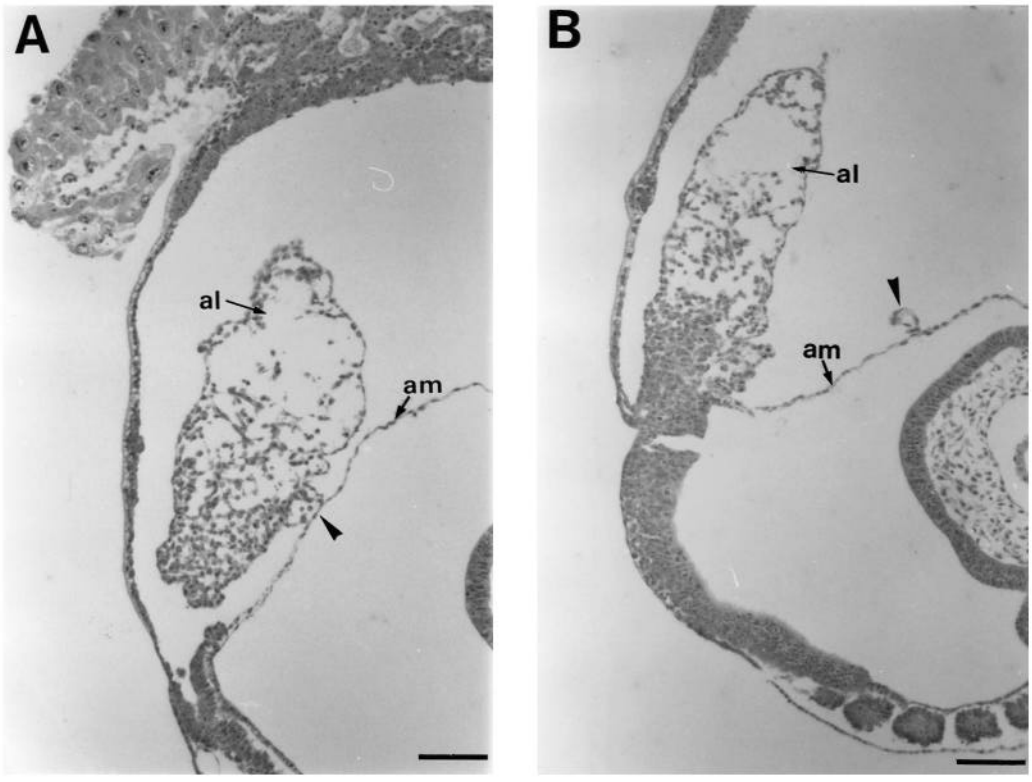

Fig. 3. Focal contacts between the allantois and the amnion in conceptuses that had developed in vivo. (A) 6-somite-stage conceptus. (B) Allantois of 6-somite-stage conceptus that was just attaching to the chorion (this is a more lateral section through the allantois). Arrowhead in A points to contact between the allantois and the amnion, and in B to a presumed remnant of a bleb of the allantois on the amnion. Scale bar: (A) 50 μm; (B) 100 μm. Abbreviations: al, allantois; am, amnion.

its attachment to the chorion at the time of dissection (Table 1). No preferential adhesion to the amnion or yolk sac in relation to stage was observed (data not shown). The sites of these adhesions, two examples of which are illustrated in Fig. 3A and B, seemed to correspond with the small blebs on the allantois noted earlier (Fig. 1B,D). Such focal adhesions between the allantois and yolk sac or amnion were rare in intact conceptuses that had been cultured for 8 hours (Table 1B). Interestingly, they were observed in nearly one-third of sham-operated conceptuses that were cultured for 2 hours, when they invariably occurred between the allantois and yolk sac, but not in those cultured for 4-6 hours (Table 1).

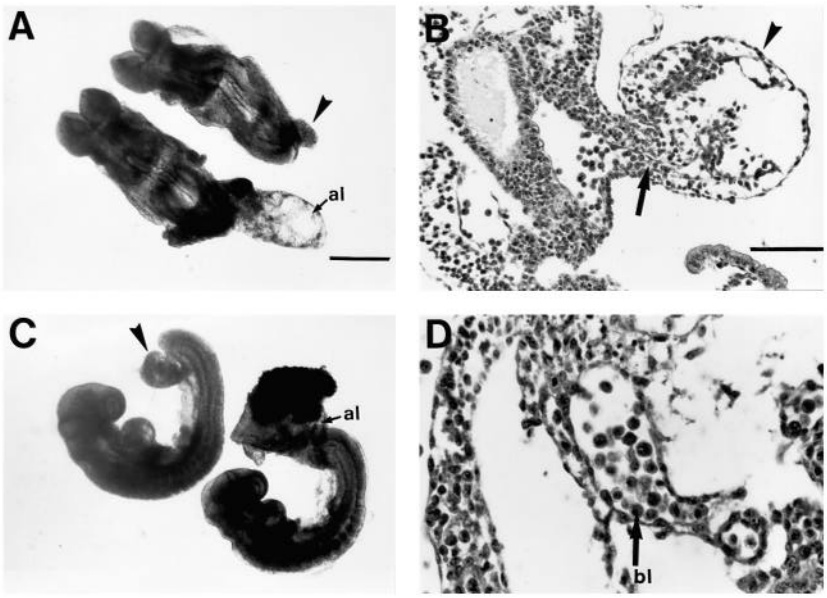

Fig. 4. Extent of regeneration of the allantois after its removal. (A) A pair of 5-somite-stage conceptuses cultured in vitro for 7 hours (final somite no: 7-8 pairs). The allantois (al) of the lower specimen had not been removed (and it was found fused to the chorion), while the allantois from the upper embryo had been excised at its base (arrowhead) prior to culture. Both conceptuses had undergone similar development, the operated one having seven somites, whilst the unoperated conceptus has eight. The yolk sac circulation was more advanced in the operated conceptus than in the unoperated one. (B) 6 μm histological section of the allantoic stump in the operated conceptus of A showing early vasculogenesis within the stump (arrowhead) and the presence of embryonic red blood cells (arrow). (C) A pair of conceptuses initially with 4-5 somite pairs, after culture for 22 hours. The allantois of the conceptus in the upper left had been removed before culture (arrowhead). Both conceptuses had 18 somite pairs and were identical in all other morphological landmarks except turning: the operated specimen was nearly fully turned, whilst the unoperated one was only half-turned. (D) 6 μm histological section of the allantoic stump of the operated conceptus to show that vasculogenesis had occurred and that embryonic red blood cells (bl) were present within one of the vesicles. Scale bars: (A), (C), 100 μm; (B), (D), 100 μm and 50 μm, respectively.

To determine the appropriate periods of culture for investigating attachment of the allantois to the chorion experimentally, final somite counts were made on conceptuses that had been incubated for 4-6.5 hours following explantation at the headfold or early somite stages. According to the results obtained from a total of sixty conceptuses cultured in five experiments, new pairs of somites were formed at intervals of approximately 2 hours (0.97±0.47 somite pairs per 2 hours).

Development in vitro of conceptuses following removal of the allantois or labelling with [^{3}H]methyl thymidine

Whether development was compromised by removal of the allantois was investigated by culturing conceptuses with and without allantoises in vitro as stage-matched pairs. Two experiments were undertaken in which a total of nineteen such pairs were cultured for 7 or 22 hours. No adverse effect of removing the allantois was discernible when control and operated conceptuses were compared (Fig. 4A,C). Comparison was made with the aid of the detailed morphological staging system described elsewhere (Downs and Davies, 1993), in conjunction with appraisal of heart beat, yolk sac circulation and state of development of the heart, branchial arches, lens and otocyst (Brown, 1990). The extent of regeneration of the allantois from the remaining stump was negligible in the operated conceptuses, and in only 2 of the 19 had it attached to the chorion. Vascularization and the presence of embryonic blood cells were evident histologically in the stumps by 7 hours of culture, when the initially 4- to 5-somite conceptuses had reached the 7- to 8-somite stage. In some cases, the regenerates could be seen to contain red blood cells when examined in the dissecting microscope (Fig. 4B,D).

Table 1. Allantoic focal contacts with the amnion or yolk sac during growth and expansion of the allantois

	(A) in vivo (n=32)	(B) in vitro (n=48)	C. in vitro sham-operated	
	0 hours	8 hours	2 hours (n=26)	4-6 hours (n=27)
Amnion	6 (18.8%)	0	0	0
Yolk sac	6 (18.8%)	4† (8.3%)	8‡ (30.8%)	0
Total	12 (37.5%)	4 (8.3%)	8 (30.8%)	0 (0.0%)

All conceptuses were scored at 1- to 5-somite pairs. See text for details. Data are from separate experiments.

†All four of these allantoises were in contact with the yolk sac blood islands.

‡1 conceptus (5-somite stage) exhibited contact of the allantois with both the yolk sac and the chorion.

In two separate experiments, the effect of [^{3}H]methyl thymidine on the differentiation of conceptuses in vitro was tested. The extent of differentiation of labelled conceptuses, both in general and specifically in relation to the staging of attachment of the allantois to the chorion, was found to be similar to that of unlabelled controls. (Details of the method used to measure the effect of tritium on growth and development in vitro will be presented elsewhere, Downs and Lawson, unpublished data.) Most cells in treated conceptuses had labelled nuclei, the exception being those of the node, which were largely unlabelled in both the headfold- and 6-somite-stage conceptuses. (A similar result was recently reported for Hensen's node in the chick (Sanders et al., 1993).)

Synchronous allantoic grafts

Having established that the [^{3}H]methyl thymidine-labelling regime did not affect development at the headfold and early somite stages, labelled conceptuses were used as donors to

ensure that grafts could be distinguished from regenerating host allantoic tissue. Since no difficulty was encountered in making this distinction, unlabelled allantoic tissue was used either as auto- or allo-grafts in later experiments. Grafted conceptuses were cultured for different periods following operation on the assumption that somite number increased at a rate of 1 pair per 2 hours (see Results, Section A).

(1) Conceptuses examined before the 6-somite stage

A comparison in the staging of chorionic fusion between grafted allantoises and those developing in situ is presented in Fig. 2. Although often wedged between the amnion and chorion, grafts were unattached in the exocoelomic cavity in the majority of conceptuses with fewer than 5 somites (Table 2; Fig. 2). In all except 5 of the 16 cases where attachment had occurred, it was to the chorion alone (Fig. 2; Table 2). In the five exceptions, the grafts were attached either to the amnion or yolk sac or, in one case, to both the yolk sac and the residual stump of the host allantois (Table 2). At the 5-somite stage, the proportion of conceptuses with grafts attached to the chorion alone was somewhat higher than earlier (27.2% at 5-somites versus 19.4% at <5-somites, Fig. 2). Two of the four grafts that were attached elsewhere at this more advanced stage were adhering to both the chorion and the amnion, and the other two were adhering to the stump of the allantois (Table 2).

Table 2. Location of synchronous donor allantoic grafts that were not adherent only to the chorion

	<5 somites *n*=36 (%)	=5 somites *n*=11 (%)	≥6 somites *n*=69 (%)
Not adherent†	24 (66.7%)	4 (36.4%)	6 (8.7%)
Ch + YS	0	0	1 (1.4%)
Ch + Am	0	1 (9.1%)	1 (1.4%)
Ch + Al	0	0	1 (1.4%)
Am	2 (5.6%)	1 (9.1%)	1 (1.4%)
YS	2 (5.6%)	0	1 (1.4%)
Al	1‡ (2.8%)	2 (18.2%)	0
Total	29 (80.6%)	8 (72.7%)	11 (15.9%)

†In at least one half of the 34 conceptuses in each category of this class, the donor allantois was wedged between the chorion and the amnion, but not adherent to either tissue.

‡The allantoic graft was adherent to both the residual stump of the host allantois and the yolk sac.

Abbreviations: Al: allantois; Am: amnion; Ch: chorion; YS: yolk sac.

(2) Conceptuses examined at or beyond the 6-somite stage

In the great majority of cases, the allantoic grafts were attached to the chorion only (Fig. 2). In the remainder, they were either loose within the exocoelom or adhering to the yolk sac or amnion, as well as, or instead of, the chorion (Table 2). In no case was the graft attached to the stump of the original allantois. Examination of autoradiographic preparations of sectioned conceptuses that received labelled grafts confirmed

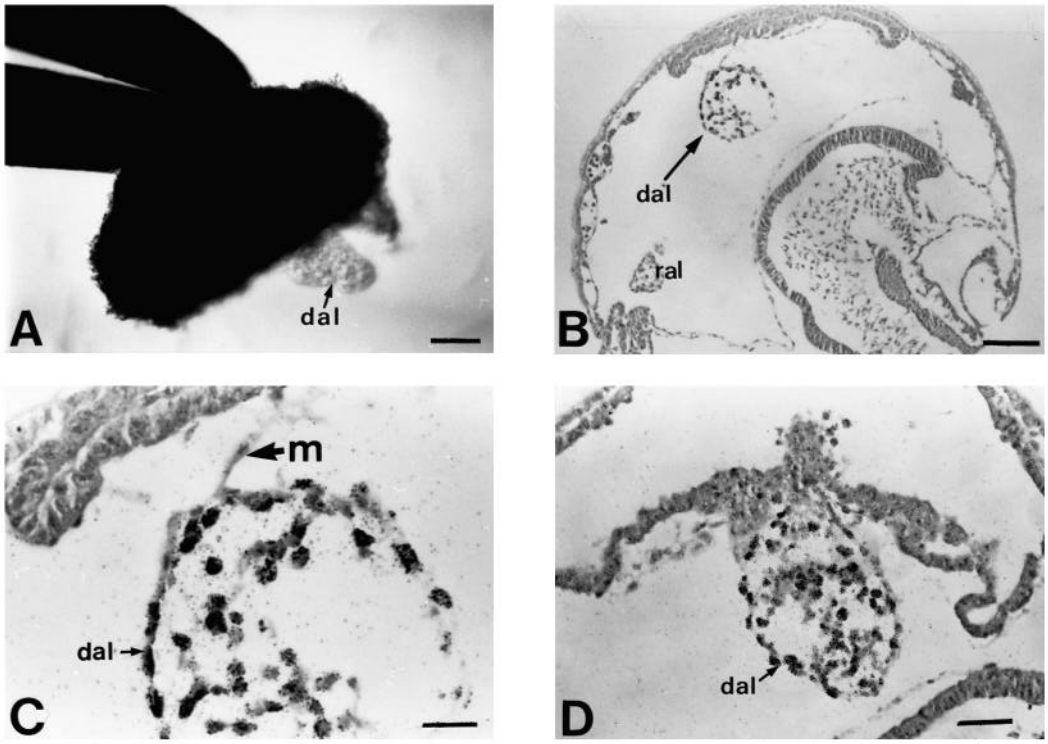

Fig. 5. Chorioallantoic attachment in synchronous conceptuses as assessed by visual inspection and autoradiography. (A) Attachment observed in an operated 6-somite-stage conceptus after removal of the yolk sac. (B,C) Bright-field autoradiographs of tethering of the allantois from a [^{3}H]methyl thymidine-labelled donor to the chorionic mesodermal component in a sectioned 6-somite-stage conceptus. The resident allantois can be seen as a small stump in B. The arrow in C points to the point of fusion between the mesodermal components of the chorion and allantois. (D) Assimilation of the labelled allantois by the chorion in another 6-somite-stage conceptus. Scale bars: (A,B), 100 μm; (C), 25 μm; (D), 50 μm. dal, donor allantois; ral, resident allantois; m, point of contact between the mesodermal surfaces of the allantois and chorion.

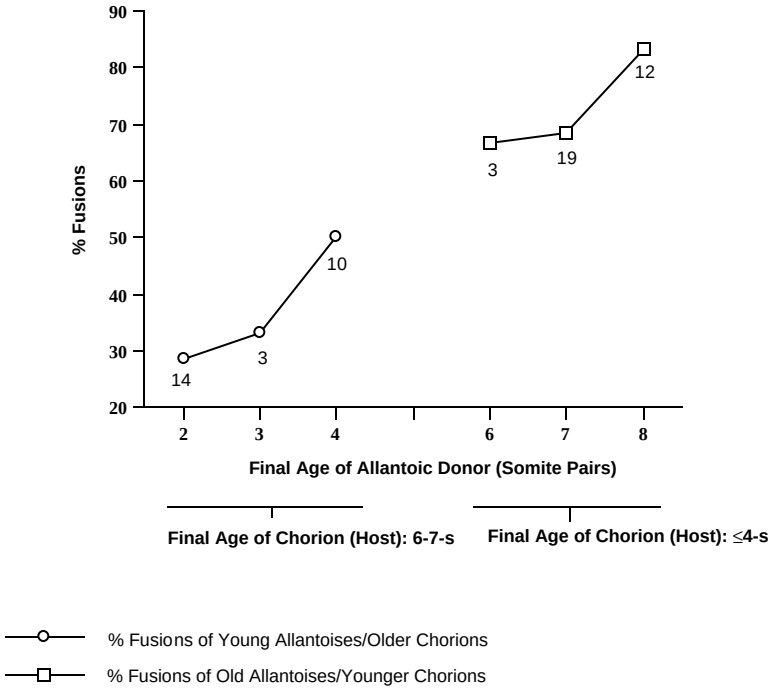

Fig. 6. Staging of attachment of of the allantois to the chorion in asynchronous conceptuses. Conceptuses were scored as in Fig. 5. The age of the allantoic donor is given as somite number on the horizontal axis, and the age of the (chorionic) host conceptus is presented similarly below the horizontal axis. The brackets encompass the age of those allantoises that were introduced into hosts of either 6-7 somites (left side), or ≤4 somites (right side). Conceptuses with allantoises or chorions of age 5-somites were not included in this analysis, because according to Fig. 2, the allantois demonstrated an approximately 50% chance of adhering to the chorion; therefore, data collected at this stage would not be meaningful in asynchronous experiments. Conceptuses at the late headfold (LHF) stage formed somites at the rate of approximately 1 pair/2 hours.

that the initial union between allantois and chorion was via their intact surfaces (Fig. 5A-C). Where fusion had progressed beyond the initial attachment stage, integrity of the surface layer of both chorion and allantois was lost (Fig. 5D).

It is notable that the grafts did not fuse with the chorion precociously, even though they are likely to have come into contact with it earlier than usual (Table 2, footnote to class labelled 'Not Adherent'). To investigate the possibility that fusion is a developmentally regulated process, additional experiments were undertaken in which allantoic grafts were made between conceptuses of different stages.

Asynchronous Allantoic Grafts

(1) Recipient conceptuses examined at or just beyond the 6-somite stage

In the first series of asynchronous grafting experiments, allantoises were taken from headfold- or early somite-stage donor conceptuses. They were grafted into 4- to 5-somite-stage hosts which were then cultured to the 6- to 7-somite stage, when the donors would be expected to have reached the 2- to 4-somite stage. The grafted allantois had attached to the chorion in just over one-third of these conceptuses, remaining completely unattached in most of the others (Fig. 6).

In the second series of experiments, the stage of donors and hosts was essentially reversed, with allantoises from 3- to 5-somite donors being grafted into early headfold- to 1-somite-stage hosts. In this series, culture was terminated when the allantoic donor conceptuses would have been expected to possess at least 6 pairs of somites. More than two-thirds of the host conceptuses showed chorioallantoic fusion, while only a few had reached the earliest stage at which it occurred in intact or sham-operated controls (Fig. 6). Conceptuses receiving synchronous grafts that were scored at or beyond the 6-somite stage were included in each asynchronous experiment as controls to ensure that culture conditions were permissive for chorioallantoic fusion (21/28, 75.0% fusion at the 6- to 7-somite stage).

These data suggest that chorioallantoic fusion depends more on the developmental stage of the allantois than the chorion. Thus, whilst the chorion could participate in fusion as early as the 1-somite stage, the allantois did not do so until it had attained the 6-somite stage.

To more closely define the period for which the chorion was competent to fuse with the allantois, a further series of asynchronous grafting experiments was undertaken in which the recipient conceptuses were cultured to the 12- to 14-somite stage. This ensured that the developmentally less advanced donor allantoises would also reach their stage of competence for fusion before the end of culture.

(2) Recipient conceptuses examined at the 12- to 14-somite stage

Since mice of the PO strain were no longer readily available, conceptuses from the F_2 generation of matings between (C57Bl/6×CBA) F_1s were used in this series of experiments. These conceptuses closely resembled those of the PO strain both in timing of chorioallantoic fusion (at 6-somites, 80.0%, n =35; at 7-somites, 100.0%, n=10; total number of conceptuses examined at the headfold- to 9-somite stages = 180) and in the rate of somite addition.

One concern about scoring conceptuses after a longer period in culture was that chorioallantoic fusion might advance to a stage at which the donor allantoic tissue was hard to identify. Therefore, whole allantoic buds rather than just apical halves were grafted in these experiments.

Donor allantoises from early headfold- to 3-somite-stage conceptuses were grafted into recipient conceptuses at the 4- to 5-somite stage. The operated conceptuses were cultured for approximately 18 hours before scoring, by which time concurrently cultured allantoic donor conceptuses had reached at

Table 3. Asynchronous experiments: recipient conceptuses examined at the 12- to 14-somite stage

Experiment type	Stage of donor allantois	Stage of host chorion	Final stage of donor allantois	Final stage of host chorion	No. conceptuses	No. adherent only to chorion (%)†	No. unknown (%)
A Asynchronous grafts	Headfold-2-somites	4-5- somites	7-11-somites	12-14- somites	26	18 (69.2%)	6 (23.1%)
B Synchronous grafts	Headfold - 5-somites	Headfold-5-somites	8-13-somites	8-13-somites	33	25 (75.8%)	4 (12.1%)
C in vitro controls	Neural plate-5-somites	Neural plate-5-somites	5-14-somites	5-14-somites	15	14‡ (93.3%)	0

The category 'Unknown' was added to this Table because long culture periods precluded the ability to identify all donor allantoises at the experimental endpoint. See Results for methods. **Experiment Type A**: Asynchronous grafts of young donor allantoises into the exocoelomic cavity of older recipient conceptuses and culture until the donor allantois was at least at the 7-somite stage. **Experiment Type B**: Synchronous grafts to monitor appropriate chorioallantoic fusion. **Experiment Type C**: In vitro untampered controls to verify appropriate culture conditions for fusion.

†Asychronous and synchronous grafts not indicated in this Table are those grafts that were identified as 'not adherent' to any structure in the exocoelomic cavity.

‡1/15 embryos was at the 5-somite stage at the end of the culture period and the allantois would not be expected to have fused to the chorion.

least the 7-somite stage. Synchronous controls were included in all four experimental runs and in vitro controls in three of them.

At the end of the culture period, chorioallantoic fusion had clearly taken place in 69% of the conceptuses with asynchronous grafts and in 78% of the controls with synchronous grafts (Table 3). As anticipated, donor allantoic tissue could not be identified in all cases, the frequency of failure being 12% in synchronous grafts and 23% in asynchronous grafts. In the corresponding in vitro controls, the rate of chorioallantoic fusion was nearly 100%. That failure to identify some of the longer-term grafts was due to their intimate union with the chorion was confirmed in a related study in which donor allantoic tissue was marked with a transgene that gives ubiquitous expression of *lacZ* (ROSAB-geo26, Friedrich and Soriano, 1991). Here, in cases where the grafts were not discernible in the dissecting microscope after culture, they were invariably found to be flattened against the chorion following staining for *lacZ* expression (K. Downs, unpublished observations).

DISCUSSION

Initiation of chorioallantoic fusion is selective, and developmentally regulated via the allantois

We have found that initial attachment of the allantois to the chorion occurs during a fairly narrow window between the 5- and 6-somite stage in the great majority of conceptuses developing in vivo in the PO strain of mice and in the F_2 generation of (C57Bl/6 × CBA) matings. Most importantly for the present investigation, this critical early step in development of the chorioallantoic placenta took place on schedule in conceptuses cultured in vitro from an earlier stage even when, as in the sham-operated controls, they had experienced puncture of the wall of the nascent visceral yolk sac and consequent loss of exocoelomic fluid. Furthermore, removal of the resident allantois did not adversely affect development of the remainder of the conceptus in vitro and was typically followed by only modest regeneration from its residual stump. Hence we believe that the combination of microsurgical and in vitro culture techniques employed in the present investigation offers a valid way of addressing the problem of how the allantois attaches specifically to the chorion. In most experiments, only the apical region of the allantois was used in grafts so as to ensure that primitive streak tissue and primordial germ cells were excluded. Possible effects on the behavior of the grafts of inclusion of the latter cells, which would no longer be appropriately sited to migrate into the fetal hindgut, were of particular concern.

Only rarely did allantoic tissue transplanted into the exocoelom attach to the residual stump formed by removal of the resident allantois. Nevertheless, in synchronous transplantation experiments, the grafts were found to attach selectively to the chorion, notwithstanding their variable siting within the exocoelomic cavity. Hence the possibility that selective attachment of the allantois to the chorion is due to sustained linear growth in the appropriate direction that is dictated by its initial orientation can clearly be discounted. Such directed growth may nevertheless facilitate homing of allantoises developing in situ.

The removal and transplantation of allantoic tissue through the wall of the yolk sac caused a marked temporary reduction in the volume of the exocoelom through loss of fluid. Both this and the fact that the conceptuses were subsequently incubated in roller culture, make it most unlikely that the grafts were more favourably placed to make contact with the chorion than with the other parts of the surface of the exocoelom. Indeed, we observed that before the 6-somite stage, grafts were generally found wedged between the amnion and chorion, but were not adherent to either tissue (Table 2). Furthermore, in the allantoises grafted before the 5-somite stage, contact between the grafted allantois and all other surface components of the exocoelom was observed, making it highly unlikely that there was a bias in the conditions of roller culture that would ensure preferential attachment to the chorion (Table 2).

It is noteworthy in this context that focal adhesions between the allantois and the amnion or the yolk sac were observed in more than one-third of conceptuses freshly recovered from in vivo before chorioallantoic fusion had begun. Similar adhesion sites were also evident in a substantial minority of both unoperated and sham-operated conceptuses that had been cultured in vitro for 2 hours, but not in those that had been cultured for

4 or 8 hours. Unlike in conceptuses developing in vivo, such foci were invariably between the allantois and the yolk sac. Again, of the operated conceptuses in the synchronous allantoic transplantation experiments that were examined before or at the 5-somite stage, only a minority had adherent grafts. Among these, the transplanted allantoic tissue was as often attached to other parts of the lining of the exocoelom in addition to, or instead of, the chorion, as to the latter alone. By contrast, once such operated conceptuses had attained 6 or more somite pairs, adhesion of the allantoic grafts to the chorion specifically was the norm.

These experimental findings clearly point to the involvement of a selective cell adhesion mechanism, which is regulated both spatially and temporally, in the initial attachment of the allantois to the chorion. The nature of such a mechanism was further illuminated by the results of an additional series of experiments in which donors of the allantoic grafts were either developmentally more or less advanced than recipient conceptuses. Here it was found that, while allantoic tissue from conceptuses that were at or near the stage of initial chorioallantoic attachment consistently attached to a younger chorion, attachment occurred much less frequently when the staging of donor and recipient conceptuses was reversed (Fig. 6). This implies that the temporal regulation of attachment of the allantois to the chorion may be dictated principally by a stage-related change in the adhesive properties of allantoic cells.

Allantoic homing

While this study clearly points to differential adhesiveness as the means whereby the allantois retains specific attachment to the chorion once the two structures have come together, the question of how they are initially brought into contact remains unanswered. In the case of the grafts, we suspect that doming of the amnion as its cavity enlarges may serve to lift the allantois towards the chorion. Consistent with this possibility is the finding that approximately half of the synchronous grafts examined before the 6-somite stage were wedged between the amnion and chorion (=14/28 in the 'non-adherent' class, Table 2). The focal attachments between allantois and yolk sac observed in conceptuses developing in vitro were clearly transient, being limited to the first 2 hours of culture. The fact that the allantois was attached to the amnion or yolk sac in only a minority of conceptuses developing in vivo suggests that these contacts, likewise, were not enduring. The occurrence of blebbing and branching of the allantois may be related to the formation of such foci which could play a role in 'homing' of the allantois under normal conditions.

Regeneration of the allantois

Conceptuses from which the allantois had been removed and not replaced with a corresponding graft continued to develop normally in culture to the 18-somite stage. In particular, formation of the hindgut, which begins to invaginate at the 5-somite stage in PO mice, was not impaired despite being the fetal structure whose primordium lies closest to the allantois at the posterior end of the primitive streak. Tam and Beddington (1987) have shown by transplantation that cells from this region of the primitive streak no longer contributed to the allantois when transplanted orthotopically into conceptuses with 3-7 somites, at approximately 8.0-8.5 dpc.

In our study, some allantoic tissue invariably regenerated from the stump that remained following removal of the allantois at the headfold and early somite stages. Interestingly, profiles of endothelium containing nucleated erythrocytes were evident in sectioned regenerates, notwithstanding the fact that very few of them had enlarged enough to reach the chorion. Hence, this confirms that contact with the chorion is not required to initiate vascularization of the allantois (Jolly and Ferester-Tadié, 1936, Fig. 43; Ellington, 1985). This finding is consistent with recent evidence from in situ hybridization that the *flk*-1 gene, which encodes a receptor tyrosine kinase that is specific for endothelial precursor cells, is abundantly transcribed in the prefusion allantois (Yamaguchi et al., 1993). Whether the endothelium and blood cells in the allantois originate from within it or through secondary invasion from elsewhere is not known.

Regional differentiation in the extraembryonic mesoderm

Our observations on the early stages of chorioallantoic fusion in the mouse support those of Ellington (1985) in the rat in showing that initial attachment between these two extraembryonic structures is via their intact surface layers. It seems most probable, but is as yet unproven, that the surface layer of the allantois is formed by an inward ballooning of part of the extraembryonic mesoderm that lines the entire exocoelomic cavity as its mesodermal core pushes into this cavity from the posterior end of the primitive streak. If this is, indeed, the case, it is part of the same cell layer that constitutes the inner surface of the chorion, yolk sac, and amnion. Hence, the selective attachment of the allantois to the chorion demonstrated in the present study implies that the mesodermal lining of the exocoelom must be regionally differentiated with regard to its adhesive properties. This mesoderm is known to be a primitive ectodermal (epiblast) derivative (Gardner, 1983) which passes into the extraembryonic region of the conceptus from the proximal part of the primitive ectoderm via the primitive streak (Lawson et al., 1991; Lawson and Pedersen, 1992). Its subsequent regional differentiation presumably depends on its interaction with distinct tissues, namely the chorionic ectoderm, yolk sac endoderm, and amniotic ectoderm, which it lines in the ceiling, walls, and floor of the exocoelom, respectively. However, this has yet to be proven experimentally.

We wish to thank Andrew Copp, Tim Davies, Kirstie Lawson and Denis New for valuable discussion and comments. We are grateful to the reviewers and to Janet Rossant for critical appraisal of our manuscript. This work was supported by the Imperial Cancer Research Fund, the EPA Cephalosporin Trust Fund (K. M. D.), start-up funds from the Anatomy Department, and the Medical and Graduate Schools of the University of Wisconsin--Madison Medical School (K. M. D.), and The Royal Society (R. L. G.).

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(Accepted 7 November 1994)