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Effect of acyclovir on mammalian embryonic development in culture*

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Abstract. 1. Acyclovir [9-(2-hydroxyethoxymethyl)guanine] interfered with embryonic development in vitro when assessed with the "whole-embryo" culture technique. The "no-observed-effect level" was at 10 μM acyclovir; 2. Minor impairment of embryonic development (retarded development of ear anlagen) was observed in vitro at 25 μM acyclovir in the culture medium. At high concentrations (100 or 200 μM) development of the ear anlagen was largely inhibited. At concentrations of 50 μM acyclovir or higher, additional disturbances of embryonic differentiation in vitro became obvious, resulting in gross structural abnormalities, especially of the brain (telencephalon); 3. Histological examinations confirmed and extended these observations: at 100 μM acyclovir alterations of the neuroepithelium of the ventricles were pronounced, the telencephalon had developed poorly or was almost completely absent, and necroses were seen in the ear anlagen, the maxillar branch and within the somites; 4. In a limb bud culture (mouse embryos, starting with day 11 of gestation) acyclovir interfered with the differentiation of cartilaginous bone anlagen at concentrations of 200 μM and more in the culture medium. A concentration of 100 μM induced no significant effect. Thus, this organ culture system is less sensitive to the action of acyclovir when compared with whole-embryo culture; 5. Contrary to the results achieved with acyclovir, physiological nucleosides (2'-deoxyguanosine and 2'-deoxyadenosine) did not interfere with embryonic development in vitro even at the highest concentration tested (500 μM).

Key words: Acyclovir – Physiological deoxy-nucleosides – "Whole – embryo culture" – Limb bud culture – In vitro techniques – Embryotoxicity

Introduction

Acyclovir [9-(2-hydroxyethoxymethyl)guanine] is used as a specific virustatic agent for the treatment of infections with herpes-type viruses. With the help of a specific, virus-coded kinase acyclovir is phosphorylated to the corresponding triphosphate (Furman et al. 1981) and incorporated into the viral genome during replication. Inhibition of DNA synthesis (Furman et al. 1979) by termination of DNA chain growth is assumed to be responsible for the virocidal effect observed. Normal, non-infected mammalian cells apparently convert acyclovir much more slowly to the triphosphate, thus explaining the relative specificity of the agent (Biron and Elion 1980). Studies on reproductive toxicity up till now apparently have given no evidence for a teratogenic effect, but appropriate in vivo studies are complicated by a nephrotoxic action seen at high doses of acyclovir, due to the poor water solubility and crystallization of the substance within the tubular system of the kidneys (Tucker et al. 1983).

In order to clarify whether acyclovir has a teratogenic potential and at which concentrations of acyclovir a teratogenic potency may result, we performed in vitro studies with mammalian embryos, using the "whole-embryo" culture technique developed by New and coworkers (New 1978; Cockroft 1976, 1977) and a limb bud culture technique. With these techniques nephrotoxic complications seen with in vivo studies can be circumvented and possible embryotoxic effects of higher acyclovir concentrations evaluated.

Materials and methods

Animal maintenance and mating procedure. Wistar rats (Bor: Wisw/SPF, TNO; Fa. Winkelmann, Borchen, Federal Republic of Germany) and mice (Han: NMRI, Zentralinstitut für Versuchstierzucht, Hannover) were kept under spf-conditions at a constant day/night cycle (light from 9.00 to 21.00 hours), at 21 ± 1 °C and 50 ± 5% relative humidity. They received a standard pellet feed (Altromin® 1324) and water ad libitum. One male was caged together with three female animals for 2 h (6.00–8.00 a.m.) and the first 24 h following this mating period (7.00) was called "day 0" of pregnancy if sperm were detected in the vaginal smears.

"Whole-embryo" culture. For the results presented in this paper rat embryos were cultured starting on day 9.5 of gestation. This corresponds to one to four somites in our strain. The experimental conditions have been described in a preceding paper (Klug et al. 1985).

Limb bud culture. Limb buds were cultured using a suspension culture technique and a chemically-defined culture

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medium (Lessmöllmann et al 1976; Neubert et al. 1977) using 11-day-old mouse embryos. The technique has been described in detail elsewhere (Blankenburg 1981). About 15 explants were used per assay (i.e. per flask); all assays were performed at least in duplicate. After a 6-day culture period the limbs were fixed in Bouin's solution, the cartilage formed and differentiated in vitro was stained with alcian blue, and the explants cleared and stored in cedar oil.

The drugs were added to the culture medium at concentrations of 100–500 µM. The limb buds were exposed to the drugs only for the first days of the culture period. Subsequently, the drug-containing medium was replaced by control medium.

Drug application. Acyclovir was supplied by the Deutsche Wellcome GmbH (3006 Burgwedel) in the form of a sodium salt, which is produced by freeze drying of a solution of acyclovir in NaOH; 2'-deoxyguanosine and 2'-deoxyadenosine were purchased from Sigma.

For our studies 250 mg acyclovir was solubilized in 10 ml aqua tridest in its original ampoule. To achieve the desired concentrations of acyclovir in the culture medium, different amounts of the stock solution were diluted with either Tyrode-phosphate buffer (for whole-embryo culture) or with culture medium (for limb bud cultures). The final concentrations tested were:

(1) whole-embryo culture:

acyclovir

(a) 200 µM ≙ 45.0 µg acyclovir/ml culture medium;
(b) 100 µM ≙ 22.5 µg acyclovir/ml culture medium;
(c) 50 µM ≙ 11.3 µg acyclovir/ml culture medium;
(d) 25 µM ≙ 5.6 µg acyclovir/ml culture medium;
(e) 10 µM ≙ 2.25 µg acyclovir/ml culture medium.

physiological deoxynucleosides

(a) 200 µM 2'-deoxyguanosine ≙ 53.44 µg/ml culture medium;
(b) 500 µM 2'-deoxyguanosine ≙ 133.60 µg/ml culture medium;
(c) 200 µM 2'-deoxyadenosine ≙ 50.24 µg/ml culture medium;
(d) 500 µM 2'-deoxyadenosine ≙ 125.60 µg/ml culture medium.

Table 1. Effect of acyclovir and physiological deoxynucleosides (2'-deoxyguanosine, 2'-deoxyadenosine) on growth and development of 9.5-day-old rat embryos cultured for 48 h

$n\Sigma = 213$	YS	CR	SOM	PROT	Score	ABN%
Control	4.56	3.60	27.0	283.5	38.0	0
$n = 44$	*4.32 (100%)*	*3.36 (100%)*	*26.0 (100%)*	*234.5 (100%)*	*37.0*	
	4.20	3.18	25.0	176.0	35.25	
10 µM Acyclovir	4.56	3.60	27.0	264.0	37.0	0
$n = 19$	*4.38 (101%)*	*3.30 (98%)*	*26.0*	*236.5 (101%)*	*37.0*	
	4.26	3.12	25.0	209.0	35.0	
25 µM Acyclovir	4.50	3.30	26.0	196.0	36.0	0
$n = 27$	*4.26 (99%)*	*3.06 ▲ (91%)*	*25.0 ▲*	*150.0 ▲ (64%)*	*36.0 ▲*	
	4.08	2.88	23.0	123.5	34.0	
50 µM Acyclovir	4.51	3.36	27.0	175.0	38.0	28%
$n = 18$	*4.35 (101%)*	*3.15 ▲ (94%)*	*26.0*	*130.0 ▲ (55%)*	*34.5 △*	
	4.17	2.88	24.0	110.0	32.75	
100 µM Acyclovir	4.80	3.36	27.0	174.5	33.0	95%
$n = 19$	*4.56 ▲ (106%)*	*3.00 ▲ (89%)*	*26.0*	*137.5 ▲ (59%)*	*32.0 ▲*	
	4.38	2.82	25.0	122.3	31.0	
200 µM Acyclovir	4.74	2.91	24.0	103.5	31.0	95%
$n = 21$	*4.56 ▲ (106%)*	*2.64 ▲ (79%)*	*23.0 ▲*	*93.5 ▲ (40%)*	*30.0 ▲*	
	4.44	2.43	20.0	63.7	29.0	
200 µM 2'-deoxyguanosine	4.65	3.76	27.75	284.0	38.0	0
$n = 16$	*4.53 (105%)*	*3.45 (103%)*	*26.5*	*252.0 (108%)*	*37.0*	
	4.33	3.25	26.0	225.0	36.0	
500 µM 2'-deoxyguanosine	4.32	3.39	26.0	206.0	36.5	6% (1/16 Shape)
$n = 16$	*4.20 △ (97%)*	*3.18 △ (95%)*	*25.0 △*	*177.0 △ (76%)*	*34.0 ▲*	
	3.93	3.01	24.0	156.0	33.0	
200 µM 2'-deoxyadenosine	4.72	3.52	26.0	250.0	37.0	0
$n = 16$	*4.53 ▲ (105%)*	*3.39 (101%)*	*26.0*	*241.0 (103%)*	*36.5 △*	
	4.39	3.15	25.0	218.0	35.0	
500 µM 2'-deoxyadenosine	4.38	3.36	26.0	231.8	37.0	0
$n = 17$	*4.26 (99%)*	*3.30 (98%)*	*25.0 △*	*197.0 (84%)*	*36.0 △*	
	4.20	3.09	24.0	183.3	33.5	

Median values (in italics) are given in this table. The figures under the median values represent the first quartile, the figures above the median values represent the third quartile

△ = significantly different from control group at $P \leq 0.01 - 0.05$ (Mann-Whitney Test)

▲ = significantly different from control group at $P \leq 0.01$ (Mann-Whitney Test)

YS = yolksac diameter [mm]; CR = crown-rump length [mm]; SOM = number of somite pairs; PROT = protein content [µg/embryo]

For culture conditions cf Klug et al. (1985)

(2) limb bud culture:

acyclovir
(a) 400 μM ≙ 90 μg acyclovir/ml culture medium;
(b) 200 μM;
(c) 100 μM.

physiological deoxynucleosides
(a) 500 μM 2'-deoxyguanosine ≙ 133.60 μg/ml culture medium;
(b) 500 μM 2'-deoxyadenosine ≙ 125.60 μg/ml culture medium.

For the whole-embryo culture, appropriate stock solutions were sterile-filtered and added to the corresponding culture bottles. The pH and osmolarity of the medium were not altered by the drug.

For the limb bud culture, certain precautions were observed when acyclovir was added in order to protect the pH-sensitive components of the culture medium. The acyclovir stock solution was added drop-wise to the medium while simultaneously gassing it with 5% CO_2 and 95% O_2. The physiological deoxynucleosides were dissolved in the culture medium; the pH and osmolarity were not altered. Subsequently, the drug-containing culture medium was sterile-filtered.

Results

Studies with acyclovir using the whole-embryo culture

With concentrations of acyclovir in the culture medium exceeding 10 μM, a dose-dependent pronounced retardation of the ear anlage was observed (i.e. the ear vesicle was smaller or not yet closed). This finding is apparently not the result of a "general" retardation, since growth and development of the other organ anlagen was normal. The decrease in the score is only caused by the disturbance of the development of the ear anlage and, at higher concentrations (beginning with 50 μM), by abnormalities in the head region. A dose-related, pronounced reduction in the protein content of the treated embryos is obvious (cf Tab. 1). Experience has shown that this is a sensitive, but rather unspecific variable.

The abnormalities occurring in the presence of acyclovir predominantly concern the cranial region of the embryos; the shape of the head is abnormal. It is, however, difficult to classify these abnormalities in a specific way: the width of the skull has decreased, resembling a beak-like visceral cranium (cf. Fig. 1).

Studies with physiological deoxynucleosides using the whole-embryo culture

With 200 μM 2'-deoxyguanosine in the culture medium no difference in growth and development was observed when compared with the control group, whereas a slight reduction in the score was seen with 2'-deoxyadenosine at this dose level. Since this effect was not dose-dependent, it may have occurred by chance. The higher doses (500 μM) of the two deoxynucleosides produced a general decrease in the embryonic score, the retardation being more pronounced with 2'-deoxyguanosine (crown-rump-length, protein content). It is noteworthy that the retardation observed in the presence of 500 μM of these natural deoxynucleosides was less pronounced than that produced by 25 μM acyclovir (protein content). Remarkably, no specific abnormality occurred even at the highest dose level; the only abnormality in the 500 μM 2'-deoxyguanosine test group concerned the shape of the embryo and is probably a spontaneous abnormality (not drug-induced) which may occasionally also be seen in controls.

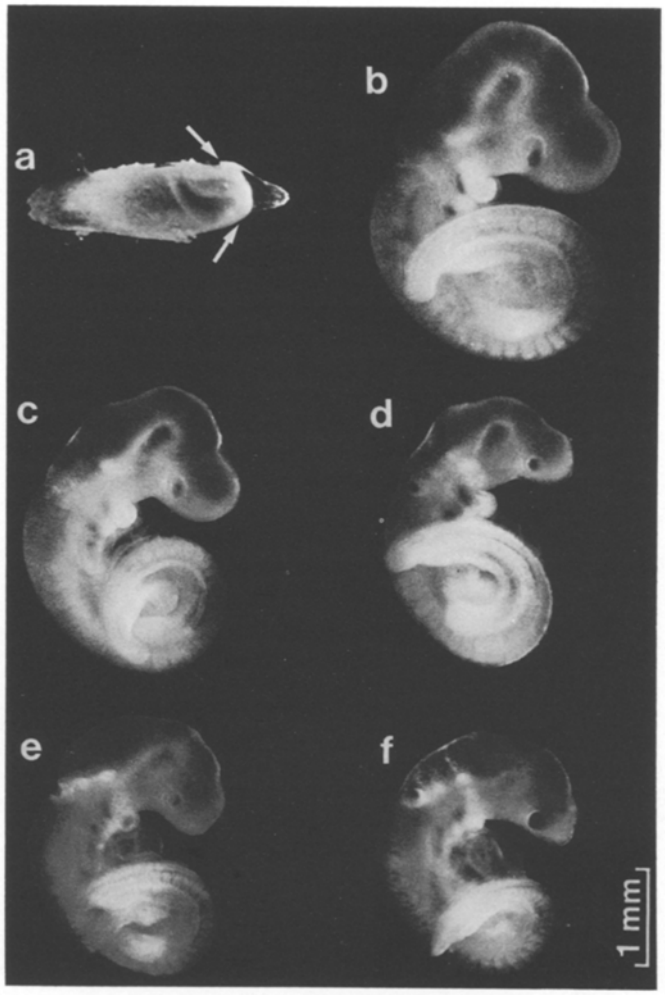

Fig. 1. a 9.5-day-old rat embryo (↓) in its membranes at the beginning of the culture. **b–f** Rat embryos after 48 h of culture in bovine serum with test substances added to the medium. Culture in the presence of: **b** 200 μM 2'-deoxyguanosine (development corresponding to controls); **c** 25 μM, **d** 50 μM, **e** 100 μM, and **f** 200 μM acyclovir, respectively

Histological evaluation

Histological examination of the embryos cultured in the presence of acyclovir shows dose-dependent abnormalities of the prospective central nervous system, the ear anlagen and the somites when compared to controls.

At the higher concentrations (100 μM, 200 μM) the ventricles of the brain, as well as the central channel of the neural tube were dilated (cf Figs. 2b and 3b); the neuroepithelium had not developed correctly to a pseudostratified or multi-layered neuroepithelium and was mono-layered (cf Figs. 2b and 3b). The most severe abnormality concerned the telencephalon: in the embryos which showed gross-structural "head abnormalities" (50–200 μM), the telencephalon vesicles were poorly developed and in some embryos the telencephalon anlage was completely missing (cf Figs. 2c, 3c and 4b). In these cases, the brain anlage

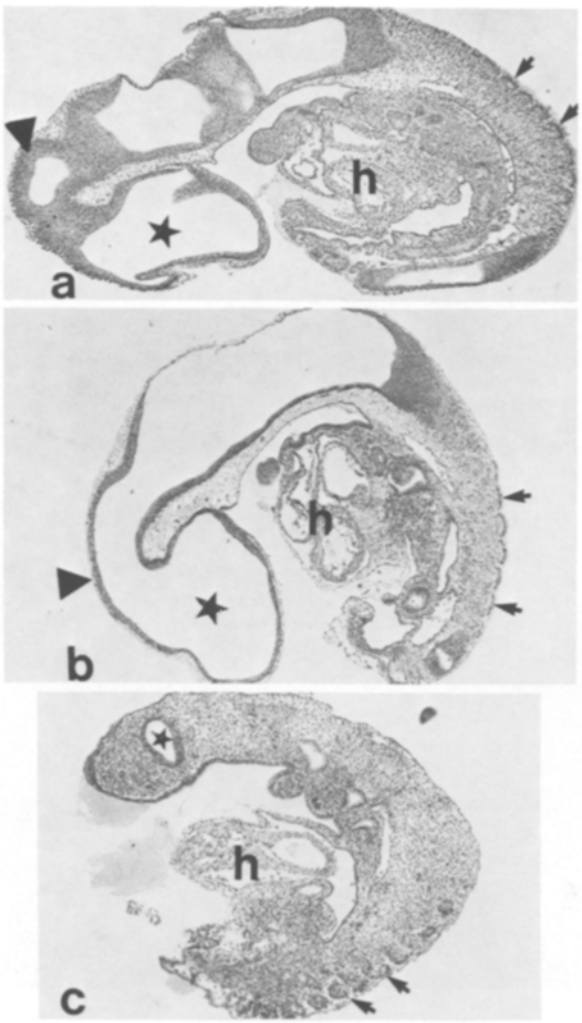

Fig. 2. Rat embryos after a 48-h cultivation period starting with day 9.5 of gestation; X20. * ventricle system; ↓ somites; *h* heart anlage; ▲ cephalic flexure. **a** Untreated control; **b** 100 μM acyclovir: enlarged ventricle system with attenuated neuroepithelium; **c** 200 μM acyclovir: missing flexure in the region of the mesencephalon, i.e. missing cephalic flexure, incomplete facial development, decrease in size of telencephalic vesicles (*), disturbed somite development (↓)

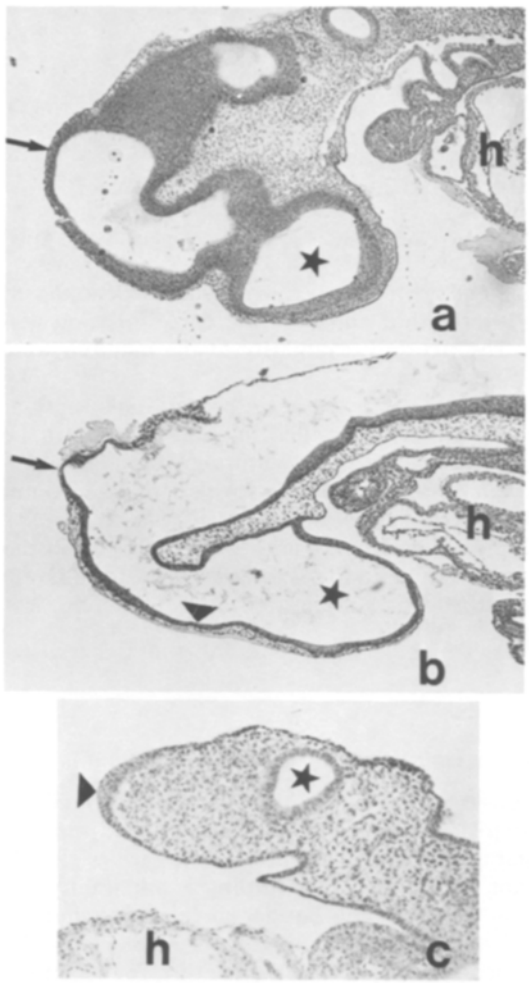

Fig. 3. Rat embryos after a 48-h cultivation period starting with day 9.5 of gestation. Head regions of the embryos with cut telencephalic vesicles (*); *h* heart anlage; ↓ cephalic flexure. **a** Untreated control; X 30; **b** 100 μM acyclovir: enlarged ventricle system (*), attenuated neuroepithelium (▲); X 30; **c** 200 μM acyclovir: very small telencephalic vesicle (*), cephalic flexure missing, thickening of the epithelium in the region of the nasal placode (▲); X 50

consisted only of the tube-like diencephalon, that extended further in the rostral direction than is normally the case (cf schematic presentation in Fig. 5). This explains the deformation of the head (beak-like deformation of the upper visceral cranium). Furthermore, the olfactory plate was thickened, but not invaginated (cf Fig. 3c). At lower concentrations of acyclovir (25 μM, 50 μM), the embryos without gross morphological abnormalities showed the typical multi-layered neuroepithelium and the brain developed correctly (cf Fig. 6a).

At 200 μM acyclovir the ear anlage was smaller, often not closed and consisted of a decreased number of cells. Many cells were necrotic. With 100 μM and less acyclovir in the culture medium fewer necroses in the ear anlage were observed, but the number of cells was still below normal (cf Fig. 7).

The somites, dose-dependently, had irregular forms and seemed to become frayed. Some somites were displaced or in some cases confluenced (cf Figs. 6c and 8). Necroses, also dose-dependent, were observed in the neuroepithelium, in the ear anlage, in the maxillar branch and in the somites.

Histological examination of embryos cultured in the presence of the physiological deoxynucleosides (2'-deoxyguanosine or 2'-deoxyadenosine) did not reveal any alterations: brain, ear anlage and somites developed normally, comparable to control embryos.

Studies with the organ culture (limb bud) system

In order to analyse whether acyclovir may interfere with different types of embryonic differentiation, even at later stages of prenatal development, we also studied the possible effect of this drug in an organ culture system. In the limb-bud system the tissue exhibits much less proliferation and growth, when compared with the whole-embryo system, but pronounced morphogenetic differentiation of cartilaginous structures occurs.

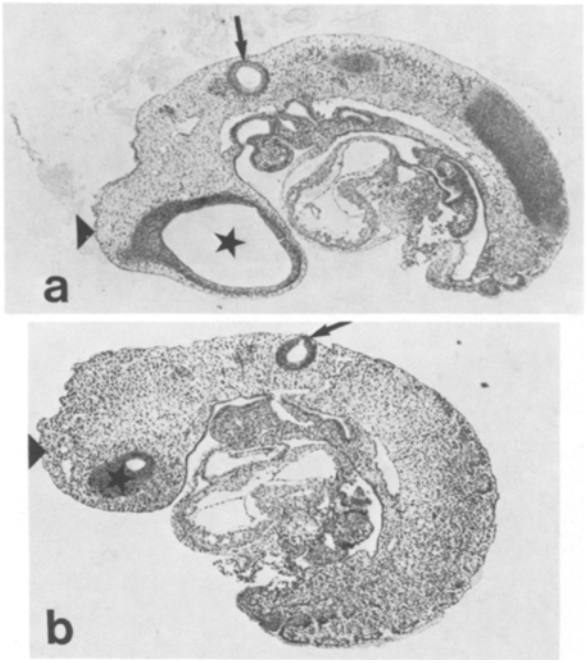

Fig. 4. Rat embryos after a 48-h cultivation period starting with day 9.5 of gestation. Lateral sagittal section through a telencephalic vesicle (*) and the otic vesicle (↓); ▲ cephalic flexure. **a** Untreated control; **b** 200 μM acyclovir: decrease in size of telencephalic vesicles (*), incomplete closure of otic vesicle (↓), cephalic flexure hardly developed, incomplete facial development; X 20

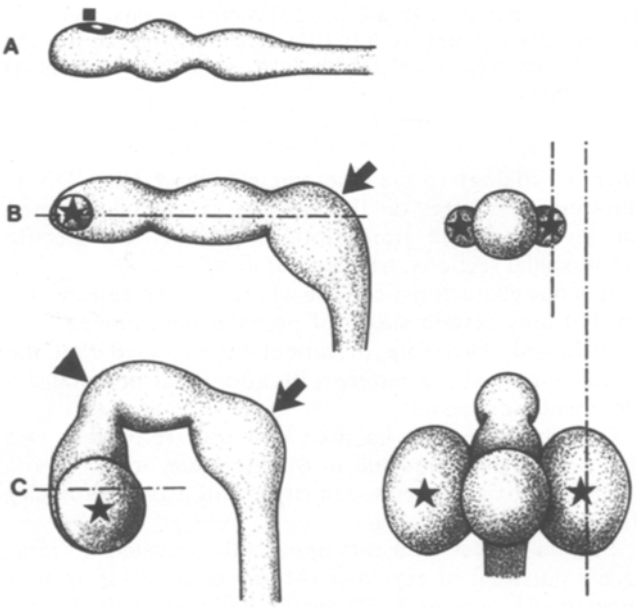

Fig. 5. Schematic presentation of the course of brain development and the formation of telencephalic vesicles. **A** Stretched neural tube with open anterior neuroporus (■). **B** Incomplete formation of the telencephalic vesicle (*) and no cephalic flexure; cervical flexure (↓) is completed. *Left:* lateral view; *Right:* frontial view. **C** More advanced development of the telencephalic vesicle (*), both cervical (↓) and cephalic flexures (▲) present. *Left:* lateral view; *Right:* frontal view; The *broken lines* show the horizontal and sagittal direction of sections. The histological sections shown in *Figs. 2–4* represent sagittal sections

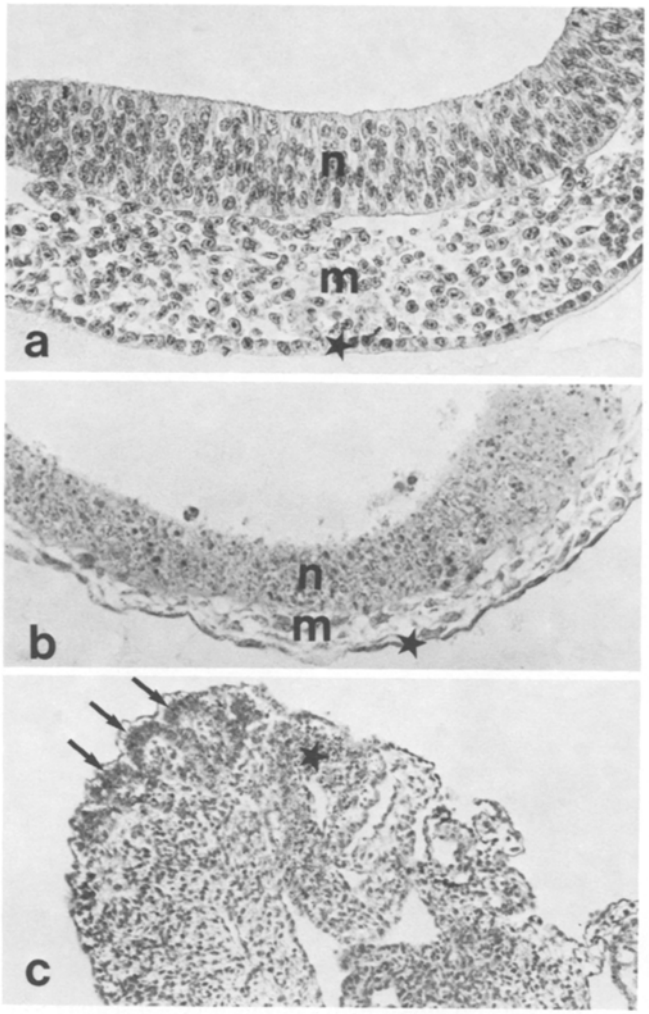

Fig. 6. Rat embryos after a 48 h cultivation period starting with day 9.5 of gestation. **a** Untreated control: neuroepithelium in the region of the telencephalic vesicle *(n)*, mesenchyme *(m)*, ectodermal epithelium (*); X 180; **b** 200 μM acyclovir: numerous necroses in the neuroeptihelium *(n)*, small amount of mesenchyme *(m)*, thin ectodermal epithelium (*); X 120; **c** 100 μM acyclovir: caudal region showing somites (↓) and necrotic areas (*); X 50

As can be seen in Fig. 9 no significant effect was observed with an acyclovir concentration of ≤ 100 μM ($\triangleq 22.5$ μg/ml). While a concentration of 200 μM acyclovir produced clear-cut interference with development, drastic impairment of limb differentiation was obtained at 400 μM. It is obvious that the interference of acyclovir with morphogenetic differentiation at low concentrations predominantly resulted in retarded and impaired development, whereas with higher concentrations (>100 μM) clear-cut structural abnormalities – mainly of the paw skeleton – were observed. These results show that limb development in vitro is much less susceptible to the action of acyclovir compared with the whole-embryo in culture at an earlier stage of embryonic development.

As observed in the whole-embryo system, the physiological deoxynucleosides (2'-deoxyguanosine and 2'-deoxyadenosine) did not interfere with limb development in vitro at as concentration of 500 μM.

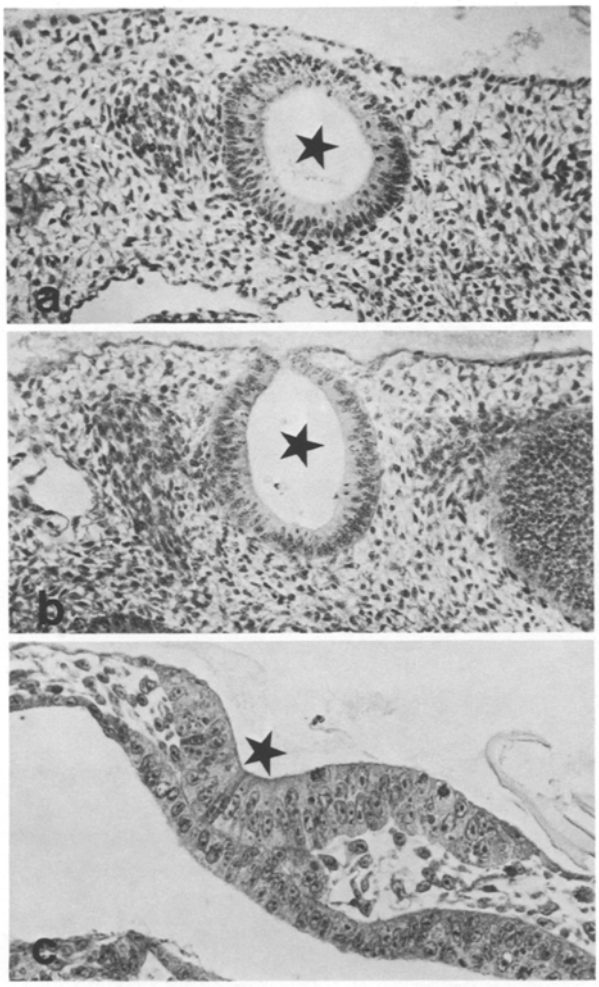

Fig. 7. Rat embryos after a 48-h cultivation period starting with day 9.5 of gestation. **a** Untreated control: showing a closed otic vesicle (*); X 130; **b** 100 μM acyclovir: otic vesicle (*) not yet completely closed; X 130; **c** 200 μM acyclovir: otic placode with incipient invagination (*); X 350

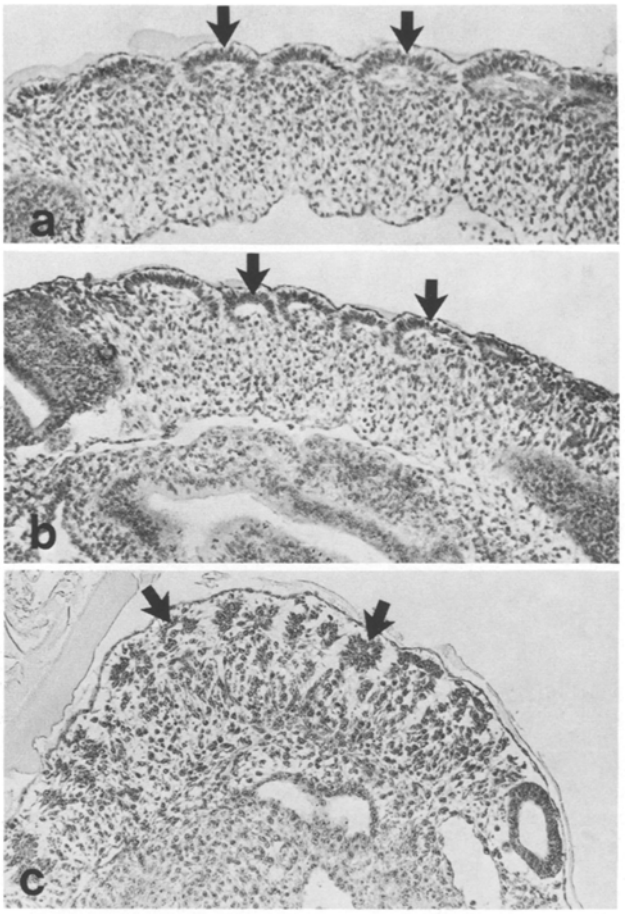

Fig. 8. Rat embryos after a 48-h cultivation period starting with day 9.5 of gestation; X 70. **a** Untreated control: normal and regular somite development (↓); **b** 100 μM acyclovir: irregular size and shape of somites (↓); **c** 200 μM acyclovir: somite development (↓) *grossly impaired*

Discussion

Using these standardized procedures we have evaluated the effect of acyclovir on embryonic development in vitro.

At a concentration of 25 μM (5.6 μg/ml) acyclovir in the culture medium a slight effect on ear development was observed. At 50 μM this impairment was more pronounced and additional signs of abnormal development (especially of the telencephalon) became obvious. A concentration of 100 μM acyclovir in the culture medium was highly toxic to the rat embryo in vitro.

The changes in the region of the prospective nervous system, the ear anlage and the somites are due to the occurrence of necroses and inhibitation of mitosis. Thus, particularly effected is that part of the brain anlage that develops during the cultivation period, the telencephalon. This causes a peculiar deformation of the head. Moreover, a special affinity of the substance for neuroepithelial cells seems to exist. Differences in the proliferation rate are not sufficient to explain the localization of the effects, since the other tissues also proliferate extensively in culture. The mechanism of the cytotoxic or cytolethal effect is not clear either. In analogy to the cytotoxic effects of other DNA-inhibiting substances on the embryo, it can be assumed that an incomplete transcription, possibly of specific chromosomal sections, triggers cell death.

It is one characteristic of the whole-embryo culture system that only certain stages of prenatal development can be evaluated. Therefore, it cannot be excluded that the substance may also interfere with additional prenatal developmental processes.

Our findings must be discussed with respect to two other sets of data reported in the literature on acyclovir: (a) embryotoxicity (teratogenicity), and (b) cytotoxicity (clastogenicity).

In vivo studies with rats apparently revealed no teratogenic potential of acyclovir (Moore et al. 1983) up to a subcutaneous dose of 2 × 25 mg/kg body wt. daily. Under these experimental conditions maternal peak plasma concentrations of only about 16 μg/ml (71 μM) were measured and peak concentrations of about 5 μg/g wet weight (23 μM) were observed in fetuses on the 10th day of treatment. Higher doses apparently could not be tested because of renal toxicity in this species. These data have to be compared (cf Table 2) with peak plasma concentrations achieved in man during chemotherapy: about 5 μM after oral treatment (5 × 200 mg per day) and about 90 μM

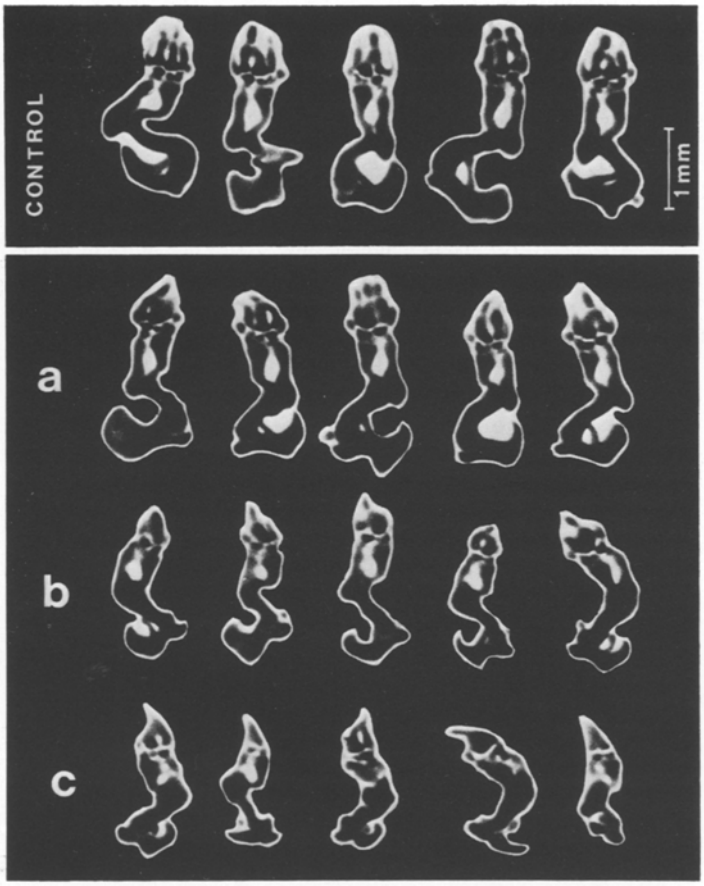

Fig. 9. Effect of acyclovir on limb development in organ culture. Forelimbs of 11-day-old mouse embryos (42 somites) were cultured for 6 days. Controls, and explants grown in the presence of: **a** 100 μM acyclovir; **b** 200 μM acyclovir; **c** 400 μM acyclovir. Experimental conditions as described under *Methods*

Table 2. Comparison of concentrations of Acyclovir affecting various biological systems and therapeutic concentrations achieved in man

	Approximate plasma concentrations in man μM	Approximate NOEL[a] in different systems μM
Human data		
Oral treatment (5 × /day 200 mg)	2 (4)	
iv infusion		
(3 × /day 5 mg/kg b.w.)	34–44[b,c]	
(3 × /day 10 mg/kg b.w.)	92 ± 45[c]	
Experimental data		
Lymphocytes in vitro[d]		550
Embryotoxicity in vivo[e]		70
Limb bud organ culture[f]		100
Whole-embryo culture[f]		10–(25)[f]

[a] No observed effect level
[b] Peak plasma concentrations in parenthesis; assuming normal kidney function (data from: De Miranda et al. 1979)
[c] Data from: Blum et al. (1982)
[d] Data from: Clive et al. (1983)
[e] Data from: Moore et al. (1983)
[f] Data from this paper; 25 μM might be accepted as NOEL if minor effect on ear development is considered to be reversible

(Blum et al. 1982) after intravenous infusion (3 × 10 mg/kg per day).

The second aspect to be considered is the cytotoxic and clastogenic effect produced by acyclovir. Using human lymphocytes in culture Clive et al. (1983) found no effect on chromosomes and mitotic index at 125 μg acyclovir/ml (550 μM) in the culture medium, but a dose-dependent clear-cut increase in chromatid breaks and a decline in mitotic index at concentrations exceeding 250 μg/ml (1110 μM).

These clastogenic effects of acyclovir have been suggested to be a property of high concentrations of nucleosides in general and not a specific characteristic of acyclovir, since natural nucleosides, such as 2'-deoxyguanosine, produce similar effects (Clive et al. 1983). Since such an effect has been reported to occur with several nucleosides (including thymidine, adenine etc.), interference with feedback control of nucleoside precursor pools and an imbalance of these pools has been proposed as a possible mechanism (Anderson et al. 1981; Fox 1982).

Whatever the mechanism of action may be, two results of our studies are of significance in this respect:

(a) Interference with prenatal development can be induced with only 25 μM acyclovir, concentrations about 40 times lower than those reported to be clastogenic, and
(b) 2'-deoxyguanosine and 2'-deoxyadenosine were inactive in this respect in our studies – up to concentrations of 500 μM.

These findings suggest specific cytotoxic or even teratogenic effects at concentrations several orders of magnitude lower than those possibly induced "inspecifically" by natural deoxynucleosides.

It is worth mentioning that interference with morphogenetic differentiation in the organ culture system used (mouse limb bud culture) was observed at 200 μM acyclovir in the medium. This is a concentration considerably higher than those necessary to interfere with development in whole-embryo culture, but still lower (cf Table 2) than the concentrations needed to produce clastogenic effects with acyclovir or natural deoxynucleosides as published in the literature.

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