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Selection of a precocious line of the rabbit coccidium *Eimeria flavescens* Marotel and Guilhon (1941) and characterisation of its endogenous cycle

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Abstract The SPF rabbits were inoculated with oocysts of *Eimeria flavescens* and the first newly developed oocysts were recovered. They were used for inoculation of other rabbits which consequently excreted oocysts sooner than in the previous passage. By repeated use of this method, the prepatent period was shortened after 18 passages by more than 60 h. The endogenous development of this precocious line (PL) differed from that of the original strain (OS). Compared to OS, two asexual generations, second (or third) and fourth, were absent in PL. The first merogony took place in the jejunum and ileum in OS and, in contrast, in the large intestine in PL. Like in other rabbit coccidia, two types of meronts (A and B) were seen in each generation. However, the ratio of B: A meronts in the last (fifth) asexual generation as well as ratio of microgamonts:macrogamonts differs in OS and PL.

each generation. The type A gave rise to a smaller number of thick polynucleate merozoites in which daughter merozoites were formed by endomerogony, while in the type B meronts slender uninucleate merozoites arose from ectomerogony. The first generation meronts were found in crypts and proximal parts of the villi of the jejunum and ileum, whereas three following generations developed in the superficial epithelium of the large intestine (caecum, vermiform appendix and colon). The last merogony as well as gamogony took place in crypts of the large intestine. In the present work, selection of a precocious line of *E. flavescens* is described and its endogenous cycle is characterised and compared with an already known cycle of the original strain (Pakandl et al. 2003).

Introduction

Although precocious lines have been already selected in four rabbit coccidia: *Eimeria coecicola*, *E. intestinalis*, *E. magna* and *E. media* (Licois et al. 1990, 1994, 1995), a precocious line has not yet been derived in *E. flavescens*, which is, next to *E. intestinalis*, the most pathogenic rabbit coccidium. The endogenous cycle of the original strain (OS) was already described (Pakandl et al. 2003). Briefly, five asexual generations were observed and, like in other rabbit coccidia (Streun et al. 1979; Licois et al. 1992; Pakandl et al. 1993; 1996a–c; Pakandl and Coudert 1999), two types of meronts and merozoites were found in

Material and methods

Animals

Specific pathogen-free (SPF) rabbits supplied by ANLAB (Prague, Czech Republic), weight category 1–1.5 kg, were used in the experiment.

Parasite strain

A pure strain of *E. flavescens* (strain Vácha) was previously used to study endogenous development of this species (Pakandl et al. 2003).

Selection of precocious line

The animals were usually inoculated with high doses of oocysts (30,000–400,000/animal).

Newly developed oocysts were recovered from contents of the caecum and stomach of the sacrificed animals (Coudert et al. 1995). In some animals the oocyst yield was low and in these cases the rabbits in the following passages were inoculated with smaller doses and sacrificed

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later after inoculation to obtain enough oocysts. The succession of passages is summarised in Table 1.

Stability test

The rabbits were inoculated with 20,000 oocysts of the precocious line (PL) and new oocysts were recovered after 9 days. The next rabbits were inoculated with the same dose of the new oocysts and the whole cycle of isolation was repeated five times. The prepatent period was then assessed.

Life-cycle study

The animals were orally inoculated with graded doses of oocysts of PL and sacrificed every 16 h (Table 2), and, in addition, two rabbits were sacrificed 70 h post-inoculation (p.i.).

The tissue samples were taken from the caecum, vermiform appendix, proximal colon, ileum (5 cm and 15 cm from sacculus rotundus), jejunum (about 40 cm from sacculus rotundus and a half of the length of the small intestine), and duodenum.

For electron microscopy, the samples were fixed with Karnovsky's fixative, postfixed with osmium tetroxide, dehydrated and embedded in Spurr. Semithin sections were stained with Warmke's polychrome for light microscopy, and ultrathin sections were contrasted with uranyl acetate and lead citrate and examined in a Jeol 1010 electron microscope.

Table 1 The intervals from inoculation to oocyst recovery in successive passages ^a Oocysts isolated from a rabbit at this time were used in the life-cycle studies. In some cases (*italics*) the rabbits were sacrificed after a longer intervals in order to obtain sufficient number of oocysts

207 h		179.5 h	
204 h	↓	<i>204.5 h</i>	↓
202 h		169 h	
<i>210.5 h</i>		<i>185.5 h</i>	
201 h		161 h	
197.5 h		148.5 h	
196 h		<i>163.5 h</i>	
<i>211.5 h</i>	↓	139.5 h	↓
187.5 h		<i>165 h</i>	
<i>205 h</i>		<i>168 h*</i>	

Table 2 Intervals of sampling of tissue samples and infective doses in the experiment

Hour p.i.	16	32	48	64	70	80	96	112	128	144
Infective doses	2×10^7	2×10^7	5×10^6 2×10^7	5×10^6 2×10^7	5×10^6 2×10^7	5×10^6 2×10^7	5×10^6 2×10^7	2×10^6 2.5×10^6	5×10^5 1.9×10^6	5×10^5

Other samples from the same intestinal segments were fixed with 10% neutral formaldehyde, paraffin-embedded and sections were stained with hematoxylin-eosin. In order to assess the ratio of B:A meronts, or respectively, macrogamonts:microgamonts, at least five hundred parasite stages were observed in histological sections.

Results

Selection of the precocious line

The design of passages and oocyst recovery is presented in Table 1. The time from inoculation to appearance of the first oocysts in the caecum was shortened by about 60 h, namely from about 201 h (at this time low oocyst production was noted at the beginning of attenuation) to 139.5 h after 18 passages including seven rabbits inoculated with small dose and sacrificed after longer interval in order to obtain enough oocysts for the following passage. Unlike other rabbit coccidia, no differences in oocysts morphology between OS and PL were noted.

Stability test

The prepatent period (respectively the time from inoculation to appearance of the first oocysts in the caecum, i.e. about 139.5 h) remained abbreviated after five consecutive passages without any selection pressure.

Endogenous development of the precocious line

In order to avoid any confusion, the asexual generations are related to those of OS and numbered according to its life cycle. Like in OS, two types of meronts characterised by polynucleate (type A) or uninucleate merozoites (type B) occurred in each asexual generation. A survey of the endogenous development of PL is included in the Table 3, whilst Tables 4 and 5 show differences between OS and PL.

No parasite stages were found until 64 h p.i. Meronts of the first generation were first seen 70 h p.i. in superficial epithelium of the large intestine. The type A meronts (Fig. 1) formed 2–4 merozoites harbouring 4–8 nuclei, whereas type B meronts (Fig. 2) gave rise to 8–20 slender uninucleate merozoites. Amylopectin granules were abundant in the cytoplasm of both A and B merozoites. Eighty hours p.i. these meronts were almost completely replaced by those of the next generation (Figs. 3–5).

Table 3 A survey of the asexual generations in the precocious line

Stage	Hour p.i.	Localisation	Type A		Type B	
			Number of merozoites/meront	Number of nuclei in one merozoite	Number of merozoites/meront	Number of nuclei in one merozoite
First generation	70	Superficial epithelium of the large intestine	2–4	4–8	8–20	1
Second generation ^a	80–96	Superficial epithelium of the large intestine	Mostly 2	2–3	Mostly 2	1
Fifth generation	96–128	Crypts of the large intestine	2–4	Up to 12	20–60	1

^a It was not possible to decide whether this stage corresponded to the second or third asexual generation of the parent strain (see the text)

Table 4 A comparison of asexual phases of the endogenous development of the original strain and precocious line

Stage	Original strain			Precocious line		
	h p.i.	Localisation	Ratio of A:B meronts	h p.i.	Localisation	Ratio of A:B meronts
First merogony	80–96	Epithelium of crypts and proximal part of the villi in the jejunum and ileum	1:1.59	70	Superficial epithelium of the large intestine	1:1.72
Second merogony	96–112	Superficial epithelium of the large intestine	^a	80–96	Superficial epithelium of the large intestine	^a
Third merogony	112–128	Superficial epithelium of the large intestine	^a	Lacking ^b		
Fourth merogony	128–144	Superficial epithelium of the large intestine	1:1.72	Lacking		
Fifth merogony	144–176	Epithelium of crypts of the large intestine	1:6.30	96–128	Epithelium of crypts of the large intestine	1:1.14

^a In this case, the A:B ratio could not be exactly assessed ^b It is not sure which generation (second or third) is actually absent

The next generation meronts occurred in the same localisation. Since the second and third generation meronts of OS are very similar, it is not sure which of them was lacking in PL (see “Discussion” section). The two types of meronts were difficult to discriminate as they formed the same number of merozoites: usually two and seldom three or four. Merozoites with more than one nucleus were relatively rarely seen. The number of nuclei visible in the merozoites certainly depends on the plane of section, but in some cases only one nucleus may be actually present in the type A merozoites. Nuclear division which was noted within merozoites (Fig. 4) suggests that only one nucleus may be initially incorporated into the merozoite. For these reasons, the number of the type A meronts and hence the ratio of A:B meronts cannot be assessed.

Meronts of the last generation (Figs. 6–9) were noted 96–128 h p.i. and they developed in the epithelium of crypts of the large intestine. The type A meronts usually

gave rise to two merozoites, which possessed up to 12 nuclei. However, large merozoites, apparently of the type A, with only one nucleus were also seen (Fig. 6). In some cases a formation of new merozoites from the type A merozoites was noted within the same parasitophorous vacuole (Figs. 7 and 8). The type B (Fig. 9) meronts formed 20–60 uninucleate merozoites.

Gamogony was first observed from 128 h p.i. in the same localisation as the last asexual generation, that is, in the crypts of the large intestine.

Discussion

The endogenous development of PL differs from that of OS in the following points: localisation of the first asexual generation, absence of the third (or second) and fourth generations, ratio of A:B meronts in the last generation (1:1.14 in PL and 1:6.30 in OS) and ratio of

Table 5 A comparison of the sexual phase of original strain and precocious line

	Hour p.i.	Localisation	Ratio of macrogamonts:microgamonts
Original strain	From 160	Epithelium of crypts of the large intestine	5.81:1
Precocious line	From 128	Epithelium of crypts of the large intestine	2.16:1

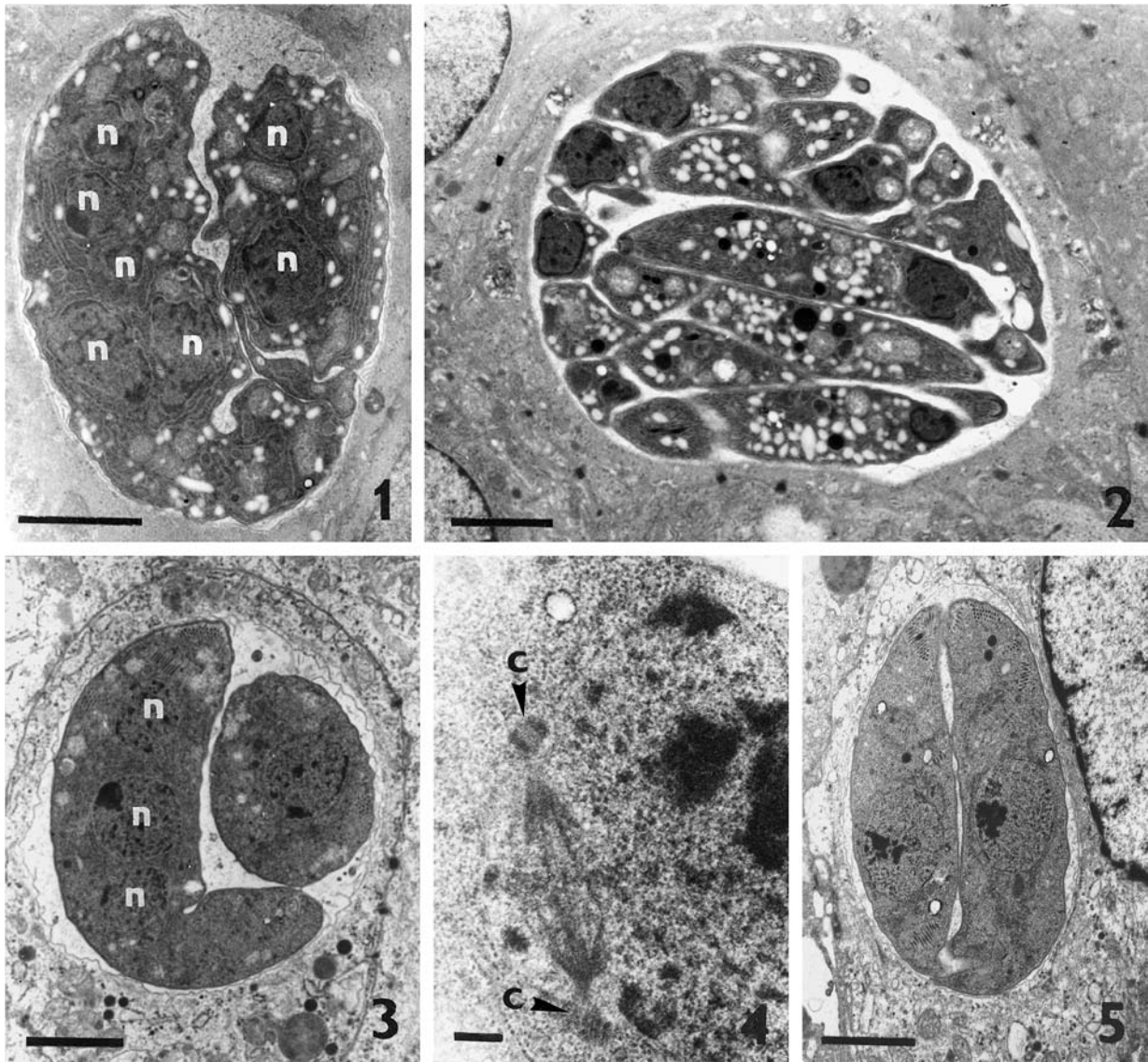


Fig. 1 Transmission electron micrograph of the type A meront of the first generation. *n* nuclei. Bar = 2 μ m

Fig. 2 Type B meront of the first generation. Bar = 2 μ m

Fig. 3 Type A meront of the second (or third) generation. Bar = 2 μ m

Fig. 4 Nuclear division within type A meront of the second (or third) generation. *c* centrioles. Bar = 200 nm

Fig. 5 Type B meront of the second (or third) generation. Bar = 2 μ m

microgamonts:macrogamonts (1:2.16 in PL and 1:5.81 in OS). In addition, the type B meronts produced 30–150 merozoites in the original strain (Pakandl et al. 2003), but only 20–60 in the PL.

Surprisingly, not the last asexual generation, like in other rabbit coccidia (Pakandl et al. 1996a, 1996c), but two preceding generations were lacking in the life cycle of PL.

As the second and third generations (in OS) are mutually very similar, it is impossible to solve which of them is present in PL. The meronts forming mostly two

merozoites were first seen 80 h p.i. and for the last time 96 h p.i. when they were accompanied by meronts of the last generation. Thus, it seems unlikely that more than one generation could develop during such a short time. Two types of meronts in this generation do not markedly differ in number, size and shape of merozoites. Only one nucleus may be often visible in the type A depending on the plane of section and, as mentioned in Results, only one nucleus may be actually present. For this reason, the ratio of A:B meronts cannot be exactly assessed in this generation.

In the fourth generation of OS, two types of meronts markedly differed since the type B produced 8–25 merozoites, whereas the type A formed 2–4 merozoites (Pakandl et al. 2003) (Fig. 9). This fact strongly suggests that absence of this generation may be a cause of the changed B:A ratio in the last generation meronts of PL (1:1.14 in PL and 1:6.30 in OS). Streun et al. (1979) were the first to postulate that the type A merozoites give rise to type A meronts of the next generation and finally

Fig. 6 Type A meronts of the fifth generation. *Above* a meront with uninucleate, *below* with polynucleate merozoites.

Bar = 2 μ m

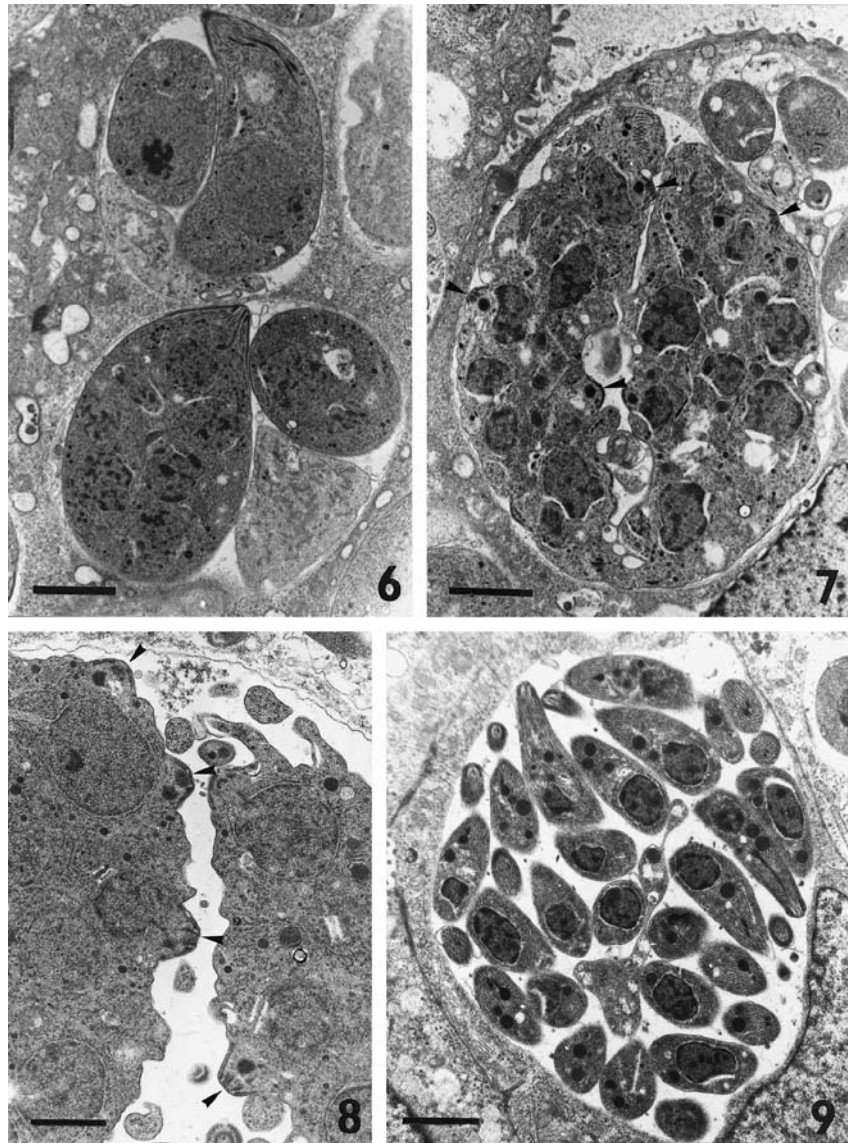
Fig. 7 Type A meront of the fifth generation. Two polynucleate merozoites are not completely separated, but a beginning of formation of new merozoites within the same parasitophorous vacuole is visible (*arrowheads*).

Bar = 2 μ m

Fig. 8 New merozoites (*arrowheads*) arising from polynucleated merozoites of the fifth generation.

Bar = 1 μ m

Fig. 9 Type B meronts of the fifth generation. Bar = 2 μ m



microgamonts. Analogously, the type B stages precede the same type in the next generation and macrogamonts. If this is true, even the sporozoites, meronts and merozoites must be sexually determined. However, this hypothesis seems to be in contradiction with the fact that patent infection can be obtained after inoculation of chickens with single sporocyst or sporozoite (Lee et al. 1977; Shirley and Harvey 1996) or mice with single merozoite (Haberkorn 1970). As the nature of sexual differentiation of coccidian endogenous stages is insufficiently known, it is impossible to speculate about a putative genetic background of two different types of meronts.

It is uncertain whether the polynucleate merozoites leave the host cell and enter another one to give rise to meronts. Our results suggest that, at least in some cases, another alternative is probable. Several new merozoites, which could subsequently penetrate into other host cells, may arise from polynucleate merozoites within the same parasitophorous vacuole. In addition, it is not sure

whether several nuclei are incorporated into the type A merozoites or single nuclei undergo division within the merozoites. As we saw nuclear division inside the merozoites and in some cases the five-generation merozoites, apparently of the type A, harboured only one nucleus, the second alternative seems to be more likely.

The PLs undoubtedly represent a very promising model for studies of regulation of coccidian life cycle. Moreover, OS and PL of *E. flavescens* exhibit different ratio of A:B meronts and microgamonts:macrogamonts and this is very interesting in connection with sexual differentiation of endogenous stages of coccidia.

The prepatent period, or respectively the time from inoculation to appearance of the first oocysts in the contents of the large intestine, was reduced by about 60 h. It is very interesting that genetically stable alteration was achieved as a result of the selection pressure during as few as 18 passages. Nothing is known about the nature of this phenomenon even in chicken coccidia,

which are the mean model in this field of research, but a selection from genetically heterogeneous background probably plays a role.

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