Parasitol Res (2001) 87: 98–106

ORIGINAL PAPER

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Tissues and cells involved in the invasion of the rabbit intestinal tract by sporozoites of *Eimeria coecicola*

Received: 27 June 2000 / Accepted: 27 July 2000

Abstract This study was designed to identify an extra-intestinal route of migration of *Eimeria coecicola* sporozoites and the types of cell harbouring the parasite during the invasion of the intestine. The presence of *E. coecicola* in blood, spleen and mesenteric lymph nodes of infected donor rabbits was demonstrated by immunohistology on donor organs and measurement of oocyst excretion by coccidia-free recipient rabbits injected with whole-cell suspensions prepared from donor tissues. Two types of donor lymphocyte, B (IgM^+) and T ($CD5^+$), were labelled using a two-colour immunofluorescence-labelling technique and separated with a cell-sorter ($FACStar^{plus}$). The presence of parasites in the sorted cells was assessed by direct examination and by using the same in vivo test after intravenous injection of IgM^+ B or $CD5^+$ T lymphocytes collected from donors at different times after inoculation. This test provided evidence that the parasites were alive and still infectious within the sorted lymphocytes. It was demonstrated that both B and T lymphocytes were infected.

Key words *Eimeria coecicola* · Rabbit · Lymphocytes · Invasion · Host cell · Flow-cytometry · Cell-sorting

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Introduction

The life cycle of *Eimeria coecicola,* which is a common parasite of domesticated rabbits, has already been extensively described by electron microscopy studies (Pakandl et al. 1993, 1996). After intraduodenal injection of sporocysts, the sporozoites excyst very quickly and enter the villous epithelial cells of the duodenum. In the following hours, they are observed in lymphoid cells of the lamina propria of this segment. From the time of inoculation until 48 h post-inoculation (p.i.) the number of parasites in the duodenum decreases, while they appear in the lymphoid cells of the gut-associated lymphoid tissues (GALT), i.e. vermiform appendix, Sacculus rotundus and Peyer's patches, in which they undergo their first merogony. The following merogonies occur in the epithelial cells of the domes. For three other rabbit Eimeria species, penetration and development of the parasite also take place in different parts of the intestine. The sporozoites penetrate in the duodenum (Horton 1967; Drouet-Viard et al. 1994; Pakandl et al. 1995), but the target organ can be very distal (the appendix in the case of *E. coecicola*, the liver for *E. stiedai*, etc.). Therefore, there is a striking difference between chicken and rabbit invasion by coccidia. In the case of chicken coccidia, it is commonly admitted that sporozoites enter the villous epithelial cells of the target organ directly. They then migrate within the same villus; and it has been demonstrated that this migration occurs either in intraepithelial lymphocytes (IEL; Lawn et al. 1982; Al-Attar and Fernando 1987; Fernando et al. 1987), or in some cases in macrophages (Doran 1966; Michael 1976; Fernando et al. 1987).

In the rabbit, we considered the distance between the site of penetration and the site of multiplication and, since the first merogony occurs in cells resembling lymphocytes, we hypothesised that sporozoites of *E. coecicola* reach the vermiform appendix via lymphocytes in an extra-intestinal route. We therefore researched the presence of parasites in various extra-intestinal tissues

and determined the types of cell harbouring sporozoites, both at their site of penetration and at their site of development, using a method adapted from that used by Kerloeguen et al. (1996). Cell preparations from blood and different organs (spleen, mesenteric lymph node, etc.) of infected donor rabbits taken at different times after inoculation were injected intravenously into coccidia-free recipient rabbits. The histology of the donor organs and oocyst excretion by the recipient animals provided evidence that the donor organs were infected. We subsequently identified the intestinal cells harbouring the sporozoites. IgM^+ B and $CD5^+$ T lymphocytes from the duodenum and appendix were sorted after double-labelling. The purified populations obtained were injected into recipient rabbits to demonstrate that the parasites were still alive and infectious in the sorted cells.

Materials and methods

Animals

New Zealand white rabbits (5–6 weeks old) homozygous for class II antigens of the major histocompatibility complex were used. They were specific pathogen-free, especially coccidia-free, and were reared under controlled conditions (Coudert et al. 1988). All the young were fed a robenidine-supplemented commercial pelleted feed until 4 days before inoculation, when non-supplemented pelleted feed (UAR 91360 Villemoison/Orge, France) was given. Housing conditions were those described by Licois et al. (1994).

Parasites

The freshly prepared inocula originated from a pure strain of *Eimeria coecicola* obtained from a mixture of seven isolates from different farms. Sporocysts were used to infect rabbits. They were obtained from oocysts crushed with 1-mm glass beads. Sporozoites were used to infect rabbits in some experiments: excystation was performed at 37 °C by the addition of trypsin and biliary salts to the medium and was stopped with cold phosphate-buffered saline (PBS) as soon as the sporozoites were released.

Inoculation and collection of samples

To synchronise the parasite cycle, anaesthetised donor rabbits were inoculated with 5×10^6 sporocysts (i.e. 10^7 sporozoites) directly injected into the proximal duodenum (Drouet-Viard et al. 1994). Euthanasia was performed at different times after inoculation (15–65 h p.i.) and the relevant tissues were prepared for injection of cells into recipient rabbits.

To verify that donor tissues were infected, in each experiment, samples of duodenum and vermiform appendix were taken and frozen at −80 °C. The presence of parasites in organ sections was detected by immunofluorescence-labelling as described by Drouet-Viard et al. (1994).

Preparation of cell suspensions from different organs for transfer to recipients

Blood and peripheral blood leukocytes

A 10-ml sample of blood was drawn from the central vein of the ear onto ethylenediaminetetraacetic acid K3. Peripheral blood leukocytes (PBL) were separated from red cells by centrifugation and the remaining red cells were lysed with an ammonium chloride solution. The PBL were resuspended in RPMI-1640 medium (Sigma, St. Louis, Mo.) containing 2% fetal calf serum (FCS; Sigma).

Vermiform appendix, mesenteric lymph node and spleen whole-cell suspensions

The vermiform appendix was opened longitudinally and flushed with PBS. Then spleen, mesenteric lymph node (MLN) and vermiform appendix were disrupted in RPMI/2% FCS medium by crushing them against a 40-μm wire mesh with a syringe piston. Spleen red blood cells were lysed with an ammonium chloride solution and leukocytes were resuspended in RPMI-1640/2% FCS.

Suspension of duodenal cells

The duodenum was flushed with PBS and the Peyer's patches and fat were removed. The duodenum was cut into 4-cm pieces that were opened longitudinally. The mucosa was scraped and then dissociated for 15 min in RPMI-1640 medium containing 10% FCS and 1 mM dithioerythritol (Sigma) by mechanical disruption using a magnetic stirring bar. The extracted cells were washed; and the tissue debris and cell aggregates were then eliminated by slow centrifugation (40 *g*). The suspension of whole cells obtained (homogenate) was centrifuged and resuspended in medium.

Electron microscopy studies of whole cells suspensions of donor appendices

To verify that parasites were present in the cells after mechanical disruption of donor tissues, we observed preparations of appendix by electron microscopy. The pelleted cells were fixed in modified Karnovski fixative, post-fixed with osmium tetroxide and embedded in Spurr (Pakandl et al. 1993). Semi-thin sections were stained with Warmke's polychrome for light microscopy; and ultra-thin sections were contrasted with uranyl acetate and lead citrate and then examined with a Philips EM 420 electron microscope.

Double-labelling of gut lymphoid cells

Lymphoid cells obtained from the duodenum and the vermiform appendix were double-labelled successively with two anti-lymphocyte antibodies in order to separate single-labelled IgM^+ B cells from single-labelled $CD5^+$ T cells and to exclude the sub-population of IgM^+ B cells which was also $CD5^+$. The cells were first labelled with an anti-IgM polyclonal antibody (Nordic Immunological Laboratories, Tilburg, The Netherlands) and a fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat immunoglobulin (Nordic) was used as secondary antibody. The $CD5^+$ cells were then labelled with the monoclonal antibody MCA800 (Serotec, Oxford, England) and revealed with a red phycoerythrin (R-PE) conjugated goat anti-mouse secondary antibody (Jackson Immunoresearch Laboratories, West Grove, Pa.).

Cell sorting and flow-cytometry analysis of cell suspensions

Cells were sorted using a FACStar^{plus} (Becton Dickinson, San Jose, Calif.) equipped with an argon ion laser operating at 488 nm and 80 mW. FITC fluorescence was analysed with a 530/30-nm band pass filter; and R-PE fluorescence was analysed with a 575/26-nm band pass filter. Sorting was performed using morphological and fluorescence criteria. For the most accurate sorting, we used the "one deflected drop sorting mode" which selected only the drops

containing one labelled cell. This method made it possible to separate single-labelled IgM^+ cells and single-labelled $CD5^+$ cells which were not cross-contaminated. Flow-cytometry analysis of the sorted cells was then performed to check the purity of each cell subpopulation to be injected.

Experimental design – "in vivo test"

Donor rabbits were inoculated with sporocysts and were sacrificed 15, 23, 34, 40, 46 and 65 h later. Organs were collected for the preparation of cells ($CD5^+$ T and IgM^+ B lymphocytes from the duodenum and GALT, leukocytes from the blood, spleen and MLN) to be transferred to coccidia-free recipient rabbits by intravenous injection. Each recipient received a single type of cell from one donor, either 10^8 cells suspended from a tissue or 10^5 sorted lymphocytes from the intestine. Thus several recipients corresponded to one donor. Only for the duodenum were cell preparations from two donors pooled to obtain enough sorted lymphocytes of each type. The recipients were kept in autoclaved isolators for 12 days to collect faeces and to determine the total oocyst output per rabbit. The prepatent period for *E. coecicola* is 9 days, so faeces were collected every day from day 6 to day 12 after injection of cells, in order to detect the first oocysts excreted and the total oocyst excretion per rabbit. A control non-injected recipient was included. Oocysts were counted as described by Coudert et al. (1995). Because the total number of sorted cells of each type was not sufficient to be injected into a uniform number of recipients for each post-inoculation time, experiments were repeated between two and five times.

Results

Sporozoites injected intravenously were able to induce infection when transferred to coccidia-free rabbits

To calibrate the sorted cell transfers, four rabbits were injected intravenously with free sporozoites and we determined how many were required to induce oocyst excretion. A rabbit inoculated *per os* with *E. coecicola* oocysts can excrete a maximum of 10^8 oocysts (Coudert et al. 1995); and this high level of excretion can be obtained with >400 oocysts, i.e. about 3,500 sporozoites or more. In this experiment, after the intravenous injection of 100,000 sporozoites, an oocyst output was achieved which was under 10^8 oocysts and thus within the dose-dependent excretion range. The parasites reached their target site within 65 h p.i., as observed in vermiform appendix sections after immunohistological labelling (Fig. 1F); and the prepatent period was normal (9 days).

Immunohistological checking of infection of donor intestines

To assess the infection of duodenums and appendices of the donor rabbits, sporozoites were searched by immunofluorescence on frozen sections (Fig. 1A–D). At 15 h p.i. and up to 65 h p.i. sporozoites could be found in duodenums. Parasites appeared in appendices from 34 h p.i. and their number increased up to 65 h p.i.

Electron microscopy study of appendix lymphocytes

Pelleted whole cells from appendices were observed by electron microscopy in order to verify the presence of parasites within lymphocytes (Fig. 2).

Infection induced in recipient rabbits injected with cell suspensions from different organs

Whole cells (10^8) from spleen, MLN, duodenum and appendix and 10^8 PBL were injected into recipients. The results of infection transmission are given in Fig. 3.

All the donor organs tested were able to transfer infection to recipients but the results were different according to the time p.i.. Up to 65 h p.i., all cell preparations except those from the appendix (at 15 h p.i.) induced infection in recipient rabbits.

Where all organs were able to transfer infection, the oocyst excretion of recipient rabbits differed, depending on the origin of the injected cells. Infection due to transferred PBL or transfused blood was obtained at all times tested but total oocyst excretion was sometimes very low, just above the sensitivity limit of the counting test ($\leq 10^4$ total oocyst output). Cell suspensions from lymphoid organs (MLN and spleen) and intestine (duodenum and vermiform appendix) induced greater oocyst excretions. Transferred duodenal cells induced infection up to 65 h p.i.. For the appendix, the highest oocyst excretion was obtained with cells recovered from donor rabbits at 40 h and 65 h p.i..

After injection of appendix sorted cells, the prepatent period in the recipient animals was shortened to 7–8 days instead of 9 days, according to the time allowed for parasite development in the donor (time p.i. before euthanasia of the donor).

Flow-cytometry analysis of sorted lymphocytes

Double-labelling compared with single-labelling is a more suitable and reliable manner to separate single-labelled IgM^+ B cells from single-labelled $CD5^+$ T cells and to obtain non-cross-contaminated cell populations (Fig. 4). In all experiments, the number of double-labelled cells was very low. Moreover, sorting gates (R_3 and R_4) were narrow to avoid contamination by both epithelial cells and non-labelled or double-labelled cells. Flow-cytometry analysis of the sorted cell suspensions confirmed that the purity of preparations was over 98% and that cross-contamination of B cell suspensions by T cells (and vice versa) was completely eliminated (Fig. 4B, C). To get such purity, we had to limit the number of sorted cells to 100,000 lymphocytes of each type from the appendix cells of one donor and 100,000 lymphocytes of each type from the duodenum cells pooled from two donors.

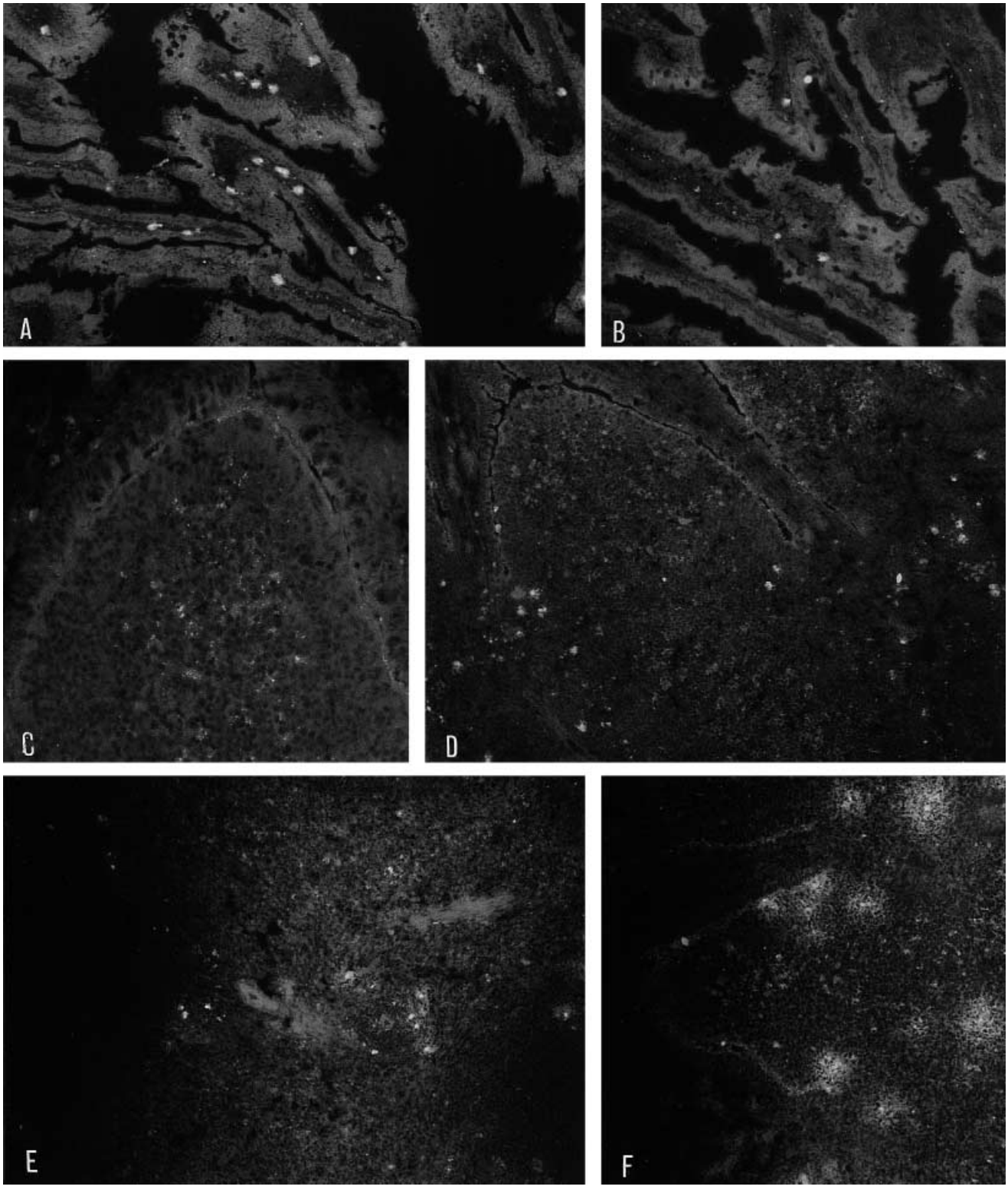

Fig. 1 Cryostat sections of organs from donor rabbits labelled with anti-*Eimeria coecicola* antibodies (×1,250): **A** duodenum at 15 h post-infection (p.i.), **B** duodenum at 65 h p.i., **C** appendix at 15 h p.i., **D** appendix at 65 h p.i., **E** spleen at 23 h p.i. **F**: appendix of a rabbit injected intravenously with sporozoites and sacrificed at 65 h p.i.

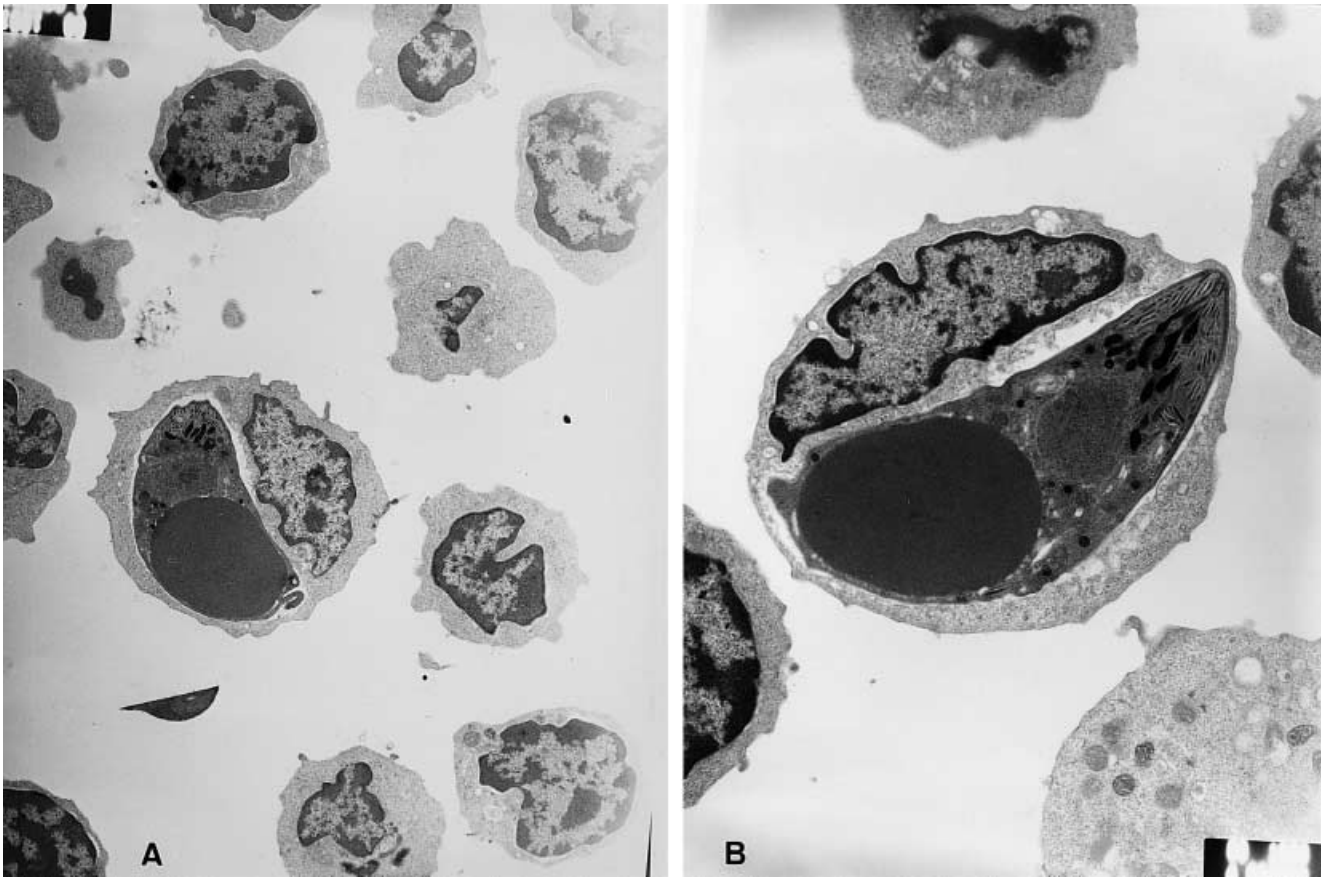

Fig. 2A Transmission electron micrograph of a suspension of appendix cells from a donor rabbit sacrificed at 65 h p.i., showing a sporozoite of *E. coecicola* within a lymphocyte (×4,750) and many non-parasitised cells; and **B** detail of another parasitised lymphocyte (× 9,100)

Infection induced in recipient rabbits injected intravenously with sorted lymphocytes (from the duodenum or the appendix of donors)

We established that 100,000 sorted lymphocytes injected intravenously were enough to induce infection in a recipient. The number of infected cells among these sorted lymphocytes was sufficient to cause infection, but did not always induce the maximum oocyst excretion of 10^8 oocysts. Thus the number of parasites injected induced an excretion which was within the dose-dependent excretion range. The oocyst excretions of recipient rabbits injected with sorted lymphocytes are presented in Fig. 5.

Duodenum B (IgM^+) and T ($CD5^+$) lymphocytes from 15 h p.i. donors were able to transfer infection. Unlabelled sorted cells which were neither T nor B (T^- B^- area) could also transfer infection, but the oocyst output was under the sensitivity limit of the counting test ($< 10^4$ oocysts) and could only be detected by using a more sensitive non-quantitative flotation technique. Thus, the excretion levels obtained with T or B lymphocytes were completely different, compared to those obtained with unlabelled (T^- B^-) cells.

Both B and T lymphocytes of the appendices from donor rabbits infected for 34–65 h caused infection in recipients at all times tested. As observed in the duodenum, unlabelled (T^- B^-) cells induced oocyst output in recipient animals, but the excretion was much lower than that induced by labelled cells.

Discussion

Our histological immunofluorescence tests performed on sections of donor organs confirmed the results obtained by Pakandl et al. (1993), who found that sporozoites were present in the duodenum (site of penetration) at 16 h p.i. and in the vermiform appendix (site of multiplication) at 48 h p.i. In view of our results, migration of parasites occurs within the 2–3 days following inoculation.

Our results also indicate that the early stages of *Eimeria coecicola* are not confined to the intestine. During this invasive period, *E. coecicola* was present in peripheral blood and various organs of the host. Such extra-intestinal localisation has already been described for chicken coccidia (Naciri et al. 1982; Kogut and Long 1984; Pittilo et al. 1986; Fernando et al. 1987). In all our experiments, the prepatent period in recipient rabbits was shortened according to the length of previous development of the parasite in the appendix of donor rabbits. This demonstrates both that the extra-intestinal infectious stages were sporozoites (because oocyst output

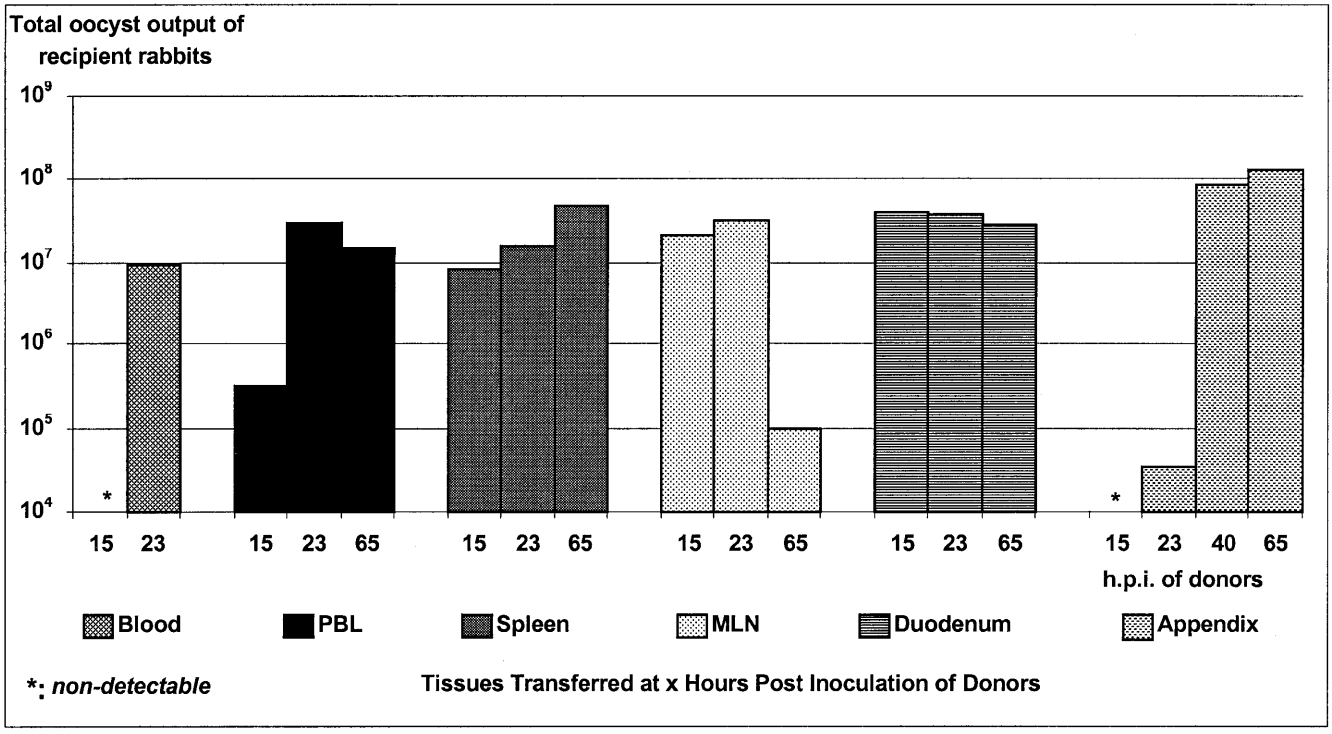

Fig. 3 Total oocyst output per rabbit injected intravenously with cell suspensions of different organs recovered from infected donor rabbits sacrificed at various times after inoculation of *E. coecicola* sporocysts. *MLN* mesenteric lymph nodes, *PBL* peripheral blood lymphocytes

began 8 days p.i.) and that the infectious stages within the appendix cells were either sporozoites (excretion starting 8 days p.i.) or merozoites (excretion starting 7 days p.i.). When transferred blood and PBL induced infection in recipients, oocyst excretion was sometimes very low; the results obtained with spleen and MLN homogenates were more constant. The presence of parasites in the MLN of infected rabbits from 15 h p.i. suggests that sporozoites might take the lymphatic system as their first migration route to reach their site of multiplication.

We also attempted to research the chronology of the invasion of the different donor organs by the parasites. In fact, since all the extra-intestinal tissues tested were already infected from 15 h p.i., we could not determine a sequence of events, i.e. which organ if any was infected first. Moreover, despite the induced synchronisation of the parasite cycle, there were still sporozoites alive in the duodenum of donors at 65 h p.i. (the time at which the first merogony occurs in the vermiform appendix). This may be due to too high a number of sporozoites being injected and to the fact that delayed parasites may no longer be able to migrate. It is tempting to speculate that extra-intestinal migration is necessary for *E. coecicola* because of: (1) the distance between the site of penetration and the site of multiplication, (2) the minimum of 23 h needed to cover this distance and (3) the fact that sporozoites cannot survive more than a few minutes in the intestinal lumen. Though the present results did not provide evidence that sporozoites have to take an extra-intestinal route, the presence of parasites in lymphoid organs and blood demonstrated in this study, associated with their absence from the lumen in tissue sections of the intestine, strengthens this hypothesis.

Having demonstrated the presence of parasites in different organs, we sought to detect the types of cell harbouring sporozoites in the duodenum and appendix. As sporozoites had already been described in cells resembling lymphocytes (Pakandl et al. 1993), we investigated their presence in different types of lymphocytes isolated by cell sorting. This technique provided single-labelled cell populations at a high grade of purity. The presence of parasites in the lymphocytes was assessed by electron microscopy.

The in vivo test performed by intravenous injection of sorted lymphocytes demonstrated that the sporozoites within both B and T lymphocytes were alive and still infectious. The experiments were not designed to identify the route of migration taken by the sorted parasitised lymphocytes to the target cells of the recipients. Though donors and recipients were homozygous on class II genes of the major histocompatibility complex, most probably part of these foreign sorted lymphocytes were destroyed; and the released parasites were taken in charge by the cells of the recipient. In any case, the excretions observed provided evidence that, at the time of sorting, the parasites were alive inside the lymphocytes.

Our findings are different from the results obtained with chicken coccidia. The presence of *E. acervulina* in T cells has already been described by Trout and Lillehoj (1993, 1995). They found sporozoites in $CD8^+$, in $CD4^+$ and in macrophages, but never in B cells; whereas in our

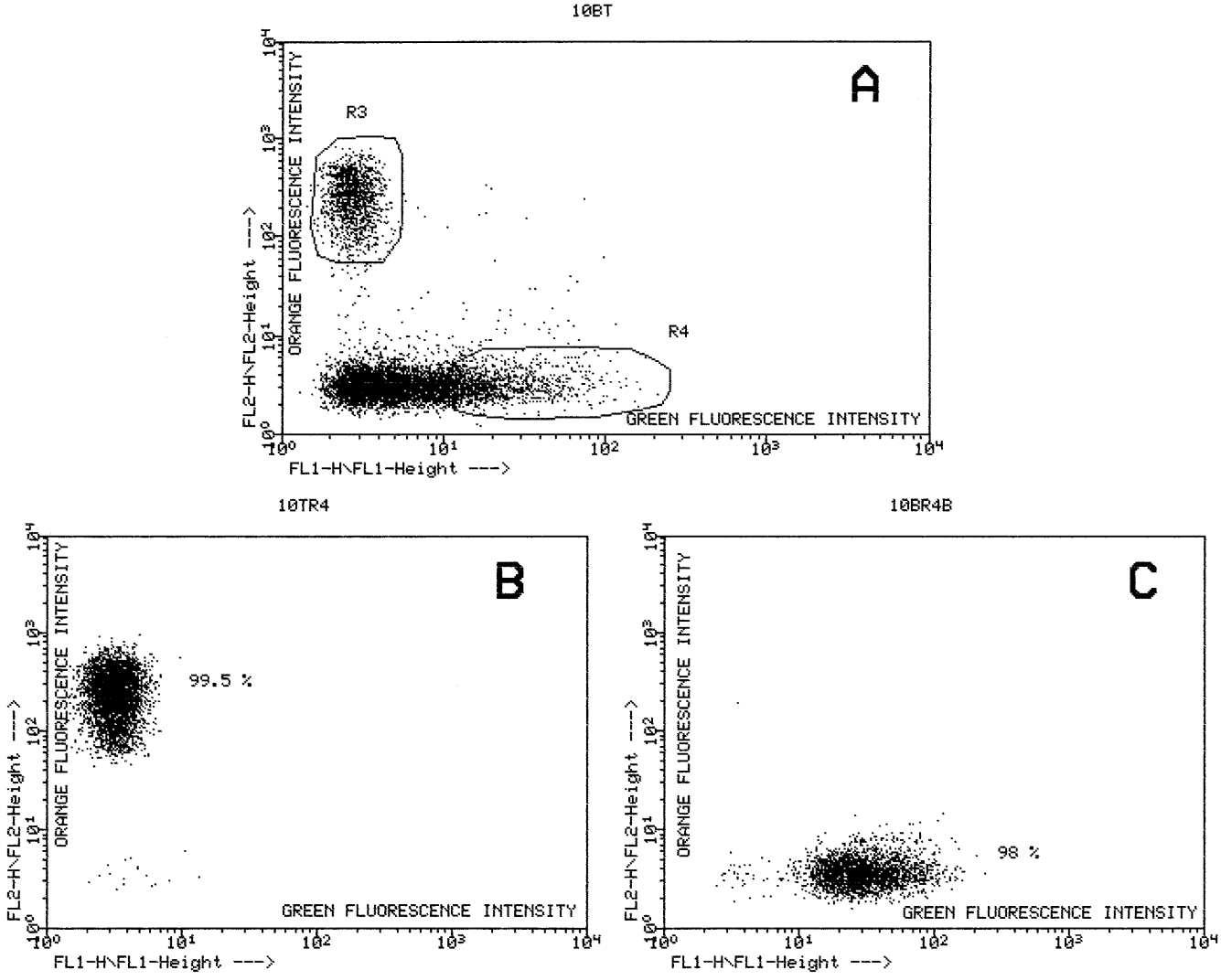

Fig. 4A–C Dot-plots of both green (fluorescein isothiocyanate, FITC) and orange (red phycoerythrin, R-PE) fluorescence of double-labelled appendix lymphocytes. **A** x-axis: lymphocytes labelled with FITC-IgM+, y-axis: lymphocytes labelled with R-PE-CD5+. *R3* and *R4* correspond to the sorting gates for CD5+ T and IgM+ B cells respectively. The *lower left region* corresponds to unlabelled lymphocytes. The *upper right region* corresponds to double-labelled lymphocytes. **B** Flow-cytometry analysis of sorted T lymphocytes. **C** Flow-cytometry analysis of sorted B lymphocytes

experiments, B and T lymphocytes were infected. Nevertheless, Vervelde et al. (1995) found a few sporozoites of *E. tenella* in B lymphocytes, more within macrophages and 50% in T cells, especially in $CD8^+$ cells. The difference in our results is probably due to the difference in the ratio of B and T cells in the tissues being studied. In fact, large numbers of B cells are present in the lamina propria of rabbit intestinal tissues, especially in the GALT (Ermak et al. 1994; personal observation). We cannot conclude whether sporozoites preferentially enter T or B cells of the lamina propria, because in most cases the differences between the oocyst outputs obtained with either type of these sorted lymphocytes were small.

In contrast, Vervelde et al. (1995) found that only a few sporozoites of *E. tenella* (about 12%) were within leukocytes in both the epithelium and the lamina propria. Further, Pakandl et al. (1993, 1996) found *E. coecicola* sporozoites almost only in lymphoid cells after the first stage of penetration; and in our study there was also evidence that the majority of sporozoites might be within B and T lymphocytes. Although occasionally other cells (unlabelled cells whose nature was not well defined) were also able to induce infection in recipients, oocyst excretion was much lower compared to that obtained with labelled cells. Moreover, compared with the duodenum, the vast majority of the vermiform appendix cells were leukocytes (~90%), so the probability of having sporozoites present in another cellular type was very limited.

Our first hypothesis was that IELs transported the sporozoites (Pakandl et al. 1995). In the mammalian intestine (and particularly in the rabbit appendix) most IELs are T cells and most are $CD8^+$ lymphocytes (Ermak et al. 1990). In our experiments, parasites were found in both B and T cells. Although sporozoites might

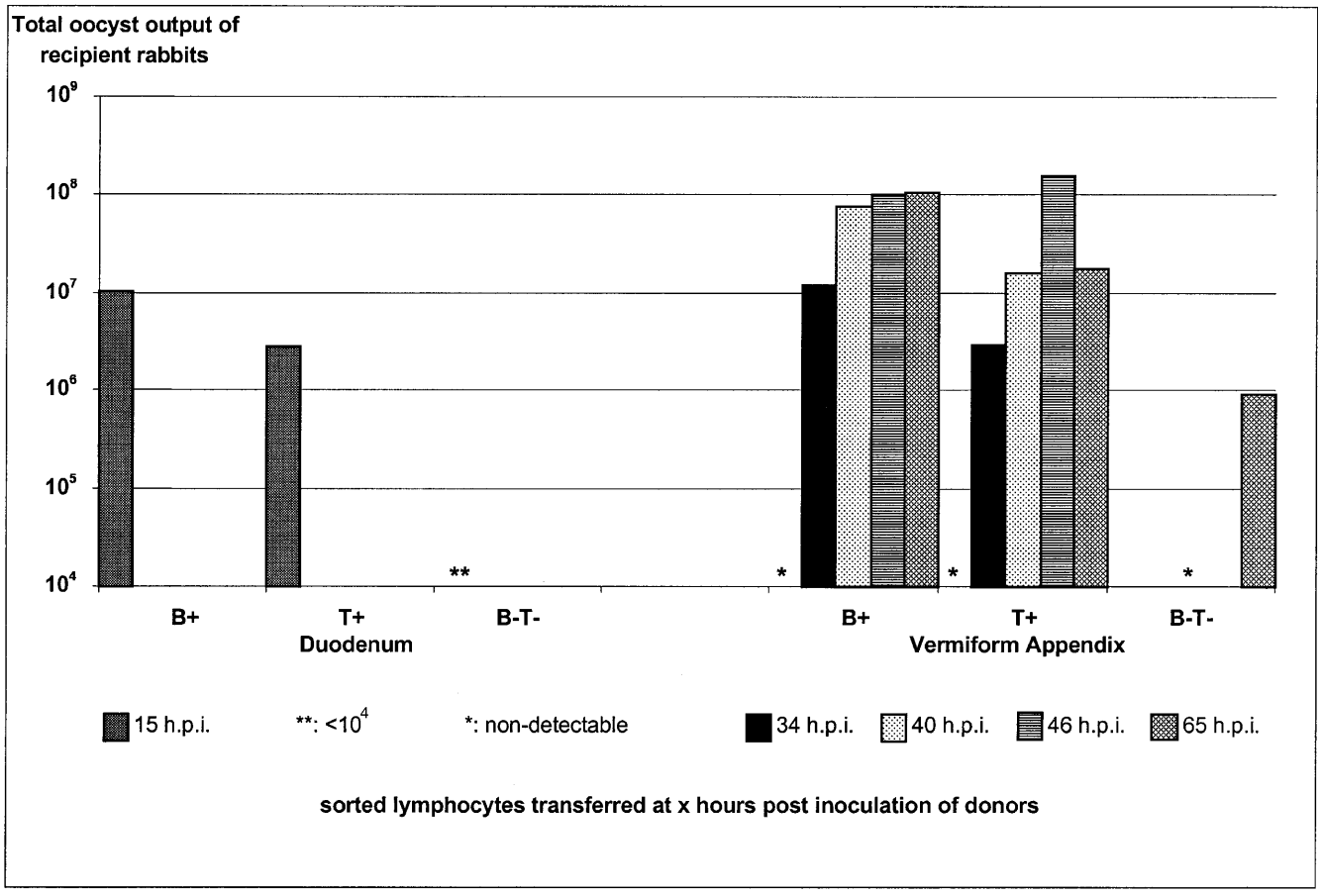

Fig. 5 Total *Eimeria coecicola* oocyst output per rabbit intravenously injected with about 100,000 sorted lymphocytes from donor rabbits sacrificed at various times post-inoculation. For experiments performed at 40 h p.i., cells were single-labelled with anti-IgM or anti-CD5 antibodies

be transported by IELs from the epithelium to the lamina propria of the duodenum, they are then able to enter B lymphocytes, which are lymphocytes of the lamina propria (LPL). Sporozoites may thus reach the vermiform appendix within LPL, rather than within IEL.

This study must be extended to other rabbit Eimeria species. In fact to date, *E. coecicola* is the only rabbit Eimeria for which the first merogony has been demonstrated to take place in lymphocytes and not in epithelial cells. *E. intestinalis* or *E. magna* sporozoites are able to travel from the duodenum to the ileum within 4–6 h (Drouet-Viard et al. 1994; Pakandl et al. 1995); it would be interesting to know by which extra-luminal route.

Acknowledgements We are grateful to Dr. J. Aycardi for his advice and help, to J. Moralès, J.P. Bouvier, J.P. Molteni, B. Sewald, A.F. Francineau and Y. Breuzin for their valuable technical assistance and to EU-COST Action 820 for their contacts. This work was supported by a grant from the Conseil Régional "Région Centre" (France). The experiments performed complied with the European rules for the care of experimental animals.

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