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Expression of multidrug resistance (*mdr*) gene(s) in primary lymphoid organs of chicken immune system during embryonic development

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Abstract. The presence of a multidrug resistance (MDR) related protein, P-170, in normal and pathological lymphoid cells has been described. The present report evaluates the expression of the *mdr1* gene by using the reverse Polymerase Chain Reaction (PCR) on cells obtained from the thymus and bursa of chicken embryos starting from day 12 until hatching. Results show that the thymic cells are positive from day 12 to the end of the observation period. In contrast, *mdr* mRNA was detected in the bursa from day 14 to day 17 of embryonic life. Possible relationships between the expression of *mdr* and the development of T and B lymphocytes are discussed.
Key words. Chicken; thymus; bursa of Fabricius; MDR; *mdr* genes.

In the recent past many research groups have focused their attention on the chemoresistance-related P-170 glycoprotein. This is a 170 kD, ATP binding, transmembrane glycoprotein widely known to be responsible for multidrug resistance (MDR) (see ref. 1 for review). P-170 is coded by the *mdr1* gene, and is thought to be an energy-dependent pump which exports several unrelated drugs out of resistant cells, thus lowering the intracellular drug concentration to sublethal levels. This protein shares extensive sequence homology with numerous bacterial and eukaryotic transport proteins, and has been considered to be a member of a protein superfamily able to transport a wide group of substances out of cell membranes[2]. P-170 belongs to a small, well-preserved multigene family. In invertebrates the number of *mdr* genes has been evaluated by Southern analysis[3]; primates, rabbit, chicken and fish contain two genes; rodents and bovines contain three genes while five genes were found in the pig[2]. In human beings, high levels of *mdr1* RNA have been found in the normal adrenal gland, kidney, colon, jejunum and liver. Expression was low in the other tissues that have been studied, which included bone marrow, muscle, skin, brain and nerve[4]. Normal peripheral blood cells express very low levels of P-170, if any.

Mdr1 gene expression has been detected by PCR in human T lymphocytes[5], and it has been suggested that the use of a highly sensitive immunofluorescence method may improve its detection in about two thirds of lymphocytes and monocytes and in about one third of granulocytes[6]. However, in this latter report *mdr1*-specific mRNA in normal pooled leukocytes was found to be either undetectable or comparable to that of a non-MDR cell line. On the other hand, expression of mRNA coded by the multidrug resistance gene *mdr1* has been found in a subset of normal bone marrow cells[7]. Its presence appears to be related to the positivity of haematopoietic stem cells that have been shown to express substantial amounts of P-170 glycoprotein. These are CD34+, CD33− cells and they are also able to discharge Rhodamine-123, a fluorescent laser dye successfully employed to test MDR activity[8]. Furthermore, the *mdr1* gene is frequently shown to be overexpressed in haematological malignancies[1] including acute leukemia[9,10], myelodisplasia[11], chronic lymphocytic leukemia[12], lymphoma[13], and myeloma [14]. In B and T neoplastic disorders P-170 expression could be related to the proliferation of cells with immature phenotypes, and attempts to demonstrate whether P-170 is expressed during differentiation could contribute to identifying the normal counterparts of neoplastic cells. The primary lymphoid organs of the chicken appear to be particularly interesting for this purpose. In birds, maturation of the immune system cells takes place in the thymus for T lymphocytes and in the bursa of Fabricius for the cells of the humoral immune response. These organs are populated by haematopoietic stem cells that mature in these sites towards the two lines of differentiation, T and B (ref. 15). In this report we evaluate the expression of *mdr1* mRNA in the thymus and bursa of Fabricius of chicken embryos from day 12 of embryonic life to hatching. mRNA expression was evaluated by reverse PCR using two primers chosen in order to amplify a highly conserved region of the *mdr1* gene, which is

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stable in several species. Primers were selected to span exon-intron boundaries in order to distinguish amplification of mRNA from any possible contamination by genomic DNA, and to amplify a 450 bp fragment. The specificity of this fragment was evaluated by Southern blot analysis using the pCHP1 probe[16] kindly supplied by Dr. V. Ling of The Ontario Cancer Institute, Toronto, Canada.

Materials and methods

Animals. Chicken eggs of the white Leghorn strain obtained from a local hatchery were incubated in the usual way and sampled every day from day 12 up to hatching. The thymus and bursa of Fabricius were removed from the embryos under sterile conditions in order to avoid contaminating the tissues with exogenous ribonucleases by manual handling.

Expression of *mdr1* mRNA was assessed in at least three repeated experiments.

PCR analysis. Total RNA from tissues was isolated by the single-step acid guanidine thiocyanate-phenol/chloroform method[17]. Each sample was then diluted to 300 ng/μl and subjected to reverse transcription to generate cDNA, according to the following procedure.

Aliquots (2 μl) of RNA sample were mixed with a solution containing 5 mM MgC12, 50 mM KCl, 10 mM Tris HCl pH 8.3, 1 mM each dNTP (Perkin Elmer), 2.5 μM Random Hexamers (Perkin Elmer), 1 U/μl RNAse inhibitor (Perkin Elmer), 2.5 U/μl reverse transcriptase (Perkin Elmer), in a final volume of 20 μl. Oligonucleotides were synthesized by Diagene Labs (Como, Italy) and purified by gel filtration. Downstream and upstream amplification primers were selected to span exon-intron boundaries in order to distinguish amplification of mRNA from any possible contaminating genomic DNA and to amplify a 450 bp fragment.

The sequence of the two amplification primers was:

Downstream: GTC-CAG-GGC-TTC-TTG-GAC-AAC
Upstream: GGC-AGC-AGT-GGC-TGT-GGG-AAG

PCR was carried out in a final concentration of 2 mM MgC12, 50 mM KCl, 10 mM Tris HCl pH 8.3, 0.2 mM of each dNTP, 2.5 U/μl Ampli Taq DNA Polymerase (Perkin Elmer Cetus), 0.15 μM upstream primer, 0.15 μM downstream primer. The mixture was overlaid with mineral oil and then amplified using a DNA thermal cycler (Perkin Elmer Cetus). The step cycle program was set to denature at 95 °C for 1 min, anneal at 58 °C for 1 min and extend at 70 °C for 1 min for a total of 35 cycles. The absence of contaminants was regularly checked by control samples that did not contain either RNA or reverse transcriptase, or contained PCR reaction buffer only.

Southern blot analysis. 10 μl of each PCR product was electrophoresed in a 5% acrylamide gel (90 V for 60 min), stained by ethidium bromide and then photographed. After incubation in a 0.25 N final concentration of sodium hydroxide (10 min at room temperature), samples were blotted on to a nylon membrane (Oncor) and dried for 1 hour at 80 °C. Hybridization was performed overnight at 42 °C using 25 ng/ml final concentration of digoxigenin labelled pCHP1 probe[16]. The pCHP1 probe was cloned from a cDNA library constructed in the expression vector λgT11 screened by C-219 monoclonal antibody. An insert was subcloned in the PUC 9 vector. It was about 600 bp long and contained no internal EcoR1 sites. It appeared to hybridize to several genes from the *mdr* family[16]. The filter was then washed twice in 2 × SSC (SSC:150 mM NaCl, 15 mM sodium citrate, pH 7) and 0.1% SDS (room temperature for 5 min), then twice at 68 °C for 20 min in 0.1 × SSC, 0.1% SDS. The filter was then developed using the 'Nucleic Acid Detection Kit' (Boehringer Mannheim), based on an enzyme-linked immunoassay using an antibody-conjugate (anti-digoxigenin alkaline phosphatase conjugate) and a subsequent enzyme-catalyzed color reaction with 5-bromo-4chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium salt (NBT).

Results

Results of Southern analysis of reverse PCR products are shown in figures 1 and 2.

The product of amplification run on polyacrylamide gels showed a clear band of 450 bp and several less evident bands. Only the 450 bp band reacted with pCHP1 after hybridization. Results indicate that the specific band was present or absent depending upon the age of embryo and the organ analysed. In the thymus a clear signal was present from the first day of analysis (day 12) to hatching (fig. 1). In contrast, the message was absent in the bursa until day 14 and then was present up to the day 17. After this time the message disappeared (fig. 2).

Discussion and conclusions

The aim of the present paper was to study the expression of the *mdr1* gene in the primary lymphoid organs

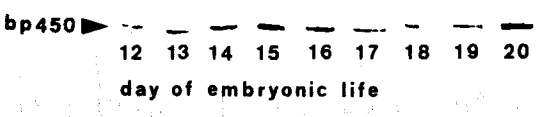

Figure 1. Southern blot analysis of reverse PCR products from thymus sampled at different days of embryonic life. Each experiment was performed at least in triplicate. In the positive samples a dark band of the expected MW was detected when PCR products were hybridized with a digoxigenin-labelled pCHP1 probe. Expression of the *mdr* gene was always detected.

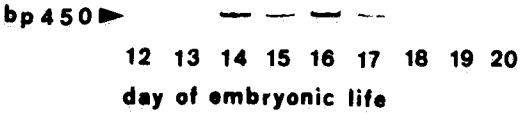

Figure 2. Southern blot analysis of reverse PCR products from bursa sampled at different days of embryonic life. Each experiment was performed at least in triplicate. In the positive samples a dark band of the expected MW was detected when PCR products were hybridized with a digoxigenin-labelled pCHP1 probe. Expression of the *mdr* gene was detected from day 14 to day 17 of embryonic life.

of the chicken during embryonic development. The results we obtained show that the *mdr1* gene is expressed in the thymus from the 12th day of embryonic life, when we started the observations, up to the time of hatching, while it is expressed in the bursa during the period between the 14th and the 17th day of embryonic development.

As far as the haematopoietic organs are concerned, studies have been carried out in humans and in other animals which have shown that low levels of P-170 glycoprotein are expressed in the bone marrow[4], and that human T lymphocytes express the *mdr1* gene. The *mdr1* gene is frequently overexpressed in haematological malignancies[1] including acute leukemia[9,10], myelodisplasia[11], chronic lymphocytic leukemia[12], lymphoma[13] and myeloma[14]. Overexpression of the *mdr1* gene product has been related to several mechanisms including gene amplification[20] and oncogene activity[21,22]. Moreover, heat shock proteins[23] and xenobiotics[24] have been shown to be able to increase the expression of P-170. In spite of these reports, conclusive results clarifying the mechanism(s) underlying the increased expression of MDR in spontaneous human cancer have not been obtained. One possible hypothesis is that the high frequency of MDR positive cells is related to an immature phenotype: stem cells have indeed been reported to be P-170 positive[8]. Recently the presence of the *mdr1* gene has been reported in some normal bone marrow-derived cells, which were shown to be CD34+, CD33−, belonging to the stem-cell lineage of blood cells.

From a genetic point of view, we think it is worthwhile to underline that the present study was intended to analyze the expression of the *mdr1* gene in the primary lymphoid organs by reverse PCR and hybridization with the probe pCHP1. However, in order to study the *mdr* gene in the chicken, we had to select primers in a highly conserved portion of the gene. This *mdr1* gene fragment is strictly related to a homologous portion in the *mdr3* gene that is expressed in the chicken, too. Although the 3′ primer has a homology of 60% with the *mdr3* sequence, the 5′ one shares a 100% homology between the *mdr1* and *mdr3* sequences. Thus the selected primers could have led to the amplification of a fragment from the mRNA coded by *mdr3*. In that case, the *mdr3* PCR product would have a length of 453 bp and it would be indistinguishable from the *mdr1* PCR product by our protocol, even after hybridization with the pCHP1 probe. Thus we cannot exclude the possibility that the present paper evaluates the expression of *mdr1* or of *mdr3*, or both in the lymphoid differentiation of the chicken.

To confirm that our results showed the expression of the *mdr* gene product we performed preliminary studies using two monoclonal antibodies directed to different epitopes of the P-170 glycoprotein (JSB-1 and MM 4.17)[18,19]. The protein was maximally expressed in the follicle on the 15th day whereas it was not found before the 13th day. After the 18th day the positivity was weak and possibly unrelated to lymphoid cells (data not shown). Studies are in progress in order to clarify which are the positive cell types.

The significance of the expression of the *mdr1* gene in immature cells or during lymphoid development is far from being explained. Considering the detoxifying activity of P-170 it is tempting to suggest that *mdr1* gene is expressed in order to protect progenitor cells during some 'critical' steps in their maturation. However, the physiological role of P-170 has not been completely explained and thus it is impossible to exclude a specific role in cell differentiation. Taking into account the chronological sequence of the events during maturation in the primary lymphoid organs of the chicken[25–27], it would be tempting to hypothesize that the early lymphopoietic stem cells may express *mdr* genes during their maturation steps.

The primary lymphoid organs are populated in the early embryonic stages (days 7–8 for the thymus, 8–9 for the bursa) by haematopoietic blood-borne stem cells (CFU cells) which are able to differentiate into the T and B cellular lineages thanks to the microenvironment in the primary lymphoid organs[25–27].

As far as the bursa is concerned, the period during which *mdr* genes become detectable coincides with the time at which the CFU cells are thought to give rise to SIg bearing cells. This striking coincidence could indicate that the SIg+ bursal cells are the cells expressing *mdr* gene(s). This could also explain the absence of *mdr*-expressing cells after day 18 of embryonic life, when SIg+ cells begin to transform into mature B cells and have started their migration towards the secondary lymphoid organs[26,27]. The positivity of thymus cells is expressed throughout the period we have observed in our experiments, and no clear connection with any critical developmental step of T cells was found. In conclusion, our data suggest that during T and B cell differentiation in primary lymphoid organs, steps exist in which lymphopoietic cells express *mdr* gene(s); further studies are in progress to identify which cell subset expresses the *mdr1* gene.

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