

Evidence of antibodies to erythromycin in serum of a patient following an episode of acute drug-induced hepatitis

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Summary

Background A patient who had taken erythromycin orally developed mild signs of hepatocellular damage and appeared to become sensitized. After a fortuitous oral intake of the antibiotic, 18 months later, the patient developed an extensive intrahepatic cholestasis.

Objective To confirm the allergic nature of this form of hepatitis by demonstrating the presence of serum antibodies directed at the drug.

Methods Primary cultured human hepatocytes were used to investigate whether erythromycin can covalently bind to hepatocytes; and to demonstrate the presence of drug-directed immunoglobulins in the serum of the patient.

Results As a consequence of its metabolism by hepatocytes, a small percentage of erythromycin remained bound to hepatocyte proteins. The extent of covalent binding varied among hepatocytes from different donors, and was not observed in metabolically inactive hepatocytes. Antibodies able to bind erythromycin were detected in the serum of the patient. When this serum was added to a human hepatocyte culture that had been previously incubated with the drug, binding of immunoglobulins was observed around the cell membrane and close to pseudo-bile canaliculi, thus indicating the presence of antibodies able to recognize erythromycin-labelled hepatocytes.

Conclusions The existence of serum antibodies directed at erythromycin haptens in this patient strongly suggests an allergic mechanism for this drug-induced hepatitis.

Keywords: erythromycin, allergic hepatitis, human hepatocytes, drug hepatitis, drug hepatotoxicity

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Introduction

Erythromycin is a macrolide antibiotic widely used for the treatment of respiratory infections caused by mycoplasma and gram-positive bacterial pathogens (i.e. *Campylobacter*, *Staphylococcus*, *Streptococcus*). Although this antibiotic was considered to be safe, hepatotoxicity

was reported quite soon after its generalized clinical use [1–12]. First case-reports indicated that this antibiotic caused hepatic steatosis [3,4,6]. However, it was soon recognized that such a toxic effect was caused by the estolate derivative rather than the erythromycin moiety. In spite of this, some clinical reports still indicated that erythromycin *per se* could also lead to hepatic injury in humans [1,2,5,9–12]. Frequently, the alterations caused by the drug were accompanied by clinical features suggestive of drug hypersensitivity and, based on those findings, an immunologically based mechanism of drug

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injury was suggested [3,11–13]. However, no direct evidence in favour of such a mechanism has been reported to date.

We have investigated the presence of serum antibodies directed at erythromycin in a patient who presumably become sensitized to the drug and, who after a fortuitous re-administration of the antibiotic, developed an episode of severe cholestasis. To this end, human hepatocytes from different donors were incubated with non-cytotoxic concentrations of the antibiotic and incubated with the patient's serum. The results of our work evidenced a variable degree of covalent binding of erythromycin to human hepatocytes, and the presence of antibodies in the patient's serum able to bind to erythromycin-labelled human hepatocytes.

Materials and methods

Clinical case

A 38-year-old woman complained of fatigue, pruritus and increasing jaundice. The patient was taking erythromycin (500 mg/8 h) orally because of a respiratory infection and had to interrupt the treatment after 4 days because of general discomfort, dark urine and clear faeces. She did not take any other drug during the period immediately before erythromycin treatment nor she had complained of liver dysfunction in the previous weeks. After admission to the hospital, bilirubin was elevated (8.5 mg/dL), while oxaloacetate transaminase (GOT) (90 U/L), and glutamate pyruvate transaminase (GPT) (121 U/L) were moderately elevated. Alkaline phosphatase (168 U/L), and gamma-glutamyl transferase (GGT) (25 U/L) were normal. Markers for hepatitis A, B and C were negative. Echography did not show pathological changes in the bile bladder, and the axial computerized tomography did not reveal the existence of tumours in the abdominal cavity. Morphological examination of a liver biopsy showed perivenous cholestasis and features compatible with an iatrogenic hepatitis. No treatment was given and bilirubin decreased to 6 mg/mL in the course of the next 2 weeks, pruritus persisted, while alkaline phosphatase, GOT and GPT remained moderately elevated. Two months later bilirubin had dropped to 1.6 mg/dL and GOT (19 U/L), GPT (24 U/L), alkaline phosphatase (71 U/L) and GGT (12 U/L) returned to normal values. Eighteen months earlier, the patient had been prescribed erythromycin (500 mg/12 h) and diame-tochlopramide (10 mg/8 h). At the end of the treatment (5 days), she showed mild signs of hepatic dysfunction (2.5 mg/dL bilirubin, 60 U/L GOT, 110 U/L GPT). Hepatitis B markers were negative, and the echography did not reveal pathological alterations. No treatment was given, and clinical parameters normalized within 1 month.

Isolation and culture of human hepatocytes

Liver biopsies were obtained from patients undergoing cholecystectomy. Biopsies were obtained after informed consent. Part of each biopsy was used for routine morphological examination and the rest (1–3 g) for research experiments, previously authorized by the Ethical Committee of the Hospital. The patients ($n = 5$) had no known liver pathology nor did they receive medication during the weeks prior to surgery. None of the patients (two men, ages 56 and 64 years, and three women, ages 36, 71, 79 years) were habitual consumers of alcohol or other drugs. Hepatocytes were isolated by a two-step micro-perfusion technique and cultured in serum-free media as previously described in detail [14].

Cytotoxicity evaluation

Cytotoxicity was evaluated in 24-h hepatocytes cultured in 96-well plates (25×10^3 cells/well) and incubated with increasing concentrations of erythromycin by measuring mitochondrial succinate dehydrogenase [15]. The experimental data were expressed as a percentage of controls and the typical sigmoid dose–effect curves were linearized using the LOGIT transformation. IC_{10} and IC_{50} values (concentrations that produce a 10% and 50% decrease in cell viability, 4.20×10^{-4} M; and 5.16×10^{-5} M, respectively, were estimated mathematically. The maximal non-toxic concentration (i.e. lowest concentration producing a statistically significant effect) was 5×10^{-5} M.

Covalent binding of erythromycin to human hepatocytes

Human hepatocytes were incubated with 5×10^{-5} M [^{14} C]-erythromycin (final specific activity 54 mCi/mmol) for variable periods of time. After incubation, plates (3.5 cm diameter, about 0.75×10^5 cells) were washed twice with ice-cold phosphate buffered saline (PBS), while the medium was fully aspirated. Cells were harvested and homogenized in 300 μ L of cold phosphate, and 2 mL of cold methanol was added and the mixture was centrifuged at 10 000 g for 10 min at 4 °C. Pellets were washed twice with ice-cold methanol and twice with ethanol and dried under an N_2 stream. After dissolving the pellet in 10% sodium dodecyl sulfate (SDS) and 0.5 mL 0.8 M NaOH, an aliquot was withdrawn for protein quantification [16]. The rest was transferred into scintillation vials, and 10 mL of scintillation liquid cocktail (Biogreen 101, Scharlau, Spain) was added for radioactive measurement.

Binding of serum antibodies to erythromycin

A sample of 20 μ L of the patient's serum was incubated

(4 °C) overnight with ^{14}C -erythromycin (0.5 μCi) and increasing quantities of non-radioactive erythromycin in a final volume of 800 μL . Samples were incubated for 3 h with protein A-sepharose (20 mg per vial), centrifuged 5 min at 9000 g , and washed three times with PBS. The pellets were resuspended in 40 μL of buffer (5% SDS, 10% mercaptoethanol in buffer Tris 100 mM pH 6.8), heated at 95 °C for 5 min. The pellets were further extracted with 100 μL of ethanol. Aliquots of the supernatants were transferred into scintillation vials, and 4 mL of scintillation liquid cocktail (Biogreen 101, Scharlau) was added for radioactive measurement.

Immunofluorescence staining

Human hepatocytes were 24-h incubated with $5 \times 10^{-5}\text{ M}$ erythromycin. Monolayers were washed twice with phosphate buffered saline (PBS), fixed with 100% ethanol for 3 min and washed twice with 70% ethanol. Thereafter, plates were incubated for 1 h at 37 °C with sera of the patient suffering from erythromycin-induced hepatitis (1/10 dilution in 0.05% Tween 20 in PBS) or with control human serum. In parallel, control hepatocytes (not exposed to erythromycin) were incubated with both sera. After incubation, monolayers were rinsed three

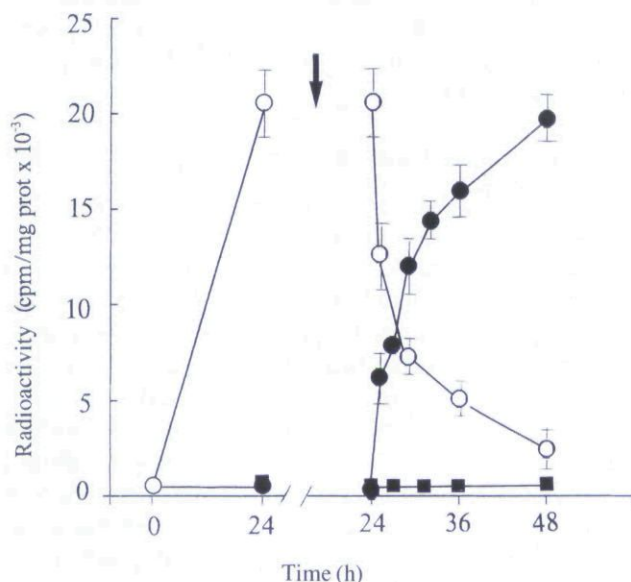


Fig. 1. Uptake and release of erythromycin by cultured human hepatocytes. Human hepatocytes prepared from donor 1 were incubated for 24 h in the presence of ^{14}C -erythromycin ($5 \times 10^{-5}\text{ M}$; final spec. act. 54 mCi/mmol). Cells were washed thereafter and shifted to drug-free culture medium (indicated by the arrow). At regular time intervals, cells (O) and medium (●) were analysed for the presence of labelled erythromycin. Squares represent covalently bound drug.

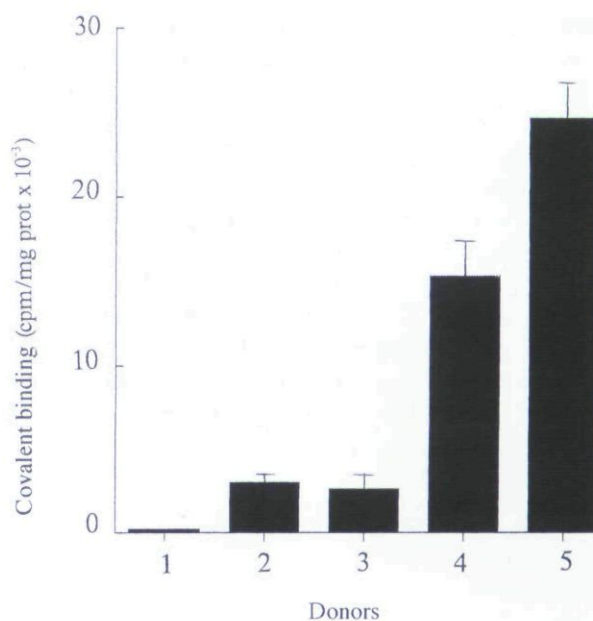


Fig. 2. Covalent binding of erythromycin to human hepatocytes. Human hepatocytes from several donors were incubated for 24 h with ^{14}C -erythromycin ($5 \times 10^{-5}\text{ M}$; final spec. act. 54 mCi/mmol). After incubation, plates were washed and cells were harvested and homogenized in 300 μL of cold phosphate. Cold methanol was added to precipitate macromolecules. After repeated extraction of unbound erythromycin from pellets, radioactivity was measured.

times with 0.05% Tween 20 in PBS, and fluorescein-labelled IgG anti-human IgG were added (1 h, 37 °C). After repeated washes (3 $\times$), plates were mounted and examined under a fluorescence microscope.

Results

Covalent binding of erythromycin in human hepatocytes

The features of the reported clinical case were strongly suggestive of a drug-mediated allergic hepatitis. Such a mechanism would require the ability of the drug or a metabolite to haptenize hepatocytes, and the existence of elements of the immune system able to recognize cells that had been in contact with the drug. Consequently, a first point to be addressed was whether erythromycin can bind to human hepatocytes in the course of its metabolism.

Incubation of hepatocytes with a non-cytotoxic concentration of radioactive erythromycin ($5 \times 10^{-5}\text{ M}$, 54 mCi/mmol) resulted in an increase in intracellular radioactivity that was rapidly released into the culture medium upon removal of the drug (Fig. 1). In most of the cell preparations (donors 1, 2 and 3), only a small

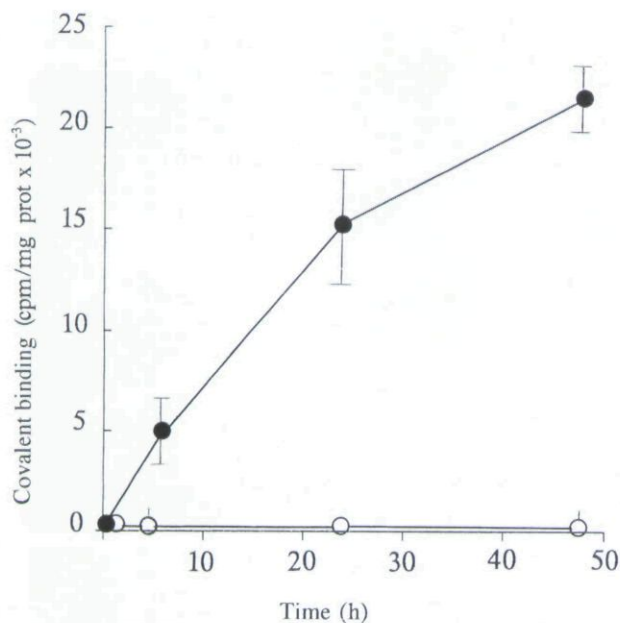


Fig. 3. Kinetics of covalent binding of erythromycin to cellular macromolecules. The kinetics of irreversible binding of erythromycin to hepatic macromolecules was investigated after incubating human hepatocyte cultures with 5×10^{-5} M [^{14} C]-erythromycin (final specific activity 54 mCi/mmol) for variable periods of time. At regular time-intervals, cells were harvested, extracted several times with cold methanol to remove unbound erythromycin, and radioactivity was determined (●). In parallel, covalent binding was determined in heat-killed cultures (○).

percentage of the drug remained associated with hepatocyte macromolecules. However, a significant amount of radioactivity was found bound to hepatocytes from donors 4 and 5 (approx. 115 and 160 pmol/mg cell protein, respectively) and could not be washed up with methanol (Fig. 2). The kinetics of this process was investigated and, as shown in Fig. 3 for donor 4, binding steadily increased with incubation time, say metabolism. This did not happen when radioactive erythromycin was incubated with metabolically inactive (heated) cells of the same donor (Fig. 3).

Presence of antibodies against erythromycin in serum of the sensitized patient

When the patient's serum was incubated with radioactive erythromycin, and the immunocomplexes separated with the aid of protein-A sepharose, radioactivity was retained in the protein-A sepharose gel, indicating that Fc-bound antibodies had retained radioactive erythromycin. To confirm the specificity of binding, increasing amounts of the non-radioactive drug were also added to the incubation mixture. As expected, a decrease in

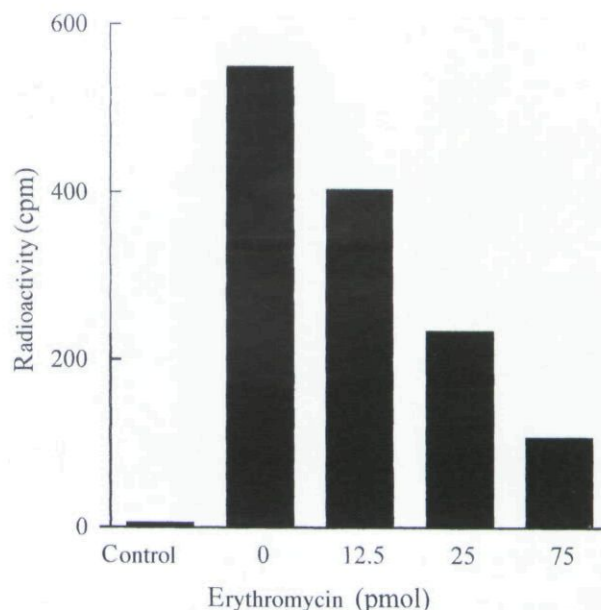


Fig. 4. Presence in serum of antibodies specific to erythromycin. A sample of 20 μ L of the patient's serum was incubated (4 °C) overnight with [^{14} C]-erythromycin (0.5 μ Ci) and increasing quantities of non-radioactive erythromycin in a final volume of 800 μ L. Samples were incubated for 3 h with protein A-sepharose, centrifuged 5 min at 9000g and, after extraction with ethanol, the radioactivity of pellets was measured.

protein-A sepharose bound radioactivity was observed (Fig. 4).

A human hepatocyte culture showing a high degree of covalent erythromycin binding (donor 4) was incubated with a non-cytotoxic concentration of the antibiotic for 24 h. Thereafter, serum from the hepatitis patient was added to the hepatocyte culture. After washing the culture plates to remove unbound immunoglobulins and adding a fluorescein-labelled second antibody, binding of human IgG to human hepatocytes became evident (Fig. 5). Fluorescence was only observed in erythromycin-treated human hepatocytes incubated with the patient's serum (A, B), while no fluorescence was observed when the same cell preparation was incubated with normal serum (C) or with the same human hepatocytes but not pre-incubated with the drug (D). As shown in detail in panel B, fluorescence granules located around the membrane of the hepatocytes, and close to pseudo-bile canaliculi, could be observed.

Discussion

The exaggerated hepatic susceptibility of an individual to a drug can be linked either to an abnormal production of a toxic metabolite or to a hypersensitivity reaction. The

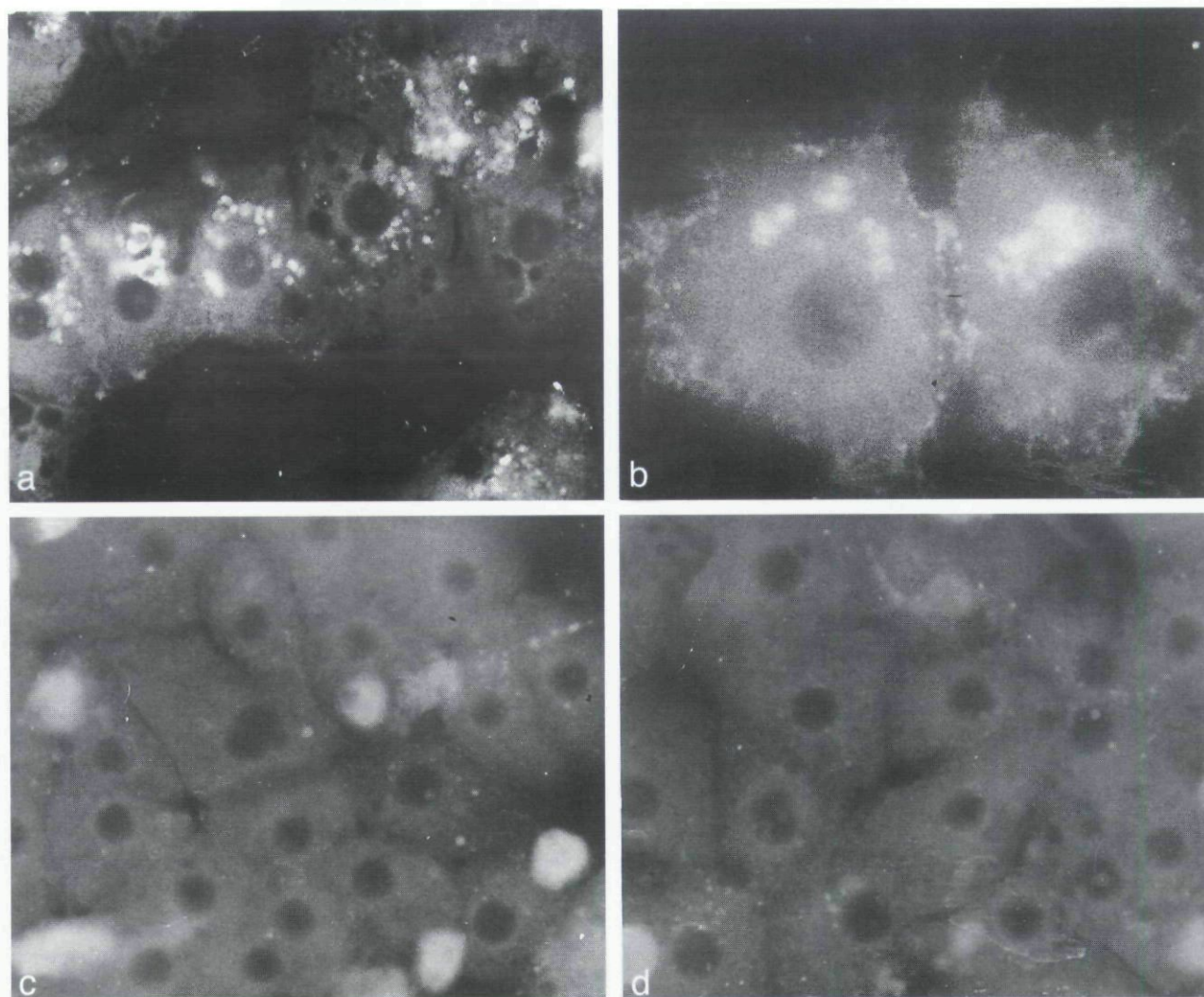


Fig. 5. Immunofluorescence staining of hepatocytes incubated with erythromycin. Human hepatocytes were 24-h incubated with 5×10^{-5} M erythromycin. Monolayers were washed twice with phosphate buffered saline (PBS), fixed with ethanol (several changes), and incubated for 1 h at 37°C with a 1/50 dilution of serum from the patient who suffered erythromycin-induced hepatitis (a, b). In parallel, hepatocytes were incubated with normal serum (c), or were not exposed to erythromycin and were incubated with patient's serum (d). After thorough rinsing, fluorescein-labelled IgG anti human IgG were added, the plates were washed again, and mounted for fluorescence examination.

former has a genetic basis and the severity of the observed hepatocellular damage is related to the administered dose of the compound. In the latter case, it is generally assumed that the drug, acting as a hapten, induces an immune response. Once the individual has become sensitized, further administration of the drug elicits an immune-mediated non-dose-dependent hepatic injury [17].

The diagnosis of an iatrogenic hepatitis is commonly based on exclusion criteria. Frequently, the drug that caused liver injury in a patient is not identified because of ethical restrictions on re-challenge experiments.

Moreover, it is difficult to distinguish between the two basic mechanisms of idiosyncratic hepatotoxicity. Certain indications help to discriminate drug-induced allergic reactions from toxicity due to the excessive production of a toxic metabolite: the existence of a previous sensitization period, prompt recurrence of hepatic injury upon fortuitous administration of small doses of the agent, fever, rash and inflammatory lesions in the liver [5]. The clinical case reported here was most valuable as it was possible to clearly identify erythromycin as the drug eliciting hepatitis. Moreover, the fact that the patient had taken the antibiotic at least once

before, the rapid onset of a severe cholestasis after an oral intake of the drug, the pathological findings in the liver biopsy, and the exclusion of other possible etiologies strongly suggested the involvement of an allergic mechanism in this case.

To find evidence in favour of this hypothesis, we investigated: whether erythromycin can bind to human hepatocytes and the presence of drug-directed antibodies in the patient's serum. Binding of erythromycin was studied in human hepatocytes incubated with the maximal non-cytotoxic concentration of the drug. From the results presented in Figs 1–3, it became evident that most of the erythromycin taken up by cells was released to the culture medium within the following hours. However, a small percentage of radioactivity was retained bound to cellular macromolecules in hepatocytes from certain donors (Nos. 4 and 5; Figs 2 and 3), while in others it was negligible. This variability is an interesting observation that might also reflect *in vivo* differences in covalent binding.

The experiment summarized in Fig. 4 revealed the presence of antibodies able to specifically recognize erythromycin. Antibodies against drug-protein adducts are not a common finding in drug-induced allergic hepatitis [19–21]. They are more often directed against cytochrome P450 isozymes [22–25] or cellular organelles [25, 26]. In the current study serum antibodies were able to bind human hepatocytes only if previously incubated with erythromycin (Fig. 5a,b), while this phenomenon was not observed in controls (Fig. 5 c,d). We interpret this to be the result of the covalent binding of the drug to cellular components (Fig. 2, donor 4), generation of drug-derived antigens, and recognition by immunoglobulins of the sensitized patient.

Little is known about the ethiopathogenic mechanism of allergic hepatitis and, in particular, the linkage between covalent binding, circulating drug-directed antibodies and the observed liver dysfunction. Covalent binding may not always be a cause of hepatocyte dysfunction *per se*. In fact, only in few cases has it been possible to demonstrate that the bond formation between a chemical and a critical biomolecule results in a well defined cell injury [18]. However, it is agreed that covalent binding must be a prerequisite for the triggering of an immunological response.

Analytical and experimental data on this hepatitis indicated a severe impairment of bile acid function without massive cell necrosis and the existence of circulating antibodies directed at erythromycin. Antibodies are large macromolecules and can not readily enter the cell. They could instead bind to haptenized hepatocyte membranes [18,19,27,28], thus altering their physicochemical properties and, indirectly, the functionality of membrane

allocated proteins (i.e. enzymes, ion pumps, carriers, etc.). This could be the origin of a reduced bile acid and anion transport and, ultimately, of less bile production. The micrograph shown in Fig. 5a, where binding around the bile canaliculi is observed, seems to lend support to this working hypothesis.

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