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ScienceDirect
Biochemical and Biophysical Research Communications 358 (2007) 325–330
BBRC
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Induction of autoantibodies to murine P-glycoprotein: Consequences on drug sensitivity in MDR cancer cells and on the expression of *mdr* genes in organs

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Received 13 April 2007
Available online 25 April 2007

Abstract

Overexpression of the 170 kDa plasma membrane P-glycoprotein (P-gp) represents the most common MDR mechanism in chemotherapy. In this work, specific autoantibodies to fragments from extracellular loops 1, 2, and 4 of the murine MDR1 P-gp were elicited in mice using synthetic palmitoylated peptides reconstituted in liposomes and alum. The highest IgG level was observed after the third immunization and the immune response against lipopeptides was still detected more than 200 days after immunizations. Immunocytochemichal studies revealed that these antibodies were specific for P-gp. When incubated with P-gp-expressing MDR cell lines, serum from immunized mice restored sensitivity to either doxorubicin or vinblastine, or had no effect in a cell type specific manner, suggesting that several mechanisms may occur in the establishment of the MDR phenotype. The expression of *mdr1* and *mdr3* genes was unchanged in organs from mice immunized with palmitoylpeptides grafted on liposomes. These results suggest that the induction of autoantibodies to P-gp is a safe strategy to overcome MDR in cancer chemotherapy.

Keywords: Multidrug resistance; P-glycoprotein; Peptides; Vaccination; Autoantibodies

Multidrug resistance (MDR) is a major obstacle that limits the effectiveness of chemotherapy in a variety of malignancies. This phenomenon is characterized by a reduced sensitivity to a broad spectrum of structurally unrelated chemotherapeutic agents with multiple subcellular targets such as anthracyclines, vinca alcaloids, epipodophyllotoxins or taxanes [1]. The MDR phenotype is often associated with the overexpression of ATP-driven drug efflux pump known as ATP-binding cassette (ABC) transporters, one of the best characterized is the 170 kDa P-glycoprotein (P-gp) [2]. It has been shown that overexpression of P-gp is sufficient to confer a MDR phenotype [3]. This membrane glycoprotein acts as an efflux pump, leading to a decreased intracellular drug accumulation and tumor cell survival. P-glycoprotein has been shown to be overexpressed in a number of solid tumors and hematological malignancies, and this overexpression is often correlated with poor prognosis [4]. Therefore, attempting to inhibit P-gp activity has been of great interest to overcome MDR.

P-glycoprotein has also been detected in normal mammalian tissues, including kidney, adrenal gland, liver, pancreas, intestine or brain [5], in which it plays an active role in excretion and bioavailability of metabolites and xenobiotics.

In mice, P-gp consists in a 1276-amino acid protein encoded by the structurally conserved *mdr* gene family [6]. The *mdr1*/*mdr1b* and *mdr3*/*mdr1a* genes can confer MDR when transfected and overexpressed in drug-sensitive cells [7], whereas *mdr2* is involved in hepatic phospholipid transport into the bile and not in the MDR phenotype [8,9].

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0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.04.117

In a previous study, we demonstrated that immunization of mice with liposome-grafted palmitoylated peptides corresponding to fragments from extracellular loops 1, 2, and 4 of the murine MDR1 P-gp induced both an increase of 77% in the survival time of mice in association with chemotherapy and in the intracellular accumulation of anticancer drugs [10]. In this study, we showed that immunization with palmitoylpeptides in liposomes triggered a long lasting humoral immune response against P-gp and that the elicited autoantibodies were specific for P-gp. We also assessed the MDR-reversing activity of these antibodies on several doxorubicin resistant cell lines and their consequences on P-gp-expressing organs in immunized mice.

Materials and methods

Cell lines. Murine leukemic (P388 and L1210) cell lines were cultured at 37 °C, 5% CO_2 in RPMI 1640 (Gibco-Invitrogen, Cergy-Pontoise, France) with10% heat-inactivated fetal bovine serum (FBS, Invitrogen). B16 melanoma and LM(tk−) fibroblastic cell lines were grown in DMEM with 10% FBS. For each cell line, a resistant subline was established by continual exposure to 0.35, 1, 4, and 10 μM doxorubicin (DOX, Teva Classics, Paris, France) for B16R, L1210R, LM(tk−)R, and P388R, respectively. Ten days before experiments, cells were cultured in drug-free medium.

Immune formulations. Synthetic tetrapalmitoylated peptides corresponding to extracellular loops 1 (mpp1), 2 (mpp2), 4 (mpp4) of the murine P-gp [10] were mixed with liposomes as previously described [11]. Two types of immune formulations were prepared: Lp1: DMPC, DMPG, cholesterol, palmitoylpeptides mpp1, 2, and 4; Lp2: DMPC, DMPG, cholesterol as a control.

Immunization protocol. Groups of nine B6D2F1 female mice (Iffa Credo, L'Arbresle, France) weighing 19–22 g were immunized by 3 i.p. inoculations at two week intervals with 200 μl of Lp1 or Lp2 formulation. Blood samples were collected by bleeding from the retro-orbital plexus. These experiments were conducted according to the animal care and use of the European Community. Samples were processed and serum was collected and stored at 4 °C less than 4 days for further experiments.

Dot blot assay. Antibodies were quantified by a dot blot assay. Each serum sample was incubated with nitrocellulose-adsorbed palmitoylated peptides as previously described [11]. After blocking and washing, the nitrocellulose was incubated with a secondary antibody and IgG specific to mpp1, mpp2, and mpp4 were detected with an enhanced chemiluminescence detection kit (Interchim, Uptima, Montluçon, France). Films were scanned and IgG concentrations were estimated from standard dilutions of purified IgG (The Binding Site, Grenoble, France).

MTT assay. To evaluate the activity of the elicited antibodies, 4.5×10^3 cells were incubated with 1.2% serum for 1 h at 37 °C and then grown 48 h with 10 μM DOX or 50 μM vinblastine (VBL, Sigma, St. Quentin Fallavier, France). Cell viability was estimated by the MTT assay. To evaluate the effect of the elicited antibodies, verapamil (VPL, Sigma), which is a specific inhibitor of P-gp, was used as a reference modulator of resistance and cell viability was analyzed under the same conditions. Percentages of cell death were determined by comparing cell viability obtained in the presence of drug only (DOX or VBL) and cell viability obtained in the presence of drug and MDR reversal agent (Lp1-induced antibodies or VPL).

Immunocytochemical labeling. Cells were fixed 1 h in 10% formaldehyde and subsequently incubated with Lp1 or control Lp2 serum (dilution 1/50) overnight at 4 °C. Immunostaining was performed by using the HistoMouse™-SP system (Zymed Laboratories, Invitrogen). A red staining was observed in positively labeled cells.

RT-PCR. Total RNA was extracted from cultured cells or from frozen tissue samples using the Qiagen RNeasy® Kit procedure (Qiagen; Courtaboeuf, France). One microgram of mRNA was used as a template for each RT-PCR. The primer sets were 5′-tgcttatggatcccagagtgac-3′ and 5′-ttggtgaggatctctccggct-3′ for *mdr1*; 5′-gaaagatggtgaactatgcc-3′ and 5′-ttaccaaaagtggcccacta-3′ for *mdr3*; 5′-gaaagatggtgaactatgcc-3′ and 5′-ttaccaaaagtggcccacta-3′ for *rRNA*.

Histological studies. Three groups of nine mice were immunized with Lp1, alum (100 μg) or physiological saline. Two months after the third challenge, mice were sacrificed and organs (liver, spleen, pancreas, and ovaries) were removed and fixed in neutral-buffered formalin. Histological studies were done on 3 μm sections of paraffin-embedded organs stained with haematoxylin-eosin.

Results

Immunization with tetrapalmitoylated P-gp derived peptides induces a long-lasting immune response against the extracellular loops of the murine P-gp

In a previous study, we showed that specific autoantibodies to extracellular loops 1, 2, and 4 of murine P-gp were elicited in mice using synthetic tetrapalmitoylated peptides reconstituted in liposomes and resuspended in alum. To demonstrate that the immune response persisted in time, a group of nine mice was immunized with Lp1 vaccine as described in materials and methods and antibody levels specific to mpp1, mpp2, and mpp4 were determined. As shown in Fig. 1, antibody synthesis was detected from day 24, i.e. 9 days after the second challenge, and the highest antibody titres were recorded after the third challenge for the three peptides. The mpp2 antigen induced the strongest immune response, as the antibody titre against mpp2 was 1.9- and 1.7-fold greater compared to antibody to mpp1 and mpp4, respectively. At days 75 and 240, the immune response decreased progressively. However, a sustained antibody titre toward mpp2 (2.65- and 3.7-fold compared to mpp1 and mpp4) remained detectable at day 240. This result shows that palmitoylpeptides presented by liposomes induces a persistence of the humoral

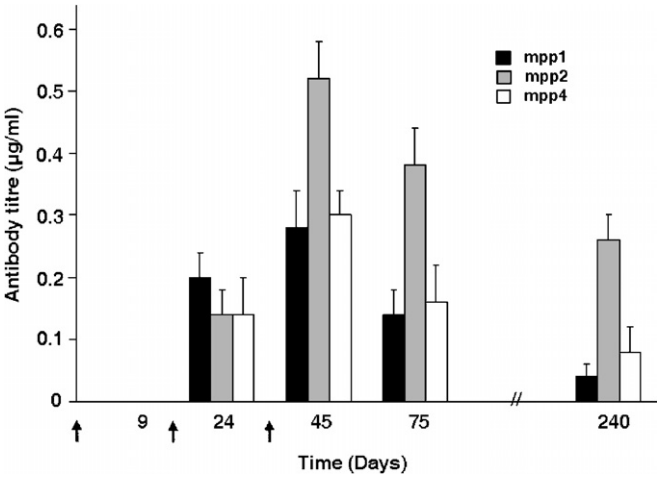

Fig. 1. Antibody titres induced after immunizations with mpp1, mpp2, and mpp4 antigens. IgG levels were measured using a dot blot assay. Means ± SD of nine mice are shown. Arrows represent immunizations.

immune response, especially toward the extracellular loop 2 of the murine P-gp.

In a previous work, we observed the development of granulomas after immunization with liposomal vaccine containing aluminium hydroxide (alum). To determine whether our Lp1 vaccine which contains alum induced the development of granulomas, Pgp-expressing organs from Lp1-, alum-, or saline-immunized mice were subjected to histological studies. Peritoneal granulomas were found in the pancreas, adrenals, spleen, and ovaries from mice that received 100 μg alum and Lp1 formulation, but not in mice injected with saline (Fig. 2). However, mice behavior and weight were not modified until eight months after the third immunization.

Expression of mdr1 and mdr3 genes in murine cell lines

Because both *mdr1* and *mdr3* gene can confer MDR, we next examined the expression of the two genes in murine cell lines by RT-PCR. We found that the *mdr1* gene was weakly expressed in all sensitive cell lines and it was overexpressed in their DXR-resistant counterparts, except in P388R cells (Fig. 3A). Overexpression of the *mdr3* gene was clearly detected in L1210R and P388R and to a lesser extent in LM(tk−)R cells, while it was not or slightly expressed in the other cell lines. These data suggest that P-gp-mediated MDR may be attributed to the co-expression of both *mdr1* and *mdr3* genes in L1210R, P388R and LM(tk−)R cells. In B16R cell line, only the *mdr1* gene was found to be expressed.

The Lp1-elicited antibodies can restore in vitro drug sensitivity and selectively bind to MDR cells

In order to characterize the MDR-reversing activity of the Lp1-elicited antibodies, we assessed their capacity to restore drug sensitivity in four resistant cell lines (P388R, L1210R, LM(tk−)R, and B16R) in comparison with the calcium channel blocker VPL. Incubation of Lp1 serum with P388R and L1210R enhanced cell toxicity induced by 10 μM DOX (15 ± 3% and 21 ± 4% of cell death com-

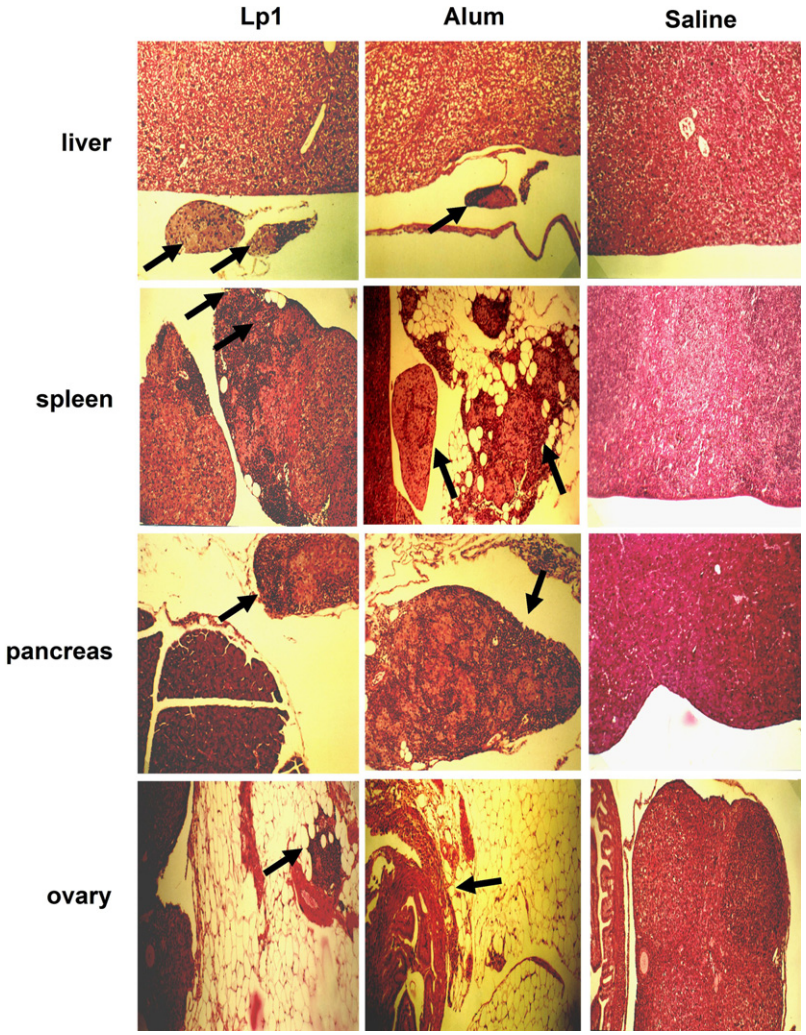

Fig. 2. Histological studies of organs from mice immunized with Lp1, alum or physiological saline. Two months after the third challenge, organs were removed and fixed in neutral-buffered formalin. Hematoxylin-eosin staining shows intraperitoneal granulomatous lesions in liver, ovary, pancreas, and spleen, or some isolated giant cells in the spleen, indicated by arrows.

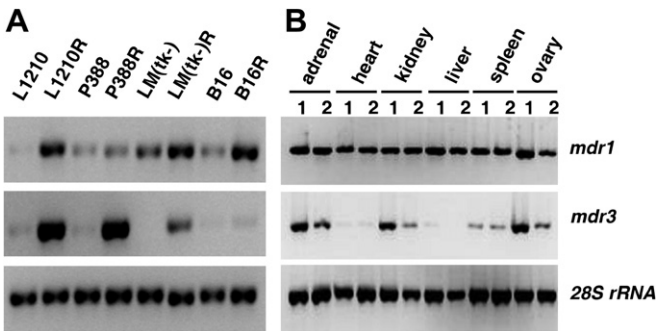

Fig. 3. Expression of the two *mdr* genes in P388, L1210, LM(tk−) and B16 cell lines and in their DOX-resistant counterparts (A), and in organs from mice immunized by control Lp2 (1) or Lp1 vaccine (2) (B). Total mRNA was extracted and the level of *mdr1* and *mdr3* was analyzed using RT-PCR.

pared to cells treated with DOX only, see Fig. 4A). This effect was less pronounced when cells were incubated with 50 μM VBL (7 ± 2% and 8 ± 3%), but remained equivalent to VPL-induced toxicity in both cell lines. In LM(tk−)R cells, the elicited antibodies modulated chemoresistance at the same level to 3 μM VPL (23 ± 4 and 18 ± 3%, respectively) when cells were cultured in the presence of 50 μM VBL. However, neither the Lp1-induced antibodies nor VPL enhanced DOX-induced cytotoxicity in LM(tk−)R cells. Although we showed that B16R cells express the *mdr1* gene, the Lp1 antibodies as well as VPL failed to reverse both DOX and VBL-induced toxicity.

To demonstrate that the activity of Lp1-elicited antibodies is associated with a specific recognition of P-gp, we performed an immunocytochemical study by incubating Lp1 or control serum (Lp2) with sensitive or DXR-resistant cell lines. Incubation with empty liposomes (Lp2) did not show any labeling (Fig. 4B). When incubated with Lp1 serum, all the resistant cell lines were positively labeled, demonstrating the binding of antibodies to cells, whereas no staining was observed in sensitive cell lines. These results show that vaccination with Lp1 formulation induced antibodies specific to P-gp-derived peptides that selectively bind to MDR cells.

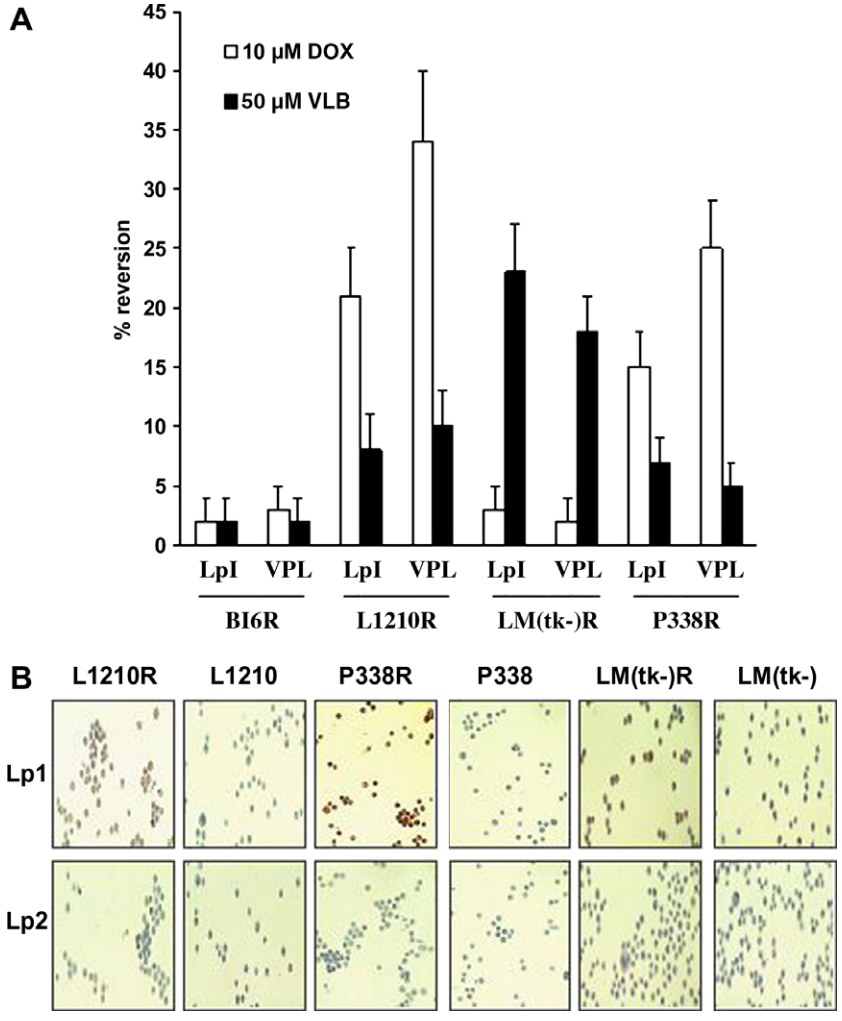

Fig. 4. The Lp1-induced antibodies can restore sensitivity to anticancer drugs in MDR cells and selectively bind to P-gp-expressing cells. (A) MDR-reversal activity of Lp1 serum and VPL in MDR cell lines. Each result represents an average of the values obtained from sera of 3 mice in triplicate. (B) After fixation in paraformaldehyde, cells were incubated with Lp1 or control Lp2 serum. Antibody binding was revealed using the HistoMouse™-SP system. Positive cells exhibit a red labeling. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The expression of mdr1 and mdr3 genes in organs is not modified after immunization with P-gp-derived peptides

mdr1 and *mdr3* genes are also naturally expressed in several organs where they are involved in the transport and excretion of endogenous metabolites or xenobiotics. To know whether anti-P-gp antibodies induced any change in *mdr1* and *mdr3* gene expression, we examined their expression in organs from mice immunized with control Lp2 or Lp1 vaccine. The results showed that *mdr1* gene was expressed in all tissues tested in mice immunized with peptide-free liposomes (Lp2, see Fig. 3B). The immunization with palmitoylpeptides anchored in liposomes (Lp1) did not induce any modifications in the *mdr1* gene expression. The *mdr3* gene was mainly expressed in the adrenal gland, kidney, and ovary, and its level of expression was unchanged after immunization with Lp1 vaccine.

Discussion

A major concern in the treatment of cancer is the development of resistance to chemotherapy and subsequent relapse. Overexpression of membrane-bound P-gp is the hallmark of several MDR cell lines [12].

A variety of compounds have been shown to reverse P-gp-mediated MDR such as the calcium channel blocker VPL or the cyclosporine derivative Valspodar [13,14], but their clinical use has been hampered by the toxic side effects that occur when non-physiological doses, which are required to achieve a significant reversal of MDR, are used [15]. Therefore, novel strategies aiming at inhibiting P-pg-mediated drug efflux should be developed.

In order to overcome MDR, several approaches using antibodies specific to P-gp have been reported. The mAb MRK16 has been shown to enhance intracellular accumulation of anticancer drugs and to reverse MDR in a model of transgenic mice whose bone marrow cells express the *mdr1* gene [16,17]. We have previously shown that liposome-grafted palmitoylated peptides mimicking the external-loops 1, 2, and 4 of the murine MDR1 P-gp reconstituted elicited specific antibodies that inhibited *in vitro* P-gp activity in multidrug resistant leukemia P388 cells [10].

We have shown here that the humoral immune response was detectable for several months after immunizations. The total antibody titre was the highest after the third challenge and was still significant up to 240 days. During the immunization period, the strongest immune response was obtained against the mpp2 antigen, a peptide fragment from the extracellular loop 2. This is in agreement with our previous data showing that the external loop 2 is the most immunogenic [10].

UIC2, a mAb which can inhibit Pgp-mediated drug efflux, recognizes a conformational epitope which involves the external loop 1 [18,19]. This justifies the use of a fragment from this loop as immunogen.

Immunocytochemical studies revealed that Lp1-induced antibodies are likely to recognize P-gp, since they bound to the four MDR cell lines tested, but not to the sensitive parental cell lines. Although the *mdr3* gene was the predominantly *mdr* gene expressed in P388R and L1210R cells, the elicited antibodies, generated by immunization with MDR1-related peptides sequences, reacted with these cell lines because MDR1 and MDR3 proteins share 84% homology [7].

We have previously shown that our Lp1 vaccine enhanced *in vitro* VBL uptake in P388R cells and improved survival of mice in combination with chemotherapy [10]. We showed here that the induction of anti-P-gp antibodies could restore doxorubicin and/or vinblastine sensitivity in several cell types expressing the MDR phenotype. The Lp1 antibodies potentiated the cytotoxic effect of DOX rather than VBL in leukemia P388R and L1210R cell lines, while VBL, but not DOX toxicity, was increased in fibroblastic LM(tk−)R cells. It was shown that the MDR phenotype could be conferred by multiple mechanisms, such as the coexpression of two drug-efflux transporters or the expression of transporters associated with down-regulation of drug targets or up-regulation of detoxification systems [20,21]. It is likely that antibodies prevented P-gp-mediated drug efflux by blocking conformational changes required to P-gp activity. However, in the two leukemia cell lines, another mechanism of resistance may be associated with P-gp expression such as drug metabolization by the cytochrome P450 enzymes, which could explain the lack of toxicity of VBL. On the contrary, in the fibroblastic LM(tk−)R cells, the lack of toxicity of DXR in the presence of Lp1 serum suggests that P-gp expression may be associated with qualitatively or quantitatively altered levels of topoisomerases. In B16R cells, neither the Lp1 antibodies nor VPL enhanced drug cytotoxicity, although these cells expressed the *mdr1* gene. P-gp overexpression may be associated in these cells with altered levels of topoisomerases or increased glutathione S-transferase activity. Therefore, several mechanisms conferring MDR may be involved in a cell type-specific manner.

The immunization with Lp1 vaccine did not induce any changes in *mdr1* and *mdr3* expression in the organs studied, and mice behavior was unmodified. P-gp is rather distributed on the luminal surface in normal secretory tissues and may not be accessible to antibodies [5]. However, peritoneal granulomas were found mainly in liver, pancreas, spleen, and ovaries. This could be attributed to the alum adjuvant, as 3 i.p. injections with 100 μg alum alone resulted in the development of similar lesions. Several other studies reported the appearance of post-vaccination granulomas in animals [22] or children immunized with aluminium-adsorbed vaccines [23]. These results suggest that inhibiting P-gp activity through immunization with our Lp1 vaccine is a safe strategy that does not interfere with the physiological role of P-gp.

In conclusion, the concept of eliciting autoantibodies by immunization with synthetic lipopeptides reconstituted in liposomes, mimicking the extracellular loops of P-pg, may be a promising and safe tool to overcome P-gp-mediated MDR in cancer therapy.

Acknowledgments

We thank Lydie Venteo for the histopathological studies. This work was supported by the region Champagne-Ardenne for P.F. Tosi and by a grant from the "Association pour la recherche contre le Cancer".

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