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Inhibition of multidrug resistance by immunisation with synthetic P-glycoprotein-derived peptides

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Abstract

Overexpression of the membrane glycoprotein (P170) represents the most common multidrug resistance (MDR) mechanism in cancer therapy. Specific auto-antibodies to extracellular loops 1, 2 and 4 of murine P170 were elicited in mice using palmitoylated synthetic peptides reconstituted in liposomes, with or without Lipid A, and resuspended in alum. IgM antibodies were detected 14 days following the first injection and IgG1 became predominant after the third challenge. Animals did not show any auto-immune symptoms or induced toxicity up to 18 months after the immunisation. Previous immunisations of mice using liposomes with MDR1 peptides increases the efficacy of chemotherapy treatments with doxorubicin and vinblastine against P388 R cells with increase of 77% in the survival half time in the immunised group. Sera from the immunised mice were also effective in reducing cellular resistance to vinblastine and doxorubicin *in vitro*. Taken together, these data suggest that this immunisation approach might have potential clinical applications.

Keywords: Multidrug resistance (MDR); P-glycoprotein 170Kd (P170); Immunisation; Liposomes; Lipopeptides

1. Introduction

Multidrug resistance (MDR) of tumour cells is known to be responsible for treatment failures in cancer chemotherapy [1,2]. Overproduction of a glycoprotein, called P170, was found to correlate with the development of resistance phenotypes to a number of unrelated drugs, including anthracyclines, epipodophyllotoxins, vinca alkaloids, actinomycin D, and taxoid derivatives. In humans, the *mdr1* gene encodes a 1280 amino acid protein with two homologous halves, each comprising six putative transmembrane domains, and one nucleotide binding site [3,4]. This glycoprotein acts as an efflux pump on a wide variety of compounds that do not have either common chemical structures or common mechanisms of pharmacological action [5]. The *mdr1* gene is expressed in all haematopoietic progenitor cells [6] and in many organs and tissues such as the liver, intestine, kidney, pancreas, blood–brain barrier and blood–testicular barrier [7,8]. This implies that in tumours arising from these tissues, resistance is intrinsic and chemotherapeutic treatments are poorly effective. In other cases, MDR is induced during treatment. Therefore, strategies have been developed in order to reverse the MDR phenotype. Indeed, successful reversal of MDR might improve the treatment approaches for different cancers. Experimental reversal of the *mdr* phenotype has been reported by chemical agents such as channel blockers (verapamil), calmodulin antagonists and different monoclonal antibodies specific to the human MDR1 [9]. Several of these monoclonal antibodies recognise epitopes of the loop 1 and one of the most efficient in mice is UIC2 [10].

The murine *mdr1* cDNA encodes a 1276 amino acid protein with a structure similar to its human homologue

Abbreviations: MDR, multidrug resistance; P 170, glycoprotein 170 Kd; mpp, murine palmitoylpeptides; Lp, liposomes; alum, aluminum hydroxide.

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[11]. Sequences of mouse and human P170 are 80% homologous, with the strongest homology occurring in the intra-cytoplasmic part of the protein, whereas extracellular sequences differ significantly [3]. Three mdr genes have been sequenced and characterised: *mdr1* (or *mdr1a*), *mdr3* (or *mdr1b*) and *mdr2* [12]. Among these isoforms, *mdr1* and *mdr3* are both active for the transport of anticancer drugs, whereas *mdr2* is not.

We showed previously that immunisation of mice with external sequences of the murine *mdr1* P170 elicited antibodies that are specific to extracellular epitopes of the protein capable of reverting *in vitro* the MDR phenotype of L1210-resistant cancer cells [13]. Such a strategy of breaking the immune tolerance to the self-protein was shown to be effective *in vivo* against β-amyloid plaques on the pancreas in transgenic NORBA mice [14].

In the present study, we aimed to investigate further the *in vivo* effectiveness of immunisation with synthetic peptides mimicking extracellular loops of murine P170 reconstituted in liposomes, without or with lipid A [15]. We hoped not only to break the immune tolerance towards the *MDR1* protein, but particularly to induce the most efficient antibodies possible, therefore we analysed the kinetics of the different isotypes.

2. Materials and methods

2.1. Synthetic palmitoylpeptides

The palmitoylpeptides (Table 1) corresponding to sequences of the extracellular loops 1 (mpp 1), 2 (mpp 2) and 4 (mpp 4) of the *mdr1* P170 [13,16] have been synthesised on an Applied Biosystems 430A peptide synthesiser using the tertiobutyloxycarbonyl/benzyl chemistry [17,18] and *in situ* (*N*-[(1H-benzotriazol-1-yl) (dimethylamino) methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide activation [19]. Each peptide coupled with 4 palmitic acid molecules was analysed by mass spectrometry: mpp 1, $[M+H]^+$, calculated (calcd): 5436, found (Matrix Assisted Lazer Desorption Ionization-Time of Flight, MALDI-TOF): 5437. mpp 2, $[M+H]^+$ calcd: 3293, found (Plasma Desorption Mass Spectrometry-Time of Flight: PDMS-TOF): 3293. mpp 4, $[M+H]^+$ calcd: 3190, found (PDMS-TOF): 3188. The peptides were also analysed by amino acid analysis following hydrolysis of an aliquot in HCl 6N/phenol at 110 °C. Results (expected/found) were as follows:

mpp 1, A (2/1.5), D (5/4.4), E (5/6.6), F (1/0.6), G (4/3.4), I (4/3.6), K (4/4.0), L (3/3.5), M (1/0.7), P (2/1.8), T (4/3.0), S (8/7.4).

mpp 2, A (2/2.3), D (1/1.2), E (2/2.4), F (1/1.0), G (2/2.1), K (6/6.0), L (2/2.1), S (1/0.9), V (1/1.0), Y (1/1.0).

mpp 4, D (5/5.0), E (3/3.1), G (2/2.0), K (4/3.7), S (1/0.9), R (2/1.9).

2.2. Immune formulation

Liposomes were prepared by mixing dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG) and cholesterol (Avanti Polar lipids, Alabaster, Alabama) at the molar ratios of 0.9; 0.1; 0.7, respectively. Monophosphoryl lipid A (MPLA) (List Biologicals, Campbell, California) was added at a concentration of 40 μg per μmole of phospholipids [20]. Three suspensions of liposomes were prepared: Lp 1: DMPC, DMPG, cholesterol, palmitoylpeptides; Lp 2: DMPC, DMPG, cholesterol and Lp 3: DMPC, DMPG, cholesterol, palmitoylpeptides, MPLA. The 3 palmitoylpeptides (mpp 1, mpp 2, mpp 4) were mixed in a constant 1/250 molar ratio with phospholipids. After evaporation of the solvent, the lipids were dried in a vacuum dessicator. The resultant film after hydration with sterile phosphate-buffered saline (PBS pH 7.4) was adjusted to a final phospholipid concentration of 4 mM. For the second and third immunisations, each batch of liposomes was kept frozen. Before the injection, the liposome suspension was mixed volume-to-volume with 0.1 ml of sterile Alum provided by Pasteur Merieux (Marcy L'Etoile, France).

Table 1
AA sequences of the synthetic lipopeptides corresponding to extracellular loops 1, 2 and 4 of the P170 mdr1

Peptides	AA sequences	AA number
mpp 1	K-G-GNMTDSFTKAEASIVLPSITNQSGPNSTLIISNSSLEEE-G-K-K-NH2 (1, 2, 3, 4)	43
mpp 2	K-G-KVLTSFTNKELQAYAK-G-K-K-NH2	21
mpp 4	K-G-SRDDDMETKRQNEN-G-K-K-NH2	19

AA, amino acid. Each peptide was coupled with 4 palmitic acids to obtain the murine palmitoylpeptides (mpp 1, mpp 2 and mpp 4). The sequences and potential glycosylation sites (in 4 boxes) are from Ref. [11]. The regions with a higher probability for induction of an immune response are overlined.

2.3. Immunisation protocol

Groups of 9 B6D2F1 female mice (Iffa Credo, L'Arbresle, France) weighing 19–22 g were immunised by 3 intraperitoneal (i.p.) inoculations at two week intervals with 200 μl of the different immune preparations. Blood samples were collected between 7 and 12 days after each immunisation by bleeding from the retro-orbital plexus. Each sample was processed and the sera were separated and stored at 4 °C for the Ig assay for less than 4 days.

2.4. Immune response analysis

Each serum sample was incubated in the presence of nitrocellulose adsorbed palmitoylpeptides as previously described in Ref. [13]. Different immunoglobulins (IgM, IgG3, IgG2a, IgG2b, IgG1) were detected by using a secondary antibody covalently linked with peroxidase (The Binding Site, Grenoble, France). The activity was detected using enhanced chemiluminescence (ECL™) reagents (Amersham Pharmacia Biotech Buckinghameshire, England) by exposure to Kodak X-O MAT film (Sigma). Films were scanned using a densitometer and concentrations were estimated from standard dilutions of purified Ig (M, G3, G2a, G2b, G1) from The Binding Site.

2.5. *In vitro* activity of the elicited antibodies

Murine lymphoid neoplasm P 388R cells (provided by G. Atassi, Laboratoire Servier, Courbevoie, France) were cultured in Roswell Park Memorial Institute (RPMI) (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and treated with 10 μM doxorubicin (Sigma). For cytotoxicity assays, cells (45×10^3) were cultured at 37 °C in a humidified atmosphere of 5% carbon dioxide/95% air and incubated with sera (0.6; 0.3; 0.15% dilution in RPMI, 10% FBS) for one hour at 37 °C and then grown for 48 h with serial dilutions of doxorubicin (DXR) or vinblastine (VLB) 50 μM in medium. Cell viability was estimated by the colorimetric dimethylthiazolyl-2,5-diphenyltetrazolium bromide (MTT) test [21]. To compare the effect of the elicited antibodies, verapamil (Sigma) was used as a reference modulator of resistance and cell viability and was analysed under the same conditions.

2.6. Uptake of [^{3}H]-vinblastine

1.6×10^6 cells in 400 μl RPMI with 10% FBS were incubated at 37 °C for 1 h, with or without effector (VLB or sera of immunised mice), then introduced in siliconed glass tube to 2 μCi of [^{3}H-VLB] (Amersham, Saclay, France) and non-radioactive VLB (final concentration 1μM) at an identical temperature [22]. After 30 min, 100 μl of the cell suspension was pipetted into plastic tubes containing 50 μl of 6% perchloric acid solution overlaid by 100 μl phtalate-ester mixing. Tubes were immediately centrifuged at 13 000g for 30 seconds and frozen in liquid nitrogen. Tubes were bisected, the bottom half was immersed in 5 ml of formula 963 (New England Nuclear) scintillation fluid and radioactivity was determined in a LKB scintillation spectrometer. Results were expressed as pmoles of VLB per 10^6 cells and as a percentage of incorporation in comparison with P 388R cells incubated with control immune sera (IS Lp 2).

2.7. *In vivo* evaluation of the immune formulations

Groups of 9 mice preimmunised either by Lp 1 or Lp 2 received by i.p. injection 10^6 P 388R cells on day zero. On days 1, 10 and 22, each mouse was injected with DXR 5.5 mg/kg and on days 4, 14 with VLB at 2.5 mg/kg. During that period, eating, drinking, alertness and

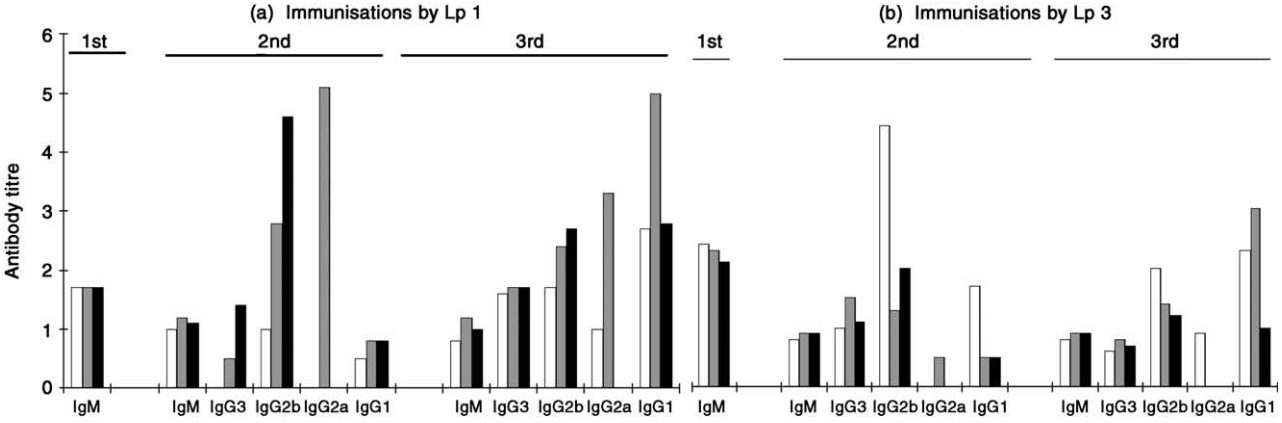

Fig. 1. Antibody titre in the sera of mice as a function of immunisation time (1st, 2nd, 3rd injection). a, Lp 1 (liposomes, palmitoylpeptides, alum); b, Lp 3 (liposomes, lipid A, palmitoyl-peptides, alum). For both figures, the anti-mpp 1 (□), -mpp 2 (▒), -mpp 4 (■) were quantified and the different isotypes detected by using secondary antibodies specific to murine Ig (M, G3, G2a, G2b, G1). Each histogram represents an average of the values obtained from sera of 5 mice bled 12 days after immunisation. One unit corresponds to 0.2 μg Ig/ml. standard deviations (SD) are not shown, they varied between 5 and 25%.

weight were noted and survival was also recorded. Before injection with P 388R cells, sera obtained between 15 to 45 days after the third immunisation were used to quantify the anti-P170 antibodies and check their activity. These experiments were conducted following the animal care and use rules of the European Community.

2.8. Histopathological studies

Mice were sacrified 13 months after the last immunisation and their organs (kidneys, adrenal glands, pancreas, spleen, liver, heart, lung, ovary) were removed and fixed in neutral-buffered formalin. Histopathology studies were done on 3 μm sections of paraffin-embedded organs stained with haematoxylin-eosin.

3. Results

3.1. Induction of the humoral immune response following administration of the different formulations

Three different immune formulations were prepared: Lp 1, Lp 2 and Lp 3. In order to quantify the antibodies specific to mpp 1, mpp 2, mpp 4 and determine their isotypes, we used to dot blot assay. The sera of mice immunised with control preparation Lp 2 were used as controls for the antibodies e.g. IgM, IgG2a, IgG2b, IgG3 and IgG1. The responses of animals immunised with Lp 1 or Lp 3 are shown in Fig. 1a and b. The preparations Lp 1 and Lp 3 after i.p. inoculation in mice, elicited IgM after the first injection. The response became predominantly IgG2b and IgG3 for Lp 1 (Fig. 1a) and IgG2b for Lp 3 (Fig. 1b) after the second injection. The titre of IgG1 shows a maximum after the third challenge for both formulations and is 65% higher in mice immunised with Lp 1. Among the titres of IgG1 antibodies induced against the different palmitoylpeptides, mpp 2 is 2.6- and 2-fold more immunogenic than mpp 1 or mpp 4, respectively. The percentage of sera with detectable antibodies against these palmitoylpeptides varied from 66 to 100%. Thus, it appears that the simplified immune formulation (Lp 1) is more efficient than the same immune preparation with lipid A (Lp 3). and these results were reproducible in 3 experiments. Using the same controls as described in Ref. [13] (flow cytometry and Western Blot analysis) indicated that the Lp 1 formulation is sufficient to reproducibly elicit polyclonal anti-P170 antibodies.

The same palmitoylpeptides were used after ten months of storage at −20 °C, to prepare a new batch of Lp 1 formulation. The immune response elicited was similar, despite the fact that antibodies specific to mpp 2 were detected in only 40% of the animals. To evaluate the potential side-effects of Lp 1 immunisation, we compared the weight and the behaviour of the immunised mice, during the 18 months after the last immunisation. During this period, the mean weight of mice immunised by 3 differents batches of immune preparation showed intergroups variations of less than 3% (data not shown) and the animals did not present with any modifications in their behaviour (i.e. eating, drinking, alertness).

Organs that express P170 (spleen, liver, kidneys, adrenal glands, pancreas, ovary, heart, lung) originating from the mice treated with the different formulations were subjected to histopathological study. We found that no intra-organic lesion or lymphocyte/monocyte infiltration in the organs investigated. However, peritoneal granulomas, mainly in the pancreas, adrenals, spleen (in 80% of the mice), and rarely in the liver (20%), was detected in both Lp 1- and Lp 2-immunised animals (Fig. 2).

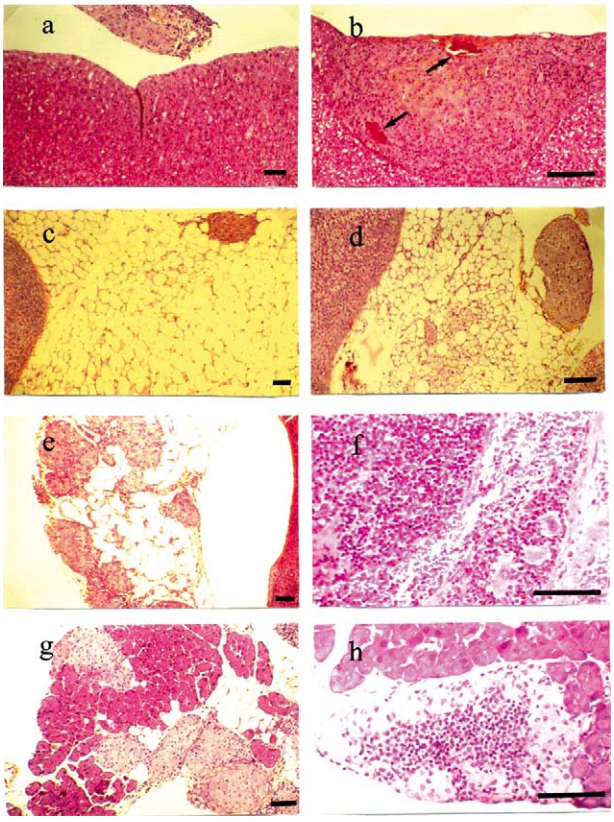

Fig. 2. Histological features of organs obtained from mice immunised by anti-P170 (a, c, e, g) or control immunisations (b, d, f, h). Haematoxylin-eosin-stained paraffin sections show: (a) a granulomatous lesion at the surface of the liver, (b) granulomas situated under the capsule of the liver with microcalcifications (arrows), (c, d) granulomas located in the adipose tissue surrounding adrenal gland, (e) granulomatous lesions in the perisplenic adipose tissue, (f) some isolated giant cells in the spleen, (g) numerous epithelioid cell granulomas present in the peripancreatic fat tissue, (h) detail of a peripancreatic granuloma. Scale bar, 100 μm. These are representative fields of organs with the most lesions and the same mice; other organs were normal.

3.2. In vivo *effect of the immune formulation on the effectiveness of chemotherapy against lymphoid neoplasms in mice*

Previously immunised mice were injected with P 388R cells and injections of doxorubicin and vinblastine were performed as described in the methods (Fig. 3). Before the injection, antibody titres in the sera of mice inoculated by Lp 1 were measured: 100%, 40% and 80% of the sera presented IgG1 anti-mpp 1, 2 and 4, respectively with average values of 0.30, 0.21 and 0.33 μg/ ml.

During treatment, the weight of the mice was measured. There was no significant weight loss in the treated mice. Mean survival time of mice immunised with Lp 1 was 39 days compared with 22 days for the control group (Fig. 3). In the Lp 2 group, we observed one mouse which survived after 70 days. From this observation, we can conclude that this animal is in cancer remission after chemotherapy treatment. Fig. 3 shows that the maximal effect of the immune formulation occurs 22 to 62 days after the injection of the P 388R cells. Haematocrit and haemoglobin concentrations were unchanged in the immunised animals compared with the control mice (data not shown).

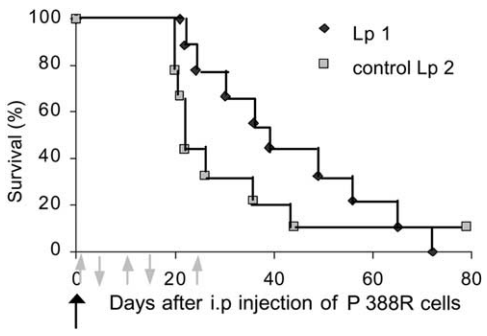

Fig. 3. Survival time of mice immunised by Lp 1 (◆) and Lp 2 (■). At day zero, 10^6 P 388R cells (↑) were inoculated intraperitoneally (i.p.). At day 1, 10, 22, doxorubicin 5.5mg/kg (↑) and on day 4 and 14, vinblastine 2.5mg/kg (↓) were injected.

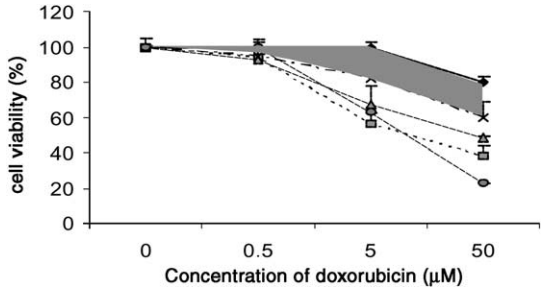

Fig. 4. Cytotoxicity induced by doxorubicin on P 388R cells in the presence of reversal agents: Control (–◆–); VRP: verapamil 3 μM (–●–); Average of 5 sera from Lp 1-immunised mice (IS Lp 1) at different concentrations (–■–: IS Lp 1 0.6%; –▲–: IS Lp 1 0.3%; –x–: IS Lp 1 0.15%). The shaded area corresponds to the variation in cell survival following incubations with IS Lp 2 (control liposomes) at concentrations of 0.15, 0.3 and 0.6%. *Points*, mean of triplicate determinations.

3.3. In vitro *anti-MDR activity of the Lp 1-elicited antibodies*

The cytotoxicity induced by doxorubicin in the P 388R cells was evaluated in the presence of verapamil and sera from Lp 1-immunised mice. In order to characterise the anti-MDR activity of the elicited antibodies, the effect of concentrations of anti-P-170 sera on the drug resistance of P 388R cells was analysed. The most efficient serum concentration was 0.6%, with an inhibitory activity similar to 3 μM verapamil (Fig. 4). After conducting cytotoxicity experiments with 50 μM vinblastine, we observed that incubation with sera of Lp 1-immunised mice at a concentration of 1.2% induced an inhibition of resistance twice that of 3 μM verapamil (Fig. 5).

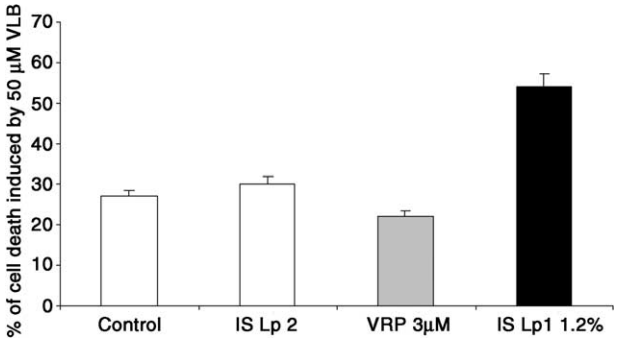

Fig. 5. Percentage of cell death induced by vinblastine (VLB) (50 μM) on P 388R cells in the presence of reversal agents: VRP 3 μM; sera (IS) of Lp 1-immunised mice 1.2% (average of triplicate measurements on 5 different sera).

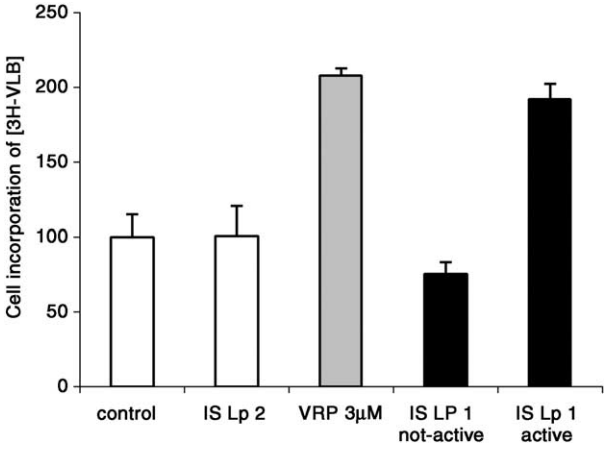

Fig. 6. Uptake of radiolabelled vinblastine [^{3}H-VLB] by P 388R cells incubated in the presence of 3 μM VRP or sera of mice immunised by Lp 1 or Lp 2. From eight sera of Lp 1-immunised mice, two groups were determined IS Lp 1 active (6 sera) and not-active (2 sera).

To extend these results, the uptake of radiolabelled vinblastine [^{3}H-VLB] by P 388R cells incubated in presence of verapamil and sera of mice immunised with Lp 1 or Lp 2 was assayed (Fig. 6). From 8 sera immunised with Lp 1 that were tested, 6 had an inhibitory effect on the VLB efflux that was similar to that observed with 3 μM verapamil.

4. Discussion

Reversal of MDR by pharmacological substances, e.g. calcium channel blockers, calmodulin inhibitors, local anaesthetics has been attempted [23]. However, clinical studies have shown that MDR modulators often have intolerably high toxic side-effects in humans and that therapeutic concentrations of MDR-modifiers can rarely be achieved in clinical practice [24–27]. A large number of multidrug-resistant cells overexpressing P170 have been described, their specific drug resistance profiles being quite heterogeneous [28]. In order to overcome MDR, antibody-directed approaches for the eradication of MDR cells have been developed. Data presented here clearly show the first demonstration of the effectiveness of an anti-P170 immunisation strategy *in vivo*. This fits with our previous data showing that palmitoylpeptides mimicking the external loops of the murine *mdr1* P170 reconstituted in liposomes containing lipid A elicited a strong immune response in mice and that sera from these mice were able to inhibit P170 activity in L1210 (MDR) cells *in vitro* [13].

For the anti-P170 immunisation, palmitoylpeptides with two palmitoyl chains at the amino- and carboxy-terminus of the peptide have been reconstituted in the liposome bilayer allowing a "loop" presentation of the antigen. This form of presentation of a β-amyloid fragment has also been used in a vaccine preventing amyloid plaque formation in young mice and plaque progression in older animals [14] confirming the capacity of inducing auto-antibodies to such self-peptides. Palmitoylpeptides, resuspended either in PBS or in PBS-Alum or reconstituted in liposomes without Alum, did not induce any auto-immune lesions in the kidney, liver, lung, adrenals and pancreas, up to 18 months after immunisation. It appears that the formulation liposomes-palmitoylpeptides-Alum is the most efficient and simplest to enhance the immunogenicity of our palmitoylpeptides. The profile of the immune response to the murine mpp 1, mpp 2, mpp 4 was similar to that observed with antigens like keyhole limpet haemocyanin or sheep erythrocytes, which presented a maximal IgG1 immune response 38 days after the first immunisation [29]. Lipid A had been used to enhance the immune response against peptides incorporated in the internal volume of liposomes [15] or to induce antibodies against small molecules such as cholesterol [30]. In our experiments, the immune preparation without lipid A elicited a higher antibody response and for future therapeutic development avoiding bacterial lipopolysaccharide (LPS) would be beneficial to the host. Alum has been described as an efficient adjuvant of the humoral immune response and is currently used in several human diphteria and tetanus vaccines [31]. Interestingly, enhancement of the cellular immune response has been described in vaccines using foreign linear peptides anchored by di- or tri-palmitoyl residues [32–34].

The anti-MDR antibodies induced a reduction in the resistance of P 388R cells to doxorubicin and vinblastine *in vitro*. For doxorubicin, concentrations of IgG1 anti-mpp 1, 2 and 4 as low as 4 ng/ml led to the same results as 3 μM verapamil. In clinical studies, the medium peak serum concentration of verapamil can not exceed 2.2 μM [35]. For vinblastine, a similar potent effect for the antibodies was observed, both in cytotoxicity and uptake of radioactive studies. When 3 μM verapamil was used as a control revertant, its activity was decreased in presence of 50 μM vinblastine, whereas uptake experiments confirmed an efficient reversal of resistance.

The *in vivo* experiments showed a 77% increase in the survival time of the immunised mice. Other authors [36], using a chemical model, obtained an increase in survival half time of only 49% with S9788 (6-[4-[2, 2-di (4-fluorophenyl)-ethylamino]-1-piperidinyl]-N, N′-di-2-propenyl-1, 3, 5-triazine-2, 4-diamine) (100 mg/kg/day). While no auto-immune symptoms were detected, our histopathological results showed peritoneal granulomas (principally in the vicinity of the pancreas, spleen and liver) in mice immunised both with control and anti-P170 immune preparations. Studies in our laboratory have shown that 3 i.p. injections of Alum alone can result in the development of similar lesions (data not shown). While we can not explain completely the lack of auto-immune lesions in tissues where P170 is abundantly expressed, it must be noted that in organs with secretory cells, P170 is present mainly at the luminal face of the cells, thus significantly reducing its accessibility to circulating anti-P170 antibodies [37,38]. The absence of LPS in the immune formulation may reduce the risk for inducing other auto-antibodies.

In other studies, passive immunisation with the monoclonal antibodies, MRK16 and UIC2, led to an increase in the uptake of anticancer drugs in MDR cells [9,39], and according to previous reports, inhibition of P170 activity might improve the prognosis of cancer patients [40,41]. The concept of eliciting therapeutic "auto-antibodies" by immunisation with synthetic lipopeptides reconstituted in liposomes might extended to other cell surface proteins such as the MRP glycoprotein [42] or tumour-associated antigens such as c-erbB-2 [43] provided that no auto-immune disease results from this therapy. From the results described herein, we

expect that breaking the immune tolerance to specific self-proteins such as MDR1, β amyloid [14] or MRP could lead to effective treatments against defined diseases such as chemoresistant cancers or Alzheimers.

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