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Promotion of tumor antigenicity in EL-4 leukemia cells by hydrostatic pressure

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Summary. Subjection of EL-4-leukemia cells to hydrostatic pressure of 1200–1500 atm for 15 min increased their weak basal immunogenicity to a potent practical level. Injection of such pressure-treated and irradiated EL-4 cells into syngeneic naive C57Bl/6 mice significantly delayed tumor development and increased survival after subsequent challenge with untreated EL-4 cells. Application of pressure of 1500 atm for a longer period of time (e.g., 120 min) resulted in cell death and a smaller increase in tumor immunogenicity which could be partially accounted for by passive shedding of membrane material. Unlike previously studied tumor cells, incorporation of cholesteryl hemisuccinate (CHS) into the plasma membrane of EL-4 cells increased their apparent tumor immunogenicity only slightly. In addition, isolated EL-4 plasma membranes, untreated, CHS-treated or pressure-treated, as well as the material shed thereof by hydrostatic pressure, were all of weak immunogenicity.

Modulation in the projection of surface antigens upon pressure treatment could account for the observed increase in tumor immunogenicity and was monitored via the Thy 1.2 antigen. Fluorescence cell sorting anylsis indicated that upon application of 1500 atm for different periods of time the projection of Thy 1.2 progressively and irreversibly increased to a maximal level of about 140% at 15 min. At longer pressurizations the availability of Thy 1.2 to antibody binding decreased sharply to levels below that of the untreated cells. It is suggested that pressure promotion of tumor immunogenicity is induced by changes in projection and surface distribution of the relevant antigens.

Introduction

Decrease in lipid fluidity of cell membranes may induce vertical displacement [1, 6] and lateral association [2, 33] of integral membrane proteins, which for membrane antigens may evoke a marked increase in immunogenic potential [23, 25]. This trend has been employed to augment the expression of tumor antigens aiming at preparations that could be used as specific tumor vaccines for immunotherapy of cancer [25, 27, 29]. In a series of in vivo studies, tumor cells treated with cholesteryl hemisuccinate (CHS), a potent nontoxic lipid rigidifier [34], were used for preimmunization [25, 26, 30] or postimmunization [28, 30, 31] of syngeneic animals bearing the same tumor, as well as for delayed hypersensitivity reaction in human patients with solid tumors [27, 29]. In most animal and human tumors, a marked increase in tumor immunogenicity upon incorporation of CHS was observed [20, 24–31]. Yet, in other tumors, both in animals [5, 30] and humans [26, 27, 29], the effect of CHS incorporation on tumor immunogenicity was small or insignificant. This not unexpected variability in potentiation of tumor immunogenicity by CHS led us to search for an alternative method for passive increase in immunogenicity which may be applied to tumor cells that fail to respond to CHS treatment.

In this study we have used hydrostatic pressure to passively modulate the organization of membrane proteins [12, 20]. The tumor studied was the chemically induced T-leukemia EL-4 which is inherently weakly immunogenic [9, 17]. As shown here, a pressure of defined magnitude and duration significantly augmented the apparent immunogenicity of EL-4 cells presumably through irreversible vertical and lateral alteration in the organization of the tumor associated antigens (TAA).

Materials and methods

Cells. El-4 tumor, a chemically induced T-leukemia in C57Bl/6 mice [7], was maintained in ascites form in the peritoneal cavity of 6 to 8-week-old male C57Bl/6 mice. A total of 10^4 cells was inoculated i. p. and the cells ($\sim 5 \times 10^8$ per animal) were harvested 6–8 days later. The melanoma line B_{16}[4] and the Rad LV induced T-leukemia 11:11 [8] were maintained i. m. and s.c. respectively, in C57Bl/6 mice.

Treatment of cell membranes. Rigidification of the cell membrane lipid layer was carried out by incorporation of CHS as previously described [27, 34]. Briefly, cells were incubated in serum-free medium consisting of 3.5% (w/v) polyvinylpyrrolidone (PVP, MW 40000 Sigma Chemical Co., St. Louis, Mo.), 1% bovine serum albumin (Grand Island Biological Co, NY) and 0.5% glucose in phosphate buffered saline (PBS). CHS 5 mg/ml in ethanol was diluted 1:100 (v/v) in the PVP medium with vigorous mixing. In the control mixture only 1% ethanol was introduced. Cells (3×10^6/ml medium) were incubated in this medium at room temperature with gentle shaking for up to 4 h [34].

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The cells were then washed three times with PBS and resuspended in PBS to a concentration of 10^7 cells/ml. Viability of the treated cells was above 95% as assessed by trypan blue exclusion. The effectiveness of CHS treatment in increasing the cell membrane rigidity was assessed by fluorescence polarization of the lipid fluidity probe 1,6-diphenyl 1,3,5-hexatriene (DPH), as described elsewhere [22].

For application of hydrostatic pressure, cells were dispersed in PBS at a concentration of 10^8 cells/ml in a capped Eppendorf plastic tube (~1.5 ml) filled to the top. A short 19-gauge needle inserted through the cap served as a vent for pressure equalization. Both the tube and the needle were filled with PBS without air bubbles to avoid cell rupture. Tubes were placed in a 40 ml pressure bomb (Aminco) filled with PBS and sealed. Pressure was applied gradually to reach after 7–8 min the maximum of 1500 at and after different times (up to 120 min) slowly released (~7–8 min). The pressurized cells were centrifuged (5 min at 1000 *g*) and both the supernatant and the cells were collected.

Isolation of cell plasma membranes. The recently published method of Maeda et al [10] was used essentially as described.

Immunization. CHS or pressure-treated EL-4 cells were irradiated (10000 rads) and then injected i.p. into groups of 10 C57Bl/6 male mice. The immunization dose consisted of 10^7 cells (irrespective of their viability before irradiation) in 1 ml PBS or the supernatant obtained from 10^8 cells. Two identical immunizations were given 8 days apart. Analogous immunization regimens were used with treated or untreated isolated membranes. Challenge with 10^4 untreated EL-4 cells/animal was given 14 days after the second immunization and animal survival was scored.

Presentation of Thy 1.2 antigens. The number of detectable Thy 1.2 antigens on the surface of EL-4 cells was assessed with a fluorescence-activated cell sorter (Becton Dickinson, FACS II Sunnyvale, CA) after indirect staining with fluorescent antibodies. Following CHS or pressure treatment, cells (6×10^6) were washed and incubated with 200 µl of 40 µg/ml monoclonal antibody to Thy 1.2 (Bio-Yeda Ltd.) for 30 min at 4 °C in PBS containing 0.01% NaN_3. The cells were then washed and stained with 1/20 dilution of fluorescein-labeled rabbit anti-mouse IgG (Bio-Yeda Ltd. Rehovot, Israel) as above. Thereafter, the fluorescien-labeled cells were washed twice with PBS and dispersed in PBS containing 2% formaldehyde for 30 min at 4 °C. The cells were then washed again with PBS and dispersed at a concentration of 3×10^6 in PBS. The distribution profile of fluorescence intensity was recorded in 10^4 cells with the cell sorter and served to evaluate the degree of accessibility of the Thy 1.2 antigens.

Results

Preimmunization of C57Bl/6 mice against subsequent challenge with untreated EL-4 cells was carried out with a series of preparations. Cell viability and membrane fluidity were determined before in vivo application.

1. Cell viability and membrane fluidity of treated EL-4 cells

Incorporation of CHS (50 µg/ml in PVP medium) into EL-4 plasma membranes was monitored by the increase in the degree of fluorescence polarization (P) of DPH [22]. CHS incorporation leveled off at 120 min (data not shown) as previously obtained with other cells [34]. As shown in Table 1, cell viability was practically unaffected by this treatment and remained above 95%. After application of hydrostatic pressure (1200 atm, 15 min) the cell viability was largely preserved (above 80%, see Table 1), however, application of higher pressure (1500 atm, 15 min) resulted in up to 80% cell death. Yet, the total number of cells, as well as their apparent morphology, was not significantly affected. At longer periods of pressurization (1500 atm, 90 min) cell death reached 100% with a significant decrease in cell volume but only a small reduction in cell number. Membrane fluiditiy of both whole cells and membrane preparations returned to its original values after pressure application (Table 1). Furthermore, the membrane rigidification induced by CHS incorporation remained unaltered after application of hydrostatic pressure.

2. Immunization and tumor challenge

Immediately after each treatment cell number and viability were measured and the cells were then irradiated. Since no

Table 1. Membrane fluidity and cell viability of treated EL-4 cells[a])

Treatment	$P(\pm 0.005)$	$2P(0.46\text{-}P)$	Cell viability
Untreated cells	0.259	2.57	>95%
CHS-treated cells (15 min)	0.268	2.79	>95%
CHS-treated cells (120 min)	0.285	3.25	>95%
Pressurized cells (15 min, 1200 atm)	0.259	2.57	>80%
Pressurized cells (15 min, 1500 atm)	0.259	2.57	20%–40%
Pressurized cells (90 min, 1500 atm)	0.257	2.53	0%
CHS-treated (15 min) and then pressurized (15 min, 1500 atm) cells	0.269	2.81	20%–40%
Plasma membranes			
a. untreated	0.330	5.07	
b. CHS-treated (60 min)	0.344	5.93	
c. pressurized (15 min, 1500 atm)	0.329	5.02	

[a] Membrane fluidity was determined at 25°C by the degree of fluorescence polarization of DPH, P, presented as mean ± SD of triplicate and assessed by the approximate linear scale of rigidity 2P/(0.46-P)

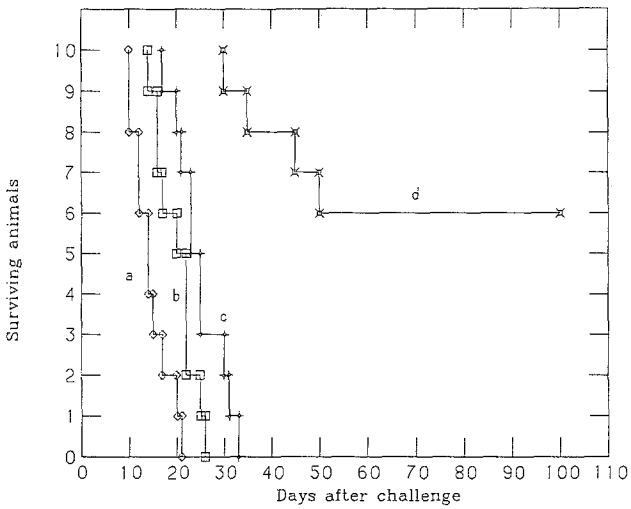

Fig. 1. Survival of C57Bl/6 mice after i.p. implantation of 10^4 EL-4 cells. Mice were twice preimmunized with 10^7 untreated cells (b), 10^7, CHS-treated cells (c) or 10^7 pressure-treated (1200 atm, 15 min) cells (d). Control experiment using animals which were not preimmunized is also shown (a)

significant loss in total cell number was observed in any of the treatments, the immunization dose of 10^7 treated-irradiated cells was according to their initial number without compensation for loss of viability.

Screening of immunization potential of cells treated with CHS and pressure, against subsequent challenge with intact EL-4 cells (see *Materials and methods*) is shown in Fig. 1. As indicated, preimmunization with untreated (b) or CHS-treated (c) cells slightly increased the survival after challenge as compared with the untreated group (a). Yet, all animals in these groups died within 35 days. Preimmunization with pressure-treated cells (1200 atm, 15 min) delayed tumor development and prolonged survival significantly (d). In this group death from tumor started only when animals of the other test groups died, while over half of them survived 100 days, which may be considered as reaching a state of remission [25].

In a large series of experiments, different preparations, which are listed in Table 2, were tested for their immunization capacity against subsequent challenge with EL-4 cells. The results are summarized in Table 2. Preimmunization with CHS-treated cells slightly prolonged the survival of the treated animals. At day 30 after challenge, 16% of them survived but none survived beyond 60 days. A marked increase in survival was achieved by preimmunizations with pressure-treated cells. Optimal results were obtained by treating the cells with 1200 atm for 15 min. After the subsequent challenge, 50% of the animals survived over 100 days. Cells treated with 1500 atm for 15 min, under which conditions viability is lost (Table 1) but morphology is retained, still induced a good protection against tumor development, resulting in 30% survival at day 100 after challenge. CHS treatment followed by pressure application did not improve the immunization capacity (Table 2). Prolonged pressurization (1500 atm, 120 min) almost completely abrogated the immunization capacity of the cells. This could be attributed to passive shedding of membrane fragments [12, 30]. However, the supernatants of the prolonged pressurized samples did not display any significant protection against tumor development. Unexpectedly, isolated plasma membranes, which were pressurized (1500 atm, 15 min) did not display the protection obtained with similarly treated whole cells.

Table 2. Survival preimmunized C57Bl/6 mice after i.p. challenge with 10^4 EL-4 cells

Pretreament	Number of experiments	Survival[a]					
		After 30 days		After 60 days		After 100 days	
No treatment	4	0/10 0/10 0/10 0/10	(0%)				
Untreated cells	4	0/10 0/10 0/10 0/10	(0%)				
CHS-treated cells	3	1/10 1/10 2/10	(16%)				
Pressurized cells (1200 atm, 15 min)	4	7/10 9/10 8/10 8/10	(80%)	4/10 8/10 5/10 5/10	(55%)	6/10 4/10 5/10 5/10	(50%)
Pressurized cells (1500 atm, 15 min)	6	7/10 8/10 7/10 9/10 7/10 7/10	(75%)	4/10 6/10 4/10 5/10 5/10 5/10	(48%)	4/10 3/10 3/10 2/10 3/10 4/10	(30%)
CHS-treated (120 min) and then pressurized cells (1500 atm, 15 min)	3	7/10 9/10 6/10	(73%)	4/10 5/10 5/10	(46%)	3/10 2/10 4/10	(30%)
Pressurized cells (1500 atm, 120 min)	3	1/10 0/10 2/10	(10%)				
Supernatant of pressurized cells	2	0/10 0/10	(0%)				
Plasma membranes – untreated – CHS-enriched – pressurized (1500 atm, 15 min)	 1 1 1	 0/10 0/10 0/10	 (0%) (0%) (0%)				

[a] Each experimental group included 10 animals. Mean number of surviving animals is indicated and the average percent survival is given in parenthesis

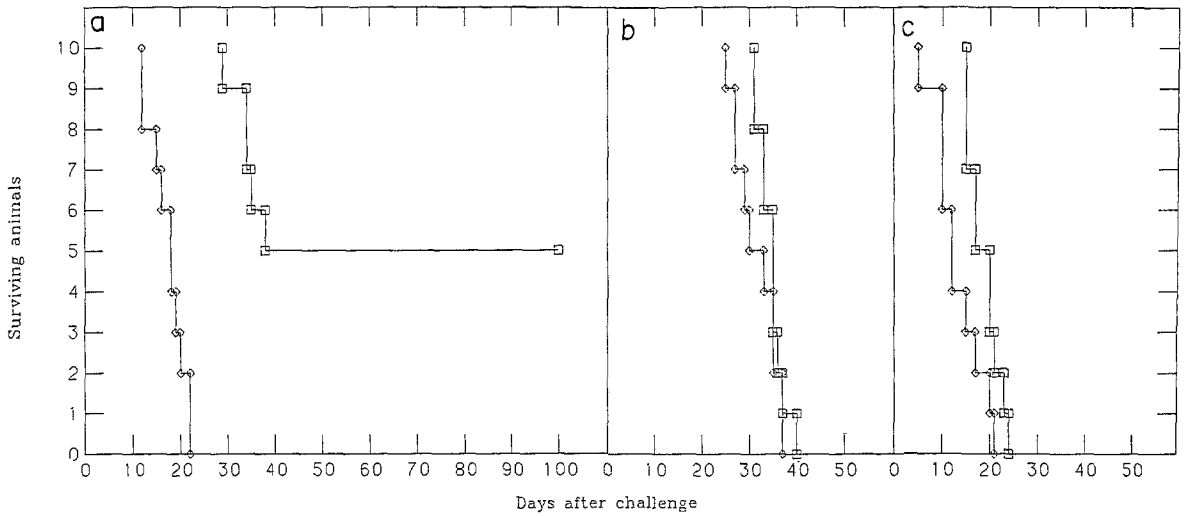

Fig. 2. Specificity of immunization induced by pressure-treated EL-4 cells. Mice were preimmunized with pressure-treated EL-4 cells and then challenged with either untreated 10^4 EL-4 (a, □———□), 10^5 11:11 T-Leukemia (b, □———□) or 5×10^4 B16 melanoma (c, □——□) cells. Control experiments with untreated animals are also shown (◇———◇)

3. Specificity of the acquired immunity

Preimmunization of C57Bl/6 mice with pressurized (1200 atm, 15 min) EL-4 cells was followed by challenge with either EL-4, 11:11 Rad LV-induced T-leukemia or B_{16} melanoma cells. As shown in Fig. 2, the protection conferred by EL-4 pressurized cells appeared to be specific for the parental tumor cell line (Fig. 2a). The pressurized EL-4 cells did not protect tumor development in mice bearing 11:11 leukemia (Fig. 2b) and B_{16} melanoma (Fig. 2c).

4. Modulation of antigen projection

The observed increase in immunogenicity of the EL-4 cells after pressurization presumably stems from vertical or lateral rearrangement of their putative TAA. Since no antibodies or other means for monitoring these antigens are yet available, we chose Thy 1.2 as an antigenic marker for the possible changes in antigenic distribution and projection which may ensue from treatment of the cell surface membrane with pressure or CHS. EL-4 cells subjected to hydrostatic pressure of 1500 atm for different periods of time displayed after 5 and 15 min, a gradual increase in availability of Thy 1.2 followed by a sharp decrease in projection to levels lower than that of untreated cells (Fig. 3). Maximal exposure of Thy 1.2 was obtained after 15 min pressurization which amounted to about 40% increase over the level observed in control cells. At long pressurization times (e.g., 90 min) both the level of Thy 1.2 and light scattering of EL-4 cells were reduced considerably (Fig. 3). The availability of Thy 1.2 to antibody binding was also monitored on EL-4 cells treated with 50 μg/ml CHS for different periods of time. In these experiments, a gradual but smaller increase of up to 15% in exposure of Thy 1.2 was also observed, but this was followed by a sharp decrease upon further incorporation of CHS (data not shown). Similar results have previously been reported for the changes in Thy 1.2 exposure on mouse thymocytes upon incorporation of cholesterol [13].

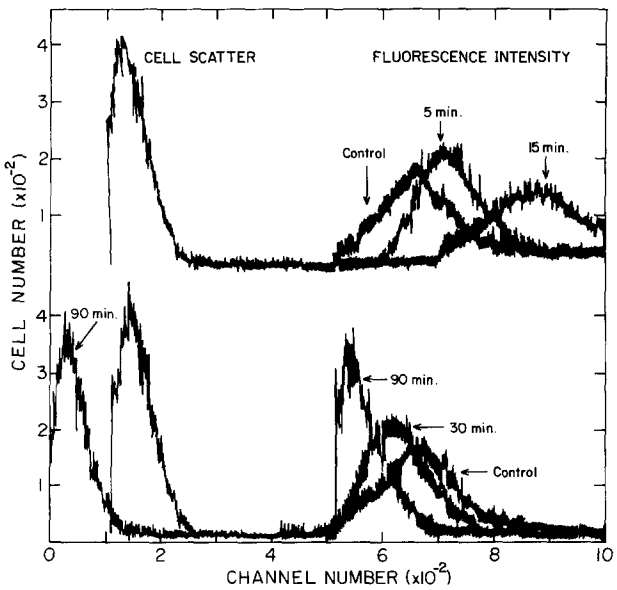

Fig. 3. Fluorescence-activated cell sorting profiles of Thy 1.2 antigens on EL-4 cells subjected to hydrostatic pressure of 1500 atm for different periods of time. After pressure treatment, cells were first labeled with anti Thy 1.2 followed by fluorescein-labeled rabbit anti-mouse IgG. Each profile was obtained with 10^4 fluorescein-labeled cells

Discussion

The mere presence of a neoantigen on the surface of an autologous cell is not always sufficient to evoke the expected immune response. The additional factors that may contribute to the immunogenic potency of a membrane antigen are proper projection, fruitful collaboration with components of the major histocompatibility complex and self-association to form immunogenic units [18, 35]. Physical or chemical manipulations of the cell membrane lipid layer that mediate structural reorganization of the integral membrane proteins, may thus modulate the immunogenic expression of cell surface antigens. This approach is especially pertinent to tumor cells of weak immunogenicity where the majority of the TAA are presumably in a cryptic position [19, 20, 24].

Rigidification of the membrane lipid layer can be effectively accomplished by incorporation of CHS [34]. Such treatment has been found to modulate the exposure and activity of membrane receptors [21], transport proteins [34] enzymes [14, 16], and antigens [13, 23], in accord with the vertical displacement hypothesis [1, 6]. Most of the tested animal and human tumor cells were found to become considerably more immunogenic after CHS enrichment, presumably due to increase in exposure of TAA to the immune system. Yet, in some other tumor cells [5, 30], incorporation of CHS did not increase the apparent immunogenicity. A few human tumors (e.g., ovarian carcinoma) have also failed to exhibit increase in immunogenicity upon incorporation of CHS (unpublished results). The following study was therefore undertaken to test an alternative method for augmentation of the antigenicity of cell surfaces which in tumor cells will induce a substantial increase in tumor immunogenicity. The extensively studied EL-4 tumor, a chemically induced T-leukemia [7], is of low apparent immunogenicity which is not significantly affected by CHS incorporation (Table 2) and could therefore serve as a model system for this study.

As clearly indicated, subjection of EL-4 cells to hydrostatic pressure at a defined magnitude augmented their immunogenic capacity as demonstrated by vaccination against subsequent challenge with viable EL-4 cells (Fig. 1 and Table 2). Optimal increase in immunogenicity was obtained by application of 1200 atm for 15 min (Table 2) on the intact EL-4 cells. Immunization with such pressure-treated EL-4 cells did not render any protection against unrelated syngeneic tumor cells (11 : 11 Rad LV T-leukemia and B_{16} melanoma) which indicates the specificity of the induced immunization (Fig. 2).

Hydrostatic pressure provides a unique tool for manipulating membrane structure. As a physical parameter which operates selectively on compressible compartments, it rigidifies fluid lipid domains [3] and dissociates quaternary assemblies of proteins [32], most prominently the cytoskeletal polymers [15], in a reversible manner. Under hydrostatic pressures of 1000–1500 atm the cell membrane lipid layer becomes considerably more rigid, reaching a microviscosity value similar to that of CHS-treated membranes [12]. In parallel, the cytoskeletal elements completely disintegrate to monomeric or small oligomeric units [15] and the restrictions for long range lateral diffusion of integral membrane proteins are, by and large, removed. Therefore it may be expected that under such conditions membrane proteins with some affinity to each other could associate due, on the one hand, to the elimination of diffusion barriers and, on the other hand, to the decrease in lipid fluidity which supports protein-protein interaction [2, 33]. In addition, membrane proteins are vertically displaced [1, 6] which under extreme conditions, e.g., prolonged imposition of high pressure or combination of cholesterol enrichment and hydrostatic pressure, may be shed off into the external medium [12, 21]. Below the shed off threshold (e.g., short application of hydrostatic pressure) the subsequent decompression leads to virtually complete restoration of both the lipid fluidity (Table 1) and the cytoskeletal assemblies [15, 32]. Yet, a substantial part of the pressure-induced protein associates may remain undissociated after release of pressure due to the formation of stable complexes which could have been formed only under conditions of unrestricted lateral diffusion. On the whole, the reshuffling of membrane proteins by pressure is expected to induce both vertical and lateral changes in the antigenic network. The results obtained for the irreversible changes on surface distribution of Thy 1.2 (Fig. 3) comply with the vertical alteration in antigenic projection. In addition, we have recently observed significant surface rearrangement of Thy 1.2 and H_2 antigens upon pressure application. The surface distribution of both of these antigens patches after pressurization (to be published).

The above arguments lead to the possibility that as long as the morphology of pressurized cells is preserved, stable and well exposed aggregates of surface proteins (e.g., antigens) could be formed. Such aggregates may possess a special immunogenic potency which is deficient in the natural distribution pattern of membrane proteins in the intact cell. Accordingly, the weak immunogenic potency of pressurized isolated membranes or cells destroyed by overpressurization (Table 2) could be partially due to the lack of such putative immunogenic assemblies.

The use of hydrostatic pressure for augmentation of tumor immunogenicity could be of practical potential. We are currently evaluating this new approach in postvaccination studies of animal tumors, as well as in immunotherapy of human cancer. In addition, our technique, as described in this paper, is currently being applied for preparation of vaccines against experimental autoimmune diseases (Lider et al. in preparation).

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