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IMMUNOLOGICAL TARGETING OF MATRIX METALLOPROTEINASE-9 AS A NOVEL APPROACH TO UNIVERSAL CANCER VACCINE: CHARACTERIZATION OF ANTI-CANCER ANTIBODIES EFFICACY IN VITRO

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Abstract – *Objective:* *Therapeutic approach to cancer based on a vaccine targeting various types of cancer compared to being cancer-type specific has many advantages and can be applied to large population. Our previous* in vivo *studies demonstrated that a vaccine aimed at curbing cancer metastasis by inhibiting matrix metalloproteinases (MMPs) – Matrix Metalloproteinase -2 (MMP-2) and Matrix Metalloproteinase -9 (MMP-9) - has potential in preventing cancer. This study investigated mechanical aspects of anti-MMP-9 antibodies with previously documented anti-cancer efficacy in vivo, for inhibition of cancer cell migration and their binding efficacy.*

Materials and Methods: *The antibodies against mouse and rat MMP-9 oligopeptides were isolated and their effects on cancer cell invasion through Matrigel were tested individually and in combinations. Binding to the corresponding MMP sequences was evaluated by Western blot.*

Results: *All tested antibodies inhibited migration of both murine and human cancer cell lines: Human Prostate Cancer (DU145), Mouse Breast cancer (4T1) and Human Pancreas Carcinoma (MIA PaCa-2). The combination of these antibodies had enhanced effect on inhibiting cancer cells invasion. Antibody binding to MMP-9 was shown for all but one of the tested antibodies by Western Blot.*

Conclusions: *Controlling matrix metalloproteinases (MMPs) is a powerful tool against metastasis - the most life-threatening aspect in all cancers. The in vitro efficacy of specific antibodies effective against MMP-9 was elucidated in this study. We showed that specific anti-MMP-9 antibodies inhibit extracellular matrix invasion of different types of cancer cells and confirmed their binding to corresponding MMP-9 peptides. Enhanced efficacy of these immunogenic peptides was observed when used in a combination. These results urge further work in this direction with its goal of developing a universal anti-cancer vaccine.*

KEYWORDS: *Cancer vaccine, MMP-9, Immunogenic oligopeptides, Tumor growth.*

INTRODUCTION

Immunotherapies in cancer are often referred to as "Therapeutic cancer vaccines". Many such therapies based on monoclonal antibodies and immune targeting drugs are currently being developed to treat already diagnosed cancers as opposed to prevent cancer from occurring.

Most approaches to cancer vaccines target various cancer specific antigens (known as Tumor Associated antigens or TAA) to activate or enhance the body's own cytotoxic T cell responses against cancer cells [1]. While immune stimulation is seen by such an approach, overall clinical response in terms of patient survival has been limited[2]. One drawback with this approach is that such cancer vaccines must be developed individually against specific tumor antigens. This greatly increases the costs of development and vaccine efficacy is uncertain since cancer cells can also evade T cell responses[3].

We implemented a different approach to cancer vaccines by targeting the universal mechanism that all cancer cells use in order to grow, invade their surroundings and metastasize, which is enzymatic

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digestion of extracellular matrix. The enzymes, associated within this process and most related to malignancy are zinc-metalloproteinases (MMPs), of which MMP-2 and MMP-9 have been associated with the progression and aggressiveness of cancer[4-6]. Our earlier studies indicated that the immunization of mice with specific oligopeptides containing MMP-9 and MMP-2 sequences from humans resulted in a significant inhibition of melanoma tumor growth and was not associated with significant peptide immune-related toxicity[7]. In addition, we also demonstrated significant inhibition of melanoma tumors in mice immunized with MMP-9 oligopeptides containing rat and mice MMP-9 sequences - a syngeneic approach[7].

In the present study, we investigated the mechanistic aspects of antibodies raised against murine MMP-9 oligopeptides and whether tumor-growth inhibition observed in our *in vivo* studies could be associated with curbing cancer cell invading ability. Therefore, we raised polyclonal antibodies against MMP-9 in rabbits by immunizing them with three oligopeptides from rat MMP-9 and two MMP-9 oligopeptides from mice and subsequently used these purified antibodies in conducting *in vitro* testing of their efficacy in inhibiting Matrigel invasion of different cancer cell lines. In addition, we tested the antibodies binding to the corresponding MMPs sequences by Western blot.

MATERIALS AND METHODS

Reagents

All reagents were of analytical grade. All solutions were prepared using pyrogen free MilliQ grade water.

Solutions of peptides were prepared into 3 ml glass vials ISO 8362-1 2R-CL-1 (Medical Glass, Bratislava, Slovakia) or PP Costar Microcentrifuge Tube (Catalog No. 3621, Corning Inc., Corning, NY, USA) depending on solution volume.

Incomplete Freund's Adjuvant (Catalog No. F5506, Sigma-Aldrich, St. Louis, MO, USA), 2 ml syringe 22Gx1$^{1}/_{2}$"(BKMI, R. Korea), Vortex Vibrofix VF1 (IKA-Werk, Germany), GP Centrifuge (Beckman, Indianapolis, IN, USA), pH/ORP Meter HI 2211 (Hanna Instruments, Smithfield, RI, USA), spectrophotometer Unico 2800 (United Products & Instruments, Inc., Dayton, NJ, USA).

Reagents for enzyme-linked immunosorbent assay (ELISA) assay: sample diluent (10 mM sodium phosphate, 500 mM NaCl, 0.5 % Bovine Serum Albumin (BSA), 0.05 % Tween-20, pH 7.4), conjugate of sheep antibody to rabbit IgG with horseradish peroxidase (HRPO), conjugate diluent (10 mM sodium phosphate, 150 mM NaCl, 0.5% Bovine Serum Albumin (BSA), 0.05% Tween-20, pH 7.4), wash fluid (10 mM sodium phosphate, 300 mM NaCl, 0.05% Tween-20, pH 7.4), substrate buffer (50 mM sodium citrate and hydrogen peroxide, pH 5.0), TMB solution (3,3',5,5'-tetramethylbenzidine), stop solution (2 M sulphuric acid) were homemade. Streptavidin from Streptomyces avidinii (MP Biomedicals LLC, Irvine, CA, USA). Plate washer ZLE201 (Amersham Inc., Amersham, UK), incubator at 37°C - Imperial II (Lab-Line Instruments Inc. Thermo Scientific, Waltham, MA, USA), ELISA plate reader - Luminometer-Photometer LM01A (Immunotech, Beckman Coulter Company, Indianapolis, IN, USA). MMP inhibitor GM6001 was obtained from Sigma-Aldrich (St. Louis, MO, USA). Affinity sorbent columns XK 16/20 were from GE Healthcare (Catalog No. 18-8773-01. Chicago, IL, USA), peristaltic pump P-1 from GE Healthcare (Catalog No. 18-1110-91. Chicago, IL, USA), and SuperFrac Fraction Collector was obtained from GE Healthcare (Catalog No. 18-1010-19. Chicago, IL, USA).

Test Peptides

Five peptides mimicking mice and rat MMP-9 sequences were synthesized at GenScript (Piscataway, NJ, USA). Each peptide was synthesized in two forms, one covalently conjugated to keyhole limpet hemocyanin (KLH) protein for immunization, the other conjugated to biotin for IgG ELISA. The both forms of peptides were dissolved in Phosphate Buffered Saline (PBS) at concentration 1.0 mg/ml and aliquots stored at -20°C until experimentation.

Peptide abbreviations and sequences are presented in Table I.

Preparation of peptide emulsion for immunization

On the day 147 of re-boosting immunization, 0.5 mL of the solution peptide covalently conjugated to KLH was gradually added to 0.5 mL of Incomplete Freund's adjuvant (IFA) and simultaneously vortexed thoroughly to get a uniform emulsion.

TABLE 1. Test peptide sequences and codes used in the study.

Peptide abbreviation	Peptide Sequence
Rat: R#7	-D-T-D-R-K-Y-G-F
Rat: R#	12-H-F-P-F-T-F-E-G-R-S-Y-L-S-C
Rat: R#18	-D-K-A-D-G-F-C-P-T-R-A-D-V-T-V
Mouse: M#11	-D-K-D-G-K-F-G-F
Mouse: M#19	-D-Q-D-K-L-Y-G-F-C-P-T-R-V-D-A
N IgG	Nonspecific rabbit IgG

Immunization and bleeding procedures

On day 147, the animals were injected 1000 μL of the mixture individual test solution emulsified with Incomplete Freund's adjuvant (IFA) subcutaneously on 4 occasions. On day 157 and 164 blood samples were withdrawn from the marginal ear vein into PP 50 mL centrifuge tubes (Catalog No. 4558, Corning Inc. Corning, NY, USA).

Immune response assay

The immune response was measured in serum samples with an indirect ELISA with streptavidin on solid-phase. (Enzyme immoabsorbent) EIA plates were coated by adding to well 100 μl of streptavidin dissolved 5 mg/ml in 50 mM carbonate buffer, pH 9.5 and incubated for 20 h at 20°C. The plates were washed 4 times with wash fluid. Biotinylated peptides 10 mg/ml, 100 ml/well in sample diluent were incubated 60 min at 37°C. Control-wells with streptavidin. Subsequently the plates were washed 4 times with wash fluid. Serum from each rabbit was diluted 1:1000 and 1:10000 in sample diluent and added to the wells, coated with corresponding peptide (100 μl per well) and incubated for 1 h at 37°C. Control - wells with sample diluent. The plates were washed 4 times with wash fluid.

Conjugates of sheep anti-rabbit IgG antibody with HRPO (dilution of 1:3000 in conjugate diluent) were added to the wells (100 μl per well). The plates were incubated for 0.5 h at 37°C and washed 4 times as specified above. 100 μl of freshly prepared substrate solution (1 volume (v) 3,3',5,5'-tetramethylbenzidine [TMB] solution + 7 v substrate buffer) were added to each well, and the plates were left at room temperature for 15 minutes in dark place after which 100 μl of stop solution was added to each well in the same sequence as the addition of substrate solution. The absorbance at 450 nm was completed within 5 minutes.

Antibody generation

All experimental procedures in the study were approved by the Institutional Animal Ethics Committee (IAEC) of Russian Cardiocenter. Male Schinschilla rabbits of 9 - 10-week-old were procured from Nursery of Lab Animals (Stolbovaya, Russia) and were acclimatized for a period of two weeks prior to the study. Animals were maintained on a standard rodent diet *ad libitum* with free access to water under controlled conditions of temperature on a natural light-dark cycle.

Rabbits ## 1, 2, 3. immunized with Peptide Rat #7+KLH

Rabbits ## 4, 5, 6. immunized with Peptide Rat #12+KLH

Rabbits ## 8, 11, 12. immunized with Peptide Rat #18+KLH

Rabbits ## 13, 14, 15. immunized with Peptide Mouse #11+KLH

Rabbits ## 16, 17, 18. immunized with Peptide Mouse #19+KLH

Preparation of antibodies
Preparation of IgG fractions

Ammonium sulphate from Merck, (Catalog No. 1217, Kenilworth, NJ, USA) was added to the serum to a final concentration of 250 mg/ml and stirred until dissolved. The mixture was incubated for 30 min at room temperature, then centrifuged at 3000 g for 30 min at room temperature. The immunoglobulin precipitate was washed several times with a solution of 1.75 M ammonium sulphate until obtaining its white color. This procedure allowed for removing albumin, transferrin, haptoglobin and hemoglobin. The final white pellet containing immunoglobins was dissolved in PBS, 0.5 M NaCl, 0.05% NaN_3.

Concentration of immunoglobulins in the solution was calculated according to the formula: Concentration of sample (mg/ml) = absorbance at 280 nm/1.4.

Purification of antibodies from total immunoglobulin fractions by affinity chromatography using peptide immobilized sorbent

A. Preparation of affinity immunosorbent. 50 ml of N-hydroxysuccinimide (NHS)-activated Sepharose 4 Fast Flow was washed with 750 ml of cold 1 mM HCl and divided into 5 equal parts. In 50 mL centrifuge tubes 10 ml of washed NHS-activated Sepharose 4 Fast Flow was mixed with 10 ml solution of the appropriate peptide dissolved in 0.2 M $NaHCO_3$, 0.5 M NaCl at 5 mg/ml. The mixture was incubated overnight on the end-over-end mixer at 4°C. Subsequently, the tubes were centrifuged for 15 min at 1000 g at room temperature and supernatant removed. The 10 ml portions of the affinity sorbents for each oligopeptide were mixed with 40 ml 0.5 M ethanolamine, 0.5 M NaCl pH 8.3 and incubated overnight on the end-over-end mixer at 4°C.

The affinity sorbents for each specific oligopeptide were mixed with 50 ml PBS, 0.5 M NaCl, 0.05 % NaN_3 (wash buffer), then with 50 ml 0.1 M glycine-HCl, 0.05 % NaN_3 pH 2.6 (elution buf-

fer) on glass Buchner funnel. This cycle of washing and elution was repeated 5 times. Finally, the affinity sorbents were packed into columns XK 16/20 and after washing with wash buffer were ready for use.

B. Purification of specific antibodies from total IgG fractions: The solution of IgG for appropriate oligopeptide was applied to the affinity column at room temperature by using peristaltic pump with flow 0.5 ml/min. The affinity sorbent was washed with PBS, 0.5 M NaCl, 0.05% NaN3 (wash buffer). The absorbance of collected fractions was monitored at 280 nm up to obtaining absorbance range 0.05-0.07, which indicates that antibodies to specific peptide were bound and retarded by immunosorbent.

Subsequently, these peptide-specific antibodies were eluted from immunosorbent by 0.1 M glycine-HCl, 0.05% NaN3 pH 2.6 (elution buffer) in 3 ml fractions and the absorbance at 280 nm was monitored. After the elution antibodies were immediately neutralized with 1 M Tris solution. Every IgG was purified in 6 cycles such affinity chromatography on the complementary sorbent. Eluted polyclonal antibodies to 5 antigens from 6 cycles of affinity chromatography were pooled in 5 pools.

Concentration of antibodies in each pooled solution was calculated using the formula:

Concentration of sample (mg/ml) = absorbance at 280 nm/1.4.

The % of purified antibody from the total amount of IgG was 0.58% for Rat #7, 0.63% for Rat #12, 0.41% for Rat #18, 0.55% for Mouse #11 and 0.47% for Mouse #19

Testing of isolated antibodies for immune specificity and affinity against corresponding peptide

Each of purified antibodies for complementary peptides was tested by ELISA. Antibody was diluted 2-fold from 100 to 0.78125 ng/ml.

Concentration of polyclonal antibodies after affinity chromatography

Ammonium sulphate from Merck (Catalog No. 1217, Kenilworth, NJ, USA) was added to the antibody solution after affinity chromatography to a final concentration of 250 mg/ml and stirred to dissolve. The mixture was incubated for 30 min at room temperature. The suspension is centrifuged at 3000 g for 30 min at room temperature and the antibody precipitate was re-suspended up to 14 ml in solution of 1.75 M ammonium sulphate.

Preparation of antibodies for cell invasion assays

Rabbit polyclonal antibodies against mouse and rat oligopeptides prepared as suspensions of serum IgG fraction in ammonium sulfate (described above) were thoroughly mixed and placed in 6x Slide-A-Lyzer MINI Dialysis units from Thermo Scientific (Catalog No. 88403, Waltham, MA, USA). 500 mcl of each antibody suspension was run against a total volume 4 x 45 ml Dulbecco's PBS with stirring at 4°C. Antibody solutions were collected and sterilized. Pierce Protein micro BCA kit was used to determine protein concentrations.

Cell cultures

Human Prostate Cancer (DU145), Mouse Breast cancer (4T1) and Human Pancreas Carcinoma (MIA PaCa-2) were obtained from ATCC (American Type Culture Collection, Rockville, MD, USA). All cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), penicillin (100 units/mL) and streptomycin (100 µg/mL). Cells were incubated at 37°C in a tissue culture incubator equilibrated with 95% air and 5% CO_2.

Invasion assay

Corning BioCoat Matrigel Invasion Chambers were used with Corning Matrigel Matrix cell culture insert in 24 - well plates (Corning, NY, USA). Plate was rehydrated for approximately two hours in the cell culture incubator by adding warm (37°C) cell culture medium without FBS to the inserts and to the lower chamber. Cells suspension was mixed with antibodies, as specified, and seeded on the insert in the well. Equal numbers of cells were added to each insert. DMEM with 1% FBS was added to the well below the insert to serve as a chemoattractant. The plates with the inserts were then incubated in a culture incubator equilibrated with 95% air and 5% CO_2 for 24 hours. After incubation, the media/antibodies were discarded from the inserts. The cells on the upper surface of the inserts were gently scrubbed away with cotton swabs. Cells that had penetrated the Matrigel membrane and migrated onto the lower surface of the Matrigel were fixed with Methanol and stained with hematoxylin and eosin. Pictures were taken under the microscope and cells were counted. For the sake of uniformity, pictures were always taken at the center of the insert.

Western Blot

Whole DU145 cell lysates were prepared using lysis buffer [50 mM Tris-HCl (pH=7.4), 1% TritonX-100, 150 mM NaCl, 1 mM EDTA, 2 mM Na3VO4, and 1X Complete protease inhibitors (Roche Applied Science, Indianapolis, IN, USA)]. The protein concentration was measured by the Dc protein assay (Bio-Rad, Hercules, CA, USA). 50 µg of protein per sample was separated by electrophoreses using standard Laemmle›s method as described[8] and transferred to a PVDF membrane. Proteins were detected with commercially available anti-MMP-9 antibody (Cell Signaling, Danvers, MA, USA) as a reference antibody, and anti-β-actin antibody as a loading control (Cell Signaling Technology, Danvers, MA, USA).

RESULTS

Immune response

The immune response to individual peptides evaluated by ELISA using complementary antiserum and expressed as an average A_{450} is presented in Table II.

The results of serum titration for two bleedings: on day 157 and 164 tested at serum dilutions 1:1000 and 1: 10000 show that test oligopeptides generated immune response of similar intensity in all rabbits (Table II). Based on the immune response, the sera from five bleedings for each rabbit immunized with a particular peptide were pooled and each pool tested for the immune response and subsequently used for the purification of polyclonal antibodies specific for each test peptide.

The immune response in pooled rabbit sera immunized with specific antigens containing MMP-9 sequences from rat and mice is presented in Figure 1. The pooled sera for each test oligopeptide were tested at two-fold dilutions ranging from 1:10,000 to 1:640,000 and immune response to the specific antigen expressed as A_{450}.

The results show that the sera from rabbits immunized with rat peptides (A1 through A3) had higher antibody titer than the sera obtained from the immunization with mouse A1 and A2 peptides.

Characterization of polyclonal antibodies against rat and mice MMP-9 sequences

Characterization of polyclonal antibodies to specific MMP-9 peptides after their subsequent purification on affinity columns are presented in Table III and Figure 2. Each antibody was tested at two-folds dilutions from 100 ng/ml to 0.78125 ng/ml and the affinity to corresponding peptide sequences expressed as A_{450}nm.

The results show approximately equal specificity and affinity of the antibodies for all antigens except Mouse peptide #11, which was two times weaker.

Inhibition of cancer cell invasion by anti-MMP-9 antibodies

The affinity purified polyclonal antibodies against individual oligopeptides mimicking specific sequences of MMP-9 in rat and mice were tested for their ability to inhibit extracellular matrix invasion of cancer cells. The results presented in Figure 3 and 4 show that all test antibodies generated against rat and mice MMP-9 oligopeptides were effective in inhibiting Matrigel invasion of both mouse breast cancer cells (4T1) and human prostate cancer cells (Du145).

TABLE 2. Immune response of rabbit antiserum to complementary antigens.

		Bleed on Day 157 Sera dilution		*Bleed on Day 164*	
Rabbit #	*Oligopeptide*	*1:1000*	*1: 10000*	*1:1000*	*1: 10000*
1	R#7	>4	1.370	>4	1.250
2	R#7	>4	2.129	>4	1.949
3	R#7	3.666	0.577	2.762	0.378
4	R#12	>4	3.804	>4	>4
5	R#12	>4	3.939	>4	3.843
6	R#12	>4	3.736	>4	3.593
7	R#18	>4	3.330	>4	3.326
8	R#18	>4	1.368	>4	1.349
9	R#18	>4	2.662	>4	2.489
10	M#11	>4	2.931	>4	2.964
11	M#11	3.707	0.853	3.911	0.911
12	M#11	>4	2.368	>4	2.550
13	M#19	>4	2.321	>4	1.968
14	M#19	>4	3.836	>4	3.725
15	M#19	>4	3.028	>4	2.131

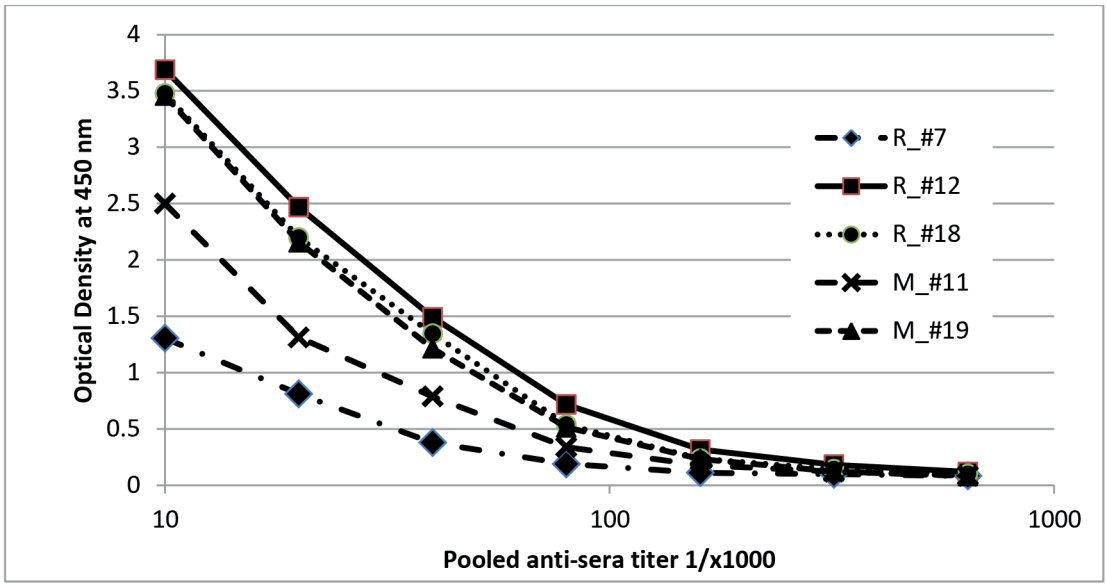

Fig. 1. Immune response of pooled rabbit antisera to their complementary antigens. Blood serum samples were collected from rabbits (three animals per group) immunized with individual peptides on five bleeding occasions following standard immunization protocol (Rockland Catalog). Immune serum samples specific to each individual peptide were pooled and tested for immune response by assaying antibody binding to peptides immobilized on plastic plates in serially diluted sera. Intensity of immune response was proportional to optical density of colored product of peroxidase enzymatic reaction measured at 450 nm. See Materials and Methods for experimental details.

As such, Figure 3 shows that the highest inhibition of breast cancer cells invasion could be obtained with antibody against mouse M#19 oligopeptide (44% inhibition) and rat R#18 oligopeptide (33 % inhibition). The antibodies directed towards these murine MMP-9s were also effective in inhibiting extracellular matrix invasion of human prostate cancer cells. As presented in Figure 4, the highest inhibition of invasion could be obtained with antibodies against mouse MMP-9 oligopeptides: M#11 (40% inhibition) and M#19 (96% inhibition), demonstrating interspecies efficacy of these antibodies (Figure 4). In general, the antisera against mouse oligopeptides were more effective in inhibiting extracellular matrix (ECM) invasion of cancer cells from human prostate than the murine 4T1 breast cancer cells.

In an effort to enhance the inhibitory effect of antibodies on cancer cells migration through ECM we applied them in various combinations. In order to correct for different protein content in various antibody mixtures we added a non-specific rabbit IgG to equalize the protein content in each test sample. We also controlled total protein concentration by adding the same amount of PBS-BSA in all Control samples. That way, antibody protein concentrations were kept constant for every tested combination.

The results (Figure 5) show that the most effective in inhibiting mouse breast cancer cells invasion was a combination of all five antibodies. The mixtures containing two and three antibodies against rat MMP-9 either without or with antibody M#11 could inhibit 4T1 cell invasion by about 60%. The

TABLE 3. Immune affinity of antibodies to their complementary antigens.

Antibody ng/ml	*A_{450} of Antibodies*				
	R_#7	*R_#12*	*R_#18*	*M_#11M_#19*	
100	2.726	2.399	2.286	1.135	2.505
50	1.823	1.388	1.28	0.569	1.51
25	0.966	0,815	0.664	0.315	0.794
12,5	0.494	0.428	0.34	0.167	0.405
6,25	0.251	0.223	0.171	0.09	0.207
3,125	0.127	0.115	0.088	0.049	0.107
1,5625	0.068	0.058	0.049	0.032	0.059
0,78125	0.038	0.038	0.032	0.027	0.039

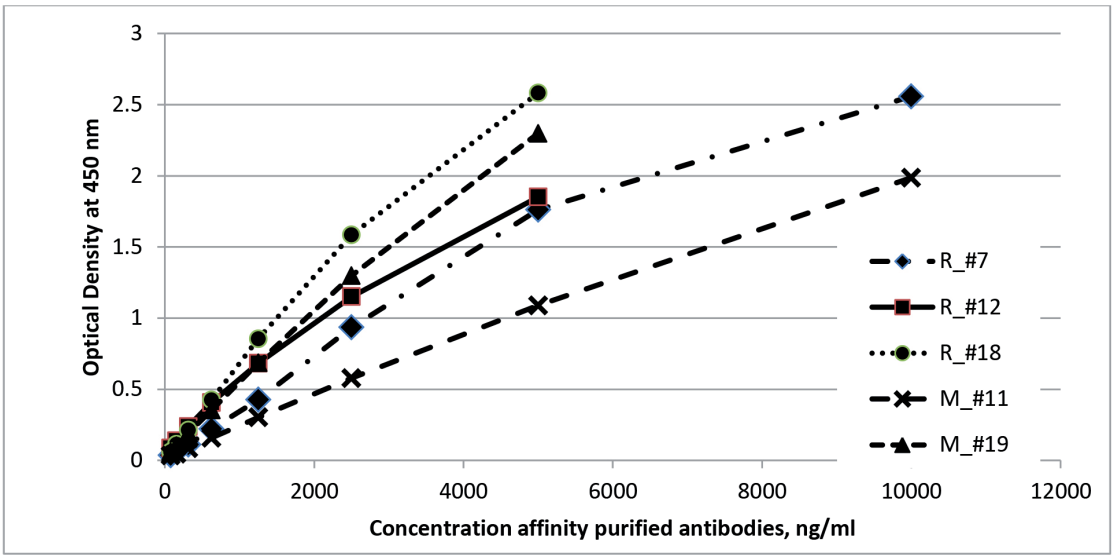

Fig. 2. Immune response of affinity purified antibodies to complementary antigens. Specific antibodies were isolated from pooled immune sera by two consecutive steps: total serum immunoglobulin precipitation with ammonium sulfate followed specific antibodies purification by affinity chromatography on Sepharose 4 Fast Flow columns loaded with individual synthetic peptides. Immune response of affinity purified antibodies was evaluated by immune response assay as described in the legend to Figure 1. See Materials and Methods for experimental details.

similar level of inhibition of 4T1 invasion could be obtained with only one individual antibody R#12.

We also tested whether these combinations of antibodies were effective in inhibiting Matrigel migration of human pancreatic cancer cells MIA PaCa-2. The results show that the ability to digest connective tissue and invade extracellular matrix gradually decreased with increasing the number of different antibodies in a combination (Figure 6). Here too, five antibodies applied together were most effective resulting in nearly 95% inhibition of ECM invasion. This confirms that antibodies against murine MMP-9 enzymes are effective in inhibiting ECM invasion of cancer cells originating from different organs in mice and humans.

The representative images of the Matrigel invading MIA PaCa-2 cells in the presence of various anti-MMP-9 antibody combinations are presented on Figure 7. The photographed inserts correspond to numerical data for each antibody combinations presented on Figure 6. All cell invasion experiments were performed in triplicates:

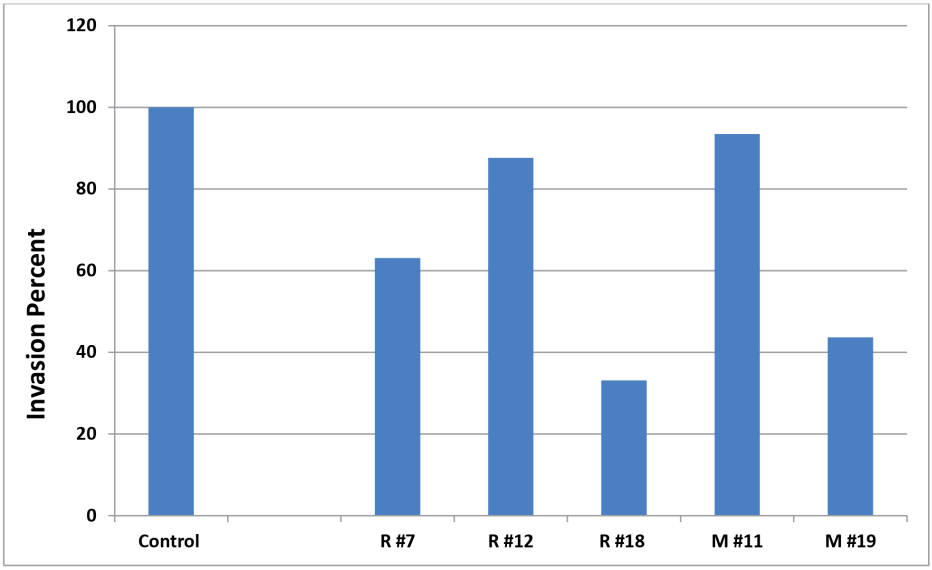

Fig. 3. Efficacy of antibodies against specific rat and mice MMP-9 oligopeptides in inhibiting murine breast cancer cells invasion through Matrigel. Inhibition is presented as the percentage of invaded 4T1 cells mixed with specified individual antibodies. Control cells were mixed with non-specific antibody at the same concentration as test peptides.

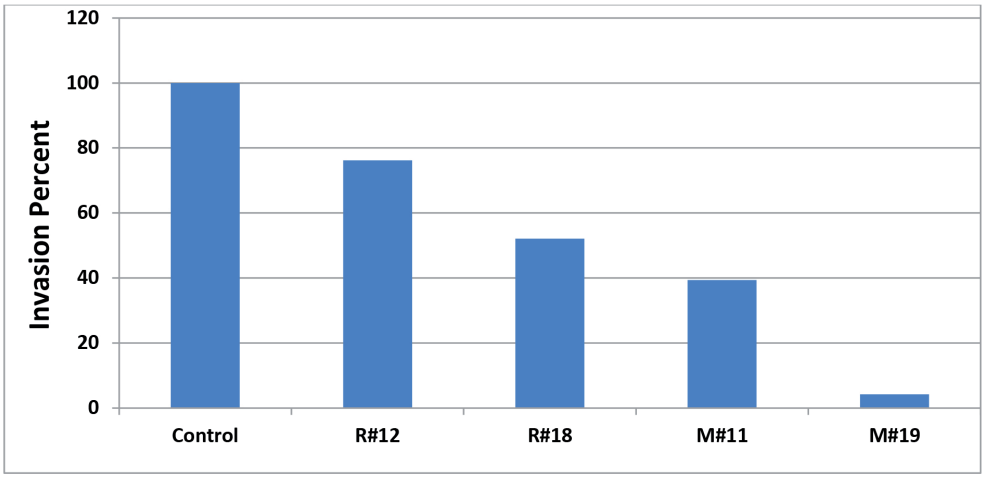

Fig. 4. Efficacy of antibodies against specific rat and mice MMP-9 oligopeptides in inhibiting human prostate cancer cells invasion through Matrigel™. The results present percentage of invaded DU145 (Human Prostate cancer) cells when mixed with specified individual antibodies as described in the Material and Methods.

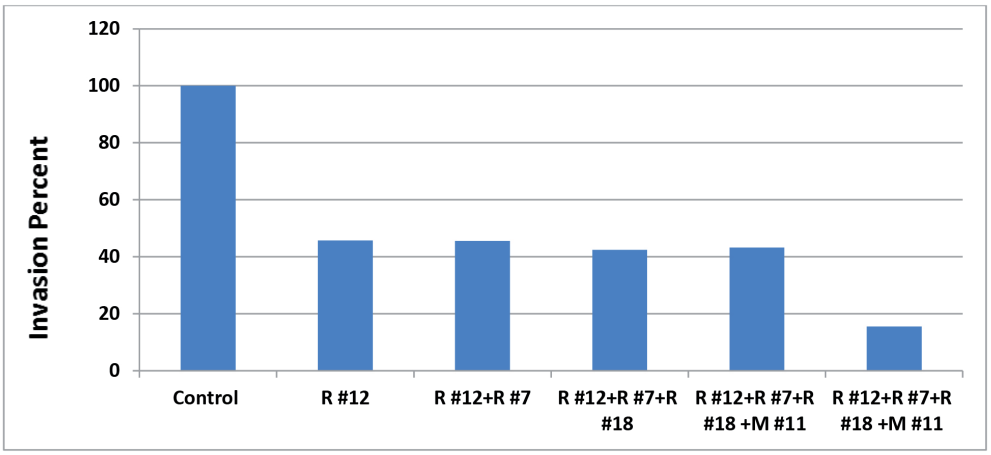

Fig. 5. Inhibition of 4T1 cells invasion through Matrigel in the presence of various combinations of antibodies generated against rat and mouse MMP-9 oligopeptides. The results show the percentage of invading cells where the Control sample is set to 100%.

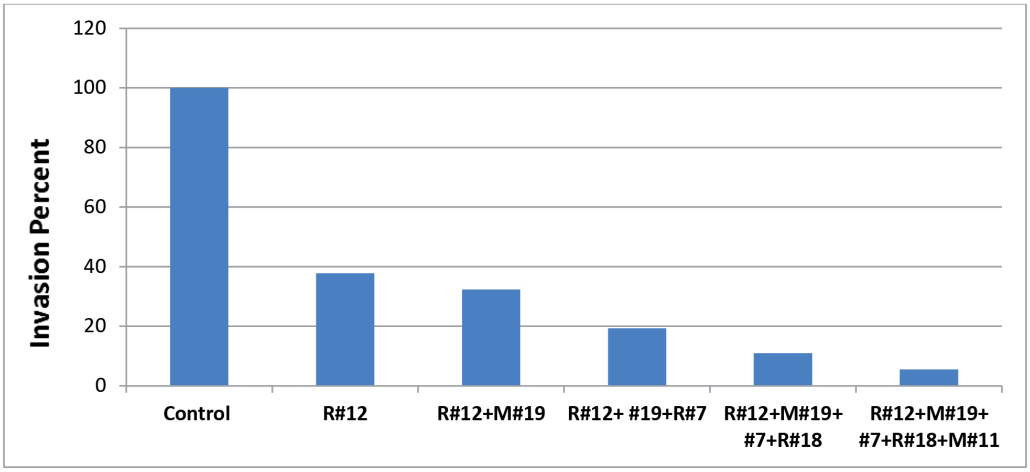

Fig. 6. Inhibition of pancreatic cancer cells (MIA PaCa-2) invasion through Matrigel in the presence of various combinations of antibodies generated against rat and mouse MMP-9 oligopeptides. The results show the percentage of invading cells where the Control sample is set to 100%.

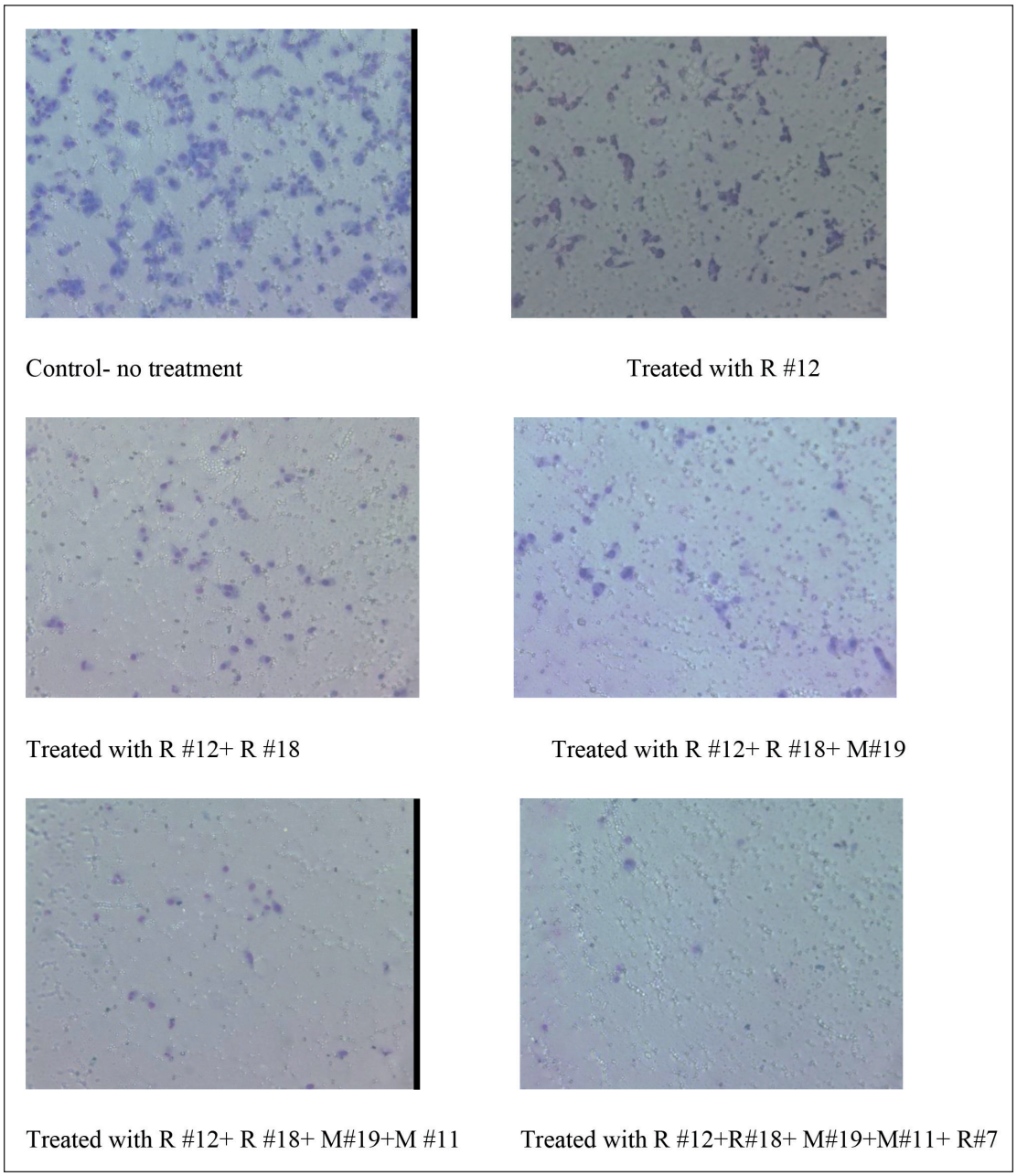

Fig. 7. Microscopic images of Matrigel invading MIA PaCa-2 cells mixed with an individual antibody (R#12) and various combinations of antibodies generated against different MMP-9 mice and rat sequences. Images taken from the center of each insert after cells were fixed and stained with Hematoxylin and Eosin.

Antibody binding to their corresponding MMP-9 peptides

The results presented in Figure 8 show the binding of specific antibodies to their corresponding MMP-9 peptides as identified by Western Blot. The R#12 showed the strongest binding to its corresponding peptide and among all tested antibodies only M#11 was found not to bind to its corresponding MMP-9 peptide sequence.

DISCUSSION

Halting the spread of cancer in tissue and its metastasis to different organs has been a 'holy grail' of cancer therapies. Current modalities used in cancer therapy are not effective against metastasis, which is responsible for over 90% death from cancer. Among different anti-cancer approaches it appears that vaccines present the biggest opportunity to succeed. In particular, when the antigen of choice can target universal mechanism involved in cancer, especially its metastasis.

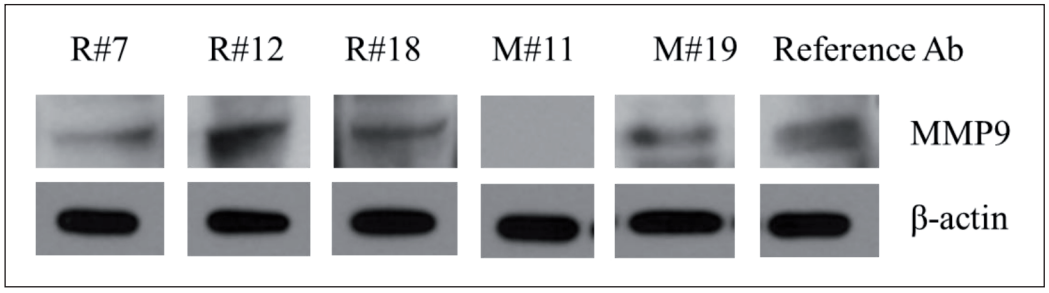

Figure 8. Western blotting showing MMP-9 binds to 4 of the 5 antibodies tested. Anti-β-actin antibody was used as loading control.

MMPs appear to be an ideal target for the development of anti-cancer vaccine. They are disease-associated and involved in cancer cell tissue invasion, angiogenesis and metastasis. Earlier attempts that aimed at the MMPs in cancer by early generation of pharmaceutical MMP inhibitors were not successful due to unacceptable side effects or insufficient efficacy of these drugs[9]. However, our earlier studies indicate that inhibiting MMP activity by vaccination is a promising therapeutic approach.

As such our earlier *in vivo* studies using specific oligopeptides containing MMP-2 and MMP-9 sequences from human, and MMP-9 peptides from rat and mice demonstrated significant inhibition of tumor growth in mice[7]. This *in vitro* study evaluated some mechanistic aspects of tumor growth inhibition by investigating the efficacy of polyclonal antibodies against oligopeptides from rat and mouse MMP-9 on inhibition of cancer cell invasion through extracellular matrix (Matrigel™).

The results obtained here provide another support for further development of effective anti-cancer vaccine. They confirm that MMP-9 activity is a viable target of anti-cancer strategy and a suitable antigen for the vaccine aiming at the universal mechanism essential in all types of cancer and associated with its ECM invasive activities. Moreover, they document universal -non-species related - efficacy of anti-MMP-9 antibodies. It is an extension of our earlier *in vivo* studies, which showed that specific oligopeptides containing human MMP sequences can generate an immune response in mice, but also oligopeptides from rat and mice MMPs can generate an immune response in a syngeneic animal model. In both cases the immunization was associated with significant anti-tumor efficacy[7].

Our Matrigel invasion study shows that antibodies raised against rodent MMP-9 are effective in inhibiting the invasion of cancer cells originating from different organs in both mice and humans, specifically the breast cancer (mice) and prostate cancer (human). We have shown that anti-invasive efficacy of these antibodies can be enhanced by using them in various combinations and as such targeting different regions of the MMP protein. This effect is plausible given that individual antibodies may target different epitopes of MMP-9 and show more efficacy when they are applied together. Specificity of various antibodies towards individual MMPs has been confirmed by their specific binding identified by immunoblot. Interestingly antibody R#12 showed the strongest binding capacity and was also associated with most significant inhibition of breast cancer and prostate cancer cells Matrigel invasion. Combination of R#12 with 2 or 3 other antibodies did not enhance its anti-invasive capacity further. The Western blot did not detect the M#11 antibody binding to its MMP-9, which may relate to its weakest binding capacity and other specificity aspects. Interestingly, this antibody was not very effective in inhibiting extracellular matrix invasion of breast cancer cells; however, it gave good results with human prostate cancer cells. This again, stresses the need to use a combination of antibodies to target cancer cell invasion for maximal effects.

CONCLUSIONS

This and our previous *in vivo* results further reinforce the urgent need for conducting specific targeted investigations towards the development of universal and effective anti-cancer vaccine.

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Conflict of interest:
Authors declare no conflict of interest.

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