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PRINCIPAL INVESTIGATOR: Jacqueline Sharon, Ph.D.

CONTRACTING ORGANIZATION: Boston University School of Medicine
Boston, Massachusetts 02118-2394

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Jacqueline Sharon, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Boston University School of Medicine
Boston, Massachusetts 02118-2394

E-MAIL:
jsharon@bu.edu

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13. ABSTRACT ***(Maximum 200 Words)***

This project proposes the development of a recombinant polyclonal antibody specific for the human breast carcinoma cell line BT-20. The proposed library would target multiple epitopes on the human cell line and also provide the genes necessary for the unlimited perpetuation of the defined antibody mixture. Ultimately, such antibody libraries could be used for treatment and diagnosis of breast cancer in humans. In the present study, V region genes for construction of a polyclonal Fab phage display library were obtained from the spleen, bone marrow, and intestine of a BALB/c mouse that had been immunized with BT-20 cells. The light and heavy chain V region genes were linked by reverse transcription-polymerase chain reaction (RT-PCR) and cloned into a bidirectional phagemid vector to express an Fab-displaying phage library. This library was then selected for binding to BT-20 cells. Direct and inhibition ELISA showed that the selected Fab phage display library bound specifically to BT-20 cells, and that this binding could be inhibited by BT-20 as well as by other human breast cancer cell lines, but not by sheep red blood cells. These results demonstrate that the first phase of generating a polyclonal antibody library can be done successfully.

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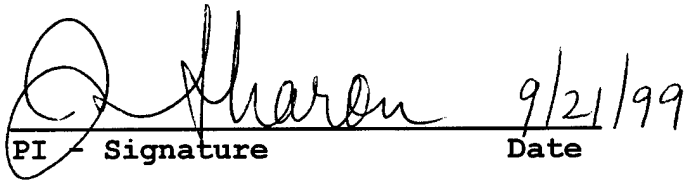

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(4) INTRODUCTION:

As an immunotherapeutic approach to breast cancer, we set out to develop recombinant polyclonal antibody libraries that would target malignant cells. Polyclonal antibody libraries combine the advantages of targeting multiple antigenic determinants (low likelihood of antigen 'escape variants') with the advantages of using monoclonal antibodies (unlimited supply of standardized reagents, and the availability of the genetic material for desired manipulations). In the present study, we have used a bidirectional *l*PP*l* cassette containing the Zeocin selectable marker (1, 2) to achieve efficient expression of Fab-displaying phage for generating a specific polyclonal Fab phage display library to the human breast carcinoma cell line BT-20.

We have previously described a system for generation of recombinant polyclonal antibody libraries (3, 4). This system involves the cloning of VL-VH region gene pairs into a bidirectional phage display vector for Fab expression, such that the VL and VH region genes of each pair are in opposite (head to head) transcriptional orientations (←→). Because the phage display vector is circular, the vector library can be opened with restriction enzymes between VL and VH to insert a bidirectional leader-promoter-promoter-leader (*l*PP*l*) cassette that allows expression of Fab on the phage surface. Furthermore, a subpopulation of Fab-phage can be selected for binding to a desired antigen or polyantigen (a multi-antigen particle such as a mammalian or microbial cell). The VL-VH region gene pairs of the selected phage subpopulation are then transferred, in mass, from the phage display vector to a bidirectional mammalian expression vector to produce whole antibodies that can mediate effector functions.

(5) BODY:

The human breast adenocarcinoma cell line BT-20 was chosen for generation of an anti-human breast cancer polyclonal antibody library (PCAL) because of the ability of BT-20 cells to form tumors in immunodeficient nude mice (ATCC catalog, Rockville, MD). This affords a system for *in vivo* efficacy testing of the library. V region genes for construction of a polyclonal Fab phage display library to BT-20 cells were obtained from a Balb/c mouse that had been immunized with BT-20 cells. The serum antibody titer was assessed, by ELISA on fixed BT-20 cells, at various days post primary immunization. As shown in Fig. 1, the serum titer rose to almost 17,000 during the immunization. Immunoblot analysis for reactivity of the antiserum with BT-20 membranes showed recognition of a wide variety of BT-20 antigens (Fig. 2), indicative of the polyclonality of the antibodies. (The antiserum also reacted with another human breast carcinoma cell line, Hs 578T, see Fig. 2)

Total RNA was prepared from spleen, bone marrow, and intestine of the sacrificed animal, and linked VL-VH region gene pairs were generated by RT-PCR as previously described (5) except that V region genes of IgA H chains (α) were also amplified, in addition to IgG and IgM H chains. The linked VL-VH region genes were ligated into the phh3 vector (see Fig. 3), resulting in a phh3-VL-VH library of about 2 x 10^7 members. For expression of Fab on the phage surface, the prokaryotic bidirectional leader/promoter cassette containing the Zeocin selectable marker (*l*P*Zeo*P*l*) was inserted between VL and VH, generating the Fab phage display library of vectors (phh3-VL- *l*P*Zeo*P*l*-VH, see Fig. 3).

To select an Fab phage sublibrary that reacts with the BT-20 carcinoma cells, phage (10^{11} cfu) were subjected to two consecutive rounds of panning on fixed BT-20 cells (with amplification of the phage by infection into bacteria after each pan). The first pan yielded 8 x 10^4 cfu of bound/eluted phage whereas the second pan yielded 3 x 10^6

cfu of bound/eluted phage, a 37.5-fold increase over the first pan. This increase indicated a sequential enrichment in anti-BT-20 Fab phage during the panning procedure. Analysis of the unpanned and panned Fab phage libraries by ELISA on fixed BT-20 cells confirmed this sequential enrichment, showing that the second pan library binds better than the first pan library which, in turn, binds better than the unpanned library (Fig. 4). Both the unpanned and second pan libraries were determined to be polyclonal by nucleotide sequence analysis of the VL and VH region genes of individual clones (data not shown).

To confirm the specificity of the second pan Fab phage library for BT-20 cells, the library was tested in a "criss-cross" inhibition ELISA in conjunction with a panned anti-sheep red blood cells (anti-SRBC) Fab phage library. BT-20 membranes inhibited the binding of the anti-BT-20 phage library to solid phase BT-20 cells in a dose-dependent manner (Fig. 5A) but not of the anti-SRBC phage library to solid phase SRBC ghosts (Fig. 5B). Conversely, SRBC ghosts inhibited the binding of the anti-SRBC phage library to solid phase SRBC ghosts in a dose-dependent manner (Fig. 5B) but not of the anti-BT-20 phage library to solid phase BT-20 cells (Fig. 5A). Thus, both the anti-BT-20 and anti-SRBC libraries showed specificity for their immunizing/panning polyantigens.

The second pan anti-BT-20 library was then tested in another inhibition ELISA to determine if it can react with other human breast carcinoma cell lines. As shown in Fig. 6, membrane preparations of both the Hs 578T and MCF-7 human breast carcinoma cell lines inhibited the binding of the second pan anti-BT-20 library to solid phase BT-20 cells, in a dose-dependent manner. Thus, the anti-BT-20 Fab phage display library can bind to other breast carcinoma cell lines.

The results presented here demonstrate that a polyclonal Fab phage display library specific for human breast carcinoma cells can be generated using our bidirectional vector system. Such libraries, after suitable negative selection to eliminate major reactivities against normal tissue, could be transferred in mass to our

bidirectional mammalian expression vector for production of libraries of whole antibodies (3, 4, 5) with constant regions of any desired isotype or species. Polyclonal antibody libraries will mediate effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), and are expected to be useful for breast cancer therapy, as well as diagnosis.

(6) KEY RESEARCH ACCOMPLISHMENTS

- Mice were immunized with human breast carcinoma cell line BT-20
- An original Fab phage library was generated from the V region genes obtained from an immune mouse
- An Fab phage display library was generated
- The Fab phage display library was positively selected on the BT-20 human breast carcinoma cell line, and shown to be specific and polyclonal

(7) REPORTABLE OUTCOMES

1 manuscript is in preparation

CONCLUSIONS

In the present study, V region genes for construction of a polyclonal Fab phage display library were obtained from the spleen, bone marrow, and intestine of a Balb/c mouse that had been immunized with BT-20 cells. The light and heavy chain V region genes

were linked by reverse transcription-polymerase chain reaction (RT-PCR) and cloned into a bidirectional phagemid vector to express an Fab-displaying phage library. This library was then selected for binding to BT-20 cells. Direct and inhibition ELISA showed that the selected Fab phage display library bound specifically to BT-20 cells, and that this binding could be inhibited by BT-20 as well as by other human breast cancer cell lines, but not by sheep red blood cells. These results demonstrate that the first phase of generating a polyclonal antibody library can be done successfully. Recombinant polyclonal antibody libraries could be used for treatment and diagnosis of breast cancer in humans.

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APPENDIX

FIGURE LEGENDS

Fig. 1 Serum antibody response of Balb/c mouse immunized with BT-20 cells. Serum titer was defined as the last dilution that gave an OD 405 reading of at least 0.6. BT-20 cells were grown to confluence in a 96 well plate, washed, and fixed with 4% paraformaldehyde for 20 minutes. ELISA procedure was performed on the fixed cells that were first blocked with 3% BSA and 1:2 dilutions of serum was added. The plate was developed with a rabbit anti-mouse Ig (Sigma, St Louis, MO) and an alkaline phosphatase-conjugated anti-rabbit antibody (Promega, Madison, WI). The ELISA results were read at 405 nm.

Fig. 2 Western analysis on BT-20 and Hs 578T membrane preps for reactivity to pre-immune and immune sera.

Fig. 3 Schematic diagram of Fab-encoding phagemid vectors. phh3-VL-*l*PZ*eo*P*l*-VH is generated by insertion of the *l*PZ*eo*P*l* cassette into phh3-VL-VH. The direction of transcription is indicated by arrow heads on promoters and V region genes. P, promoter; *l*, leader sequence; *l*mod, leader sequence with modified nucleotide sequence; Stop, translation termination. Amino acids encoded by the vectors are shown in one letter code.

Fig. 4 ELISA of the mouse anti-BT-20 phage on BT-20 cells. As negative control, phage encoding no Fab on its surface was used, and the value of 0.755 was subtracted from the graphed values as background binding of phage.

Fig. 5 Inhibition ELISA on **A)** BT-20 membrane-coated ELISA plate using Balb/c anti-BT-20 Pan II phage ; and **B)** SRBC-coated ELISA plates using Balb/c anti-SRBC Pan II phage. Phage were preincubated with either 0, 0.1, 1, or 4 μg of either SRBC ghosts or crude BT-20 membranes for one hour prior to adding to the plate.

Fig. 6 Inhibition ELISA on other breast carcinoma cell membranes.

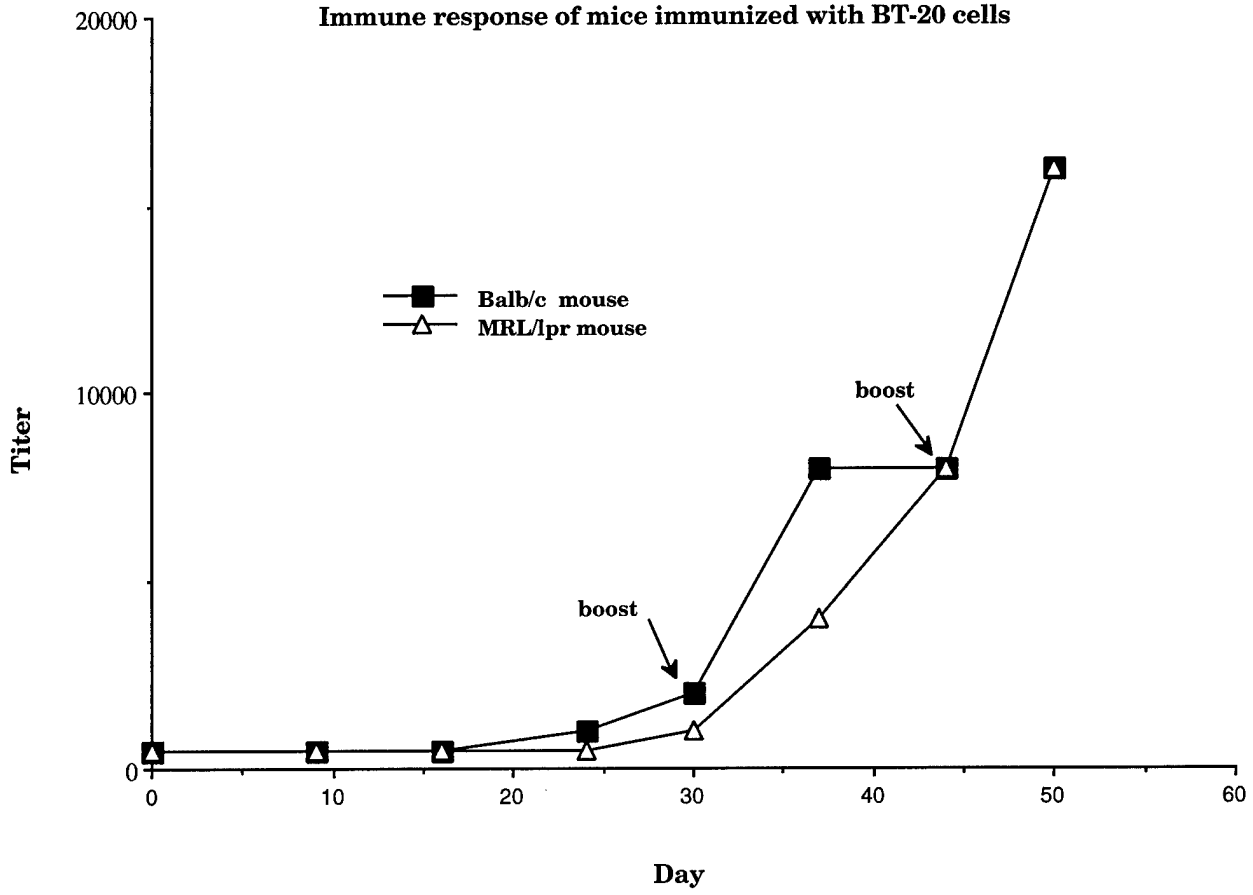

Immune response of mice immunized with BT-20 cells
20000
10000
0
Titer
Balb/c mouse
MRL/lpr mouse
boost
boost
0
10
20
30
40
50
60
Day

Fig. 1

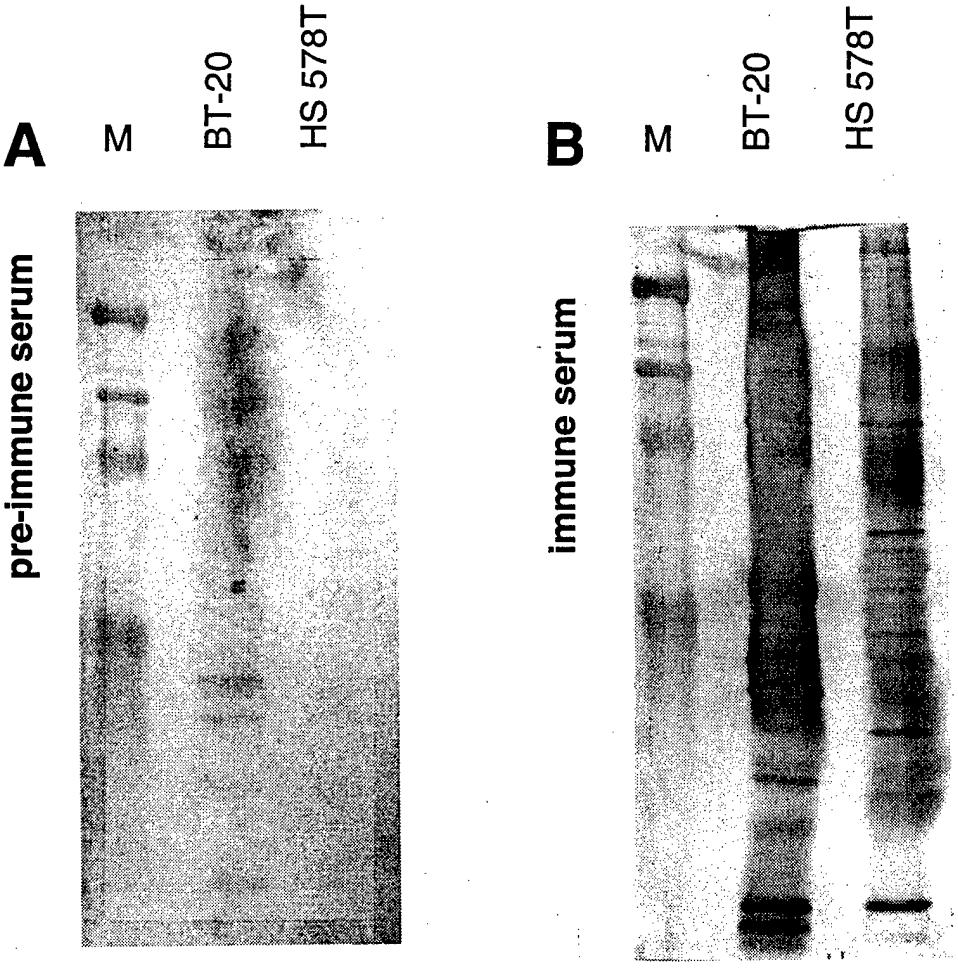
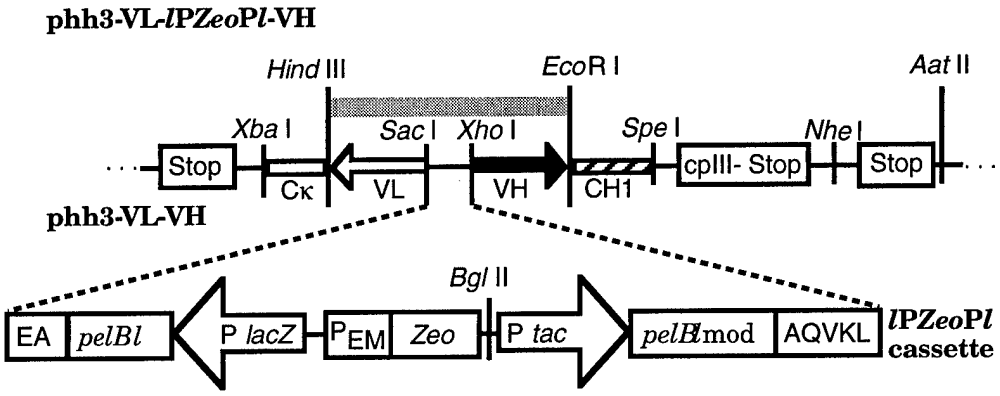


Fig. 2


phh3-VL-lPZeoPl-VH
Hind III
EcoR I
Aat II
Xba I
Sac I
Xho I
Spe I
Nhe I
Stop
Cκ
VL
VH
CH1
cpIII- Stop
Stop
phh3-VL-VH
Bgl II
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pelBl
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Zeo
P tac
pelBmod
AQVKL
lPZeoPl
cassette

Fig. 3

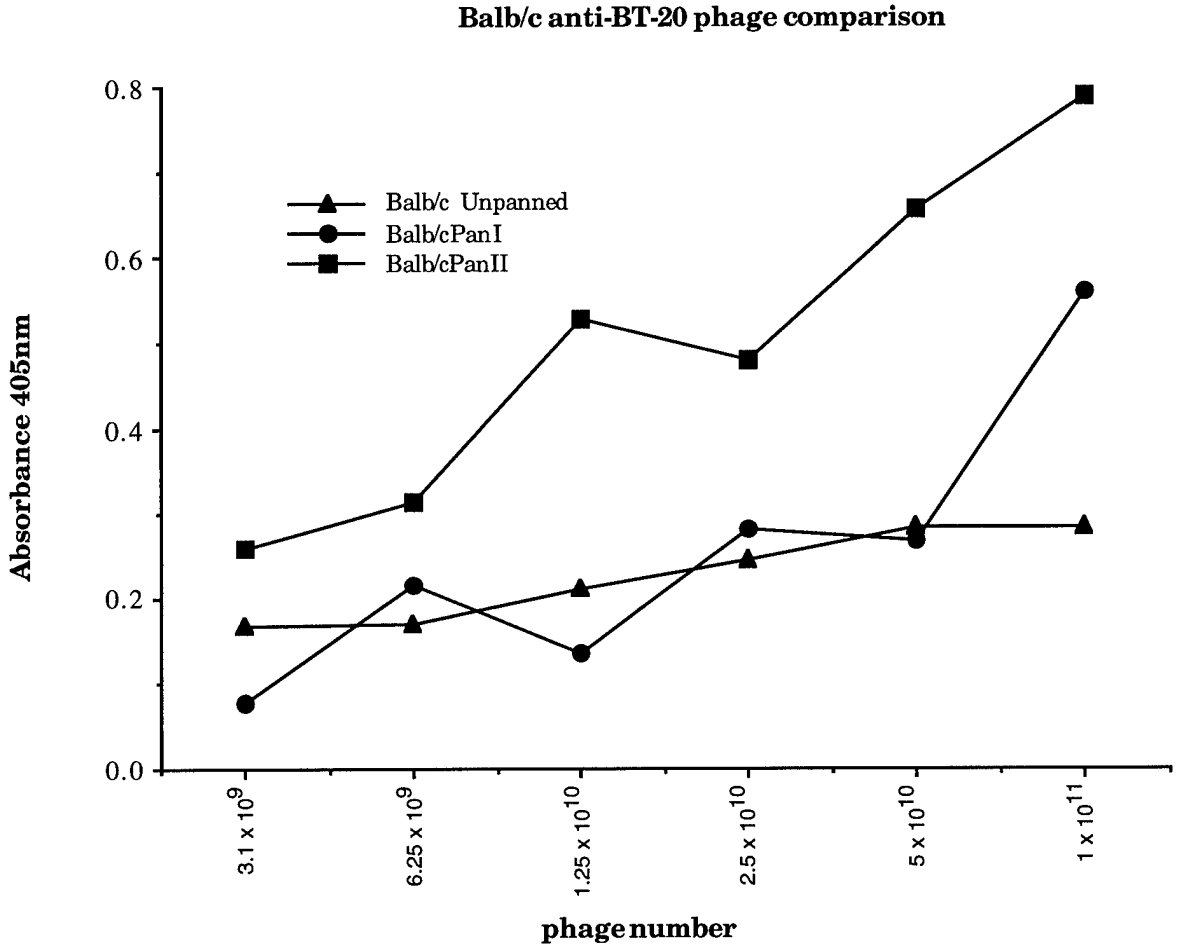
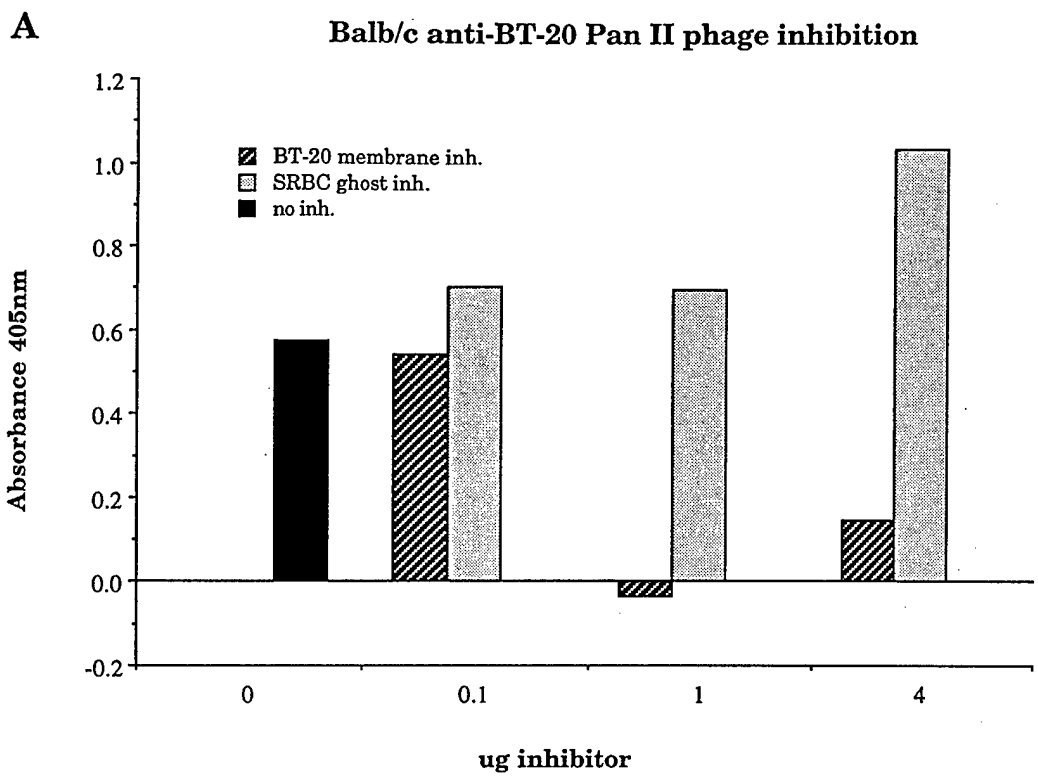

Balb/c anti-BT-20 phage comparison
Absorbance 405nm
0.8
0.6
0.4
0.2
0.0
Balb/c Unpanned
Balb/cPanI
Balb/cPanII
3.1 x 10^9
6.25 x 10^9
1.25 x 10^10
2.5 x 10^10
5 x 10^10
1 x 10^11
phage number

Fig. 4

A


Balb/c anti-BT-20 Pan II phage inhibition
BT-20 membrane inh.
SRBC ghost inh.
no inh.
Absorbance 405nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
0
0.1
1
4
ug inhibitor

B

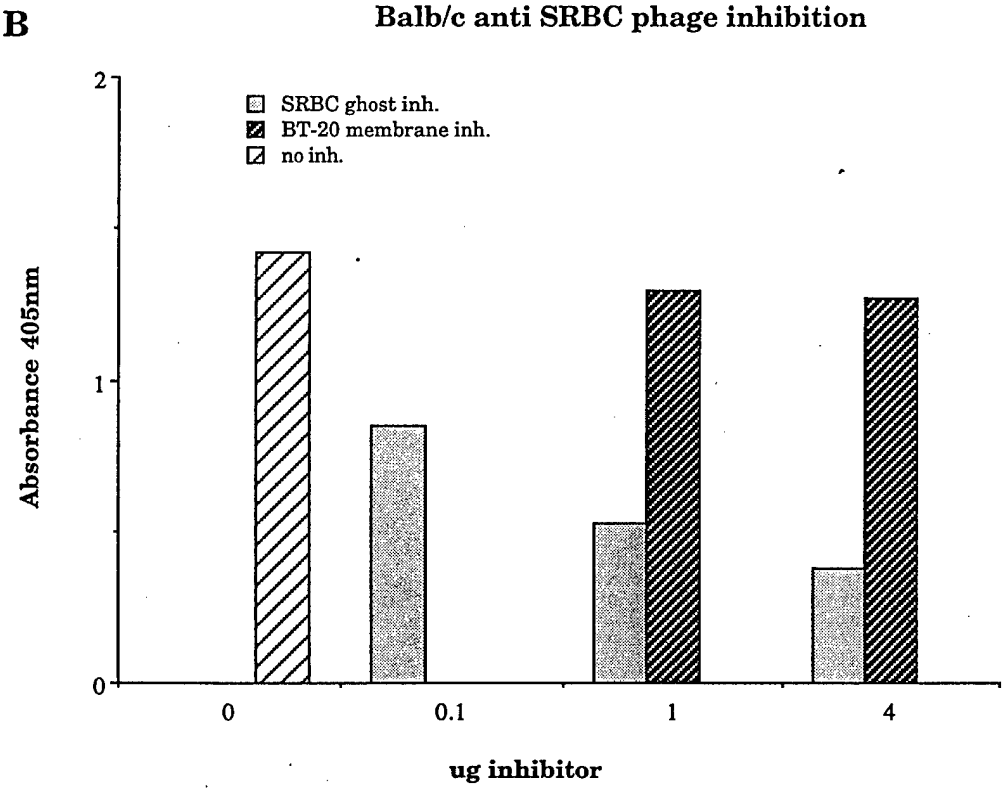

Balb/c anti SRBC phage inhibition
SRBC ghost inh.
BT-20 membrane inh.
no inh.
Absorbance 405nm
2
1
0
0
0.1
1
4
ug inhibitor

Fig. 5

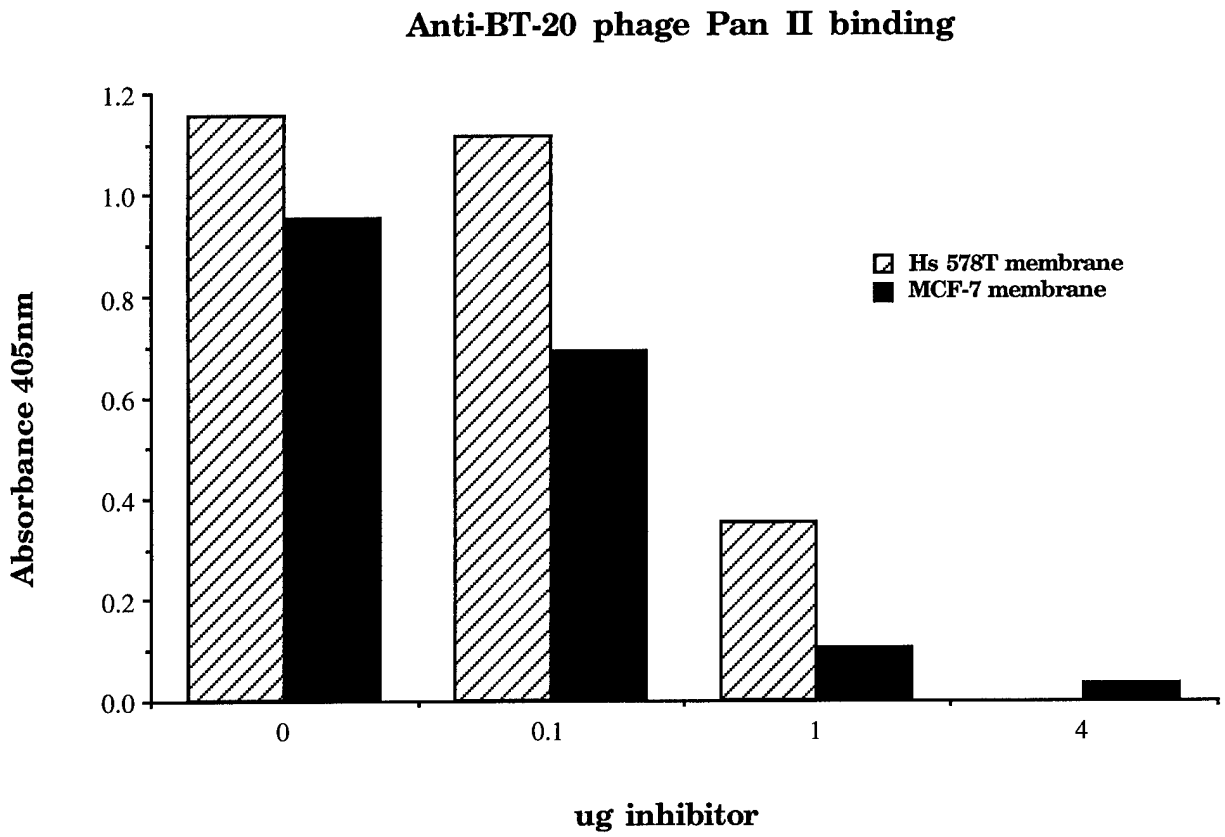

Anti-BT-20 phage Pan II binding
Absorbance 405nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
0.1
1
4
ug inhibitor
Hs 578T membrane
MCF-7 membrane

Fig. 6