Modulation of the Immune Response by Systemic Targeting of Antigens to Lymph Nodes

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Abstract

Factors that determine the immunogenicity of an antigen in vivo are still largely unknown. Direct administration of antigens into lymphatic organs appears to enhance immune response. We hypothesized that systemically targeting antigens to lymphatic tissue in vivo might modulate immunity. To test this hypothesis, we measured the humoral immune response elicited by bacteriophage vaccination. We show that the responses against a lymph node-targeted phage are significantly higher than those against control untargeted phage; the effect is specific because it is inhibited by coadministration of the cognate synthetic peptides displayed. Our data suggest that systemic targeting of antigens to lymph nodes through the circulation modulates humoral immune response. This strategy may have broad applications in the development of vaccines, production of antibodies, and immunotherapy.

Introduction

Variations in the immune response have puzzled scientists since 1796, when Jenner introduced vaccination. Because lymphoid organs were recognized as primary sites of antibody production, many strategies have been used to strengthen the immune response. Over the years, approaches ranging from the direct inoculation of antigens into lymph nodes (1) or spleen (2) to the cell targeting of receptors expressed on dendritic (3) and other antigen-presenting cells (4) were attempted, often with mixed results.

Despite a well-documented antigen-presenting function of endothelial cells (5–9), systemic targeting of antigens to lymphoid organs through the circulation has not yet been explored as a strategy to modulate immune response (10). Here we tested whether targeting an antigen to lymph nodes would affect the immunogenicity of that antigen. We show that systemic targeting of a defined test antigen (an M13-derived bacteriophage) to lymph nodes via the bloodstream by means of novel homing peptides in vivo promotes a specific enhancement of humoral response against that antigen relative to untargeted bacteriophage. These results suggest that targeting may augment humoral immune response after vaccination. A connection between the heterogeneity of blood vessels, antigen presentation in vivo, and the immunotherapy against inflammatory and malignant diseases may be established based on clinical applications of this approach. Targeting of antigens that are specifically expressed in neoplastic but not normal tissues may have broad implications in the development of effective anticancer vaccines, production of therapeutic antibodies, and immunotherapy of a wide variety of human cancers and other diseases.

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3 The abbreviation used is: TU, transducing unit(s).

In Vivo Phage Display. A total of 10^7 TU of a fd-tet-based random phage display peptide library with the general peptide insert arrangement X_CX_CX (C, cysteine; X, any residue) were injected into the tail vein of female 2-month-old nude BALB/c mice under deep anesthesia with Avertin (11–14). Five min after the i.v. phage administration, the mice were euthanized by perfusion of 5 ml of DMEM through the heart (11). To recover the bound phage, the axillary lymph nodes and control organs (brain, kidneys, and pancreas) were surgically removed, weighed, and ground with a glass Dounce homogenizer in 1 ml of DMEM plus protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 1 μg/ml leupeptin). The tissues were washed three times with 1 ml of ice-cold washing media (DMEM plus 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 1 μg/ml leupeptin, and 1% BSA). After three washes, the tissues were incubated with 1 ml of starved competent Escherichia coli K91kan, and serial dilutions of the bacterial cultures were spread onto LB agar plates containing 40 μg/ml tetracycline and 100 μg/ml kanamycin. Standard phage amplification, purification, and selection of individual clones were performed as described previously (11). In brief, three rounds of selection were performed pooling 10^4 individual colonies obtained from the first round. Single colonies were grown separately for 12 h in 5 ml of NZY medium containing 40 μg/ml tetracycline. Bacterial cultures were pooled, the phage preparations were purified, and 10^9 TU were reinjected into mice. Phage libraries may contain mutant phage with a replicative advantage, such as phage that has lost the peptide-encoding insert; insectless phage tend to overgrow desirable ones during successive amplification and selection steps. For this reason, we have amplified selected phage separately and then pooled individually amplified phage for the subsequent in vivo selection step.

This procedure sets a practical limit of a few hundred phage to the number that can be processed from one selection to the next. However, our success in identifying homing peptides shows that this does not pose a serious limitation.

Validation of Lymph Node Targeting. After three rounds of selection, enrichment of phage homing to the lymph nodes was observed relative to control organs [measurements were as follows after the third round: brain, 2,200 TU/g tissue; kidney, 4,266 TU/g tissue; pancreas, 11,105 TU/g tissue; and lymph nodes, 27,555 TU/g tissue; TU/g tissue was calculated based on titrte platings of four serial dilutions (SEs were <10% of the mean for all data points)]. Phage displaying motifs and/or peptides repeated multiple times in successive rounds were used for further analysis. The selectivity of a given phage was calculated as follows. First, the overall number of TU of the selected phage recovered from the lymph nodes was compared with the number of phage TU recovered from the control organs (normalized by mass). We used brain and pancreas as controls; brain is a suitable control for in vivo phage display because of its relatively low background postperfusion (11–14). Second, lymph node-homing phage TU counts were compared with either insectless control phage or unselected X_CX_CX phage library TU counts. To evaluate homing, four axillary lymph nodes were harvested in each experiment. Two phage clones (displaying the peptides PTCAYGWCA and WSCARPLCG) that yielded the best lymph node:control ratios were then evaluated for specificity by comparing their homing individually to negative control phage. Other phage displaying peptides with the motifs CAY and SCAR (data not shown) were also recovered from the lymph nodes during multiple rounds of in vivo selection. To test individual clones for the ability to home to the lymph nodes, phage clones displaying either PTCAYGWCA or WSCARPLCG were compared with unselected phage library or insectless phage as a
negative control. Individual phage clones were injected i.v. into female 2-month-old nude BALB/c mice; phage recovery was done as described previously (11). To confirm specificity and show that the displayed peptides mediate homing to lymph nodes, the cognate soluble peptides (PTCAYGWCA and WSCARPLCG) were synthesized, purified, cyclized (Anaspec), and tested for the ability to inhibit phage homing. Competition of phage homing with the cognate peptide in vivo was performed by coadministration of 1 mg of each of the synthetic peptides per experiment.

**Vaccination Protocol.** To compare the immunogenicity of phage displaying lymph node-homing peptides (PTCAYGWCA or WSCARPLCG phage) with that of the insertless control phage (fd-tet phage), we defined phage vaccination as the i.v. injection of a phage clone into the tail vein of a female 2-month-old BALB/c immunocompetent mice. To avoid potential biases in the preparations before vaccination (15). Every experiment was performed with an independent phage preparation of each of the lymph node-homing phage clones and the negative control phage (fd-tet). We used increasing numbers of phage particles (from 10^6 to 10^8 TU, as indicated). Phage were administered to 2 mice/sample at 2-week intervals. The mice were bled 6 days after the first vaccination and 5 min before the lymph nodes and control organs were processed for phage recovery (5).

**Results and Discussion**

We have developed an in vivo screening method in which peptides homing to specific vascular beds are selected after i.v. administration of a phage display random peptide library (11–14). This work uncovered a vascular address system that allows organ-specific targeting to normal blood vessels and angiogenesis-related targeting to tumor blood vessels (16).

We hypothesized that the targeting of antigens to the vascular endothelium of lymphatic tissue may modulate the immune response. By using in vivo phage display technology (11), we selected phage clones that home to lymph nodes. In contrast to a control phage lacking a peptide insert (fd-tet phage), phage clones displaying lymph node-homing peptides showed preferential homing to axillary lymph nodes compared with homing to control organs after systemic i.v. administration in mice (Fig. 1).

To test our hypothesis, we used the phage particles themselves as defined antigens because they have long been established as strong immunogens (17). To compare the relative immunogenicity of each phage clone, we vaccinated mice with the two lymph node-homing phage or with the insertless control phage. Anti-phage antibody titers were determined after each phage vaccination by an ELISA. In three independent experiments performed, mice vaccinated with each of the lymph node-homing phage consistently had a significantly higher titer of anti-phage antibodies than did mice vaccinated with the control phage (Fig. 2).

We have determined the amount of peptide required to inhibit the homing of the phage to the lymph nodes based on titration experiments. Dose dependence was observed, attesting to specificity (data not shown). To show that the enhanced immune response is dependent on the targeting of the phage to the lymph nodes, we coadministered the cognate peptides displayed by the lymph node-homing phage; the synthetic peptides PTCAYGWCA or WSCARPLCG inhibited the homing of the corresponding phage to the lymph nodes and abrogated the enhanced immunogenicity. Anti-phage antibody serum titers in mice vaccinated with lymph node-homing phage alone were again...
significantly higher than the serum titers observed in mice vaccinated with fd-tet phage. However, the mice pretreated with the cognate synthetic peptides and then vaccinated with lymph node-homing phage had a serum titer similar to that of fd-tet-vaccinated mice; such inhibition of anti-phage antibody titers by i.v. coadministration of the corresponding synthetic peptides strongly indicates that these effects are specific (Fig. 3). The immune response against the phage particles was enhanced when the phage were targeted to the lymph nodes. When homing was blocked by coinjection of the cognate synthetic peptides, no modulation of the immune response to phage was observed. It has long been established that short synthetic peptides (less than at least 14 residues) are not immunogenic; therefore, they can be used as competitive inhibitors to block the phage homing to the lymph nodes. Taken together, our results show that the phenomenon is mediated by selective targeting of phage particles to the lymph nodes.

Modulating the immune response through systemic targeting of antigens to lymph nodes has the potential to lead to novel biotechnology applications. Recently, striking cytolytic responses against HIV-1 were observed by displaying viral peptide epitopes within the phage capsid (18); exploiting a combination of in vivo phage display-based strategies may improve vaccination and immunotherapy against infectious and malignant diseases.

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References

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