Induction of Human Breast Cancer-specific Antibody Responses in Cynomolgus Monkeys by a Murine Monoclonal Anti-Idiotype Antibody

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ABSTRACT

We have generated and characterized a murine monoclonal anti-idiotype (Id) antibody, designated 11D10, which biologically and antigenically mimics a distinct and specific epitope of the high molecular weight human milk fat globule primarily expressed by human breast and some other tumor cells at high density. This epitope is identified by mAb BrE1, which was used as the immunizing antibody or Ab1 to generate the anti-Id (Ab2) 11D10. 11D10 induced antitumor immune responses across species barriers, i.e., in mice and rabbits. In preclinical studies, cynomolgus monkeys were immunized with 11D10 and the idiotypic- and allotypic-matched control Ab 2H1 3H1 after precipitation with aluminum hydroxide. All monkeys developed high titers of antibodies against the immunizing mouse immunoglobulin. Immunization with 11D10 induced anti-anti-idiotype antibodies (Ab3) which reacted with breast cancer cell lines but not with control T-cell and melanoma cell lines. The Ab3 shared idiotypes with BrE1 (Ab1), as demonstrated by their ability to inhibit 11D10 binding to BrE1. The Ab3 obtained with 11D10 bound specifically to human milk fat globule antigen and competed with BrE1 for binding to breast cancer cell lines, suggesting that Ab1 and Ab3 may bind to the same epitope. In addition, Id-specific cellular immune responses were demonstrated in monkeys immunized with 11D10 by T-cell proliferation assays. These results indicate that aluminum hydroxide-precipitated anti-Id 11D10 can induce breast cancer-specific antibodies in nonhuman primates and can serve as a potential network antigen for breast cancer patients.

INTRODUCTION

Breast cancer is the leading cause of cancer deaths in women. Over the years the incidence of these neoplasms has steadily increased, and according to the National Cancer Institute statistics nearly 50,000 women will die of breast cancer in 1994. The goal of this study has been to develop a new immunotherapeutic approach for breast cancer with the use of the network antigen (1) which is based on the Id network concept. The idea of using anti-Id antibodies (Ab2) as antigen substitutes was first proposed by Jerne in his network hypothesis (2). Immunization with Ab2s that mimic the structure of external antigens has been found to induce active and specific immunity against viruses, parasites, and bacteria (reviewed in Ref. 3). Active immunotherapy with such anti-Id antibodies (Ab2B) has proven to be effective in modulating tumor growth in experimental systems (4, 5), and encouraging results have been obtained in recent clinical trials (6–8).

A series of murine mAbs that recognize components of the HMFG membrane have been described that are primarily associated with human breast carcinomas and not with most normal tissues (8, 9). Among these mAbs, BrE-1 is the most restricted and specific, reacting with a large molecular weight (Mr 400,000) mucin-like protein present at high density and on >80% breast cancer cells and minimally expressed on a few normal tissues, such as the epithelial lining of lung and kidney tubules (9, 10). We have used purified mAb BrE-1 to generate a mAb Id cascade (Ab1–Ab2–Ab3) for this system (11, 12). To this end, we have generated and characterized several murine monoclonal anti-Id antibodies that bind to the antigen-binding site of BrE1 (12).

One of these Ab2s, designated 11D10, induced specific antitumor responses in mice and rabbits. As a step toward a more clinically relevant model, we have investigated the effect of anti-Id 11D10 on the induction of breast cancer-specific humoral responses in cynomolgus monkeys (Macaca fascicularis). In this preclinical study, we have used the same adjuvant and dose of 11D10 which will be used subsequently in clinical trials. These studies, therefore, are likely to predict the safety and efficacy of this anti-Id to induce antitumor antibodies in breast cancer patients.

MATERIALS AND METHODS

Cell Lines

The human breast carcinoma cell line MCF-7, which expresses HMFG antigen, was grown in RPMI 1640 medium supplemented with 10% FCS, 1% l-glutamine, penicillin, and streptomycin, and was used for the detection of antitumor responses. The human melanoma cell line M211P6 (kindly provided by Dr. Ralph Reisfeld, Scripps Research Institute, La Jolla, CA) and the T-cell line MOLT-4, both of which are HMFG negative, were grown in the same medium and were used as negative controls.

Antibodies

The Ab1 mAb BrE1 (IgG2b, k), which recognizes a distinct and specific epitope on the Mr 400,000 HMFG molecule, was used to immunize syngeneic BALB/c mice for the production of anti-Id mAb 11D10 (IgG1-k). 11D10 is specific for an idiotope of BrE1 and behaves as an internal image (3). The mAb 2H1 (IgG1-k) is a murine anti-Id mAb which mimics the human CEA (13) and was used as a control.

Adjuvant

To augment the immunogenicity of the anti-Id vaccine an adjuvant is required. Aluminum hydroxide is approved by the United States Food and Drug Administration for use as an adjuvant in humans and will be used in our clinical trials. We therefore immunized monkeys in this preclinical study with Ab2 11D10 precipitated with aluminum hydroxide (6).

Aluminum Hydroxide Precipitation

Briefly, to 5-mg aliquots of purified mAb anti-Id (Ab2), 1 ml of 2% Alu-Gel S (Serva Fine Biochem, Inc., Garden City, Long Island, NY) was added. The volume was then adjusted to 10 ml with Dulbecco's-PBS and the mixture was incubated on a vortex for 1 h at room temperature. The mixture was then centrifuged at 2000 rpm at 24°C for 10 min. The amount of mAb bound in the gel layer was determined by measuring spectrophotometrically the amount of unbound antibody in the supernatant. The Alu-Gel-precipitated antibody was...
stored at 4°C until use. These procedures were performed aseptically in a laminar flow hood, and the final product was sterile and clearly labeled as anti-Id 11D10 Alu-Gel and aliquoted into pyrogen-free, sterile glass vials.

### Immunization of Monkeys

Cynomolgus monkeys were immunized with aluminum hydroxide-precipitated anti-Id 11D10, as well as with control aluminum hydroxide-precipitated anti-Id 3H1 (specific for CEA). Monkeys were housed at the White Sands Research Institutes (Alamogordo, NM). A pair of male and female monkeys, weighing 3–4 kg, was immunized with either 2 mg of 11D10 or 3H1 i.e. at four different sites on days 0, 14, 28, and 42, respectively. Only two monkeys were used for each anti-Id (Ab2) at a single dose for financial reasons. The 2-mg dose was selected based on previous preclinical and clinical studies with different anti-Id vaccines. Blood samples were collected before immunization and 10 days after each immunization.

### Development of Humoral Immunity Induced by Immunization with Aluminum Hydroxide-precipitated Ab2

**Specific Ab3 Response to Ab2.** Sera from immunized monkeys were tested for the presence of anti-anti-Id antibodies. Sera were preincubated with normal murine immunoglobulin to block monkey antibodies against isotopic and allotypic determinants and then checked for the presence of anti-anti-Id (Ab3) by reaction with the immunizing anti-Id (11D10) coated onto microtiter plates, by RIA. Unrelated Ab2 was used as the control. After washing, the antigen-antibody reaction was tagged with the use of 125I-labeled anti-Id reagent in a homogeneous sandwich RIA. Preimmune sera and sera from monkeys immunized with control Ab2 3H1 were also used in these assays. In addition, 125I-labeled monoclonal Ab2 3H1 was used as control.

**Idiotope Analysis of Ab3.** If a positive reaction is obtained in the method described above, Ab3 sera from those monkeys were checked for their ability to inhibit the binding of 125I-labeled 11D10 to BrE1 (Ab1) bound to microtiter plates or vice versa (inhibition of the binding of radiolabeled BrE-1 to 11D10 on the plate). An unrelated Ab1-Ab2 system was used as a control (13, 15). This demonstrated whether Ab3s in monkey sera share idiotopes with BrE1 (Ab1). This inhibition assay of Ab1-Ab2 binding by Ab3 sera also demonstrates whether Ab3 is a true anti-anti-Id.

**Binding of Ab3 to Tumor Antigen.** To assess humoral immune responses directed against native target antigens, monkey Ab3 sera were tested for reactivity with cell lines known to express HMFG in a RIA. The sera were checked for reactivity against a solubilized semipurified preparation of HMFG antigen coated onto microtiter plates. The antigen-antibody reaction was detected by using 125I-labeled anti-human immunoglobulin reagents or alkaline phosphatase labeled anti-human immunoglobulin in ELISA. Preimmune sera was used as a control. The unrelated CEA was also used as a control in this assay. The isotype of monkey Ab3 sera binding to HMFG antigen was determined by ELISA using anti-human isotype-specific reagents.

**Binding of Ab3 to tumor cell lines** was also checked by immune flow cytometry. Antigen-positive MCF-7 cells (1 × 10^6 per well) were reacted with Ab1 (BrE1) and Ab3 at 100 µl at 4°C for 60 min. After washing, the cells were incubated with either goat anti-mouse or goat anti-human F(ab')2 IgG-FITC labeled antibody (Tago, Burlingame, CA) for 30 min at 4°C. They were then washed twice, fixed in 2% paraformaldehyde, and analyzed by immune flow cytometry (FACStar, Becton Dickinson, San Jose, CA). Antigen-negative MOLT-4 cells were used as a control in this assay.

### Purification of Anti-anti-Id Antibody (Ab3) from Hyperimmunized Monkey Sera

Twenty ml of hyperimmune serum was passed over an immunoadsorbent column consisting of immunizing anti-Id immunoglobulin (11D10-IgG1) coupled to Sepharose 4B. Anti-anti-Id antibodies (Ab3) were eluted with 0.1 M glycine-hydrochloric acid buffer (pH 2.4) and neutralized to pH 7.0 with 3 M Tris. The eluted antibody was then passed over an immunoadsorbent column consisting of an unrelated isotype-allotype-matched anti-Id mAb coupled to Sepharose 4B to remove anti-isotype and antiallotypic activities. Antibody that passed through was concentrated and used as purified Ab3. The isotype of Ab3 was determined by ELISA using human anti-isotype-specific reagents (Tago).

### Epitope Analysis of Ab3.** To demonstrate that Ab3s generated in monkeys and Ab1 (BrE1) bind to the same antigenic determinant, inhibition of BrE-1 binding to the antigen-positive tumor cell line MCF-7 or HMFG antigen by purified Ab3 was checked by RIA as described (13).

### Slot Blot Analysis of Purified Ab3 with HMFG

Polyvinylidene difluoride membrane was activated in methanol for 5 min and transferred to 0.02 M PBS, pH 7.0. Different concentrations of proteins (5 µg, 2 µg, 1 µg) were adsorbed on the membrane using the Hybrislot instrument (BRL Life Technologies, Gaithersburg, MD). The membrane was then blocked with 2% BSA in PBS for 2 h with shaking, followed by incubation with 5 ml of a solution of 20 µg/ml Ab1 or Ab3 for 3 h with shaking. After that, the membranes were washed 5 times with 1% BSA in PBS and incubated with alkaline phosphatase-labeled goat anti-human immunoglobulin or goat anti-mouse immunoglobulin (1:100 dilution) for 90 min, washed, and developed with substrate supplied in the Bio-Rad kit.

### Assay for Id-specific Proliferative Response

Fresh peripheral blood mononuclear cells were isolated by standard Ficoll-Hypaque density gradient centrifugation methods, and 5 × 10^5 cells/well were incubated with different concentrations of 11D10 and control 3H1 (10 ng to 2 µg) in RPMI 1640 medium with 5% heat-inactivated FCS, penicillin, and streptomycin. The nonisotypic mitogen phytohemagglutinin-P was used as a positive control at 2 and 1 µg/well. After the cells were incubated for 5 days at 37°C in an atmosphere containing 5% carbon dioxide, they were pulsed with [³H]thymidine (1 µCi/well) for 20 h. Data are expressed as mean counts (triplicate wells)/min of [³H]thymidine incorporation. The SD of the data was <10% for each determination.

### RESULTS

#### Induction of Anti-anti-Id (Ab3) Responses in Monkeys

The sera from monkeys were obtained 10 days after the fourth immunization and analyzed for Ab3 responses by sandwich RIA and inhibition of Ab2 binding to Ab1 (Table 1). For these assays, the sera were pretreated with normal mouse immunoglobulin (500 µg/ml) to block anti-isotypic and antiallotypic reactivities. Ab3 sera from monkeys

### Table 1 - Analysis of monkey anti-anti-Id sera generated with anti-Id mAb 11D10

<table>
<thead>
<tr>
<th>Assay</th>
<th>Plate coated with</th>
<th>125I-labeled anti-Id</th>
<th>Sample</th>
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<tr>
<td></td>
<td></td>
<td>11D10</td>
<td>PRO 723</td>
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<tr>
<td>Sandwich RIA</td>
<td>11D10 (Ab2)</td>
<td>12,913*</td>
<td>PRO 872</td>
</tr>
<tr>
<td></td>
<td>3H1 (unrelated Ab2)</td>
<td>301</td>
<td>Preimmune sera (pool)</td>
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<tr>
<td></td>
<td>11D10 (Ab2)</td>
<td>1,074</td>
<td></td>
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<tr>
<td>Inhibition</td>
<td>BrE-1 (Ab1)</td>
<td>91</td>
<td>PRO 541</td>
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<td>95</td>
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*Ab3 sera obtained after the fourth immunization was diluted with PBS containing normal mouse immunoglobulin (500 µg/ml). The sera were preincubated with normal mouse IgG prior to the assay. Ab3 was a 1:10 dilution was incubated with anti-Id mAb 11D10 or 3H1, coated on the microtiter plate, and then reacted with 125I-labeled 11D10 or 3H1 (50,000 cpm) in a sandwich assay. The results are expressed as bound cpm in a sandwich assay. The results are presented as mean cpm (n = 3). The SD of the data was <10%.

*For the binding inhibition assay between Ab2 and Ab1, purified Ab1 BrE-1 was used to coat the plate (250 ng/well), and the binding of radiolabeled Ab2 to Ab1 was tested in the presence of different dilutions of Ab3 sera. The sera were preincubated with normal mouse IgG prior to the assay. The results are expressed as percent inhibition at a dilution of 1:40.

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(PRO 723 and PRO 872) immunized with 11D10 bound specifically to the immunizing Ab2 (11D10) with minimal reactivity with unrelated Ab2 (3H1). Monkey Ab3 sera also inhibited the binding of radiolabeled Ab2 to Ab1 by 91 and 95%, respectively, even at a dilution of 1:40 (Table 1). There was no inhibition with preimmune sera or sera obtained from monkeys (PRO 541 and PRO 667) immunized with the unrelated Ab2 3H1. The kinetics of the Ab3 response are shown in Fig. 1 using sera from monkey PRO 723, demonstrating inhibition of the binding of radiolabeled Ab1 to Ab2. Similar reactivity was seen with sera from monkey PRO 872. These results indicate that monkey Ab3 sera share idiotypes with the Ab1.

**Induction of Antitumor Cell Antibody Response.** To determine whether 11D10 immunized monkey sera bound specifically to HMFG-positive breast carcinoma cells, the binding of monkey Ab3 sera to the breast cancer cell line MCF-7 was tested by ELISA. As shown in Fig. 2, Ab3 sera, obtained after the fourth immunization at different dilutions, reacted with MCF-7 cells but not with the antigen-negative melanoma cell line M21/P6. The Ab3 sera also bound specifically to semipurified HMFG coated onto microtiter plates by ELISA (Fig. 3). Control sera from preimmune monkeys or monkeys immunized with unrelated Ab2 (3H1) did not show appreciable binding to HMFG. In parallel experiments, the same Ab3s from monkey PRO 723 were compared on a plate coated with CEA and were negative.

To determine the reactivity with cell surface HMFG, MCF-7 cells were tested by immune flow cytometry. As shown in Fig. 4, Ab3 from Ab2-immunized monkeys showed distinct binding (Fig. 4A) that was similar to the binding pattern obtained with Ab1 (not shown). Significant binding was not obtained with MOLT-4 cells which do not express HMFG (Fig. 4B).

The Ab3 antibodies were then purified from sera as described in "Materials and Methods." The reactivity of purified Ab3 was checked by ELISA (Fig. 5). Monkey Ab3 reacted specifically with HMFG coated onto microtiter plates, whereas no reactivity was obtained with control CEA-coated plates.

The specificity of purified Ab3 for HMFG was further confirmed by Slot blot analysis (Fig. 6). In Fig. 6, Lanes 1 and 2 were coated with semipurified HMFG, and Lanes 3 and 4 were coated with purified CEA; reactivity with HMFG and not with CEA was demonstrated.

**Competition of Murine Ab1 and Monkey Ab3 for MCF-7 Cell Binding.** If Ab3 has a similar binding site as Ab1, it should compete with Ab1 for binding to HMFG on MCF-7 cells. A fixed amount of

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**Fig. 1.** Inhibition of Ab1 (mAb BrE-1) binding to Ab2 (11D10) by monkey (PRO 723) Ab3 sera by RIA. Purified 11D10 was used to coat the plate (250 ng/well), and the binding of radiolabeled BrE-1 (~50,000 cpm) to 11D10 was tested for inhibition in the presence of different dilutions of Ab3 sera. In a parallel control experiment, an unrelated Ab1-Ab2 system (mAb 8019-3H1) was used as the control.

**Fig. 2.** Binding of monkey Ab3 sera to the breast cancer cell line MCF-7 by ELISA. Ab3 sera at different dilutions were added to confluent monolayers of cells grown in 96-well microtiter plates and developed with goat anti-human IgG (H and L chain specific) enzyme-labeled antibodies. The absorbance (OD) was read after 1 hr. Monkey PRO 723 was immunized with Ab2 11D10. Monkey PRO 667 was immunized with control unrelated Ab2 (3H1). Preimmune monkey sera was also used as control. Binding of monkey Ab3 sera (..) to the melanoma cell line M21/P6 by ELISA was tested following the same protocol. The results are presented as the mean absorbance at 405 nm (n = 3). The SD of the data was <10% for each assay.

**Fig. 3.** Binding of monkey Ab3 sera to semi-purified HMFG by ELISA. The plate was coated with HMFG (2 μg/well) and reacted with Ab3 sera (1:100 dilution) from the monkey (PRO 723) immunized with Ab2 11D10 and from monkey (PRO 667) immunized with unrelated Ab2 3H1 of the same isotype and allotype. Preimmune sera and PBS-BSA were also used as controls. The results are presented as the mean absorbance at 405 nm (n = 3). The SD of the data was <10%. The differences between experimental and control values were statistically significant.

**Fig. 4.** Reciprocal inhibition of Ab1 binding to Ab2 by monkey Ab3 sera in immune flow cytometry. The flow cytometer blot analyser was used to evaluate the binding of Ab1 to Ab2. The results are presented as the mean percentage of inhibition at 405 nm (n = 3). The SD of the data was <10%.
Toxicity. The induction of Ab3 responses in monkeys did not cause any apparent side effects in animals. Only mild local swelling and irritation were observed at the injection site as a result of multiple immunizations. The monkeys were routinely checked by physical examinations and weight measurements. They did not show any signs of abnormalities.

DISCUSSION

This study was conducted to further assess the efficacy of anti-Id 11D10 to generate antitumor immune responses in a more clinically relevant model. Aluminum hydroxide precipitation, although considered weakly immunogenic, was a suitable adjuvant for eliciting immune responses. Perhaps cross-linking of soluble idiotypic determinants by aggregation or precipitation helped to increase its antigenicity (16). Also, anti-Id 11D10 was used as an intact immunoglobulin and it is likely that the Fc portion of the murine immuno-

radiolabeled Ab1 was coincubated with different amounts of purified Ab3 or control Ab3 preparations and MCF-7 cells (Fig. 7). One hundred ng of purified Ab3 inhibited binding by 60%, and 1 μg of purified Ab3 gave over 80% inhibition, whereas the control Ab3 used at a 5-μg concentration did not produce any inhibition. These results indicate that Ab2-immune monkey antibody binds to the same antigen as Ab1; therefore, the Ab3 preparation contains antibody molecules with Ab1' properties.

Cellular Responses to Anti-Id. Cellular immune responses were measured by the proliferation of peripheral blood mononuclear cells incubated with aluminum hydroxide-precipitated anti-Id antibody (11D10) and aluminum hydroxide-precipitated control anti-Id antibody (3H1). Positive proliferative responses were seen in both monkeys PRO 872 and PRO 723 (Fig. 8) that were nearly comparable to the responses to the mitogen phytohemagglutinin-P. Preimmune cells had no proliferative response to the anti-Id antibody, while postimmune cells had a significant response. There was also a minor but significant response to the isotype-matched 3H1 anti-Id antibody that was significantly less than that of the 11D10 response; this likely represents a response to the non-Id components of the immunoglobulin molecule. Proliferative responses were first noted after the third injection, and similar reactivity was obtained after the fourth immunization. Due to a limited supply of blood, we could not test T-cell proliferation in the presence of semipurified HMFG antigen and thus establish the antigen specificity of the cellular immune responses.

induced in the lymphocytes, as well as the phenotypes of these lymphocytes.
globulin molecule served as a "carrier" to help promote the immune responses. The results described here demonstrate that a murine anti-Id mAb, 11D10 raised against a HMFG-specific mAb BrE1, can induce a specific anti-HMFG humoral response in cynomolgus monkeys. The antibody concentration was quite high, from 30 ml of immune serum about 1.32 mg of purified Ab3 was recovered (44 μg/ml serum).

As little as 100 ng of this purified Ab3 was able to inhibit the binding of >60% of radiolabeled Ab1 to the HMFG-positive breast cancer cell line MCF-7. We were unable to quantitate the true Ab1' concentration (i.e., antibody that bound to HMFG) because we did not have enough semipurified HMFG for an immunoaffinity column to purify the Ab1'. However, the results demonstrated in Fig. 7 suggested that a major portion of the purified Ab3 was Ab1-like in nature.

Semipurified HMFG is available in small quantities from several sources and can be used in serological assays (17). However, HMFG is extremely difficult to isolate and purify, and purified HMFG is not available for patient immunization or animal studies. Inasmuch as some of the epitopes on HMFG are shared by normal tissues, specifically by nonpenetrating glycoproteins, immunization with intact HMFG molecule might trigger potentially harmful autoimmune reactions, whereas an Ab2β generated against an anti-HMFG mAb that recognizes a HMFG-specific epitope such as that developed in our laboratory would be theoretically safer and more effective. Furthermore, an Ab2β which is expressed in a different molecular environment can overcome host immunosuppression by stimulating "silent clones," and/or allowing active T-cell help (18, 19). Therefore, an anti-Id would be an excellent candidate to induce antitumor immunity in BrE-1 antigen-positive breast cancer patients.

In addition to humoral immune responses, Id-specific cellular immune responses were also noted in these animals by T-cell proliferation assays. Previously, in 11D10-immunized rabbits we demonstrated some delayed type hypersensitivity skin reactions against a semipurified preparation of HMFG as opposed to a purified CEA vaccine. Peripheral blood mononuclear cells obtained from monkey PRO 723 were stimulated in vitro with 11D10 (1 μg/ml); control, unrelated Ab2 3H1 (1 μg/ml). Similarly, peripheral blood mononuclear cells obtained from monkey PRO 872 were stimulated in vitro with 11D10 (1 μg/ml); unrelated Ab2 3H1 (1 μg/ml). Stimulation was measured by the degree of incorporation of a pulse of [3H]thymidine. Culture medium without any antigens was also used as a control. The postimmunization peripheral blood mononuclear cells were collected 10 days after the third immunization. The results are expressed as mean cpm of triplicate wells. The SD of the data was <10% for each determination. The differences between experimental and control values were statistically significant.

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One other concern is that we do not know whether cynomolgus monkeys contain trace amounts of HMFG in their serum or tissues, whereas HMFG will be present in breast cancer patients. However, from our own experience with the human CEA system, we know that anti-Id mimicking CEA in the presence of aluminum hydroxide was able to break tolerance and induced CEA-specific immune responses in advanced colorectal cancer patients who had high levels of circulating CEA (20). The information obtained from this study may have immediate relevance to the design and implementation of clinical trials. Recently, we have obtained an Investigational New Drug application from the United States Food and Drug Administration for 11D10. The potential role of this internal image anti-Id to induce humoral and/or cellular immune responses in patients with breast cancer can thus be investigated directly in a clinical trial.

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5 Unpublished data.
ANTI-IDIOTYPE MIMICKING A BREAST CANCER ANTIGEN IN MONKEYS

REFERENCES


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