

Antitumor Effect of the Idiotypic Cascade Induced by an Antibody Encapsulated in Poly(*d,l*-lactide-*co*-glycolide) Microspheres

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A major difficulty encountered during development of antibody vaccines is their weak immunogenicity. In this study, a monoclonal antibody CS20.5 to human breast cancer antigen CA15.3 was coencapsulated in poly(*d,l*-lactide-*co*-glycolide) microspheres with monophosphoryl lipid A. The antitumor effect of this formulation was investigated in a murine model. The induced Ab2 biologically mimics antigen as it competed with CA15.3 for the same idiotope on Ab1. Ab3 induction was also observed. After five sequential administrations of encapsulated antibody, mice showed statistically significant tumor regression. These results indicate that this formulation may serve as a potential treatment for breast cancer.

Key words: Anti-idiotype — Microsphere — Breast cancer

Drug delivery systems are increasingly becoming a fundamental component of pharmaceutical development. This issue is particularly important for proteins because of their remarkably short half-lives *in vivo*. A controlled-release system can protect proteins from degradation and introduce new therapies for prolonged clinical use. Recently, biodegradable poly(*d,l*-lactide-*co*-glycolide) (PLGA) microspheres have been widely investigated for this purpose. Studies have shown that microsphere-based vaccines could induce both humoral and cellular immune responses.^{1,2} In addition, the use of microspheres can dramatically reduce the amount of vaccine protein required to achieve a therapeutic effect.³ The present study was undertaken to develop a new immunotherapeutic approach for breast cancer with the use of microspheres.

Although chemotherapy has been extensively used for breast cancer, poor quality of life is always associated with this treatment. Immunotherapy based on immune network theory, however, is an alternative to avoid this problem. In accordance with the idiotypic network hypothesis, the infused antitumor antibody (Ab1) elicits an anti-idiotypic response against the idiotype of Ab1. Part of the variable region of anti-idiotypic antibodies (Ab2 β) resembles the nominal tumor antigen, which subsequently induces an anti-anti-idiotypic response conferring immunity against the nominal tumor antigen. It has been proposed that the idiotypic network responses, induced by mAb treatment, could be of importance for eradication of tumor cells.⁴ Active immunotherapies with Ab1 have afforded encouraging results in recent clinical trials.⁵ However, induction of Ab2 β has proven to be less effective in modulating

tumor growth in experimental systems. This might be due to the lack of T cells induced by Ab2. Hence induction of anti-tumor T cells through the idiotypic network is an even more important effector function.⁶

Our previous research showed that the combination of PLGA microspheres and monophosphoryl lipid A (MLA) could enhance T cell response as well as humoral response.⁷ Therefore, in this report, we coencapsulated anti-breast cancer cell antibody CS20.5 and MLA into PLGA microspheres. The anti-tumor effect of this formulation was observed in a murine tumor model.

MATERIALS AND METHODS

Antibody and polymers CA15.3 is elevated in approximate 75% of patients with metastatic breast cancer.⁸ The Ab1 mAb CS20.5 (IgG1), which was raised against CA15.3 (Calbiochem, La Jolla, CA), was used to immunize mice for the induction of the immune network. To augment the immunogenicity of mAb CS20.5, the biodegradable PLGA 50/50 polymer (BPI, Birmingham, AL) was used for Ab-microencapsulation. Monophosphoryl lipid A (RIBI ImmunoChem. Res., Inc., Hamilton, MO) was selected as the adjuvant to enhance T cell response. Fab fragments of mAb CS20.5 were prepared by papain digestion as described elsewhere.⁹

Preparation of microspheres A water-in-oil-in-water emulsion/solvent evaporation method, as we reported before, was used to prepare PLGA microspheres containing mAb CS20.5.⁷ Briefly, 60 μ l of mAb CS20.5 (3 mg/ml) was emulsified with PLGA (100 mg) and MLA (200 μ g) in 440 μ l of chloroform for 15 s using a sonicator homogenizer (Heat Systems, Inc., Farmingdale, NY). Then 2 ml of 9% poly(vinyl alcohol) (PVA, Aldrich, Mil-

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waukee, WI) was added and the mixture was sonicated for another 30 s. The emulsion was poured into 8 ml of 9% PVA and stirred gently for 2 h. The microspheres were washed with water and collected by centrifugation at 15 000 rpm for 10 min. The microspheres averaged approximately 5 μm in diameter and contained an antibody load of 3 μg of Ab/1 mg of microspheres.

Immunization of tumor-bearing mice 413BRC, which is a mouse mammary cell line transfected with human *MUC-1* gene was kindly provided by Dr. El-Nazir Lalani (Dept. of Histopathology, Royal Postgraduate Medical School, London). Twenty BALB/c mice were transplanted subcutaneously (s.c.) with 2.5×10^6 413BRC cells and separated into 5 treatment groups: PBS, mAb CS20.5 or MOPC-21 (an irrelevant mouse Ab, Sigma, St. Louis, MO) conjugated with keyhole limpet hemocyanin (KLH) by the method described by Jennemann *et al.*,¹⁰ mAb CS20.5 or MOPC-21 and MLA coencapsulated in microspheres. One week after tumor transplantation, the mice received the first treatment s.c. Thereafter, two intraperitoneal (i.p.) injections, one s.c. and one i.p. injection were processed. The interval between treatments was 7 days. Thirty micrograms or 15 μg of Ab in 0.2 ml of phosphate-buffered saline (PBS) was injected s.c. or i.p., respectively.

Blood samples were collected at certain time points: before immunization, a week after the first immunization, two weeks after the second immunization and two weeks after the fourth immunization. Tumor volumes were measured with a precision caliper and calculated according to the following equation.

$$\text{volume} = (\text{smaller diameter}^2 \times \text{larger diameter} \times \pi) / 6^{11)}$$

Tumor volume was used as the end point for statistical analysis of immunotherapeutic response. Mice were terminated when the tumor size reached 1 cm^3 .

Humoral immunity induced by immunization with encapsulated mAb CS20.5

1. Specific Ab2 response to Ab1: Sera from immunized mice were tested for the presence of Ab2 in an ELISA. Briefly, 96-well Maxisorp plates (Nunc, Roskild, Denmark) coated with 125 ng/well of Fab fragments of mAb CS20.5 were incubated with 100 μl of sera (1/100 dilution) for 1 h at room temperature. Then the plates were washed and filled with 100 μl of peroxidase-labeled goat anti-mouse Ig (Southern Bio. Assoc., Inc., Birmingham, AL). After 1-h incubation, the plates were rinsed and developed with 100 μl of Peroxidase Substrate Solution (ABTS). The absorbance was measured on a microplate reader at 405 nm.

To estimate the specificity of Ab2 obtained, an ELISA inhibition assay was performed using day 21 serum from mice immunized with encapsulated mAb CS20.5. The method used was identical to that mentioned above except

that a mixture of serum and CA15.3 (at various concentrations) was added to plates for the first incubation. CA125 was selected as the irrelevant control.

2. Binding of Ab3 to tumor antigen: Ab3 was assayed in ELISA as described above except that plates were coated with CA15.3 (0.35 U/well).

3. Isotype analysis of Ab2 and Ab3: For isotype detection, ELISA was carried out by using peroxidase-labeled goat anti-mouse IgM, IgG1, IgG2a, IgG2b or IgG3 (Southern Bio. Assoc., Inc.) as second antibodies. The rest of the assay procedure was the same as described above.

In vivo release studies The release of antibody from PLGA microspheres *in vivo* was evaluated in BALB/c mice. Goat anti-rabbit IgG (Sigma) encapsulated in PLGA microspheres was administered following the same schedule and dosage as for immunization. The sera of mice were collected at certain time points to detect goat antibodies released from microspheres by ELISA. Briefly, the Maxisorp plates were coated at 4°C overnight with 100 μl of normal rabbit serum (1/100 dilution). The wells were blocked with 3% bovine serum albumin (BSA)-PBS, 100 μl of mouse serum (1/100 dilution) was added to each well, and the plates were incubated for 1 h at room temperature. Then the plates were washed and reacted with peroxidase-labeled mouse anti-goat IgG (Sigma) for 1 h at room temperature. The wells were washed again and developed by adding 100 μl of ABTS. The absorbance was measured at 405 nm.

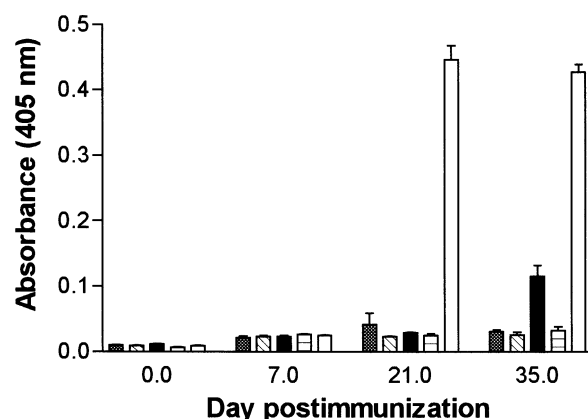


Fig. 1. The effect of PLGA microsphere delivery of antibody on Ab2 production. Mice were immunized with PBS (▨), MOPC-21 conjugated with KLH (▩), mAb CS20.5 conjugated with KLH (■), MOPC-21 and MLA coencapsulated in microspheres (□), or mAb CS20.5 and MLA coencapsulated in microspheres (◻). Serum samples (1:100 dilution) collected on day 0, 7, 21 and 35 after the first immunization were analyzed for Ab2 production by ELISA. The data represent mean absorbance \pm SD.

RESULTS

Induction of anti-idiotypic and anti-anti-idiotypic antibodies None of the mice showed an anti-idiotypic or anti-anti-idiotypic response before receiving treatment (Figs. 1 and 4). After two immunizations, mice that had received encapsulated mAb CS20.5 and MLA showed a positive Ab2 response. In one week, the post-treatment value of Ab2 was 10 times higher than the pretreatment value and clearly above the values of IgG1 groups. Moreover, comparing the mAb CS20.5 encapsulated group with the mAb

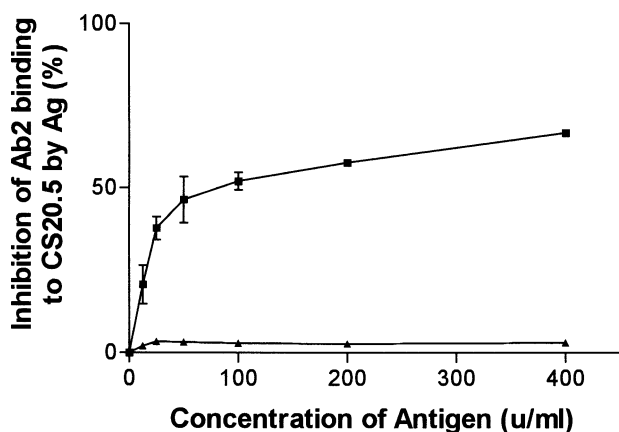


Fig. 2. The inhibition of Ab2 binding to Fab of mAb CS20.5 by CA15.3. Serum sample (1:100 dilution) collected on day 21 from mice immunized with mAb CS20.5 coencapsulated with MLA in microspheres was used to detect the binding of CA15.3 (■). An irrelevant antigen CA125 was selected as control (▲).

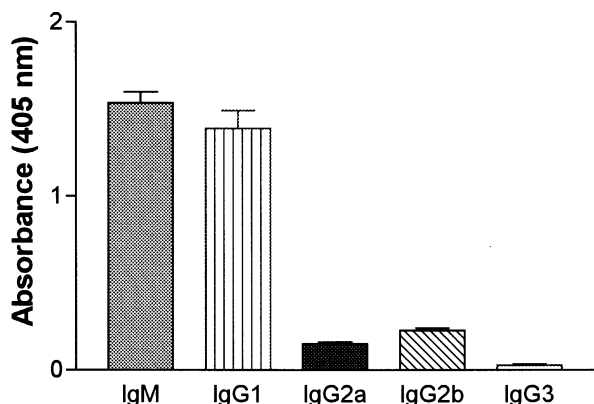


Fig. 3. Ab2 isotypes. Day 21 serum sample (1:100 dilution) from mice immunized with mAb CS20.5 coencapsulated with MLA in microspheres was analyzed for Ab2 isotypes by ELISA. The data represent mean absorbance±SD.

CS20.5 conjugated group, the immunogenicity of the former was 4 times that of the latter after 5 immunizations.

It is worth noting that Ab2 should compete with CA15.3 for binding to the same idiotope on mAb CS20.5 if it contains a similar structure to the antigen. As shown in Fig. 2, the inhibition of Ab2 to Ab1 was CA15.3 dose-dependent and as little as 100 U/ml of CA15.3 was able to inhibit binding by 50%. The irrelevant antigen CA125 could not inhibit the binding of Ab2 to Ab1. This result indicates that Ab2 shares the same binding site as CA15.3. The isotype of this Ab2 was a combination of IgM and IgG1, whereas little of IgG2a and IgG2b was observed (Fig. 3).

After three immunizations, detectable Ab3 reacting with CA15.3 was observed in the encapsulated mAb CS20.5 group, whereas no reactivity was obtained in other groups. Ab3 appeared one week after Ab2 (Fig. 4). The isotype of Ab3 was determined to be IgM (Fig. 5).

Release of antibody from microspheres *in vivo* Since the presence of free Ab1 in serum can interfere with the detection of Ab3, an *in vivo* release experiment was performed. As shown in Fig. 6, there was no detectable Ab released in mouse serum. This result assured us that the Ab appearing in mouse serum and recognizing CA15.3 was Ab3.

Antitumor effect of mAb CS20.5 encapsulated in microspheres A trend for tumor regression was noted in mice treated with mAb CS20.5 coencapsulated with MLA

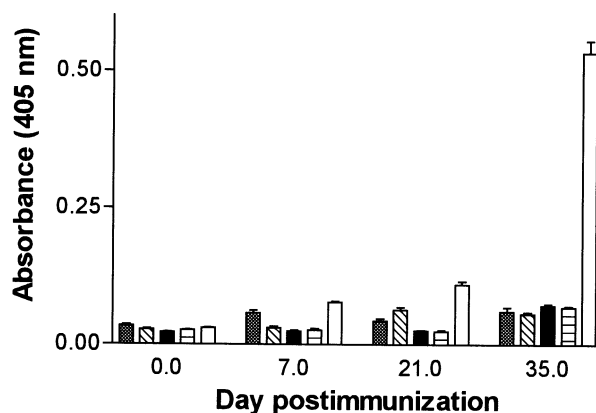


Fig. 4. The effect of PLGA microsphere delivery of mAb CS20.5 on Ab3 production. Mice were immunized with PBS (▨), MOPC-21 conjugated with KLH (▩), mAb CS20.5 conjugated with KLH (■), MOPC-21 and MLA coencapsulated in microspheres (▧), mAb CS20.5 and MLA coencapsulated in microspheres (□). Serum samples (1:100 dilution) collected on day 0, 7, 21 and 35 after the first immunization were analyzed for Ab3 production by ELISA. The data represent mean absorbance±SD.

in microspheres ($P < 0.05$), while mice in other treatment groups had no tumor response compared to the PBS group (Fig. 7). Six weeks after tumor inoculation, the tumor reached the highest volume in all other control groups and the mice were put down. Among mice given microspheres coencapsulated with mAb CS20.5 and MLA, the tumors of two mice continued to grow, though the growth rate was significantly slowed down. The mice were put down at seven weeks after tumor inoculation. Tumors in two other mice of this treatment group had collapsed to a quarter of their volume at seven weeks after inoculation and reached almost unmeasurable dimensions later. Eight weeks after the first immunization, two mice of this group cleared their tumors and remained disease-free.

DISCUSSION

Microsphere technology developed for reliable, preprogrammed release of antigen for immunization has the potential benefits of enhancing the immune response and reducing the antigen dose required to achieve immune protection.¹²⁾ Reports by ourself and others described immunopotentiality by microspheres in anti-idiotypic induction for immunotherapy of cancer and infectious disease.^{1,3)} The present study was conducted to further assess the anti-tumor efficacy of Ab1 coencapsulated with MLA in microspheres on a murine model.

When specific Ab1 was delivered *in vivo*, a number of direct and indirect actions may take place in terms of tumor growth.¹³⁾ Within the idiotype network, the presence of Ab2 β , which mimics Ag but is expressed in a different molecular environment, can overcome host immunosuppression by stimulating "silent clones." Thus, induction of Ab2 β is one goal of antibody therapy. In clinical research,

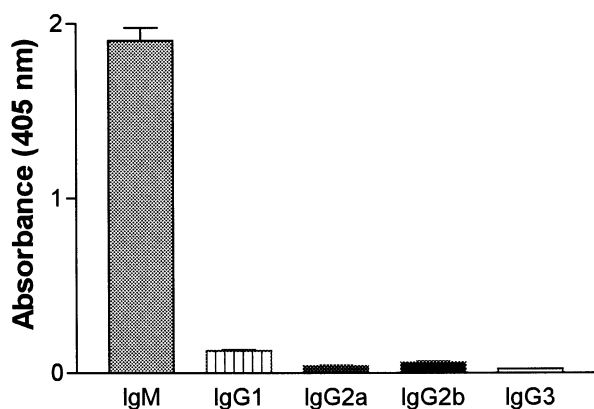


Fig. 5. Ab3 isotypes. Day 35 serum sample (1:100 dilution) from mice immunized with mAb CS20.5 coencapsulated with MLA in microspheres was analyzed for Ab3 isotypes by ELISA. The data represent mean absorbance \pm SD.

Ab2 has been induced by mAb treatment in cancer patients and was suggested to be of benefit for the patients.¹⁴⁾ The optimum approach to enhance Ab2 production by mAb is to utilize the effects of both microspheres and adjuvant. Following immunization with mAb CS20.5 coencapsulated with MLA in microspheres, the immune

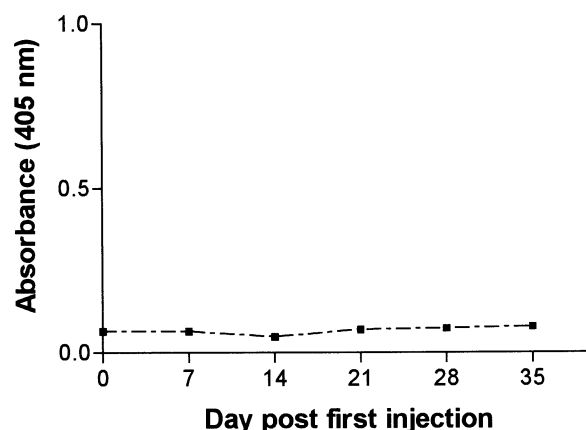


Fig. 6. Release of goat anti-rabbit IgG from microspheres *in vivo*. Three mice were injected (day 0, s.c.; day 7, i.p.; day 14, i.p.; day 21, s.c.; day 28, i.p.) with goat anti-rabbit IgG encapsulated in microspheres. The serum samples (1:100 dilution) collected on days 0, 7, 14, 21, 28 and 35 were analyzed for Ab presentation by ELISA. The data represent mean absorbance \pm SD.

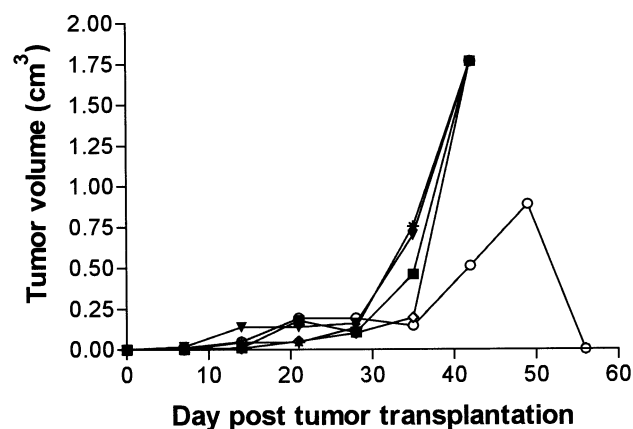


Fig. 7. Therapeutic effect of mAb CS20.5 coencapsulated with MLA in PLGA microspheres monitored in terms of tumor growth. Mice were immunized (day 7, s.c.; day 14, i.p.; day 21, i.p.; day 28, s.c.; day 35, i.p.) with PBS (■), MOPC-21 conjugated with KLH (*), mAb CS20.5 conjugated with KLH (▼), MOPC-21 and MLA coencapsulated in microspheres (◇), mAb CS20.5 and MLA coencapsulated in microspheres (○) starting at one week after tumor inoculation.

response observed was as expected. A significantly high level of Ab2 in Ab-microsphere-treated mice was noted. The high Ab2 production was most likely due to the effect of sustained release of Ab from microspheres.^{15–18)} Thus, Ab can be retained for a long period of time *in vivo*. In addition, microspheres sized between 1.2 to 7.0 μm could be taken up by antigen-presenting cells.¹⁹⁾ Therefore, a combination of prolonged release and phagocytosis is a possible mechanism of immunopotentiality by microspheres.

The structural basis for the molecular mimicry of Ab2 β to Ag may be shared primary sequences or may lie at the conformational level, involving either V_L or V_H determinants.²⁰⁾ The binding activity of Ab2 to Fab fragments of mAb CS20.5 was inhibitable by CA15.3, indicating that there were antibodies against the variable regions of mAb CS20.5, i.e., true anti-idiotypic antibodies.

Ab2 β resembling the native antigen can elicit an anti-anti-idiotypic immune response (Ab3).²¹⁾ Induction of Ab3 recognizing the nominal antigen has been proposed to correlate favorably with the clinical outcome of mAb-exposed cancer patients. Fagerberg *et al.* showed that patients with a high increase in Ab3 induced by the Ab1 therapy lived significantly longer than patients with no or a low level of Ab3.⁵⁾ The Ab3 production after microsphere-encapsulated Ab1 therapy is in accordance with our previous report.⁷⁾ However, argument about how to distinguish Ab3 from Ab1 is always an issue in idiotypic treatment. Therefore, an *in vivo* release experiment became necessary. No detectable circulating Ab1 was found when the blood samples for Ab3 analysis were drained. Isotype analysis of Ab3 also indicates that it was IgM instead of IgG1, which was the isotype of Ab1.

In the therapeutic effect study, the Ab1-microsphere treatment group clearly exhibited reduced tumor growth compared with other groups. Complement-dependent cytol-

ysis by Ab3 probably takes place as a mechanism of tumor lysis.²²⁾ Specific tumor destruction was suggested to happen in mAb therapy depending on the Ig class of the Ab3.²³⁾ The pentameric structure of IgM allows more effective complement binding on the tumor membrane, where cell lysis is induced. Activation of cells that release cytokines such as tumor necrosis factor (TNF) and INF- γ may also affect tumor lysis. MLA promotes INF- γ production by T cells and TNF production by macrophages.²⁴⁾ The fine specificity of the T cell response in this study was not evaluated because mice were terminated at different time points. However, a similar experiment we performed before showed the T cell response against idiotypic determinants.⁷⁾ A recent study in our laboratory found that T3, induced by Ab2, could lyse tumor cells (unpublished data). Therefore, T cells might be of therapeutic benefit in the present system as well. The relation between tumor regression and T cell response needs further study.

Overall, we have observed a correlation between induced immune response and tumor growth dependent on the nature of treatment in tumor-bearing mice. Our results indicate that PLGA microspheres may be a suitable Ab1 delivery system for eliciting immune responses. The high level of immune response exhibited *in vivo* encourages the use of microspheres for clinical studies.

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