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14. ABSTRACT

A humoral immune response to breast cancer cells is generally not therapeutically effective, likely due, at least in part, to overexpression of complement inhibitors expressed on tumor cell surfaces. Here we proposed to investigate two novel fusion proteins aimed at overcoming complement inhibition of breast tumor cells. During year one it was proposed to construct plasmids encoding MUC1 and MUC1-C3d; express and purify MUC1, MUC1-C3d and the recombinant fusion protein CR2-Fc; characterize CR2-Fc in vitro; and perform the MUC1 vaccination study. Construction and purification of MUC1 and MUC1-C3d and the MUC1 vaccination study has been achieved. A small, but significant, increase in the immune response was seen in mice vaccinated with MUC1-C3d. This indicates that C3d is functioning as an effective molecular adjuvant when linked to a tumor associated self antigen. Further experiments are needed to determine if this effect is protective and to possibly further increase the immune response if it is not. Technical difficulties have delayed expression and purification of mouse CR2-Fc, however efforts are underway to construct a new plasmid and in vitro characterization will proceed as soon as the problem is resolved.

15. SUBJECT TERMS
complement, immunotherapy, monoclonal antibody

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Introduction

A humoral immune response to breast tumor cells is generally not therapeutically effective. Breast and other cancer cells are resistant to antibody responses, both naturally occurring and monoclonal antibody therapy, at least in part due to complement inhibitory proteins expressed on the tumor cells surface. It is hypothesized that overcoming the complement resistance of breast tumor cells will significantly enhance the outcome of monoclonal antibody therapy. Our goal is to investigate novel approaches to enhance antibody therapy of breast cancer based on amplifying complement activation. Specifically, we proposed to prepare and characterize two types of novel fusion proteins aimed at modulating complement to enhance the humoral immune response.

Expression and Purification of CR2-Fc (Tasks 2 and 3)

Following several attempts to isolate and purify mouse CR2-Fc, it was determined that the original cell line transfected with the plasmid encoding mouse CR2-Fc (IgG2a) was not accurately expressing the recombinant protein. Due to this technical difficulty construction of a new plasmid encoding mouse CR2-Fc for expression was necessary. A new construct was designed and is currently being prepared. Once a stable cell line is reestablished the recombinant protein will be expressed and purified. Experiments to characterize mouse CR2-Fc in vitro and analyze its effect on the immune response will then proceed as originally planned.

MUC1 vaccination study (Tasks 1 and 4)

Protein constructs of five tandem repeats of MUC1 (MUC1/TR5) and five tandem repeats of MUC1 linked to three repeating units of C3d (MUC1/TR5-C3d) for vaccination studies have been prepared (Figure 1), expressed and purified (Figure 2) and characterized in the vaccination study. MUC1 transgenic mice on a C57BL/6 background were immunized intramuscularly with MUC1/TR5 or MUC1/TR5-C3d and boosted with the respective construct on day 28 and day 69. One week after the first boost (day 35), serum was taken and the antibody response was analyzed by ELISA (Figure 4). Mice were boosted a second time on day 69 and serum and spleen taken on day 78. The complete immunization schedule is depicted in Figure 3. The antibody response was analyzed and splenocytes used in an ELISPOT assay to examine the T cell immune response (Figure 5).

We found that MUC1 transgenic (MUC1-Tg) mice treated with either MUC1/TR5 or MUC1/TR5-C3d recombinant fusion protein had both IgG and IgM anti-MUC1 antibodies present in their sera at day 28. However, while both groups had similar IgM responses, mice vaccinated with MUC1/TR5-C3d had significantly higher IgG titers at days 28, 35 and 78 (Figure 4). The IgM titer in both groups increased to day 78. To further investigate the difference in IgG levels in the sera of mice inoculated with MUC1/TR5 or MUC1/TR5-C3d we titrated IgG levels in sera from mice on days 35 and 78. It was found that the relative IgG titer was 4 times higher at day 35 and 8 times higher at day 78 in mice immunized with MUC1/TR5-C3d compared to those immunized with MUC1/TR5 (Figure 4).

At day 78 spleens were harvested from MUC1-Tg mice immunized with either MUC1/TR5 or MUC1/TR5-C3d recombinant fusion protein. The isolated splenocytes were used in an interferon gamma ELISPOT to evaluate the T cell response. Gamma-

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irradiated EO771 cells transfected with vector or MUC1 were used as targets. Immunization with MUC1-TR5 did not elicit a strong T cell response against MUC1 transfected EO771 cells. However, a strong T cell response was seen against MUC1 and vector transfected EO771 cells from mice immunized with MUC1/TR5-C3d (Figure 5). This indicates that the T cell response elicited by immunization with MUC1/TR5-C3d is not MUC1 specific.

From these studies we concluded that C3d does function as a molecular adjuvant when linked to a cancer associated antigen. The anti-MUC1 humoral immune response was significantly enhanced following vaccination with the MUC1/TR5-C3d construct. A T cell response was seen in response to MUC1/TR5-C3d, while only a very weak T cell response was elicited by vaccination with MUC1/TR5. While promising, the immune response to vaccination with MUC1/TR5-C3d was not as strong as expected. In order to achieve protective immunity the immune response to the vaccine may need to be increased. To this end, we are producing adenoviral constructs expressing MUC1/TR5 and MUC1/TR5-C3d.

Adenoviral vectors are highly immunogenic. They have been shown to induce an innate immune response that is followed by an adaptive response, leading to immunological memory (Muruve 2004). This innate immune response has been shown to be a potent adjuvant for use in vaccine development (Molinier-Frenkel *et al* 2002 and Geutskens *et al* 2000). Molinier-Frenkel *et al* (2002) showed that administering adenovirus led to a strong T cell response directed against the transgene product. Further, it has been shown *in vivo* by Geutskens *et al* (2000) that replication deficient adenoviral vectors can act as an adjuvant in combination with recombinant IL-2 treatment. Importantly, this adjuvant effect was determined to be due to the presence of T cells specifically recognizing tumor antigen (Geutskens *et al* 2000). These properties of rAd vectors made them a good candidate to improve the immune response seen in our studies and produce protective, long lasting immunity against tumors.

Using adenoviral vectors may improve our MUC1/TR5-C3d immunization results and lead to protective immunity and memory. Therefore, we are currently producing recombinant adenoviral (rAd) constructs expressing MUC1/TR5 and MUC1/TR5-C3d. The immune response (humoral and cellular) following vaccination with these rAd constructs will be analyzed and compared with the protein vaccination responses above. It is hypothesized that the rAd constructs will elicit a stronger and more specific immune response and if this is seen a protective immunity study will follow.

If the results are not as expected in the rAd vaccination studies several other methods to increase the immune response could be attempted. First, administration of cytokines, such as IL-2, may lead to a stronger immune response. Further, it has been shown that a potent T cell response can be induced through a heterologous prime-boost immunization strategy (Vuola *et al* 2005 and Park *et al* 2003). In the study by Vuola *et al* (2005) they showed that priming with plasmid DNA or attenuated fowlpox strain (FP9) and boosting with modified vaccinia virus Ankara (MVA) induced a significantly stronger cellular immune response than boosting with the same vector used to prime. Further, this study showed that the priming delivery system affects the type of T cell response elicited (Vuola *et al* 2005). This indicates that it may be advantageous to investigate priming with the recombinant protein MUC1/TR5-C3d and boost with the rAd MUC1/TR5-C3d construct to achieve a protective and long lasting immune response

to MUC1$^+$ tumors. Alternatively, it may be useful to use the DNA constructs of MUC1/TR5 and MUC1/TR5-C3d to prime followed by an adenoviral boost, even though the DNA constructs alone did not elicit a significant immune response in our hands.

Key Research Accomplishments:
- Construction, expression and purification of MUC1/TR5 and MUC1/TR5-C3d
- Completion of MUC1 vaccination study
- Determined that C3d does function as a molecular adjuvant but does not elicit a strong enough immune response to provide long lasting, protective immunity.
- Determined that T cell response elicited by MUC1/TR5-C3d vaccination is not MUC1 specific.

Reportable Outcomes:
- None

Conclusions:

In the first year of these studies we have had one minor setback in that expression of the mouse CR2-Fc construct was technically difficult. Therefore, it was necessary to create a new construct to carry out these studies. This task is expected to be completed soon and these studies will then proceed as planned. MUC1/TR5 and MUC1/TR5-C3d were successfully expressed and purified and the vaccination study completed. It can be concluded from the results that when linked to a cancer associated antigen, C3d does have the potential to function as a molecular adjuvant. We saw a significant enhancement in the anti-MUC1 humoral immune response (IgG) following vaccination with the MUC1/TR5-C3d construct, as well as a cell mediated immune response. However, the T cell response was not MUC1 specific. Leading us to hypothesize that further modulation of the immune response may lead to more protective and long lasting immunity to tumor associated antigens.

References:

Geutskens SB, van der Eb MM, Plomp AC, Jonges LE, Cramer SJ, Ensink NG, Kuppen PJK and Hoeben RC. Recombinant adenoviral vectors have adjuvant activity and stimulate T cell responses against tumor cells. *Gene Therapy*; 2000, 7:1410-1416.

Molinier-Frenkel V, Lengagne R, Gaden F, Hong S-S, Choppin J, Gahery-Segard H, Boulanger P and Guillet J-G. Adenovirus Hexon Protein Is a Potent Adjuvant for Activation of a Cellular Immune Response. *Journal of Virology*; 2002, 76:127-135.

Park S-H, Yang S-H, Lee CG, Youn J-W, Chang J and Sung YC. Efficient induction of T helper 1 CD4$^+$ T-cell responses to hepatitis C virus core and E2 by a DNA prime-adenovirus boost. *Vaccine* 2003; 21: 4555-4564.

Vuola JM, Keating S, Webster DP, Berthoud T, Dunachie S, Gilbert SC and Hill AVS. Differential Immunogenicity of Various Heterologous Prime-Boost Vaccine Regimens Using DNA and Viral Vectors in Healthy Volunteers. *The Journal of Immunology;* 2005, 174: 449-455.
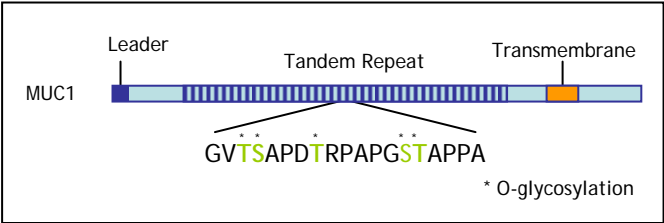
Supporting data:

Figure 1. MUC1/TR5 and MUC1/TR5-C3d constructs. Both constructs contain five tandem repeating units of MUC1. MUC1/TR5-C3d is fused to three repeating units of C3d.
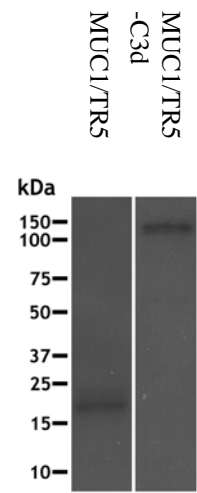

Figure 2. Purification of MUC1/TR5 and MUC1/TR5-C3d recombinant proteins. Recombinant proteins were purified from culture supernatant of COS7 transfected cells by DEAE FF Sepharose ion-exchange. The purified protein was confirmed by SDS-PAGE and Western blot, revealing proteins with in the expected molecular weight range.
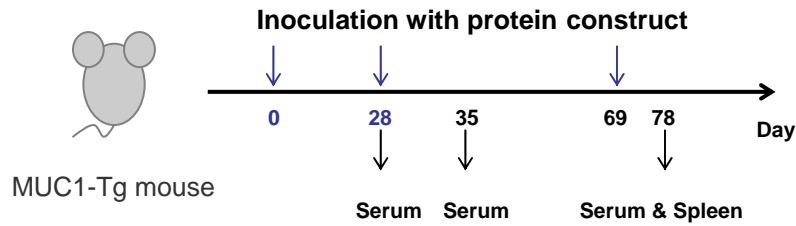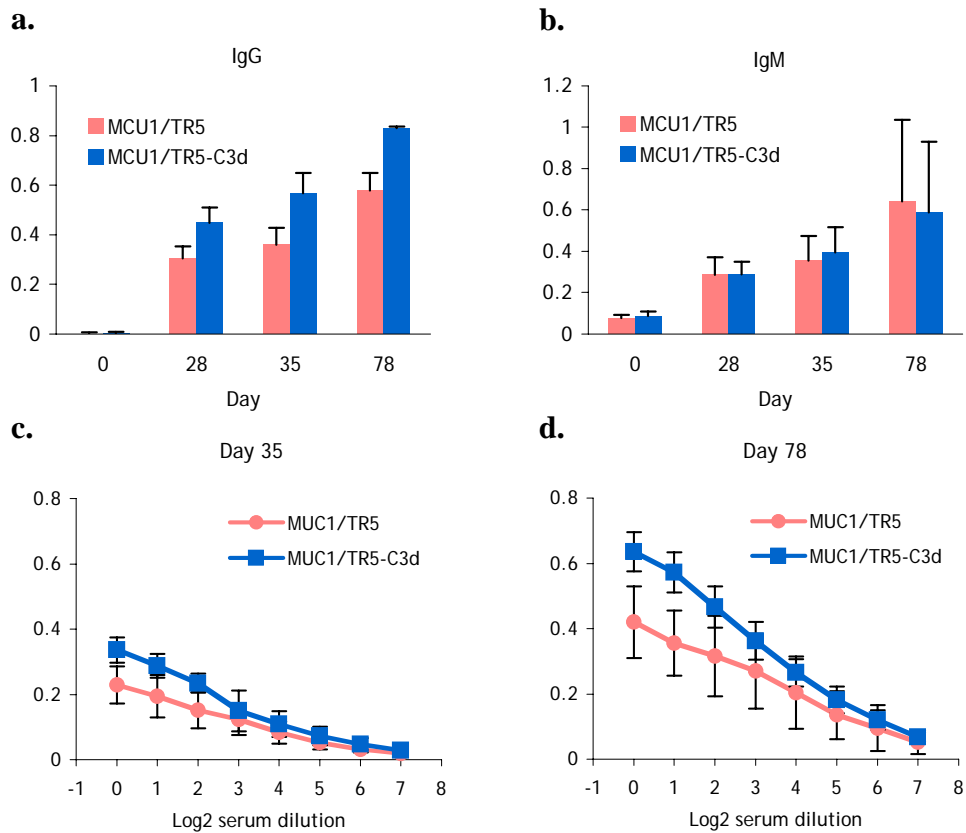
Figure 3. Immunization strategy.

Figure 4. Humoral immune response to MUC1/TR5 and MUC1/TR5-C3d in MUC1-transgenic mice. MUC1 transgenic mice on a C57BL/6 background were immunized intramuscularly with 500 pmol recombinant protein and boosted on days 28 and 69. Panels a and b). The IgG (a) and IgM (b) titers from the serum were analyzed on days 28, 35 and 78 by ELISA. Panels c and d). IgG levels from sera on days 35 (c) and 78 (d) were titrated to investigate the difference between MUC1/TR5 and MUC1/TR5-C3d immunized mice.
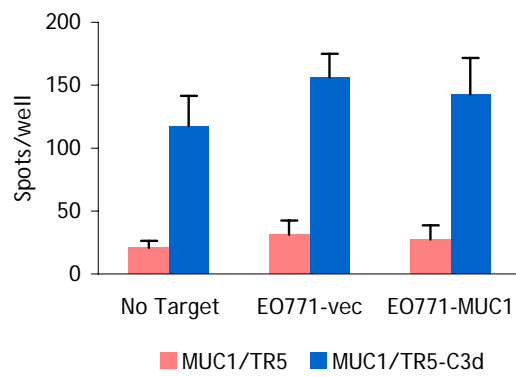
Figure 5. T cell response to MUC1/TR5 and MUC1/TR5-C3d in MUC1-transgenic mice. T cell response was analyzed on day 78 by interferon gamma ELISPOT. Gamma irradiated vector or MUC1 transfected EO771 cells were used as targets. Results are representative of two independent experiments.