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Immunology

Oxidative/reductive conjugation of mannan to antigen selects for T_1 or T_2 immune responses

(mucin/cytotoxic T lymphocytes/immunotherapy/cancer)

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ABSTRACT **The induction of $CD8^+$ cytotoxic T lymphocytes (CTLs) is desirable for immunization against many diseases, and recombinant-synthetic peptide antigens are now favored agents to use. However, a major problem is how to induce CTLs, which requires a T_1-type response to such synthetic antigens. We report that T_1-type (generating high CTL, low antibody) or T_2-type (the reciprocal) responses can be induced by conjugation of the antigen to the carbohydrate polymer mannan: T_1 responses are selected by using oxidizing conditions; T_2 responses are selected by using reducing conditions for the conjugation. Using human MUC1 as a model antigen in mice, immunization with oxidized mannan–MUC1 fusion protein (ox-M-FP) led to complete tumor protection (challenge up to 5×10^7 $MUC1^+$ tumor cells), CTLs, and a high CTL precursor (CTLp) frequency (1/6900), whereas immunization with reduced mannan–MUC1 FP (red-M-FP) led to poor protection after challenge with only 10^6 $MUC1^+$ tumor cells, no CTLs, and a low CTLp frequency (1/87,800). Ox-M-FP selects for a T_1 response (mediated here by $CD8^+$ cells) with high interferon γ (IFN-γ) secretion, no interleukin 4 (IL-4), and a predominant IgG2a antibody response; red-M-FP selects for a T_2-type response with IL-4 production and a high predominant IgG1 antibody response but no IFN-γ.**

With the production of antigens by recombinant DNA methods, there is now interest in how to deliver these antigens to the immune system to generate the appropriate immune response for vaccination and therapy. Synthetic peptides and recombinant fusion protein antigens have been linked to a variety of carriers to generate satisfactory antibody responses but little cellular immunity (1–3). However, it is often necessary to induce cellular immunity in diseases such as influenza and mycobacterial infections and possibly for certain stages of malaria and human immunodeficiency virus (HIV). We now demonstrate that conjugation of a recombinant antigen derived from the protein core of Mucin 1 (MUC1) to the polysaccharide mannan (polymannose) can stimulate cellular immune responses (refs. 4 and 5; unpublished data). The unusual finding is that if the conjugation is done under oxidizing conditions, then cell-mediated immunity is selectively stimulated, compared with using reducing conditions for conjugation when antibody responses occur.

A successful vaccine for cancer immunotherapy requires a suitable target antigen and production of cytotoxic T lymphocyte (CTL) responses (6). In adenocarcinomas, it appears that mucins, particularly MUC1, may provide a suitable target as there is a 10-fold increase in mucin expression on the cell surface, and altered glycosylation leads to exposure of normally hidden peptide sequences (e.g., APDTR). These changes in cancer generate a potential new target for immunotherapy that is apparently absent in normal mucin (7–10). The APDTR sequence [from the variable number of tandem repeats (VNTR) region] is immunogenic in mice, leading to antibody formation, whether the antigen is administered as $MUC1^+$ cancer cells, purified mucin (HMFG), or as peptides (11, 12). Such studies of immunogenicity in mice would be of little relevance to humans were it not for the findings that tumor-specific CTL precursors (CTLp) exist in the lymph nodes of patients with cancer of breast, ovary, or pancreas, or in multiple myeloma patients (13–16). The CTLp can be stimulated by antigen and interleukin 2 (IL-2) *in vitro* to become functional $CD8^+$ CTLs, the target antigen being the APDTR sequence of MUC1 (13–16). Such CTLs are unusual in that they are not major histocompatibility complex class I restricted (13–16). Thus, theoretically, patients could be immunized with MUC1 peptide sequences to convert their anti-MUC1 CTLp into functional CTLs, which would have a therapeutic anticancer effect. Using a murine $MUC1^+$ tumor model, we now demonstrate that immunization with human MUC1 fusion protein (FP) (with glutathione *S*-transferase attached) under reducing conditions is immunogenic in terms of antibody production, but it has little tumor-protective effect; there were no CTLs produced and a low frequency of CTLp. By contrast, FP coupled to oxidized mannan (ox-M-FP) gave protection against $MUC1^+$ mouse tumors and a high CTLp frequency. By measuring the appropriate cytokines [interferon γ (IFN-γ) and IL-4], it is clear that the results translate into stimulation of either T_1- or T_2-type helper T cells, depending on the chemical state of the immunogen.

MATERIALS AND METHODS

Chemical Studies. MUC1 FP containing 5 VNTR of human MUC1 and peptide (Cp13-32) were produced as described (2). FP was conjugated to mannan in two ways. (*i*) Ox-M-FP: Mannan (Sigma), at 14 mg/ml in 0.1 M phosphate buffer (pH 6.0), was oxidized with sodium periodate (0.01 M) for 60 min at 4°C. Ethanediol (10 μl) was added and incubated for a further 30 min at 4°C, and the mixture was passed through a Sephadex-G25 column equilibrated in bicarbonate buffer (pH 6.0–9.0). The oxidized mannan that eluted in the void volume (2 ml) was mixed with 900 μg of MUC1 FP, incubated overnight at room temperature, and used without further purification. (*ii*) Reduced (Red)-M-FP: The ox-M-FP mixture was treated with sodium borohydride (1 mg/ml) for 3 hr and used without further purification.

Immunizations and Tumors. BALB/c mice (females; 8 weeks old) were immunized intraperitoneally with either ox- or

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Abbreviations: CTL, cytotoxic T lymphocyte; CTLp, CTL precursor(s); DTH, delayed-type hypersensitivity; FP, human MUC1 fusion protein; ox-M-FP, mannan conjugated to FP in oxidized form; red-M-FP, mannan conjugated to FP in reduced form; VNTR, variable number of tandem repeats; IL, interleukin; IFN, interferon.

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red-M-FP conjugate (containing 5 μg of FP), 50 μg of FP, or 5×10^6 MUC1$^+$ 3T3 tumor cells weekly for 3 weeks (1). Murine 3T3 BALB/c cells and MUC1$^+$ 3T3 cells (containing the transfected cDNA encoding human MUC1) were obtained from D. Wreschner (Tel Aviv University, Israel; ref. 17) and used to challenge mice. The murine DBA/2 mastocytoma cell lines P815 and MUC1$^+$ P815 (containing the cDNA encoding human MUC1) were obtained from B. Acres (Transgene, Strasbourg, France; ref. 18) and used as target cells in CTL and CTLp assays.

ELISA: Antibody Production and Isotype. An ELISA was performed as described (12) to determine antibody production, and an ELISA for isotype determination with peptide (Cp13-32)-coated wells was set up (12). Sera from immunized mice were added to wells for 2 hr; 50 μl of a 1:1000 dilution of biotin-conjugated rat anti-mouse immunoglobulin subclass specific for isotypes M, G1, G2a, and G2b (PharMingen) were added and incubated for 1 hr; then a 1:500 dilution of rabbit anti-mouse streptavidin horseradish peroxidase conjugate (Dako) was added for 1 hr and the plate was developed and read.

CTL and CTLp Frequency Determination. CTLs were determined as described (1) and CTLp frequencies were determined using a minimum of 32 replicates of at least 6 responder cell doses by culturing with 5×10^5 BALB/c stimulator spleen cells treated with mitomycin C (25 μg/ml; 1–1.5 hr) (Kyoma, Japan), 5 mM synthetic MUC1 peptide (Cp13-32), and recombinant human IL-2 (10 units/ml). Seven days later, each microculture was assayed for cytotoxicity by replacing 100 μl of culture medium with 100 μl of target cell suspension containing 10^4 ^{51}Cr-labeled MUC1$^+$ P815 tumor target cells. Wells were regarded as containing cytotoxic activity if they yielded specific ^{51}Cr release 3SD above the mean isotope release obtained from 10^4 target cells added to responder cells cultured alone or with stimulator cells only, peptide only, or recombinant IL-2 only (19–21).

Cytokine Production. Cultures of spleen cells (4×10^6 cells) obtained from immunized mice were established as follows: (*i*) synthetic peptide Cp13-32 (90 μg/ml) (20 μM); (*ii*) red-M-FP (90 μg/ml) (corresponding to the amount of FP in the M-FP complex); (*iii*) ox-M-FP (90 μg/ml); or (*iv*) mannan (amount of mannan in M-FP) (1.5 mg/ml), together with 4×10^6 mitomycin C (25 μg/ml; 1–1.5 hr) (Kyoma, Japan)-treated normal spleen cells added as antigen presenting cells. Cells were cultured for 72 hr and supernatants were collected and tested for IFN-γ and IL-4 production. Cytokine assays performed by ELISA, using commercial kits (Endogen, Cambridge, MA), were used for detection of IFN-γ and IL-4.

RESULTS

***In Vivo* Growth of MUC1$^+$ 3T3 Tumor Cells and Immune Responses to MUC1$^+$ 3T3 Cells and FP in Immunized Mice.** MUC1$^+$ 3T3 cells (17) grow as tumors but are rejected by syngeneic BALB/c mice in ≈20 days due to the immune response to human MUC1 (1). Thus, there is a window between 0 and 11 days in which to observe either accelerated rejection or absence of tumor growth in immunized BALB/c animals. In syngeneic mice, MUC1$^+$ tumor rejection is accompanied by generation of CD8$^+$ CTLs specifically reactive with MUC1$^+$ target cells (1) (Fig. 1*A*), a high CTLp frequency with a mean of 1/14,600 compared with 1/782,500 in nonimmune BALB/c mice (Table 1), little antibody formation (Fig. 1*B*), and delayed-type hypersensitivity (DTH) mediated by CD4$^+$ cells (1). Furthermore, such mice are resistant to rechallenge with large doses ($>5 \times 10^7$) of MUC1$^+$ cells (Fig. 2) and this is entirely due to conventional H2-restricted CD8$^+$ cells (ref. 1; unpublished data). Thus, the ideal type of antitumor immunity is indicated by the presence of CD8$^+$ CTLs and not

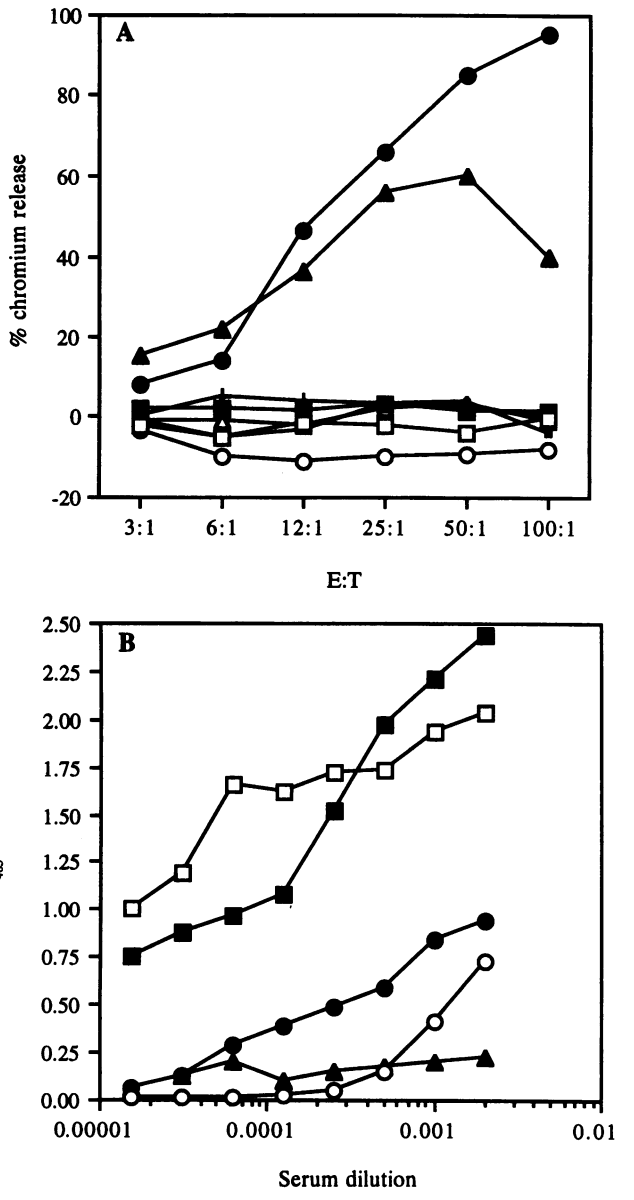

FIG. 1. CTL and antibody production in immunized BALB/c mice. (*A*) CTL assay using MUC1$^+$ P815 and control P815 target cells; % specific ^{51}Cr release vs. effector/target cell (E:T) ratio is shown using splenic lymphocytes from mice immunized with either MUC1$^+$ 3T3 cells (▲), ox-M-FP (●), red-M-FP (■), or FP (+) testing on MUC1$^+$ P815 (solid symbols) or on control P815 target cells (open symbols). (*B*) Antibody test by ELISA. Titration of sera from mice immunized with MUC1$^+$ 3T3 cells (▲), ox-M-FP (●), red-M-FP (■), FP (□), or PBS (○) on plates coated with FP (20 μg/ml). Absorbance at 405 nm vs. serum dilution is shown.

antibody. A DTH response mediated predominantly by CD4$^+$ cells is present but appears to be irrelevant (1).

Three different strategies were used to induce antitumor immunity with the MUC1 antigen model. Mice were immunized with either a MUC1 synthetic peptide (Cp13-32; a 20-amino acid sequence of the VNTR plus cysteine, leading to dimer formation) coupled to keyhole limpet hemocyanin as a carrier (1), with a MUC1-containing FP produced in the pGEX system (2) consisting of 5 VNTR, or with purified natural mucin (HMFG). Three injections containing 50 or 5 μg of the mucin component of the immunogen were injected intraperitoneally at weekly intervals (1). Each of these procedures induced a similar immune response, and the data for FP antigen suffice to illustrate this. There was some degree of

Table 1. Splenic CTLp frequency tested on MUC1$^+$ P815 cells in variously immunized BALB/c mice

Immunogen	CTLp frequency*			Mean frequency
	1	2	3	
None	1/850,000	1/725,000		1/782,500
MUC1$^+$ 3T3	1/12,500	1/17,500		1/14,600
Ox-M-FP	1/8800	1/6200	1/6300	1/6900
Red-M-FP	1/84,000	1/92,000		1/87,800
FP (50 μg)	1/85,000	1/90,000		1/87,400
FP (5 μg)	1/150,000			

*Analysis of individual BALB/c female mice.

protection when the mice were challenged with 10^6 MUC1$^+$ 3T3 cells [tumors grew more slowly and were smaller (Fig. 2*A*)] but not when the tumor challenge dose was increased to 5×10^6 cells (Fig. 2*B*). No CTLs could be detected in the spleen of immunized mice (Fig. 1*A*), but there was high antibody production (Fig. 1*B*) predominantly of the IgG1 subclass (Fig. 3). The tumor protection observed by challenging mice with 10^6 cells may have been mediated by the few CTLs induced [note the low CTLp frequency of 1/87,400 (50 μg of FP immunogen) or 1/150,000 (5 μg of FP immunogen) (Table 1)], which were too few to detect in the standard CTL assay.

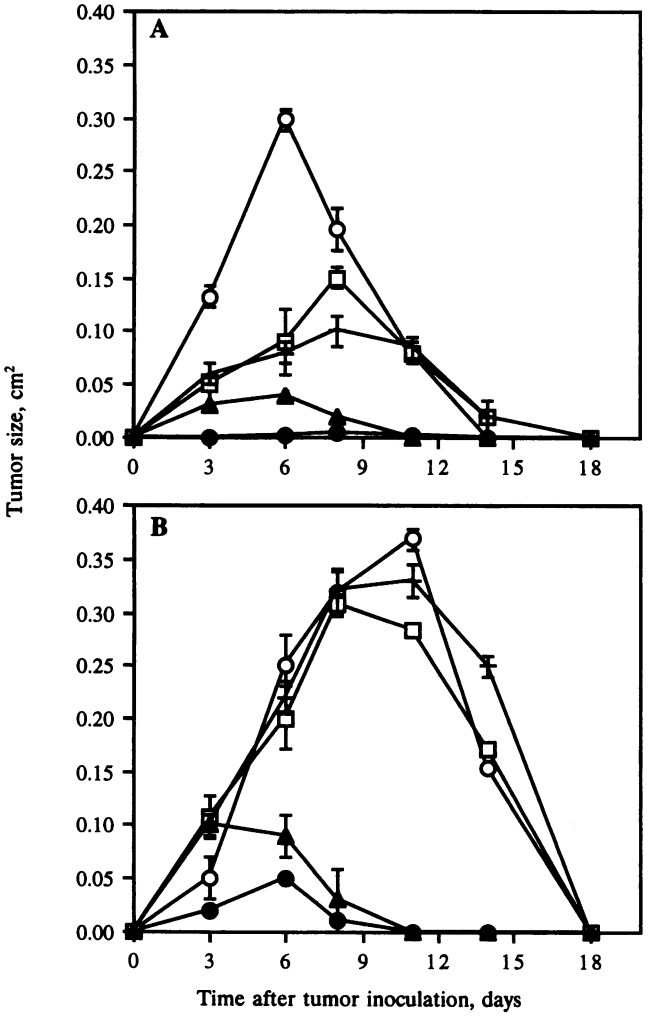

FIG. 2. Growth of MUC1$^+$ 3T3 tumors in immunized BALB/c mice. Immunization with 5×10^6 MUC1$^+$ 3T3 tumor cells (▲), ox-M-FP (●), red-M-FP (+), FP (□), or PBS (○). Mean size of tumors (product of two diameters) is shown ±SD ($n = 5$) vs. days after tumor inoculation. (*A*) Challenge with 10^6 MUC1$^+$ 3T3 cells. (*B*) Challenge with 5×10^6 MUC1$^+$ 3T3 cells.

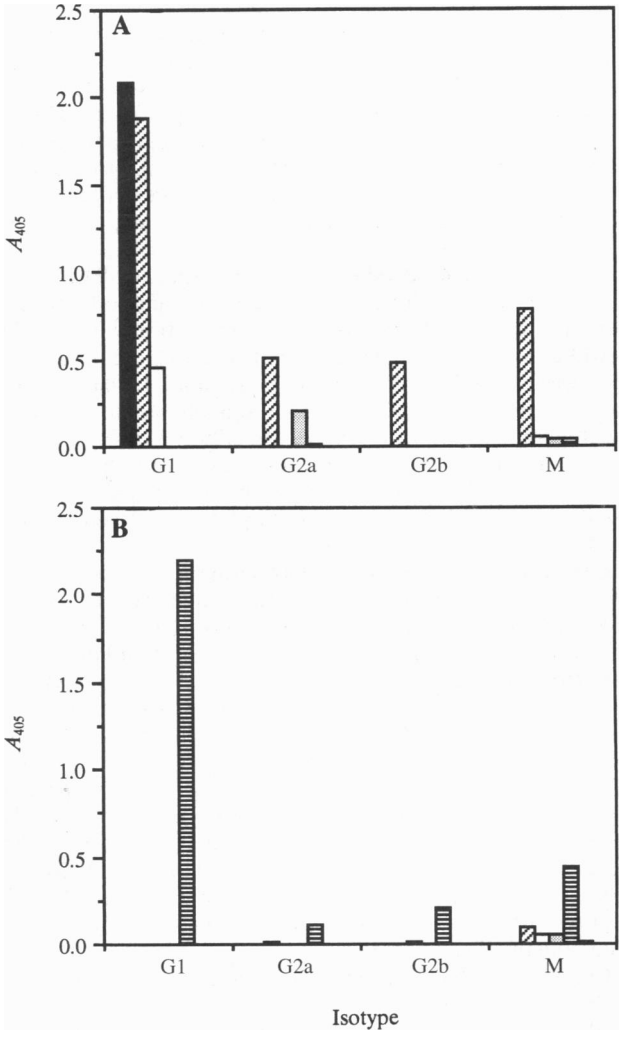

FIG. 3. Serum immunoglobulin isotype in immunized mice tested for anti-MUC1 activity. Mice were immunized with FP (▨), red-M-FP (□), ox-M-FP (▧), T4N1 peptide (▤), or MUC1$^+$ 3T3 cells (▥). Isotype and specificity were determined on plates coated with FP (*A*) and the irrelevant peptide T4N1 (*B*). Absorbance values are at 1:100 serum dilution. Assay controls for each assay plate were the anti-MUC1 IgG1 monoclonal antibody VA2 (■) (2) and sera from mice immunized with the foreign peptide T4N1. Absorbance at 405 nm vs. antibody isotype is shown.

Immunization with Ox- or Red-M-FP. Mice were then injected with ox-M-FP (5 μg of the FP component) intraperitoneally at weekly intervals (three times) and their immune status was analyzed prior to tumor challenge; there were CD8$^+$ CTLs present (Fig. 1*A*), a high CTLp frequency (mean, 1/6900) compared with 1/782,500 in nonimmune BALB/c mice (Table 1), a low antibody titer (Fig. 1*B*) of the IgG2a isotype (Fig. 3), and a DTH response (data not shown). When these ox-M-FP immunized mice were challenged with MUC1$^+$ tumor cells, they were totally resistant to tumors at doses of 10^6–5×10^7 (Fig. 2); indeed, we have not been able to find a dose of tumor cells to which the mice are susceptible. These mice appear to have immunity at least equivalent to those that had previously rejected a tumor. However, when the level of immunity was quantitated by CTLp measurement (Table 1), it was clear that ox-M-FP induced greater tumor immunity than did the tumor inoculum (1/6900 vs. 1/14,600). Thus, immunity with ox-M-FP led to the best *in vivo* antitumor immunity found.

When mice were immunized with red-M-FP, there were no detectable CTLs on direct testing (Fig. 1*A*) and there was a low CTLp frequency of 1/87,800 (Table 1). There was a high titer

of antibody (Fig. 1*B*) of the IgG1 subclass for >90% of the antibody (Fig. 3). When these mice were challenged with tumor, there was some resistance to 10^6 tumor cells (Fig. 2*A*)—likely to be due to the weak CTLp response (1/87,800 vs. 1/6900)—but no resistance to challenge with 5×10^6 tumor cells (Fig. 2*B*). Thus, oxidizing conditions for M-FP led to significant cellular, but little antibody, immunity, whereas reduction of the ox-M-FP complex with sodium borohydride led to little cellular immunity; indeed, red-M-FP stimulated immunity similar to FP injected alone.

Cytokine Production in Ox-M-FP and Red-M-FP Immunized Mice. It is known that $CD4^+$ T cells give rise to two distinct profiles of secreted cytokines when cultured *in vitro*. T_1 cells give rise to cellular immunity, induce little IgG2a antibody, and secrete IFN-γ and other cytokines (IL-2, tumor necrosis factor α, granulocyte–macrophage colony-stimulating factor). By contrast, T_2 cells give rise to antibody (IgE in many models, IgG1 in others) with little cellular immunity and secrete IL-4 but no IFN-γ (22, 23). Recently, $CD8^+$ cells have also been given a similar T_1/T_2 classification (24, 25). Clearly, ox-M-FP appeared to give a T_1 response, whereas red-M-FP induced a T_2 response. The reciprocal nature of cell-mediated immunity and antibody was noted in the early 1970s for flagellin (26) and the immune responses to MUC1 responses are consistent with this paradigm. To confirm this, cytokine measurements were performed. Spleen cells from ox-M-FP-immunized mice produced large amounts of IFN-γ when stimulated *in vitro* with Cp13-32 peptide (400 ng/ml) or ox-M-FP (225 ng/ml) (Fig. 4*A*) but no IL-4 (Fig. 4*B*)—i.e., a typical T_1 response. By contrast, cells from red-M-FP-immunized mice did not give rise to IFN-γ (Fig. 4*A*) when stimulated *in vitro* with Cp13-32 peptide (50 ng/ml) or red-M-FP (20 ng/ml) (Fig. 4*A*) but produced IL-4 (Fig. 4*B*)—i.e., a T_2 response. Thus, mannan conjugation with FP can give a T_1 or a T_2 response depending on the chemical nature of the conjugation: oxidation leads to T_1 responses (like $MUC1^+$ 3T3) and reduction leads to T_2 responses.

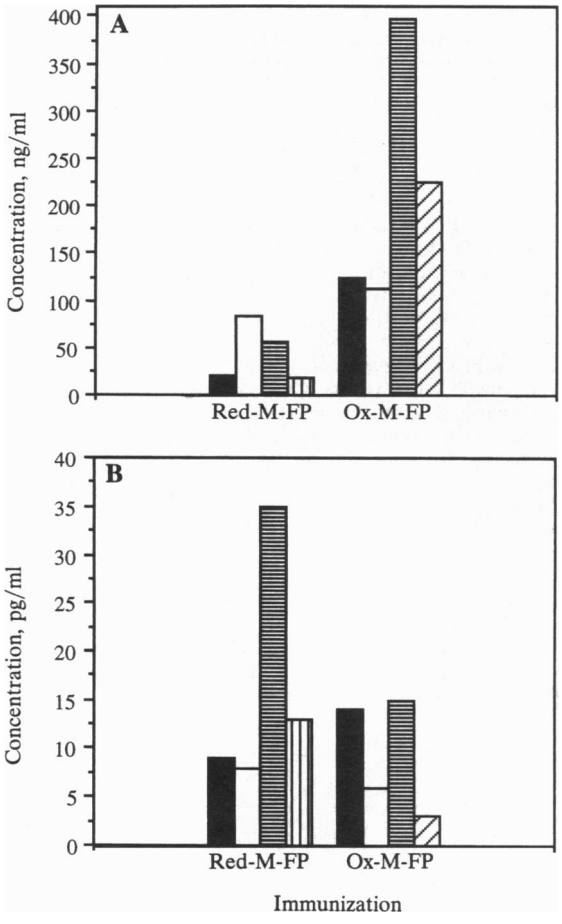

FIG. 4. Cytokine assays to detect IFN-γ (*A*) and IL-4 (*B*) using spleen cells from mice immunized with red-M-FP or ox-M-FP in the absence (□) or presence of Cp13-32 (▤), red-M-FP (▥), ox-M-FP (▨) or mannan (■). Amount of IFN-γ or IL-4 vs. mouse immunizations is shown.

DISCUSSION

We show that conjugation of MUC1 FP to mannan under oxidizing conditions gives rise to a high cellular immune response, leading to total resistance to experimental tumors in mice. Tumor resistance was accompanied by induction of a high frequency of CTLp, little antibody, and production of IFN-γ but not IL-4, indicating a T_1-type immune response. The level of immunity generated is high, higher than that generated by rejection of the tumor, which is usually taken as the standard in these experimental procedures. A number of controls for target cell and immunization specificity were used and no protection was observed after immunization with (*i*) a non-conjugated mixture of mannan and FP; (*ii*) mannan conjugated with another, irrelevant FP; or (*iii*) mannan injected alone (data not shown; see ref. 1). This work describes specific tumor cell killing generated by immunization with synthetic MUC1 antigens. By contrast, a similar conjugation performed under reducing conditions gives rise to the "opposite" type of response with little cellular immunity, strong antibody responses, and, after *in vitro* stimulation, secretion of IL-4 but no IFN-γ—now called a T_2 response (although it should be noted the original $T_{1/2}$ classifications were based on $CD4^+$ effector cells only, whereas the CTL response here is likely to be mediated by $CD8^+$ cells).

Clearly, in this model, conjugation of antigen to mannan under the appropriate oxidizing conditions gives rise to a superior antitumor response; indeed, it is one of the strongest immune responses we have seen, as it is usually difficult to immunize mice against a tumor cell challenge other than by using sublethal doses of tumor cells themselves. The cytokine profiles indicated that ox-M-FP proteins stimulate T_1-type responses, whereas red-M-FP or FP gives a T_2-type response and is almost certainly dependent on $CD4^+$ cells. In addition, the isotype of the antibody response indicates a T_1/T_2 deviation by the ox/red state of the mannan; after ox-M-FP immunizations, >90% of the antibody was IgG2a, whereas red-M-FP or FP produced IgG1 responses. However, several observations are not consistent with this T_1/T_2 characterization, such as generation of DTH by any immunization program (data not shown; see ref. 1) and that the T_1-type response was mediated by $CD8^+$ cells, whereas no $CD8^+$ effectors were detected in the T_2 response, which therefore probably involved $CD4^+$ cells. It should be noted that the phenotype of the cells secreting the cytokines is not proven at present but the following points are clear: (*i*) the antitumor response is mediated by $CD8^+$ cells; (*ii*) there is a close correlation between the number of CTLp and tumor rejection (V.A., unpublished data); the same correlation appears to extend to CTLp frequency and IFN-γ production (unpublished data). The cytokine secretion profile in separated $CD4^+$ and $CD8^+$ cells must be examined to prove this point. Similar comments apply to the IL-4-secreting cells. Thus, at this point, we can confidently present cytokine secretion profiles as T_1 type for ox-M-FP and T_2 type for red-M-FP (20, 26). The studies clearly show that the desired type of immune response—at least to MUC1—can be selected by varying the oxidizing conditions of the chemical reaction with mannan, with oxidation leading to a CTL cellular response and little antibody response and with reduction leading to antibody and not cellular responses.

It is not clear how ox-M-FP complex induces such a strong cellular immune response. It is likely that the presence of mannan serves to target the antigen to mannose receptors on the surface of antigen presenting cells such as macrophages and dendritic cells. Indeed, it is the splenic sinusoidal cells in marginal sinuses that have the highest density of receptors for mannose (27, 28). Mannan is clearly a critical component, for when FP was linked under oxidizing conditions to dextran (a polysaccharide that lacks mannose residues), no antitumor immunity was induced (data not shown). However, it would be expected that conjugation of FP to mannan under reducing conditions should have the same targeting effect to mannose receptors. The possibility that the oxidizing/reducing conditions affect the mannan in such a way that targeting is altered remains to be examined.

In the past, mannan has been used to induce cellular immunity to murine candidiasis (which already contains a significant amount of mannose) to enhance the action of lipopolysaccharides on T-cell proliferation and to induce anti-peptide antibody responses (4, 29, 30). None of those studies used the same conjugation method described here and did not lead to the same type or degree of cellular immunity. Perhaps the oxidizing conditions serve to target the antigen to the intracellular processing pathway for presentation with class I antigens, whereas the red-M-FP or FP is likely to follow the usual endocytic pathways to be processed and presented by class II. With the knowledge now available on intracellular processing pathways, it should be possible to determine how oxidation leads to such a significant improvement in the immunizing agent. There are several further points to note—oxidizing conditions for conjugation lead to formation of Schiff bases and aldehyde groups, and Schiff bases form between antigen presenting cells, antigen, and T-cell receptors (31). This may be of relevance to our study, but it is difficult to see the survival of Schiff bases after intracellular trafficking and processing of the antigen.

We also note that anti-peptide antibodies have been made by conjugating a peptide to the surface antigen of hepatitis B virus with cyanogen bromide (8), yielding a product as analogous to the red-M-FP. Recently, another study involving the coupling of synthetic peptides by reductive amination to mannan produced antibody responses (5), findings in accord with our results for a T_2-type response. In neither of these studies was the conjugation performed under appropriate conditions to induce cellular T_1-type responses. Because the method described here was able to induce significant anti-MUC1 CTLs, we are hopeful that mannan conjugation under appropriate conditions will generate cellular immunity to other antigens such as HIV, malaria, and influenza as well as agents of interest in veterinary practice. If our studies reported here can be reproduced with other antigens, the use of oxidized mannan conjugation represents a major step forward in production of vaccines to a range of antigens of interest in cancer and infectious diseases.

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