

ORIGINAL ARTICLE

Growth inhibition of myeloma cells by anti-idiotypic antibodies in the absence of membrane-bound immunoglobulin

Shiri Moshitzky, Tova Kukulansky, Joseph Haimovich and Nurit Hollander

Immunoglobulins are expressed as membrane-bound or secreted forms. Plasma cells produce little or no membrane immunoglobulin but secrete immunoglobulin molecules in large amounts. Immunoglobulin idiotypes of malignant B cells are tumor-specific antigens that may be targeted for immunotherapy. Thus, idotype vaccination is being evaluated in clinical trials to control residual disease in multiple myeloma and non-Hodgkin's lymphoma. It is traditionally considered that anti-idiotypic antibodies are not effective against plasma cell tumors, because the large amounts of immunoglobulin molecules secreted by the tumors block anti-idiotypic antibodies, and because the absence of membrane immunoglobulin on the surface of these tumor cells renders them resistant to the effect of anti-idiotypic antibodies. While the obstacle of abundant circulating idotype may be obviated by reducing tumor burden to minimal residual disease, the absence of membrane immunoglobulin has been considered as a limiting factor that prevents tumor eradication by anti-idiotypic antibodies. We demonstrate here that murine plasmacytoma cells can produce small amounts of membrane immunoglobulin M (IgM) heavy chains. However, the latter are precursor molecules that do not reach the cell surface. Although membrane-bound IgM is absent, the cells stain positively for surface IgM, reflecting molecules of the secreted form in the process of secretion. In spite of the relatively low levels of secreted immunoglobulin on the cell surface, anti-idiotypic antibodies are effective in retardation of tumor growth *in vivo*. Thus, while there is no doubt that idotype-specific cell-mediated responses are very important, myeloma patients in complete remission may additionally benefit from idotype-specific humoral responses.

Immunology and Cell Biology (2008) **86**, 261–267; doi:10.1038/sj.icb.7100153; published online 15 January 2008

Keywords: B cells; immunoglobulin; idiotypes; myeloma; vaccination

Immunoglobulins are expressed as membrane-bound or secreted forms. Whereas B lymphocytes express immunoglobulin as antigen receptor on the cell surface, terminally differentiated plasma cells secrete immunoglobulin molecules in large amounts. The membrane-bound and secreted forms differ in the amino-acid sequence of their heavy-chain C-terminal domain.¹ The two forms of heavy chains are encoded by the same gene. As has been shown for the μ chain, mRNAs for the membrane-bound μ chain (μ_m) and the secreted μ chain (μ_s) are transcribed from the μ gene by alternative usage of two polyadenylation sites.^{2,3} The two different mRNAs are generated in a developmentally ordered fashion. Thus, both μ_m and μ_s are transcribed in B-cell lymphomas, while in plasmacytomas, μ_s is found nearly exclusively.^{2,3} Myeloma cells do not express the membrane forms of other immunoglobulin isotypes as well. Plasma cells cannot express membrane-bound immunoglobulin on the cell surface not only due to the absence of relevant mRNA, but also because they lack expression of immunoglobulin- α/β (Ig α/β) polypeptides that control immunoglobulin transport to the membrane. With the exception of some rare myeloma variant clones,⁴ when expression

vectors coding for μ_m were introduced into myeloma cells, cell surface immunoglobulin M (IgM) was not expressed, although fully assembled intracellular IgM molecules were demonstrated.^{5,6}

Immunoglobulin idiotypic determinants of malignant B cells are tumor-specific antigenic determinants that may be targeted for immunotherapy. Following successful preclinical studies in animal models, induction of idotype (Id)-specific antitumor immune responses by vaccination with autologous immunoglobulin has been initiated in clinical trials to control residual disease in B-cell lymphoma and multiple myeloma (reviewed by Armstrong *et al.*,⁷ Vose⁸ and Reichardt and Brossart⁹). Although targeting Id requires individualized therapy, recent advances in genetic engineering techniques made it possible to produce personalized vaccines for large numbers of patients.^{7,8} The Id-directed effector mechanisms (humoral versus cell mediated) responsible for destruction of B-cell tumors are a controversial issue. Whereas antibodies may play a predominant role in destruction of B-cell lymphoma,^{10–12} it is traditionally considered that they are not effective against plasma cell tumors, because the large amounts of Id protein in the circulation block anti-Id antibodies, and

because the absence of membrane immunoglobulin on the surface of these tumor cells renders them resistant to the effect of anti-Id antibodies. While the obstacle of high Id concentration in the circulation may be obviated by reducing tumor burden to minimal residual disease,¹³ the absence of membrane immunoglobulin has been considered as a limiting factor that prevents tumor eradication by anti-Id antibodies.

Although immunoglobulin-secreting cells express little or no membrane immunoglobulin, myelomatous plasma cells of about 35% of myeloma patients are positive for surface immunoglobulin staining.¹⁴ The nature of this surface immunoglobulin is debated. Surface IgA of MOPC-315 plasmacytoma cells was reported to be an integral membrane protein, distinct from secreted MOPC-315 IgA.¹⁵ However, this has neither been confirmed nor demonstrated for other immunoglobulin-secreting cells. Therefore, several reports that anti-Id antibodies had an inhibitory effect on myeloma tumor growth¹⁶ were perplexing, because it has been suggested that for the induction of cell death, antibodies need to bind to an integral membrane target.

Although we have previously demonstrated that protection against myeloma in Id-vaccinated mice can be entirely mediated by T cells,¹⁷ we later observed that in some instances anti-Id antibodies may also play a role. In this report we demonstrate that anti-Id antibodies mediate protection against the D2 IgM-secreting tumor that expresses low levels of surface IgM. Taking advantage of the experimental model whereby the 38C-13 B-cell lymphoma and the D2 hybridoma share the same IgM with an identical Id specificity, we demonstrate that the surface IgM of D2 represents the secreted form rather than the membrane IgM form. Hence, although myeloma cells do not express membrane-bound immunoglobulin, they can be targeted by anti-Id antibodies.

RESULTS

Anti-Id antibodies inhibit growth of the D2 plasma cell tumor

The 38C-13 B-cell lymphoma and the D2 plasmacytoma share the same IgM with an identical Id specificity. Mice inoculated with 38C-13 lymphoma, D2 plasmacytoma or D2-C11 plasmacytoma (specificity control) were passively immunized with 0.4 ml of immune serum containing anti-Id antibodies. The immune serum was collected from Id-vaccinated mice. Anti-Id antibody concentration in the immune serum, determined by ELISA, was 100 $\mu\text{g ml}^{-1}$ (data not shown). Hence, each animal was passively immunized with an antibody dose of 40 μg . Serum of mice, inoculated with Freund's adjuvant (same protocol as inoculation of Id in adjuvant), served as control serum. As shown in Figure 1, a single dose of 40 μg anti-Id antibodies induced growth inhibition of D2 plasma cell tumor ($P < 0.001$) that was comparable to the inhibition of 38C-13 lymphoma ($P < 0.001$). In contrast, the Id-negative D2-C11 tumor cells were not affected by the passively transferred antibodies. The finding that protection against D2 is somewhat more effective than protection against 38C-13 is attributed to the higher aggressiveness of 38C-13 tumor cells, as reflected by the survival time of untreated animals. It should be noted that tumor-bearing mice were intentionally treated with only a single low dose of antibodies, to find out whether anti-Id antibodies would inhibit tumor growth despite this stringent condition.

D2 cells express surface IgM

Immunofluorescence with anti-IgM and with anti-Id antibodies revealed that D2 cells express surface IgM with the correct Id specificity, although the level of surface IgM on D2 plasmacytoma is much lower than surface IgM on 38C-13 lymphoma (Figures 2a–d). Because 38C-13 lymphoma and D2 plasmacytoma express the same

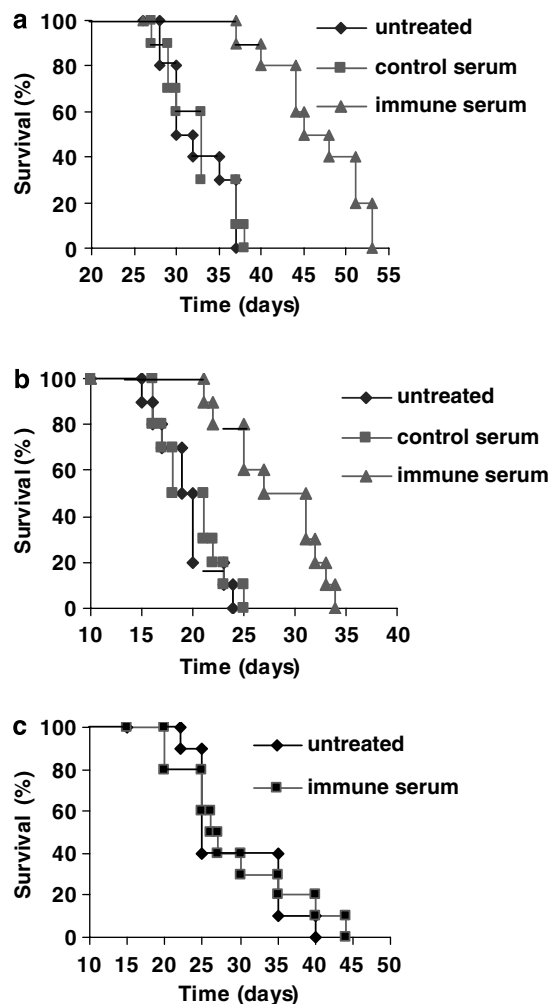


Figure 1 Anti-idiotypic (Id) antibodies prolong survival of D2 tumor-bearing animals. Mice were inoculated intraperitoneally with 1×10^4 of D2 cells (a), 38C-13 cells (b) or D2-C11 cells (c), followed 1 day later by intraperitoneal injection of serum from mice immunized with Id in adjuvant or with adjuvant alone (control serum). Survival of the antibody-treated groups in (a, b) was prolonged compared to the control groups ($P < 0.001$).

Id, we stained both cells with the rat anti-Id monoclonal antibody 2B6 that was generated against the 38C-13 Id. To ascertain that staining did not result from antibody binding to Fc receptors, we (1) pre-incubated the cells with aggregated human IgG to block Fc binding, and (2) used F(ab')_2 anti-Id fragments instead of intact antibodies. We use the heterologous human IgG because previous studies demonstrated its binding to murine Fc receptors.¹⁸ Blocking Fc receptors with aggregated IgG or replacing intact antibodies with F(ab')_2 fragments did not affect the fluorescence staining of D2 cells (Figures 2e and f). The isotype control monoclonal antibody PC61 (rat anti-CD25 IgG1) as well as R2.438.8, a rat monoclonal antibody to an unrelated Id, did not bind to D2 cells (Figures 2g and h), indicating the Id specificity of the staining. In addition, competitive inhibition studies demonstrated that excess of soluble Id protein blocked binding of anti-Id antibodies to D2 cells (Figure 3), further supporting the specificity of cell staining. It should be noted that we were unable in the past to demonstrate binding of anti-Id antibodies to D2 cells by immunofluorescence.¹⁹ This failure resulted from the treatment of stained cells with formaldehyde before analysis, a procedure that we are frequently

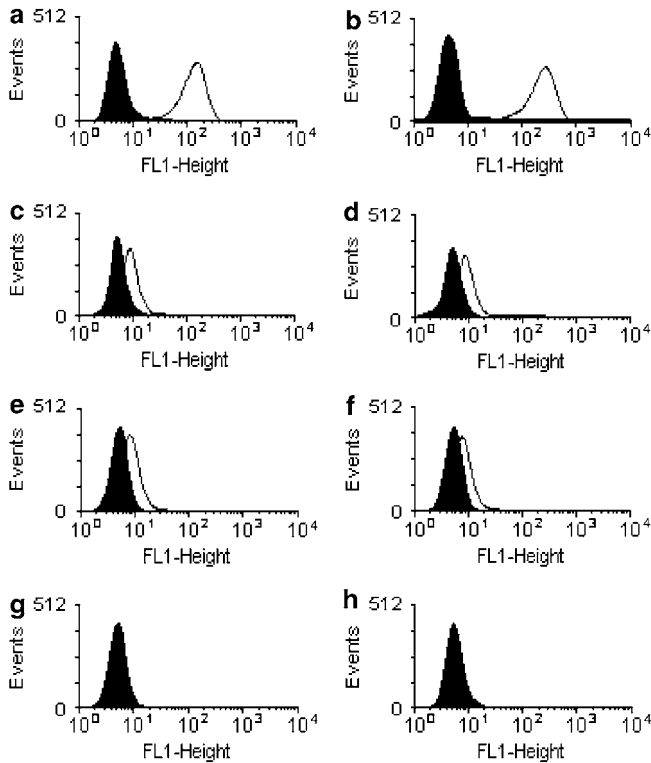


Figure 2 D2 cells express surface immunoglobulin M (IgM). (a) Binding of anti-IgM antibodies to 38C-13 cells. (b) Binding of the anti-idiotype (Id) monoclonal antibody 2B6 to 38C-13 cells. (c) Binding of anti-IgM antibodies to D2 cells. (d) Binding of the anti-Id monoclonal antibody 2B6 to D2 cells. (e) Binding of the anti-Id monoclonal antibody 2B6 to D2 cells following Fc receptor blocking with aggregated human IgG. (f) Binding of F(ab')₂ fragments of the anti-Id monoclonal antibody 2B6 to D2 cells. (g) Binding of isotype control monoclonal antibody (anti-mouse CD25 rat IgG1) to D2 cells. (h) Binding of the monoclonal antibody R2.438.8 (against an unrelated Id) to D2 cells. Following incubation with the indicated antibodies, cells were washed and incubated with fluorescein isothiocyanate (FITC)-labeled mouse anti-rat IgG antibodies. Shaded histograms depict control staining. Open histograms depict staining with the indicated antibodies.

using. In some cells, when levels of surface antigens are very low, fixation of stained cells may dim the fluorescence.

Surface IgM of D2 is not a membrane-bound IgM

Surface expression of B-cell membrane-bound IgM requires its assembly with the Ig α /Ig β heterodimer. Plasma cells lack expression of Ig α /Ig β polypeptides, hence are unable to transport IgM to the membrane.^{5,6} Since rare plasma cell variants expressing Ig α /Ig β may emerge,⁴ we determined expression of Ig α . As shown in Figure 4, while 38C-13 lymphoma cells express Ig α , D2 plasma cells show no detectable Ig α .

It has been reported that IgD but not IgM can be anchored in the plasma membrane of B cells²⁰ and rare variant clones of plasma cells²¹ through a glycosylphosphatidylinositol (GPI) linker. The GPI-anchored IgD can be transported to the cell surface in the absence of Ig α . To determine whether D2 may be a rare variant that expresses IgM in an Ig α -independent GPI-anchored form, we treated the cells with PI-PLC, an enzyme that releases GPI-anchored proteins from the cell surface. The cells were then analyzed for surface IgM. CD48, a GPI-anchored protein, served as a positive control for enzyme

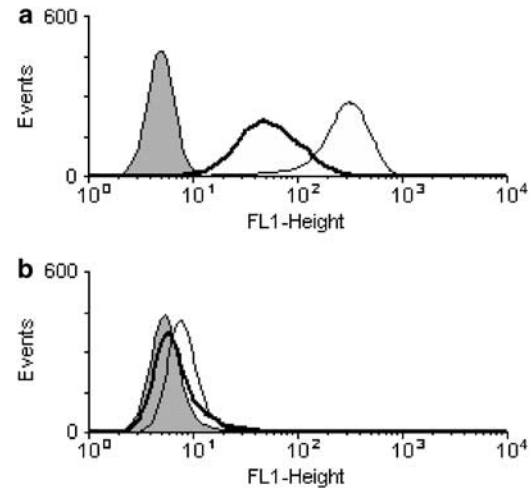


Figure 3 Soluble idiotype (Id) protein inhibits anti-Id antibody binding to D2 cells. The anti-Id monoclonal antibody 2B6 was incubated for 15 min with (open bold histograms) or without (open histograms) a 10-fold excess of Id protein. The mixture was then added to 38C-13 cells (a) or to D2 cells (b) for a 45 min incubation followed by three cell washes and incubation with fluorescein isothiocyanate (FITC)-labeled mouse anti-rat immunoglobulin G (IgG) antibodies. Shaded histograms depict control staining.

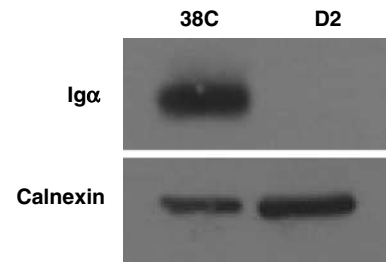


Figure 4 D2 cells do not express immunoglobulin- α (Ig α). Cell lysates of 5×10^5 38C-13 (38C) and D2 cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by western blot analysis with rabbit anti-Ig α antibodies and with rabbit anti-calnexin antibodies (loading control).

reactivity. Figure 5 demonstrates that in contrast to CD48, IgM was not released from the cell surface of D2 cells, indicating that it is not expressed as a GPI-linked molecule. It should be noted that CD48 was not entirely released by the enzyme. GPI-anchored proteins in certain cells exhibit some degree of resistance to PI-PLC cleavage due to inositol acylation of the GPI anchor.²²

IgM molecules were then analyzed by western blotting. Immuno-precipitated IgM resolved by SDS-PAGE was probed with rabbit anti-mouse IgM antibodies or with the μ_m -specific 5F8 monoclonal antibody that selectively recognizes the membrane form of IgM. When probed with anti-IgM antibodies, two μ heavy-chain species are demonstrated in 38C-13 (Figure 6a), the slower migrating band representing the mature membrane-bound μ_m and the faster migrating band representing intracellular (μ_i) precursor heavy chains.^{23,24} The μ_i fraction contains immature μ_m and μ_s molecules that cannot be resolved by SDS-PAGE. Although D2 cells produce large amounts of IgM, mature membrane-bound IgM, colocalizing with the upper band in 38C-13 cells, is not detected (Figure 6a). The IgM in D2 cells consists entirely of intracellular IgM. Typically, the mature μ_s heavy chains migrate in SDS-PAGE to a position which localizes in between

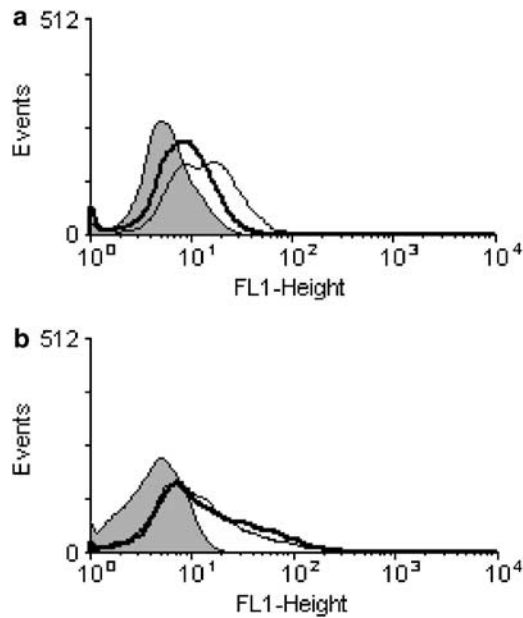


Figure 5 Immunoglobulin M (IgM) in D2 cells is not expressed as a glycosylphosphatidylinositol (GPI)-linked membrane protein. D2 cells were incubated with (open bold histograms) or without (open histograms) 0.5 U of phosphatidylinositol-specific phospholipase C (PI-PLC) for 1 h at 37 °C. The cells were then washed and analyzed by immunofluorescence staining with anti-CD48 antibodies (a) or with anti-idiotypic (Id) antibodies (b). Shaded histograms depict control staining.

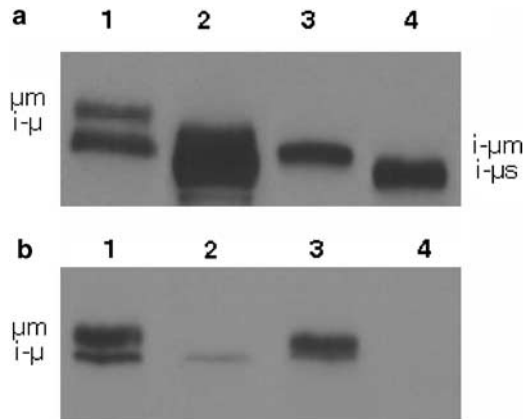


Figure 6 D2 cells produce small amounts of μ_m heavy chains. Cell lysates of 38C-13 (lane 1), D2 (lane 2), JL μ_m (lane 3) and JL μ_s (lane 4) were immunoprecipitated with rabbit anti-mouse IgM antibodies. Immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by western blot analysis with rabbit anti-IgM antibodies (a) or with the μ_m -specific 5F8 monoclonal antibody (b). Lysates were of 5×10^6 cells in (a) and 4×10^7 cells in (b).

the mature μ_m and the $i\mu$ bands of 38C-13 cells, while the immature μ_s chains migrate faster.²⁵ The mature μ_s comprises only a small fraction while the immature μ_s comprises the majority of intracellular μ_s . Due to the large amount of IgM, the resolution of the two μ_s species in D2 is poor. When probed with the μ_m -specific 5F8 monoclonal antibody, mature surface μ_m (upper band) and intracellular immature μ_m (lower band) are demonstrated in 38C-13 cells (Figure 6b). A very small amount of μ_m is produced in D2 cells. This

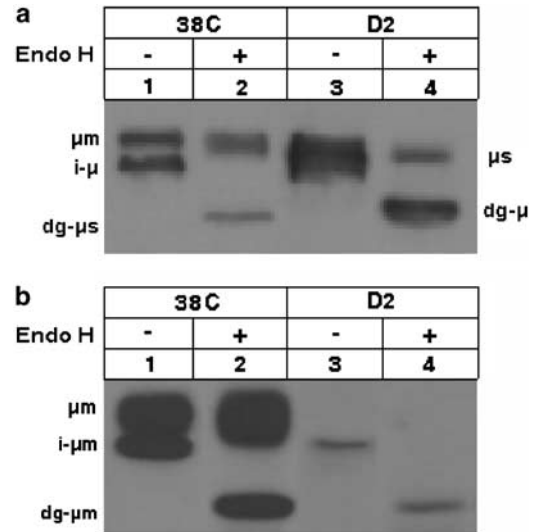


Figure 7 The μ_m heavy chains in D2 cells are immature intracellular molecules. Cell lysates of 38C-13 (lanes 1, 2) and D2 (lanes 3, 4) were immunoprecipitated with rabbit anti-mouse immunoglobulin M (IgM) antibodies. Immunoprecipitates were incubated with or without Endoglycosidase H (Endo H). Following enzyme treatment, immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by western blot analysis with rabbit anti-IgM antibodies (a) or with the μ_m -specific 5F8 monoclonal antibody (b). Lysates were of 1×10^7 cells in (a) and 4×10^7 cells in (b). Deglycosylated is abbreviated as dg.

μ_m migrates as the immature precursor, while no mature surface μ_m is demonstrated. JL μ_s cells and JL μ_m cells, producing either μ_s or μ_m respectively, were included to verify the μ_m specificity of the 5F8 monoclonal antibody. As mentioned above, while JL μ_s cells secrete IgM, JL μ_m cells produce substantial amount of IgM, which is neither deposited on the cell surface nor secreted, and is therefore degraded.

To verify the intracellular location of μ_m in D2 cells, immunoprecipitated IgM was treated with Endo H prior to analysis by SDS-PAGE. Glycoproteins in the rough endoplasmic reticulum are characterized by high-mannose glycosylation that is sensitive to cleavage by Endo H, whereas glycoproteins reaching the Golgi apparatus become terminally glycosylated and thus resistant to the enzyme. As expected, the mature surface μ_m of 38C-13 lymphoma cells (upper band) is Endo H resistant, while the intracellular μ is sensitive to the enzyme (Figure 7). Although the $i\mu$ fraction contains μ_m and μ_s molecules, probing with anti-IgM antibodies revealed only μ_s precursors because they comprise the majority of $i\mu$ molecules in B cells. Detection of intracellular μ_m requires higher cell numbers (Figure 7b). In D2 myeloma cells, the majority of IgM is of the secreted form, part of which (the mature μ_s) is Endo H resistant, while the rest (the μ_s precursor) is Endo H sensitive (Figure 7a). Only a very small fraction of IgM in D2 cells is of the membrane form. These μ_m molecules, however, have the size of immature μ_m and are Endo H sensitive (Figure 7b). Hence, although the myeloma cells produce small amounts of μ_m heavy chains, they are unable to express them on the cell surface.

Anti-Id antibodies induce complement-mediated lysis of D2 cells

Since D2 cells express very low levels of surface IgM, it was questioned whether antibody binding can possibly recruit effector mechanisms that are known to be involved in antibody-mediated cell killing. Since complement is such an effector mechanism, we studied its effect on

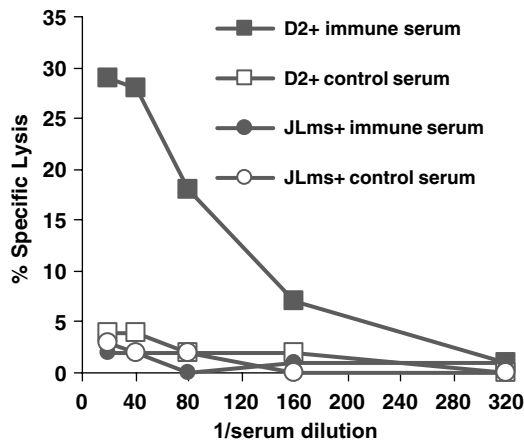


Figure 8 Complement-mediated cytotoxicity induced by anti-idiotypic (Id) antibodies. ^{51}Cr -labeled D2 cells (■, □) and JL μ s cells (●, ○) were treated with immune serum of Id-vaccinated mice (closed symbols) and with pre-immune serum as control (open symbols). Rabbit complement was added, and specific ^{51}Cr release was determined after 1 h of incubation.

D2 cells bound with anti-Id antibodies. Figure 8 demonstrates that complement is indeed activated in response to anti-Id binding to D2 cells. This response is antigen specific, since JL μ s plasma cells, expressing a different Id, were not affected.

DISCUSSION

Plasma cells produce the secreted immunoglobulin form nearly exclusively.^{2,3} The minute amounts of membrane immunoglobulin that may be generated in plasma cells cannot be transported to the cell surface because they lack expression of Ig α /Ig β polypeptides, which are needed to sheath the relatively hydrophilic transmembrane segment.²⁶ Only few exceptions to this rule have been reported. In the case of one myeloma variant clone, IgD has been shown to be inserted into the membrane by a GPI anchor.²¹ In addition, when expression vectors coding for δ m and γ 2bm (but not for other isotypes) were introduced into myeloma cells, membrane IgD and IgG2b were transported to the cell surface without either Ig α /Ig β sheath or GPI anchor,²⁷ most probably due to the intrinsic hydrophobicity of their transmembrane segments.²⁶

We demonstrate here that plasmacytoma cells can produce small amounts of immunoglobulin μ _m heavy chains. However, the latter are Endo H-sensitive μ _m precursor molecules that cannot reach the cell surface. These results are in agreement with previous reports that plasma cells do not express membrane IgM on the cell surface.^{2,3} Although membrane-bound IgM is absent, D2 cells stain positively for surface IgM, reflecting molecules of the secreted form in the process of secretion. This is not unique for the D2 tumor, as immunoglobulin staining can frequently be demonstrated in myelomatous plasma cells.¹⁴ We show that in spite of the relatively low levels of surface immunoglobulin, anti-Id antibodies were effective in retardation of tumor growth *in vivo*. These results question the common notion considering the low surface immunoglobulin and the absence of membrane-bound immunoglobulin in myeloma as a limiting factor that prevents tumor eradication by anti-Id antibodies. Although studies showing that anti-Id antibodies can inhibit myeloma growth were previously published,^{13,16} these reports did not elaborate on the possibility that the myeloma tumors represented unusual clones that express membrane-bound immunoglobulin, as has been reported.^{21,27}

We, however, demonstrate that myeloma cells expressing only the secreted immunoglobulin form are affected by anti-Id antibodies.

The mechanism of tumor growth inhibition by anti-Id antibodies has not been identified. Studies, such as the demonstration that murine IgG2a anti-Id had greater antilymphoma effects than IgG1 and IgG2b,²⁸ or that Fc γ receptor polymorphisms correlated with the clinical outcome of Id vaccination in lymphoma patients,²⁹ implicated a role for antibody-dependent effector mechanisms such as complement-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity (ADCC). However, other studies contradicted these findings, suggesting additional mechanisms. Thus, clinical trials in lymphoma patients revealed that clinical responses were induced by antibodies of the IgG1, IgG2a or IgG2b subclass, and that the presence of antitumor response or the magnitude of that response could not be correlated with the subclass of antibodies.³⁰ In addition, passive immunization with F(ab')₂ fragments of anti-Id antibodies, which lack the Fc domain necessary for recruitment of complement and ADCC, protected animals against the growth of 38C-13 tumor.^{31,32} Moreover, Id vaccination studies in the 38C-13 model demonstrated that the IgG isotype of anti-Id antibodies did not correlate with protective immunity.³³ Hence, effector mechanisms other than complement-mediated cytotoxicity and ADCC are involved in lymphoma protection by anti-Id antibodies. These mechanisms have not been precisely identified. Since anti-Ids have regulatory functions in the immune system,^{34–36} it has been suggested that inoculation of anti-Id antibodies may have an antitumor effect by altering the Id-anti-Id network that plays a role in the regulation of B-cell clones. Alternatively, it has been suggested that anti-Id antibodies exert direct antiproliferative effects, leading to programmed cell death.^{37,38} Because direct induction of cell death by anti-Id antibodies involves signal transduction by immunoglobulin molecules, it seems unlikely to be involved in growth inhibition of myeloma cells, which do not express the BCR-Ig α /Ig β complex on the surface. The other proposed effector mechanisms may however be involved. Thus, our demonstration that complement is activated by anti-Id antibodies to kill D2 cells may indicate possible involvement in tumor growth retardation.

Id vaccination with autologous immunoglobulin is being evaluated in clinical trials to control residual disease in multiple myeloma.^{9,39–41} Whereas antibodies may play a predominant role in destruction of B-cell lymphoma,^{10–12} it was traditionally considered that they are not effective against plasma cell tumors, because the large amounts of immunoglobulin molecules secreted by the tumors block anti-Id antibodies, and because the absence of membrane immunoglobulin on the surface of these tumor cells compromise their capacity to bind and be affected by anti-Id antibodies. The obstacle of high Id levels in the circulation may be obviated by reducing tumor burden to minimal residual disease.¹³ Complete response to high-dose therapy and peripheral blood stem cell transplantation in multiple myeloma patients was defined as the absence of the monoclonal immunoglobulin and no evidence of clonal plasma cells in bone marrow.⁴² With regard to the limitation of the idiotypic immunoglobulin being predominantly cytoplasmic and the absence of membrane immunoglobulin, we show here that low levels of secreted immunoglobulin on the cell surface can be targeted by anti-Id antibodies that may therefore play a role in myeloma patients achieving complete remission. Although the plasma cell is the predominant malignant cell type in multiple myeloma, several studies have shown that B cells, which are clonally related to the malignant plasma cells, are present in the bone marrow, peripheral blood and lymph nodes of myeloma patients.⁴³ The significance of these clonotypic B cells in the disease process remains unknown. However, it has been suggested that they include

drug-resistant reservoirs that may underline relapse.⁴⁴ Anti-Id antibodies may eradicate the clonotypic B cells.

Because cell-mediated responses play a predominant role in destruction of myeloma cells, the design of Id vaccines is presently focusing on vaccines that elicit Id-reactive T cells. Accordingly, assessment of vaccine efficacy focuses on T-cell responses. While induction of anti-Id antibodies was demonstrated in some Id-vaccinated myeloma patients,⁴⁵ it is generally difficult to detect anti-Id antibodies in patients with advanced disease because of the abundant circulating Id. However, their detection in patients in complete remission should be feasible. Vaccination strategies optimized for induction of both cell-mediated and humoral responses may therefore be considered. In summary, while there is no doubt that Id-specific cell-mediated responses are very important, myeloma patients in complete remission may additionally benefit from Id-specific antibodies triggered by Id vaccines.

METHODS

Mice

C3H/eB×BALB/c (8-week-old) female mice were obtained from the animal facility of Tel Aviv University. All procedures were approved by the Institutional Animal Care and Use Committee.

Cell lines

38C-13 is a carcinogen-induced B-cell tumor that expresses IgM on the cell surface.⁴⁶ D2 hybridoma has been obtained by somatic cell hybridization between 38C-13 and an Ig-negative myeloma cell line.³ This 'rescue' hybridoma secretes large amounts of IgM with idiotypic specificity identical to that of 38C-13. D2-C11 hybridoma is an Id-negative derivative of D2 that secretes only free light chains. JLM_s and JLM_m are transfectoma cell lines derived from the heavy-chain-deficient light-chain-producing J558L⁴⁷ transfected with the 17.2.25 μ chain.⁴⁸ JLM_s and JLM_m were obtained by transfection with plasmids coding for either the secreted (μ_s) or the membrane (μ_m) form. Thus, while JLM_s cells secrete IgM, JLM_m cells produce a substantial amount of IgM, which is neither deposited on the cell surface nor secreted. Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin and 50 μ M mercaptoethanol.

Antibodies and reagents

5F8 is a μ_m -specific monoclonal antibody that was generated from BALB/c mice immunized with the μ_m -specific peptide KEDEVNAEEEGFEY coupled to keyhole limpet hemocyanin (KLH). The synthetic peptide was obtained from Tana Laboratories (Houston, TX, USA). R2.438.8, a rat monoclonal antibody specific to the 17.2.25 Id of JLM_s,⁴⁹ was kindly provided by Dr T Imanishi-Kari (Tufts University School of Medicine, Boston, MA, USA). 2B6 is a rat monoclonal antibody specific to the Id of 38C-13.⁵⁰ Rat anti-mouse CD25 was purchased from BD Biosciences Pharmingen (San Diego, CA, USA). Hamster anti-mouse CD48 and fluorescein isothiocyanate (FITC)-conjugated anti-hamster IgG were purchased from eBioscience (San Diego, CA, USA). Rabbit anti-mouse Ig α was kindly provided by Dr J Cambier (University of Colorado Health Sciences Center and National Jewish Medical Research Center, Denver, CO, USA). Rabbit anti-calnexin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rat anti-mouse IgM monoclonal antibody was purchased from Zymed Laboratories (San Francisco, CA, USA). FITC-conjugated mouse anti-rat IgG and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Human IgG, HRP-conjugated protein A and phosphatidylinositol-specific phospholipase C (PI-PLC) were purchased from Sigma-Aldrich (St Louis, MO, USA). Endoglycosidase H (Endo H) was purchased from New England Biolabs (Ipswich, MA, USA). Protein G-Sepharose was purchased from GE Healthcare-Pharmacia (Uppsala, Sweden). Rabbit complement was purchased from Cedarlane Laboratories (Burlington, Ontario, Canada).

Immunization and tumor challenge

38C-13 IgM was purified from ascites of mice inoculated with D2 cells. It was coupled to KLH using 0.1% glutaraldehyde as described.⁵¹ C3H/eB×BALB/c mice were immunized subcutaneously with Id-KLH emulsified in complete Freund's adjuvant (25 μ g IgM per mouse), and boosted 2 weeks later with the same conjugate in incomplete Freund's adjuvant. Sera of immunized mice were collected 2 weeks after second immunization. They were assessed for anti-Id antibodies by enzyme-linked immunosorbent assay (ELISA) as described.¹⁷ For passive immunization, naive mice were inoculated intraperitoneally with 1×10^4 38C-13, D2 or D2-C11 tumor cells. After 1 day, 0.4 ml of immune serum was injected intraperitoneally.

Immunoprecipitation, treatment with Endo H and western blotting

Cells were lysed with 1% Triton X-100 in 0.15 M NaCl, 25 mM Tris-HCl (pH 8.0) containing protease inhibitors. Lysates were centrifuged at 12 000 g for 15 min to remove insoluble material. IgM was immunoprecipitated from cell lysates by incubation with rabbit anti-mouse IgM for 18 h at 4 °C with constant shaking, followed by incubation with protein G-Sepharose. Immune complexes were eluted from washed beads by boiling in Laemmli sample buffer, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For treatment with Endo H, immune complexes were eluted from beads by boiling with 0.5% SDS and 1% mercaptoethanol for 10 min. About 50 mM sodium citrate and 500 U of Endo H were then added and the samples were incubated for 2 h at 37 °C. Sample buffer was added, and proteins were resolved by SDS-PAGE. Resolved proteins were transferred to nitrocellulose membranes. The membranes were blocked for 2 h at room temperature and then probed with primary antibodies for 1 h at room temperature (rabbit polyclonal antibodies) or for 18 h at 4 °C (mouse monoclonal antibodies) followed by HRP-conjugated protein A or HRP-conjugated rabbit anti-mouse IgG, respectively. The probed proteins were visualized by enhanced chemiluminescence.

Immunofluorescence

Cells were incubated for 45 min at 4 °C with different antibodies. After washing, cells were incubated with FITC-conjugated secondary antibodies for 45 min at 4 °C. Cells were washed and analyzed on a FACSort flow cytometer (BD Biosciences, Mountain View, CA, USA).

Cell treatment with PI-PLC

Cells were suspended in 50 mM Tris, 0.25 M sucrose, 10 mM EDTA (pH 7.5) and incubated with or without 0.5 U of PI-PLC for 1 h at 37 °C. The cells were then washed and analyzed by immunofluorescence as described above.

Complement-mediated cytotoxicity

Cytotoxicity was assessed by a chromium (Cr) release assay.⁵¹ Cr-labeled tumor cells were placed in 96-well U-shaped microtiter plates at 1×10^4 cells (100 μ l) per well. Serum of immunized mice (50 μ l) was added at different dilutions at 4 °C for 30 min. Rabbit complement was then added (50 μ l) and microplates were incubated for 1 h at 37 °C. After centrifugation, supernatants were harvested, their radioactivity was counted and specific cytotoxicity was calculated.

Statistical analysis

Statistical analysis on survival curves was performed using the log-rank test.

ACKNOWLEDGEMENTS

This research was supported by The Israel Science Foundation (grant no. 635/02) and by a project grant from the Israel Cancer Research Fund.

1 Kehry M, Ewald S, Douglas R, Silbey C, Raschke W, Fambrough D *et al*. The immunoglobulin μ chains of membrane-bound and secreted IgM molecules differ in their C-terminal segments. *Cell* 1980; **21**: 393–406.

- 2 Alt F, Bothwell A, Knap M, Siden E, Mather E, Koshland M *et al*. Synthesis of secreted and membrane-bound immunoglobulin μ heavy chains is directed by mRNAs that differ at their 3' ends. *Cell* 1980; **20**: 293–301.
- 3 Nelson KJ, Haimovich J, Perry RP. Characterization of productive and sterile transcripts from the immunoglobulin heavy-chain locus: processing of μ m and μ s mRNA. *Mol Cell Biol* 1983; **3**: 1317–1332.
- 4 Wienands J, Hombach J, Radbruch A, Riesterer C, Reth M. Molecular components of the B cell antigen receptor complex of class IgD differ partly from those of IgM. *EMBO J* 1990; **9**: 449–455.
- 5 Sitia R, Neuberger MS, Milstein C. Regulation of membrane IgM expression in secretory B cells: translational and post-translational events. *EMBO J* 1987; **6**: 3969–3977.
- 6 Hombach J, Sablitzky F, Rajewsky K, Reth M. Transfected plasmacytoma cells do not transport the membrane form of IgM to the cell surface. *J Exp Med* 1988; **167**: 652–657.
- 7 Armstrong AC, Cheadle EJ, Hawkins RE. Toward personalized immunotherapy for non-Hodgkin lymphoma. Targeting the idiotype immunoglobulin. *Biodrugs* 2005; **19**: 289–297.
- 8 Vose JM. Personalized immunotherapy for the treatment of non-Hodgkin's lymphoma: a promising approach. *Hematol Oncol* 2006; **24**: 47–55.
- 9 Reichardt VL, Brossart P. Dendritic cells in clinical trials for multiple myeloma. *Methods Mol Med* 2005; **109**: 127–136.
- 10 George AJT, Tutt AL, Stevenson FK. Anti-idiotypic mechanisms involved in suppression of a mouse B cell lymphoma, BCL1. *J Immunol* 1987; **138**: 628–634.
- 11 Syrengelas AD, Levy R. DNA vaccination against the idiotype of a murine B cell lymphoma: mechanism of tumor protection. *J Immunol* 1999; **162**: 4790–4795.
- 12 Cesco-Gaspere M, Benvenuti F, Burrone OR. BCL1 lymphoma protection induced by idiotype DNA vaccination is entirely dependent on anti-idiotypic antibodies. *Cancer Immunol Immunother* 2005; **54**: 350–358.
- 13 Croese JW, Van den Enden-Vieeen MH, Radl J. Immune regulation of 5T2 mouse multiple myeloma. II. Immunological treatment of 5T2 MM residual disease. *Neoplasma* 1991; **38**: 467–474.
- 14 Ocqueteau M, San Miguel JF, Gonzalez M, Almeida J, Orfao A. Do myelomatous plasma cells really express surface immunoglobulin? *Haematologica* 1996; **81**: 460–463.
- 15 Jarvis MR, Voss EW. Determination of dissociation constants and ligand specificity of detergent solubilized surface membrane immunoglobulin A from MOPC-315. *Mol Immunol* 1983; **20**: 125–136.
- 16 Kodama K, Ghanta VK, Hiramoto NS, Hiramoto RN. Regression of MOPC 104E plasmacytoma with monoclonal anti-idiotypic antibodies. *J Biol Response Mod* 1989; **8**: 385–396.
- 17 Cohen S, Haimovich J, Hollander N. B-cell lymphoma and myeloma protection induced by idiotype vaccination with dendritic cells is mediated entirely by T cells in mice. *J Immunother* 2005; **28**: 461–466.
- 18 Haefner-Cavaillon N, Klein M, Dorrington KJ. Studies on the Fc γ receptor of the murine macrophage-like cell line P388D $_1$. I. The binding of homologous and heterologous immunoglobulin G. *J Immunol* 1979; **123**: 1905–1913.
- 19 Heyfets A, Haimovich J, Hollander N. Determination of idiotype-specific T cells in idiotype-vaccinated mice. *Immunol Lett* 2002; **80**: 207–213.
- 20 Chaturvedi A, Siddiqui Z, Bayiroglu F, Rao KVS. A GPI-linked isoform of the IgD receptor regulates resting B cell activation. *Nat Immunol* 2002; **3**: 951–957.
- 21 Wienands J, Reth M. Glycosyl-phosphatidylinositol linkage as a mechanism for cell-surface expression of immunoglobulin D. *Nature* 1992; **356**: 246–248.
- 22 Wong YW, Low MG. Biosynthesis of glycosylphosphatidylinositol-anchored human placental alkaline phosphatase: evidence for a phospholipase C-sensitive precursor and its post-attachment conversion into a phospholipase C-resistant form. *Biochem J* 1994; **301**: 205–209.
- 23 Bergman Y, Haimovich J. B lymphocytes contain three species of μ chains. *Eur J Immunol* 1978; **8**: 876–880.
- 24 Rabinovich E, Bar-Nun S, Amity R, Shachar I, Guri B, Taya M *et al*. Different assembly species of IgM are directed to distinct degradation sites along the secretory pathway. *J Biol Chem* 1993; **268**: 24145–24148.
- 25 Eshhar Z, Blatt C, Bergman Y, Haimovich J. Induction of secretion of IgM from cells of the B cell line 38C-13 by somatic cell hybridization. *J Immunol* 1979; **122**: 2430–2434.
- 26 Williams GT, Venkitaraman AR, Gilmore DJ, Neuberger MS. The sequence of the μ transmembrane segment determines the tissue specificity of the transport of immunoglobulin M to the cell surface. *J Exp Med* 1990; **171**: 947–952.
- 27 Williams GT, Dariavach P, Venkitaraman AR, Gilmore DJ, Neuberger MS. Membrane immunoglobulin without sheath or anchor. *Mol Immunol* 1993; **30**: 1427–1432.
- 28 Kaminski MS, Kitamura K, Maloney DG, Campbell MJ, Levy R. Importance of antibody isotype in monoclonal anti-idiotypic therapy of a murine B cell lymphoma: a study of hybridoma switch variants. *J Immunol* 1986; **136**: 1123–1130.
- 29 Weng WK, Czerwinski D, Timmerman J, Hsu FJ, Levy R. Clinical outcome of lymphoma patients after idiotype vaccination is correlated with humoral immune response and immunoglobulin G Fc receptor genotype. *J Clin Oncol* 2004; **22**: 4717–4724.
- 30 Brown SL, Miller RA, Horning SJ, Czerwinski D, Hart SM, McElderry R *et al*. Treatment of B-cell lymphoma with anti-idiotypic antibodies alone and combination with α interferon. *Blood* 1989; **3**: 651–661.
- 31 Perek Y, Hurwitz E, Burowski D, Haimovich J. Immunotherapy of a murine B cell tumor with antibodies and F(ab') $_2$ fragments against idiotype determinants of its cell surface IgM. *J Immunol* 1983; **131**: 1600–1603.
- 32 Cohen S, Haimovich J, Hollander N. Anti-idiotypic $\times$ anti-LFA-1 bispecific antibodies inhibit metastasis of B cell lymphoma. *J Immunol* 2003; **170**: 2695–2701.
- 33 Singh G, Parker S, Hobart P. The development of a bicistronic plasmid DNA vaccine for B-cell lymphoma. *Vaccine* 2002; **20**: 1400–1411.
- 34 Jerne NK. Towards a network theory of the immune system. *Ann Immunol* 1974; **125C**: 373–389.
- 35 Geha RS. Regulation of the immune response by idiotype-anti-idiotypic interactions. *N Engl J Med* 1981; **305**: 25–28.
- 36 Kearny JF, Vakil M. Idiotype-directed interactions during ontogeny play a major role in the establishment of the adult B cell repertoire. *Immunol Rev* 1986; **94**: 39–50.
- 37 Vuist WM, Levy R, Maloney DG. Lymphoma regression induced by monoclonal anti-idiotypic antibodies correlates with their ability to induce Ig signal transduction and is not prevented by tumor expression of high levels of BCL-2 protein. *Blood* 1994; **83**: 899–906.
- 38 Renschler M, Bhatt RR, Dower WJ, Levy R. Synthetic peptide ligands of the antigen binding receptor induce programmed cell death in human B-cell lymphoma. *Proc Natl Acad Sci USA* 1994; **91**: 3623–3627.
- 39 Liso A, Stockerl-Goldstein KE, Auffermann-Gretzinger S, Benik CJ, Reichardt V, van Beckhoven A *et al*. Idiotype vaccination using dendritic cells after autologous peripheral blood progenitor cell transplantation for multiple myeloma. *Biol Blood Marrow Transplant* 2000; **6**: 621–627.
- 40 Yi Q. Immunotherapy in multiple myeloma: current strategies and future prospects. *Expert Rev Vaccines* 2003; **2**: 391–398.
- 41 Bendandi M, Rodriguez-Calvillo M, Inoges S, Lopez-Diaz de Cerio A, Perez-Simon JA, Rodriguez-Caballero A *et al*. Combined vaccination with idiotype-pulsed allogeneic dendritic cells and soluble protein idiotype for multiple myeloma patients relapsing after reduced-intensity conditioning allogeneic stem cell transplantation. *Leuk Lymphoma* 2006; **47**: 29–37.
- 42 Reichardt VL, Okada CY, Liso A, Benike CJ, Stockerl-Goldstein KE, Engelman EG *et al*. Idiotype vaccination using dendritic cells after autologous peripheral blood stem cell transplantation for multiple myeloma—a feasibility study. *Blood* 1999; **93**: 2411–2419.
- 43 Rasmussen T, Lodghal M, Hance S, Johnsen HE. In multiple myeloma clonotypic CD38-/CD19+/CD27+ memory B cells recirculate through bone marrow peripheral blood and lymph nodes. *Leuk Lymphoma* 2004; **45**: 1413–1417.
- 44 Bergsagel PL, Smith AM, Szczeppek A, Mant MJ, Belch AR, Pilarsky LM. In multiple myeloma, clonotypic B lymphocytes are detectable among CD19+ peripheral blood cells expressing CD38, CD56, and monotypic Ig light chain. *Blood* 1995; **85**: 436–447.
- 45 Titze S, Christensen O, Mancke O, Tesch H, Wolf J, Emmerich B *et al*. Vaccination of multiple myeloma patients with idiotype-pulsed dendritic cells: immunological and clinical aspects. *Br J Haematol* 2000; **108**: 805–816.
- 46 Bergman Y, Haimovich J. Characterization of a carcinogen-induced murine B lymphocyte cell line of C3H/EB origin. *Eur J Immunol* 1977; **7**: 413–417.
- 47 Oi VT, Morrison SL, Herzenberg LA, Berg P. Immunoglobulin gene expression in transformed lymphoid cells. *Proc Natl Acad Sci USA* 1983; **80**: 825–829.
- 48 Lo DY, Bothwell AL, White-Scharf ME, Imanishi-Kari T, Baltimore D. Molecular basis of a mouse strain-specific anti-hapten response. *Cell* 1983; **33**: 85–93.
- 49 Cascalho M, Wong J, Steinberg C, Wabl M. Mismatch repair co-opted by hypermutation. *Science* 1998; **279**: 1207–1210.
- 50 Hurwitz E, Burowski D, Kashi R, Hollander N, Haimovich J. A synergistic effect between anti-idiotypic antibodies and anti-neoplastic drugs in the therapy of a murine B-cell tumor. *Int J Cancer* 1986; **37**: 739–745.
- 51 Maloney DG, Kaminski MS, Burowski D, Haimovich J, Levy R. Monoclonal anti-idiotypic antibodies against the murine B cell lymphoma 38C13: characterization and use as probes for the biology of the tumor *in vivo* and *in vitro*. *Hybridoma* 1985; **4**: 191–209.