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The ability of human bispecific anti-idiotype antibody to elicit humoral and cellular immune responses in mice

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ABSTRACT

Our goal is to compare the immunogenicity and the extent of immunologic reactivity between bispecific and mono anti-idiotype vaccines. We previously obtained two human anti-Id antibody fragments fuse5-G22, fuse5-I50 by phage display technology which were mimics of the antigens from nasopharyngeal carcinoma cell line (HNE2). In this study, we developed and characterized a bispecific anti-Id antibody vaccine G22–I50 and its parent monovalent antibody vaccines G22 and I50. The efficacy of G22–I50, G22, and I50 as tumor vaccines was evaluated in Balb/c mice with three injections of these vaccines adjuvanted with Freund's adjuvant. Mice immunized with G22–I50 exhibited comparable levels of antibody titers and stronger binding inhibition capabilities. Spleen cells from G22–I50-immunized mice gave a significant proliferative response and higher expression level of IFN-γ and IL-2.These results suggested that bispecific anti-Id antibody vaccine was able to induce more powerful humoral and cell-mediated immune responses, which might make it to be a potential vaccine candidate for the therapy of nasopharyngeal carcinoma.

1. Introduction

Nasopharyngeal carcinoma (NPC) is endemic in Asia and is etiologically associated with Epstein–Barr virus. Although radio-chemotherapy can cure most patients with early-stage disease, it seems that the conventional therapy has reached a therapeutic plateau and a significant number of patients present with metastatic or refractory disease or develop relapses [1,2]. Thus, alternative therapeutic strategies need to be established.

According to Niels Jerne's idiotypic network hypothesis, antigenic epitope structures can be mirrored through an anti-idiotypic cascade of antibodies. Of these, the so-called internal image anti-idiotypic (Ab2β) antibodies can mimic epitopes of self-antigens and serve for tumor vaccination strategies [3–5]. The use of anti-idiotype (Id) antibodies as vaccines to stimulate anti-tumor immunity is one of the several promising immunologic approaches to the therapy of cancer. Clinical studies have shown the induction of specific antibody (Ab3) responses by Ab2β anti-Id antibodies in tumor patients bearing a variety of tumors [6–10].

During the past two decades, a number of Ab2β monoclonal antibodies (mAbs) had been produced and applied into the therapy of cancer patients. However, therapy with these mAbs was limited by development of human antibodies against this large mouse protein (HAMA) [11–13]. Antibody fragments have been proposed to address these problems [14,15]. The single chain antibody (scFv) fragments are heterodimers made up of the antibody variable regions of heavy and light chain (VH and VL) domains and are the smallest antibody fragments that contain all of the structural information necessary for specific antigen binding [16]. ScFvs to several well-characterized tumor antigens have been developed and studied. However, decreased immunogenicity and univalent antigen binding of scFvs have limited its application [17–19]. To overcome these limitations, both covalent dimeric scFv (di-scFv) and noncovalent diabody constructs have been studied; as bivalent molecules, they exhibit greater avidity than the smaller univalent scFv fragments [20–23].

In our previous experiment, He et al. [24] had constructed a human phage anti-Id antibody library with 1.5×10^8 clones by in vitro immunization with Ab1(FC2) and EBV transformation of peripheral blood mononuclear cells (PBMC) from NPC patients. 5 clones which might display β type Ab2 were selected by binding inhibition test. Among these, two human anti-Id antibody fragments fuse5-G22, fuse5-I50 had stronger inhibition of binding of Ab1(FC2) to the surface antigen of HNE2 cells and thus, we selected clones G22 and I50 to construct a bispecific antibody molecule [25]. In general, bispecific and multivalent antibodies which have more antigen binding sites will improve their immunogenicity. In order to know whether bispecific antibodies would improve their immunogenicity or not, we obtained a bispecific anti-Id antibody vaccine G22–I50 and its parent monovalent anti-Id vaccines G22 and I50 by genetic engineering method. In order to adequately measure the effectiveness of these vaccines, both humoral and cellular immune responses had been quantitated in animal models. Also, the extent of immunologic reactivity between bispecific and mono anti-Id vaccines had been determined. This finding is important for the ultimate

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utility of these anti-Id protein vaccines as part of the novel therapeutic strategy for NPC.

2. Materials and methods

2.1. FC2 mAb

One of the highly reactive hybridoma cell lines (FC2) [26] was injected intraperitoneally into female Balb/c mice(6 to 8 weeks old, Shanghai Slac, China) and the ascites were collected, centrifuged to remove the debris and stored at 4 °C until use. Purified FC2 mAb was labeled with horseradish peroxidase (HRP) by improved method of $NaIO_4$ [27]. HRP-FC2 was employed to analyse the inhibitory activity of Ab2β anti-Id antibodies.

2.2. Construction of anti-Id protein vaccines

To construct anti-Id protein vaccines, prokaryotic expression vector pET25b(+) (Invitrogen) was used. The gene encoding anti-Id antibody fragment G22 was firstly amplified with Pfu polymerase (MBI) from fuse5-G22 [24,25] using primers G22-1 and G22-2 (Table 1) and was then cloned into EcoRI and HindIII sites of pET25b(+).The resulting construct, pET25b–G22 was transformed into *E. coli* DH5α (Invitrogen) and confirmed by amplification, digestion and DNA sequencing (Invitrogen). The gene encoding anti-Id antibody fragment I50 was amplified from fuse5-I50 [24,25] using primers I50-1, I50-2 and I50-3, I50-4, respectively (Table 1). The products were then cloned into EcoRI and XhoI sites of pET25b or HindIII and XhoI sites of pET25b–G22. The correct constructs were referred as pET25b–I50 and pET25b–G22–I50.

2.3. Expression of anti-Id protein vaccines in E. coli BL21 (DE3)

Protein expression was done using *E. coli* BL21 (DE3) (Invitrogen) previously transformed with above recombinant pET25(+) plasmids, respectively. Bacteria were grown in Luria–Bertani medium with 50 μg/ml ampicillin (Sigma, St. Louis, MO) to an absorbance of 0.6 (λ = 600 nm) after which β-D-thiogalactopyranoside (IPTG) (Dingguo Bio Inc, Beijing, China) was added to a final concentration of 0.5 mM. Cultures were incubated at 30 °C for additional 6 h. Subsequently, cells were harvested by centrifugation (12,000 g, 4 °C, 15 min) and disrupted by sonication on ice and centrifuged (12,000 g, 4 °C, 15 min). Aliquots of both the soluble and insoluble protein fraction (inclusive bodies) were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The insoluble materials were purified by Ni^{2+}-NTA-agarose beads (Toyobo, Japan) under the denaturing condition and then renatured by dialysis refolding method for western blot analysis and immunization.

2.4. SDS-PAGE and western blot analysis

Proteins were separated by PAGE on 15% polyacrylamide gels under denaturing condition. Gels were either developed with Coomassie brilliant blue G protein stain or blotted onto nitrocellulose membranes (Dingguo) using a wet blotter (Liuyi Instrument Factory, Beijing, China) for 2 h at 100 mA. For western blot analysis, membranes were blocked for 2 h in phosphate-buffered saline (PBS) with 5% nonfat dried milk. The detection of proteins were carried out by incubating the membranes with Ab1 (FC2) (1:1000) or anti-His monoclonal antibody (1:1000; Novagen) diluted in PBS overnight at 4 °C and followed by the incubation of HRP-coupled goat anti-mouse IgG (Novagen) as secondary antibody. Three washes of nitrocellulose membranes were performed using PBS with 0.05% (v/v) Tween 20 (PBST) for 5 min each. Finally, protein bands were detected by ECL chemiluminescence (Beyotime, Shanghai, China).

2.5. Animals and immunization schedule

Female Balb/c mice, 6–8 weeks old, were purchased from Shanghai Slac Animal Center and used for in vivo studies. The mice were immunized with three proteins dissolved in endotoxin-free PBS at approximately bi-weekly intervals, respectively. They were subcutaneously injected as an emulsion of FCA (Freund's complete adjuvant; primary immunization, 100 μg) (Sigma) or FIA (Freund's incomplete adjuvant; two booster immunizations, 50 μg) (Sigma). Control group received mixtures containing endotoxin-free PBS with Freund's adjuvant. Serum samples from mice were taken prior to the first immunization and 14 days after each immunization to evaluate the antibody responses. The mice were sacrificed and spleens were harvested for cellular immunity assessment.

2.6. Measurement of antibody response by ELISA

The presence of Ab3 in sera was determined in a solid-phase ELISA (enzyme-linked immunosorbent assay) [28]. In brief, Ab2–Ab3 interaction was determined by coating the microtiter plates (Sigma) with 10 μg/ml of the respective protein, blocking with 2% bovine serum albumin (BSA; v/v; Dingguo) in PBS, pH 7.2 for 2 h at 37 °C. After three washes with PBST, 100 μl of serial two-fold dilutions of mouse sera in PBS was added and incubated at 37 °C for 2 h. The plates were then washed three times with PBST, and 100 μl of HRP-labeled goat anti-mouse IgG (Novagen) diluted 1:1000 was added. After an hour of incubation at 37 °C, 100 μl of fresh ABTS (2′,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) substrate solution (0.3 g/l ABTS and 0.03% H_2O_2 in a glycine/citric acid buffer, pH 4.0; w/v, v/v) was added for color development. The optical density (OD) of the plate was measured at 405 nm.

Inhibition of Ab1 (HRP-FC2) binding to the surface antigen of HNE2 cells (QK10903, cell library, China) by Ab3 sera was measured by competitive ELISA, where 50 μl of different sera at sequential dilutions was incubated for 2 h at 37 °C in HNE2 cells coated microtiter plates (Sigma), together with 50 μl of pre-determined dilution of HRP-FC2 which had given an absorbance value of 0.8–1.2 in a preliminary titration. The binding of HRP-FC2 was detected by adding ABTS complex and developed as described above. The adsorbance was measured at 405 nm in an ELISA reader (Bio-Tek, USA). The inhibitory activity was expressed as percentage of inhibition and determined as follows: Percentage of inhibition = $100 \times (A_{405\,nm}$ of HRP-FC2 − $A_{405\,nm}$ of HRP-FC2 bound to the surface antigen in the presence of Ab3 sera)/$A_{405\,nm}$ of HRP-FC2 [29].

Table 1
Primers for amplification to construct prokaryotic expression vectors.

Primer name	Sequence	Role
G22-1	TATAA*GAATTC* EcoRI TGACGTGGCCCAGGTACAGC	Introduction of EcoRI into G22 to construct pET25–G22
G22-2	GCTCT*AAGCTT*HindIII CAGGTCCCTTCGCCGAATACC	Introduction of HindIII into G22 to construct pET25–G22
I50-1	TATAA*GAATTC* EcoRI TGCCCAGGTACAGCTGGAGG	Introduction of EcoRI into I50 to construct pET25–I50
I50-2	ATATA*CTCGAG* XhoI GACCTTGGTCCCTTTGCCC	Introduction of XhoI into I50 to construct pET25–I50
I50-3	GCTCT*AAGCTT* HindIII **TCTAGTGGATCCGGT** linker GCCCAGGTACAGCTGGAGG	Introduction of HindIII and linker into I50 to construct pET25–G22–I50
I50-4	ATATA*CTCGAG* XhoI GACCTTGGTCCCTTTGCCC	Introduction of XhoI into I50 to construct pET25–G22–I50

2.7. Lymphocyte proliferation assay

Spleen cells were obtained from both immunized and control mice. Spleen cells at 2×10^5 cells/well were cultured in 200 μl of RPMI 1640 containing 10% (v/v) fetal bovine serum and supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 25 mM HEPES and L-glutamine (2 mM) (all from Hyclone, USA) in 96 well round-bottom plates (Sigma). Cells were stimulated with purified recombinant Ab2β proteins at 0.1, 1 and 10 μg/ml and incubated at 37 °C in a 5% CO_2 humidified atmosphere. Cells stimulated with PHA 1% (v/v) (Sigma) were used as a positive control, while cultures in RPMI served as background growth controls. T cell proliferation assays were set up in triplicate. After incubation for 5 days, 20 μl MTT of (methyl thiazolyl tetrazolium) solution (5 mg/ml) (Sigma) was added into each well and incubated for another 4 h at 37 °C. The medium was discarded from each well and 150 μl dimethyl sulfoxide (DMSO) (Genview, USA) was added. After being shaken in a micro-blender (Liuyi) for 10 min, the absorbency which correlates to the number of cells was measured on a microplate reader (Bio-Tek, USA) at 570 nm wavelength.

2.8. Detection of cytokine gene expression

Spleen cells were resuspended in complete RPMI-1640 (Hyclone), and seeded at 10^6 cells/ml in 24-well plates (Sigma). Cells were stimulated with corresponding Ab2β protein at a concentration of 1 μg/ml, for 18 h at 37 °C in a 5% CO_2 humidified atmosphere. Cells from non-immunized mice were used as negative controls. Spleen cells incubated with PHA 1% (v/v) (Sigma) for 2 h were used as positive control. The cultures were collected after 18 h and total RNA extracted to test the expression of mRNA for interferon-γ (IFN-γ), interleukin-2 (IL-2), IL-4, and IL-10.

Total RNA was isolated according to the TRIzol (Invitrogen) reagent instructions. The resultant RNA was resuspended in water and quantified by spectrophotometry at 260 nm. Complementary DNAs were prepared from 1 μg of total RNA using a RT kit (MBI) with oligo (dt) primer according to the manufacturer's instructions. The primers for IFN-γ, IL-2, IL-4, IL-10 and β-actin genes were as follows: IFN-γ (forward primer) 5′ CACACTGCATCTTGGCTTTG 3′; IFN-γ (reverse primer) 5′ ACTCCTTTTCCGC TTCCTGA 3′; IL-2 (forward primer) 5′ GGACTTAGTACTT ATCTCAATTGAA 3′; IL-2 (reverse primer) 5′ TCCTTGTTGTTCTG-AAGGTTCG 3′; IL-4 (forward primer) 5′ TCTCAA CCCCCAGCTAGTTGT 3′; IL-4 (reverse primer) 5′ TTGCATGATGCTC TTTAGC 3′; IL-10 (forward primer) 5′ TGCTATGCTGCCTGCTCTTA 3′; IL-10 (reverse primer) 5′ AATCACTCTTCACCTGCTCCA3′; β-actin (forward primer) 5′TCTGGCAC-CAC ACCTTCTACA 3′; β-actin (reverse primer) 5′ TTCTCCAGGG AGGAA-GAGGAT 3′. The housekeeping gene β-actin was also transcribed and amplified in each PCR reaction to normalize the reactions. After the appropriate number of PCR cycles, reaction products were visualized by electrophoresis of 5 μl reaction mixture at 100 V for 30 min in a 1% agarose gel, photographed and quantitated by densitometry using Scion Image software (Scion Corp, Frederick, MD, USA).

2.9. Statistical analysis

To compare the levels of antibodies and proliferative responses, the Mann–Whitney *U*-test was used. p values <0.05 were considered statistically significant. Assays of cytokine mRNA gene expression were considered significant when the experimental expression reached a value at least twice that of the negative control.

3. Results

3.1. Expression and verification of anti-Id protein vaccines

The expression of the recombinant proteins in *E. coli* BL21 (DE3) was induced by 0.5 mM IPTG for 6 h at 30 °C. Up to 10 μg recombinant proteins per milligram wet weight with a level of purity higher than 90% were obtained as judged from comparing known amounts of marker proteins with bacterial extracts on SDS-PAGE gels after Coomassie Blue staining. The expression of proteins was verified by western blot. Western blot analysis using monoclonal antibody FC2 as primary antibody, showed bands of around 42 kD, 27 kD, and 15 kD which correspond to the molecular weight for G22–I50, G22, and I50 (Fig. 1). The presence of the His-tag in the recombinant proteins was confirmed by western blot with an anti-His-tag antibody (data not shown).

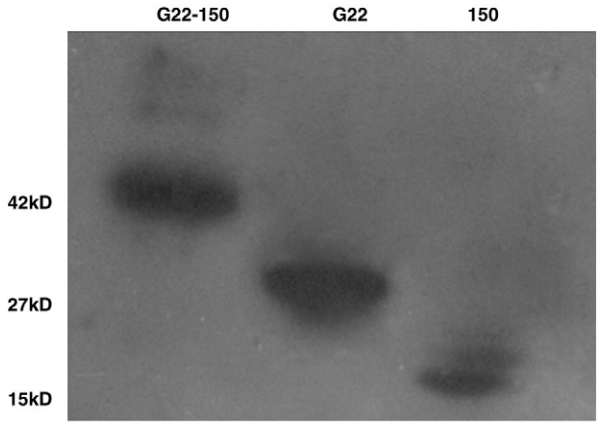

Fig. 1. Western blot analysis of the expression of recombinant proteins in *E. coli* BL21 (DE3) FC2 mAb at 1:1000 dilution was used as the detection antibody. Lane 1, G22–I50 (about 42 kD); lane 2, G22(about 27 kD); lane 3, I50 (about 15 kD).

3.2. Humoral immune response

In order to test the ability of different protein vaccines to induce specific anti-Ab2 response (Ab3) in vivo, five animals in each group were immunized using equimolar amounts of the recombinant proteins. Sera were obtained from the animals before and 14 days after every immunization and tested by ELISA against the corresponding protein. Sera from animals of control group did not show any specific reactivity (data not shown). By contrast, animals of all immunized groups demonstrated specific antibodies only 2 weeks after primary immunization. The antibody titer rose rapidly from the second to 6th week in mice of G22–I50 group and was above 1:10,000 at the end of the schedule. However, the titer of G22 group in the induction phase ranged from 1:2000 to 1:8000 as measured by ELISA. The mean titer of mice immunized with I50 remained stable 1:1000 at

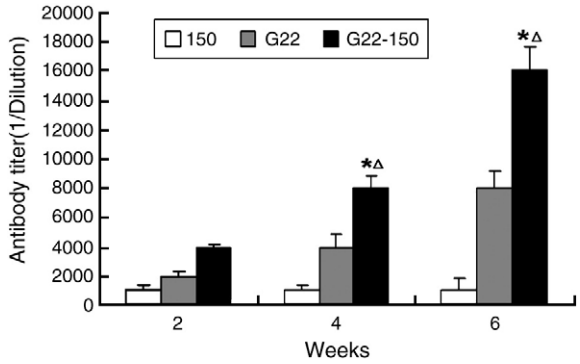

Fig. 2. The humoral immune responses in mice immunized with different Ab2β protein vaccines. The presence of Ab3 antibodies in the sera of mice immunized with G22–I50, G22, and I50 at 2nd, 4th, and 6th week of the schedule was detected using ELISA plates coated with respective protein, respectively. Data shown are averages of the absorbances obtained for the five individual animals in each group, along with standard deviations. $^{*}P<0.05$ vs.G22 group; $^{\Delta}P<0.05$ vs. I50 group.

the 6th week (Fig. 2). The antibody response generated in mice immunized with monovalent vaccines was lower than the bispecific one ($p<0.05$, Fig. 2).

To confirm that a specific response against FC2 idiotype was generated by the immunization, the same aliquot of HRP-FC2 and mouse sera competed for the surface antigen of HNE2 cells, and the remnant reactivity against the surface antigen was measured by ELISA. As shown in Fig. 3, sera from animals of immunized groups exhibited high inhibitory activity during the schedule and the inhibitory activity increased gradually along with the schedule. The percentage of inhibition of G22–I50 group rose to 64.4% at the end of the schedule which was stronger than that of monovalent vaccines (48.8%, 39.8%, Fig. 3C).

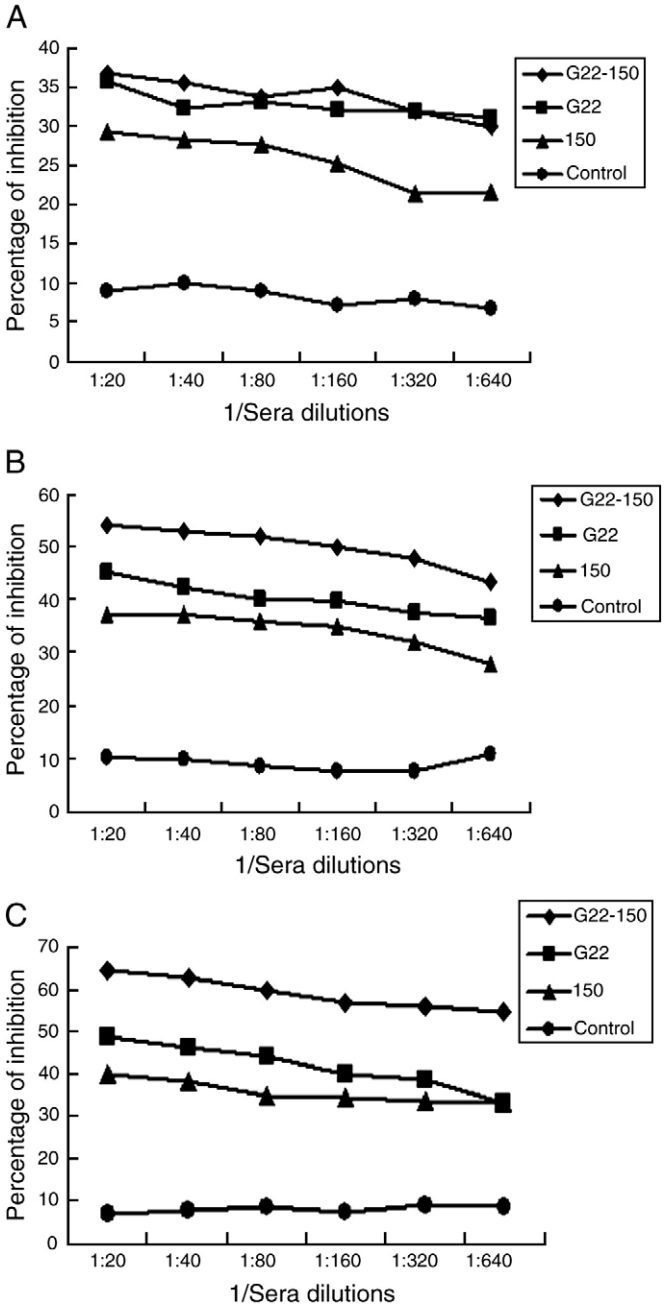

Fig. 3. Specific inhibition of binding of Ab1 (FC2) to the surface antigen of HNE2 cells by mice sera. Binding of FC2 to HNE2 cells coated plates was tested in the presence of serial dilutions of pooled mice sera at 2nd (A), 4th (B), and 6th (C) week of the schedule. The percentage of inhibition was relative to the binding of HRP-FC2 mAb to the surface antigen of HNE2 cells in the absence of serum.

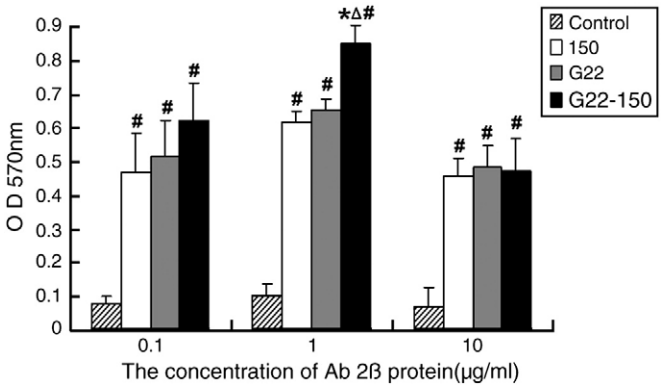

Fig. 4. Lymphocyte proliferative response to respective Ab2β protein. Spleen cells were obtained from both immunized and control mice. The cells from each group were cultured with respective protein at the concentrations indicated above for 5 days. The concentration of Ab2β protein is indicated on the *x*-axis, and groups are represented as follows: G22–I50 group (black bars), G22 group (gray bars), I50 group (white bars), and control group (oblique hatched bars). OD of background (cells cultured in medium alone) had been automatically subtracted. The results are presented as the mean ± SD for duplicate cultures. $^{\#}P<0.05$ vs. control group; $^{*}P<0.05$ vs. G22 group; $^{\Delta}P<0.05$ vs. I50 group.

3.3. Proliferative responses to anti-Id protein vaccines

Spleen cells primed in vivo were stimulated with corresponding Ab2β protein at different concentrations. As shown in Fig. 4, all groups of immunized mice had specific proliferative responses significantly higher in all cases than the control group ($p<0.05$). The proliferative response of G22–I50 group at the stimulation of 1 μg/ml Ab2β protein was statistically significant when compared with G22 and I50 group ($p<0.05$), but did not differ significantly when the concentrations were 0.1 μg/ml and 10 μg/ml. These results suggested that proliferative responses were to some extent dependent of the concentration of Ab2β protein.

3.4. Cytokine gene expression profile

Spleen cells from immunized and control groups were stimulated with respective protein and expression levels of cytokine genes were measured as described above. As was shown in Fig. 5, cells from all immunized groups showed an increase of IFN-γ and IL-2 gene expression after stimulation which indicated that spleen cells had been primed and displayed a predominantly Th1 cytokine profile, especially in G22–I50 group IL-4 and IL-10 gene expression was, at most, only marginally affected. This finding is important since it is known that in mice, IFN-γ and IL-2 are the predominant cytokines

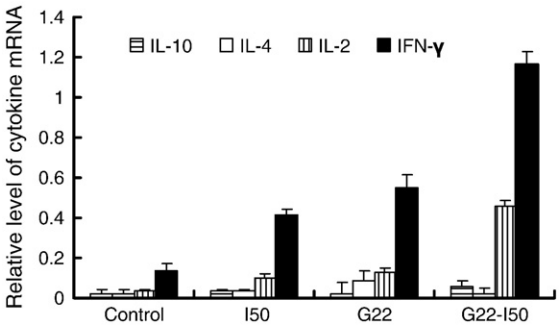

Fig. 5. Effect of different Ab2β proteins on IFN-γ, IL-2, IL-4, and IL-10 mRNA expression. Spleen cells (3×10^7) from both immunized and control mice were grown in the presence of 1 μg/ml of respective protein. At 18 h, cells were collected and total RNA was isolated and complementary DNAs were prepared by RT-PCR. The expression of IFN-γ, IL-2 mRNA, IL-4, and IL-10 mRNA was assayed by PCR using specific primers. Levels of β-actin mRNA were used to normalize mRNA quantities.

produced during cellular responses of a Th1 profile, with the corresponding implication of immunomodulation towards a cell-mediated response pattern.

4. Discussion

Immunotherapy is rapidly accelerating as an approach to selectively reach, identify and eliminate tumors. The use of anti-Id antibodies as surrogate of antigens in the immunization has been considered a promising vaccination strategy. Up to now, Ab2β mAbs had been applied into the therapy of many tumors [30–35], however, HAMA response limited their extensive application severely.

Genetic engineering technology and phage libraries had predicted new avenue to the therapy of cancer patients with small antibody fragments without invoking a HAMA response. Although the Fvs are the smallest functional modules that confer specific antigen binding, they lack one vital property of the whole antibody [36]. The Fvs are monovalent as compared with a bivalent IgG or even a decavalent IgM. In theory, the immunogenicity of multivalent antibodies is higher than that of corresponding Fvs. The present study was designed as an exploratory study to determine whether bivalent bispecific anti-Id vaccine would enhance the immune response in Balb/c mice. In this study, we compared the antibody responses produced by immunization with bispecific anti-Id vaccine and its parent small fragment vaccines. We found that all animals developed anti-mouse immunoglobulin antibodies, but the serological response against bispecific G22–I50 was stronger when compared with that achieved with monovalent molecules.

The anti-idiotypic antibodies elicited by these vaccines in Balb/c mice were true Ab3 antibodies, as evidenced by the strong inhibition of Ab1 (FC2) binding to the surface antigen of HNE2 cells by mouse immune sera, as this is indicated by the presence of antibodies sharing idiotopes with FC2 mAb. Furthermore, the magnitude of the anti-idiotypic response of G22–I50 group was significantly higher than the response detected in the mice of G22 and I50 group.

The idiotypic network idea, originally proposed by Jerne, hypothesized that the interactions of Id and anti-Id determinants select and regulate the available repertoire in immune systems [37]. Participation of T lymphocytes in Id-based regulatory systems is the one of the proposed mechanisms by which this is achieved. Our study had shown that T lymphocyte cells of all immunized mice had been primed and possessed the ability to recognize and react to stimulation ex vivo and the effect of bispecific vaccine was significantly more potent than that of monovalent ones. Also, a significant expression of the IFN-γ and IL-2 gene was found after immunization with bispecific vaccine, suggesting that the predominant subset of Th cells is of the Th1 type [38]. These observations suggested that Ab2β protein could activate $CD8^+$ T cells into IFN-γ-producing CTL effectors and offer new evidence for the concept that proinflammatory cytokines, such as IFN-γ and TNF-α, sensitized the tumor cells to be lysed by cytotoxic T cells which could play an important role not only in the induction but also in the effector phase of the immune response [39].

In conclusion, the bispecific anti-Id antibody vaccine had been shown to be a better candidate to the therapy of nasopharyngeal carcinoma, able to induce more powerful humoral and cell-mediated immune responses. However, achieving immune responses in the presence of immunosuppressive factors secreted by tumor cell is the critical question for any anti-tumor immunotherapeutic regimen, and thus, it would be of interest to use a hu-PBL-SCID model to assess the ability of this bispecific anti-Id antibody vaccine.

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