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BASIC STUDY AND PHASE I CLINIC TRIAL OF ANTI-IDIOTYPIC ANTIBODY MIMICKING NASOPHARYNGEAL CARCINOMA CELL ANTIGEN

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An anti-idiotypic monoclonal antibody 2A9 (Ab2) was prepared, which mimicked the nasopharynegeal carcinoma (NPC) cell antigen defined by anti-NPC McAb Fc1. The abilities of 2A9's inducing humoral and cellular immunity against NPC cell antigen were studied in syngeneic mice by inducing anti-Ab2 sera (Ab3) and delayed-type hypersensitivity. Two periods of phase I clinical trials were carried out, stage IV NPC patients receiving radiotherapy were chosen. Human anti-mouse antibodies (HAMA), anti-anti-idiotypic antibodies (Ab3), and anti-NPC cell antibodies (Ab1') were detected by ELISA. TNF-α,IL-2, IFN-γ levels in sera were determined by ELISA Kits. IL-2 mRNA expression in peripheral blood mononuclear cells (PBMC) were shown by *in situ* hybridization. The results showed that 2A9 was safe in applying on NPC patients and induced some humoral and/or cellular immune responses.

Key words: NPC, Anti-idiotypic, McAb, Clinical trial

Because of the special role of anti-idiotypic (anti-id) antibodies (Abs) in immune network, its potential role in tumor immunotherapy and immunoprevention has drawn wide interest. Anti-id Abs mimicking colorectal carcinoma-associated antigen and human high molecular weight melanoma-associated antigen (HMW-MAA) have been prepared and tried for active immunotherapy on tumor patients.[1-6] But there hasn't been any report about anti-id Ab of nasopharyngeal carcinoma (NPC). NPC is a tumor with high epidemic incidence in China and Southeast Asia, there hasn't been much study on its immunotherapy. Here we have prepared an anti-id Ab 2A9 (Ab2) directed against monoclonal anti-NPC cell antibody Fc1 (Ab1) and proved 2A9 to be the "internal image" of NPC cell antigen defined by Fc1. Two phase I clinical trials of specific active immunotherapy using the anti-id Ab 2A9 in advanced NPC patients have been undertaken. The aim of this paper is to describe the preclinical basic studies and the clinic trials designed to test toxicity and immune responses in patients.

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MATERIALS, PATIENTS AND METHODS

Antibodies

Monoclonal antibody Fc1 (Ab1) directed against NPC cell line CNE-1 was prepared. Fc1 had fairly good specificity and affinity to NPC cell lines (CNE1, CNE-2 and HNE2). Consequently monoclonal anti-idiotypic antibody 2A9 (Ab_2) against antigen-binding

area of Fc1 was prepared. 2A9 shared antigen epitope on NPC cells defined by Fc1. After purification, 2A9 was formulated in alum- precipitation for clinic use. Tests for mycoplasm, bacteria, pyrogen and toxicity were passed. Purity was >95% as monitored by SDS-PAGE.

Patients

Stage IV NPC patients were selected according to TNM staging criteria made by UICC in 1987.[7] Patients must have normal systemic function, white blood cell count must be no less than 3×10^9/dl. Ethics committee approval was obtained prior to the start of the study. Patients entered into the study after giving personal consent. Patients first accepted 20 μg 2A9 as skin test, those with negative reaction after 24-hour observation were chosen. One immunization protocal was: Patient accepted intradermous injections of alum- 2A9 (2mg 2A9/2υ0 μl) at 0, 1, 2, 5 week (wk). Another protocal was: Patient accepted subcutaneous injections of alum-2A9 (2mg 2A9/1ml) at 0, 7, 28 day. Before and two wks after the immunization schedule, blood samples were taken. Sera were separated, peripheral blood mononuclear cells (PBMC) were separated on Lymphoprep (Sigma). Both were cryopreserved. The clinic outcome was defined according to guidelines previously described by the National Cancer Institute. Briefly, complete remission (CR) was defined as complete disappearance of all measurable tumors for at least 4 wks; partial remission (PR) as >50% reduction of the bidimensional diameters of all measurable lesions for at least 4 wks.

Humoral Immunity against NPC Cell Antigen Induced in Mice by 2A9

Ab3 anti-sera were got by immunizing Balb/c mice with Ab2 2A9. To determine whether Ab3 could bind to original NPC cell antigen, competitive inhibition test was carried out: anti-sera containing Ab3 were added at double dilutions to 96-well microtiter plate coated with CNE1 cells, 50 μl to each well. 50 μl Ab_1(FC1) Labelled with horseraddish peroxidase (HRP) at working dilution was added to each well at the same time. The plate was incubated at 37 ℃ for 2 hours (h), washed with phosphate buffered saline-Tween (PBST), followed by addition of HRP substrate: 2, 2' azino- di- 3- ethyl-benzthiazoline sulfonate (ABTS). 37 ℃ for 20 minutes (min). Absorptions at wave 405 nm (OD_{405}) were determined on an ELISA reader. The antigen binding site of Ab3 on CNE1-cells was determined by immunohistochemistry test with indirect peroxidase staining method, and compared with those of Ab1.

Cellular Immunity against NPC Cell Antigen in Mice Induced by 2A9

This was investigated *in vivo* by induction of a delayed-type hypersensitivity (DTH) response. A group of ten Balb/C mice were immunized intraperitoneally with 100μg Ab2 (2A9) conjugated to keyhole limpet hemocyanin (KLH) and mixed with complete freund's adjuvant (CFA) on day 0, with incomplete freund's adjuvant (IFA) on day 14, and with PBS on day 28. Cultured human NPC cells (HNE-2 cell line) irradiated by ^{60}Co(200Gy) and human B lymphocyte cells (5×10^5) were injected into the right and left hind footpads on day 50. The thickness of each footpad was measured with a Digit Outside Micrometer (unit: 10^{-2}mm) at time 0 and time 24, 48 and 72 h after the injection of cells. The swelling induced by injection of cells was determined by subtracting the thickness of footpad at time 0 from that after the injection of cells. Results are expressed as $\bar{\chi} \pm s$ of swelling. Differences in swelling values obtained after the injection of NPC and B lymphoid cells were calculated using the Student's t-test. The control group of 5 mice was immunized with normal mouse IgG (mIgG) with the same immunization and cell-injection schedule as described above. Local transfer of delayed hypersensitivity was also demonstrated using lymphocytes from mice primed with Ab2.

Detection of Antibodies in Patients' Sera[8]

Detection of human anti-mouse antibody (HAMA): ELISA plates (96-well microtiter) were coated with 2A9 (3μg/well), blocked with 1% BSA-PBS, patients' sera were 10 times diluted from 1: 1 to 1: 1000 with PBS. 100μl of dilutions were added to each well, incubated for 1 h at 37 ℃, washed with PBST followed by addition of substrate ABTS, stayed at 37 ℃ for 20 min, then OD_{405} values were determined. Sera before therapy and PBS were as controls at parallel wells with sera after therapy. In below assays the controls were designed the same way.

Anti-2A9 antibody (Ab3): Patients' sera were preincubated with non-immune normal mouse IgG at 300 μg/ml at 4 ℃ O/N with gentle shaking to block the human Abs binding the constant region determinants of mouse IgG present in sera of patients treated by Ab2. Blocked sera lost their binding reactivity to non-immune mouse IgG. Thus, the serum assay may detect human Abs that bind specifically to variable region of Ab2, i.e., Ab3, ELISA plates were coated with 2A9, preincubated sera were 2 times diluted from 1: 10 to 1: 80, the left procedures were the same as described above.

Anti-NPC antibody (Ab1'): 96-well plate coated with 10^5 HNE2 cells/well was incubated with 200μl/well DMEM containing 10% FCS O/N at 37 ℃ and 5% CO_2 in air. Adherent cells were washed and plates were blocked, and then fixed with 0.15% glutaraldehyde in PBS for 15 min at RT, washed three times with PBST followed by the addition of 100μl patients' sera at double dilutions from 1: 1 to 1: 8. The following procedures were the same as described in **1.**

Detection of Cytokine Levels in Patients' Sera

TNF-α, IFN-γ and IL-2 levels were detected following the guidelines of PREDITA™ Tumor Necrosis Factor-α ELISA kit, Inter Test-γ™ Human IFN-γ ELISA kit and Inter Test-2X™ Human IL-2 ELISA kit (Genzyme Co.).

Expression of IL-2 mRNA in patients' PBMC

This was demonstrated by *in situ* hybridization. IL-2 probe was prepared according to methods described.[9] E. coli HB101 were transformed by vector plasmid pBR322 inserted with IL-2 gene segment (a gift from Beth Israel Hospital of Harvard University). Then the bacteria were cultured in LB containing benzylpenicillin, positive clones were selected. Plasmid DNA was extracted, and incised with BamH I enzyme. The 0.94 Kb IL-2 segments were separated and recovered, then labeled with Dig-II-dUTP (Genzyme Co.). Thawed PBMC were adjusted to a concentration of 5×10^6 /ml, dropped onto dry, clean, Apes gel covered slides, fixed with 4% formaldehyde buffer. Then the detection and analysis of IL-2 mRNA expression were done according to general *in situ* hybridization potocal. Smear of PBMC from normal donor stimulated with PHA *in vitro* was as positive control. The hybridization fluid depleted of Dig-IL-2 probe was designed as negative control.

RESULTS

After Ab2 2A9 immunizations in syngeneic Balb/C mice, polyclonal Ab3 anti-sera were got. Ab3-containing sera could compete with Ab1 Fc1 for binding with original NPC cell line CNE-1 (Figure 1). Thus Ab3 shared antigen binding paratope of Ab1 Fc1. So 2A9 is the mimicry of NPC cell antigen defined by Fc1 and could induce specific humoral immune response. Ab3 acted at the same antigen determinant on tumor cells as Ab1 did, shown by the same membrane staining in immuno-histochemistry assay.

A marked swelling of footpads was observed 24h after the injection of NPC cells in 2A9 immunized mice, and the swelling persisted 72h afterward. The swelling at 24h was significantly higher than that observed in the contralateral footpad injected with B lymphoid cells, which do not express NPC antigen (Figure 2A). The DTH reaction to NPC cells induced by Ab2 2A9 was specific, because no reaction was detected in mice immunized with the irrelevant mouse IgG (Figure 2B). The DTH response induced by 2A9 was shown to be cellular immunity in nature by local adoptive transfer to naive recipients. Peritoneal exudate lymphocytes from mice primed with 2A9 mixed with NPC cells were injected into hind footpads of syngeneic naive mice and induced marked swelling, which was significantly higher than those induced by mixture of normal mouse IgG primed peritoneal exudate lymphocytes and NPC cells (Figure 2C).

In preliminary clinical trial 11 patients accepted 2A9 immunizations. None of the immunizations of Ab2 was associated with toxicity or allergic reactions. Minor itching was noted in some patients. 9 patients who went through the whole therapy process all produced HAMA, 8 produced Ab3 (patient 4 lacking). It seems all patients originally had significant amounts of surface-reactive anti-NPC Ab1' level, after Ab2 immunotherapy (patient 4 and 8 failing) (Figure 3). No significant differences existed among antibody titers produced by two different immunization protocals.

Finally, 4 patients obtained complete remission of tumors (patient 1, 3, 6, 9), 5 patients obtained partial remission (patient 2, 4, 5, 7, 8).

In secondary clinical trial, 20 patients were randomly divided into two groups. Samples were able

to be collected only in 16 patients. 7 patients were from the group receiving radiotherapy plus 2A9 immunization, 9 patients were from control group receiving radiotherapy plus normal saline injection. Detection of antibodies: All 2A9-treated patients produced HAMA and Ab3, similar to what happened in preliminary trial. All 16 NPC patients had preexistent anti-NPC cell antibody Ab1' in their sera, coherent with what was observed in preliminary trial. But the level increased in 2A9 immunized group (t=5.834, $P<0.001$), whereas no increase in radiotherapy alone treated group (t=1.234, $P>0.2$). Cytokine detections: Of 7 patients in 2A9 immunization group, the TNF-α in 0/7 patients' sera were detected before 2A9 treatment, 2/7 were detected after treatment, with average of 283.2 pg/ml (patient 4,5); 3/7 patients had detectable IFN-γ level before treatment with average of 157.6 pg/ml (patient 1, 2, 3), after 2A9 immunization 6/7 had detectable IFN-γ and the average level increased to 359.2 pg/ml (patient 1, 2, 3, 4, 6, 7); IL-2 level in 3/7 patients' sera were detectable before treatment with average of 281.0 pg/ml (patient 4, 5, 7), 5/7 increased the level after treatment, with an average of 809.7 pg/ml (patient 2, 3, 4, 5, 7). In control group, TNF-α, IL-2 were under detection level before and after radiotherapy. IFN-γ in 7/9 patients were detectable (patients 2, 4, 5, 6, 7, 8, 9), the average before radiotherapy was 126.9 pg/ml, the average after radiotherapy was 69.9± 83.5 pg/ml. There seemed to be a decreasing tendency after radiotherapy, but no significant difference ($P>0.2$). Expression of IL-2 mRNA: According to the analysis of PC Vision Plus (85 type) General Mapping Analyzing System, there were significant differences between the grey-levels of *in situ* hybridization before and after therapy in 2A9 immunization group (average before therapy: 117.84± 21.71, after therapy: 169.68± 43.14, t=2.840, $P<0.02$), while no such difference in control group (average before radiotherapy: 105.21± 9.97, after radiotherapy: 108.25± 6.6, t=0.7634, $P>0.2$), whose average level was close to probe-lacking negative control (92.00± 10.4). There existed positive relationship between difference of IL-2 mRNA expression and difference of IL-2 level in sera before and after 2A9 immunotherapy (r=0.9940, $P<0.001$).

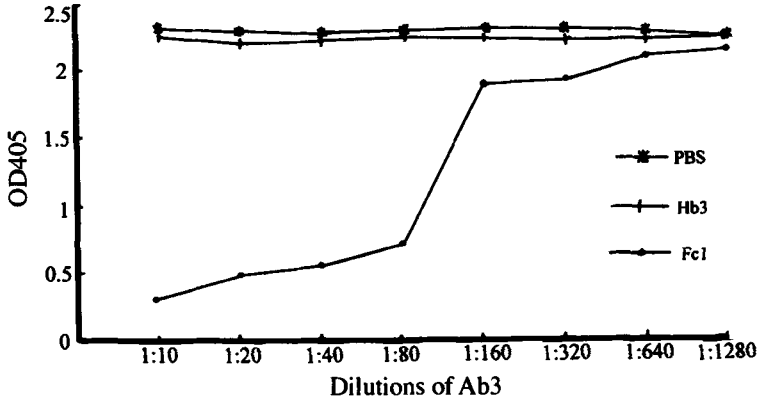

Fig 1. Inhibition of the binding of anti-NPC mAb Fc1 to cultured NPC cells CNE-1 by serum from Balb/C mice immunized with the syngeneic antiidiotypic mAb 2A9 Anti-colon cancer mAb Hb3 was used as a specificity control.

In the group receiving radiotherapy plus 2A9 immunization, patient 3, 4, 5, 7 obtained CR, patient 1, 2, 6 obtained PR. In the control group, patient 1, 4, 6, 7 obtained CR, patient 2, 3, 5, 8, 9 obtained PR.

DISCUSSION

Nisonoff and Lamoyi have proposed, on the basis of Jerne's network theory, that anti- idiotypic

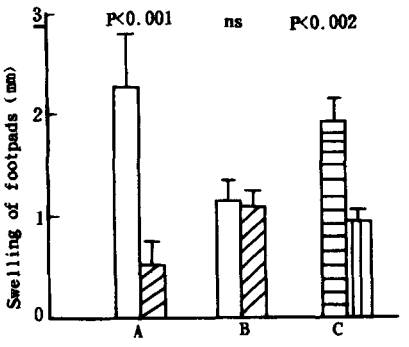

Fig 2. (A and B) Anti-id Ab 2A9 immunization for delayed-type hypersensitivity in mice. Mice were immunized with Ab2 (A) or normal mouse IgG (B) and challenged with injections of HNE2 NPC cells (□) or human B lymphocytes (□) given into the right and left hind footpads. (C) Local adoptive transfer of delayed-type hypersensitivity in mice. Lymphocytes from mice immunized with Ab2 2A9 (□) or normal mouse IgG (□) were mixed with HNE2 NPC cells and injected into right hind footpads of naive mice. Before and twenty-four hours after injection of cells, footpad thickness was measured. Increase in footpad thickness compared with that of before injection of cells (swelling) is shown. ns=not significant.

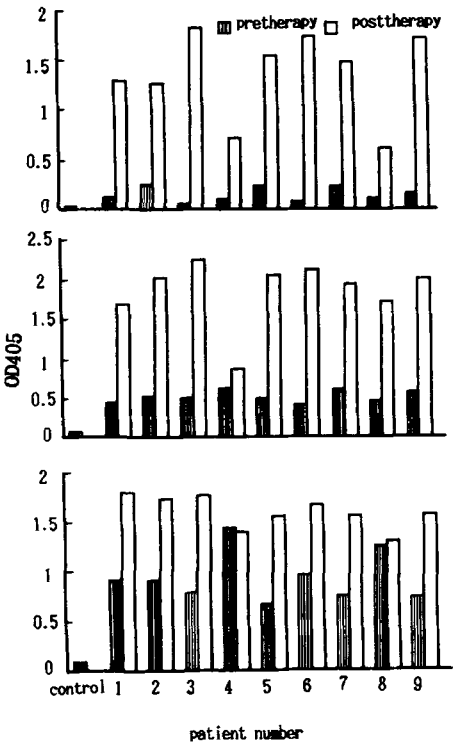

Fig 3. Binding reactivities of human anti-sera.
(A) Binding reactivities of patients' sera (diluted 1: 100) to mouse Ab2 2A9. (HAMA). (B) Binding of human antibodies (patients' sera to normal mouse IgG. (Ab3). (C) bingding reactivities of patients' sera (diluted 1: 8) to NPC cells HNE2. (Ab1').

antibody (Ab2), which idiotope, like the antigen with its epitope, combined with the paratope of Ab1 consequently can replace external and self-antigen in inducing antigen-specific responses.[10] This Ab2 is socalled "internal-image" anti-id Ab. 2A9 could compete with NPC cell for binding with anti-NPC cell antibody Fc1 (Ab1), so it shared antigen epitope of NPC cell defined by Fc1. Later it was proved 2A9 could indeed induce NPC cell specific humoral and cellular immune response in syngeneic mice. So 2A9 might substitute for NPC cell antigen and be a good NPC vaccine candidate for clinical evaluation.

2A9 was a mouse-origined antibody, production of HAMA in 2A9 immunized patients was not surprising. But it showed no relation with occurrence of intense side effects. Ab3 was generated specifically against the idiotype on Ab2. It was detected in almost all patients who accepted Ab2 2A9 immunization. NPC patients clearly had produced significant amount of surface-reactive anti-NPC Abs (Ab1') before treatment with Ab2. Similar situation had been observed in colorectal carcinoma patients.[3] In these patients treated with Ab2, it is possible that priming of B cells by self-tumor cells greatly facilitated induction of antigen-specific Ab3 (we called it Ab1'), since in most of the Ab2-treated patients, the Ab1' level increased markedly while in patients receiving radiotherapy alone the level didn't increase. Production of Ab3 had been shown to be positively related to remission of small tumor lesions and/or prolongation of patients' survival time[1-3]. Ab1' might as well take part in Ab-dependent, cell-mediated cytotoxicity or complement dependent cytotoxicity against tumor cells.

After accepting 2A9 active immunotherapy, 2/7 patients produced TNF. IFN and IL-2 level were increased in most patients' sera, while cytokine levels in patients accepting radiotherapy alone weren't increased. Recently, cellular immune responses in cancer patients treated with anti-id Ab other than

humoral immune responses have been reported. Six patients who were operated for colorectal carcinoma were immunized with human monoclonal anti-id Abs, all developed a long-lasting T-cell immunity, which were shown *in vitro* by specific cell proliferation assay as well as IL-2 and IFN-γ production, *in vivo* by DTH reaction.[6] In two colon carcinoma patients who did not demonstrate GA733 antigen (Ag) /Ab2-reactive T cells before therapy, Ab2 administration induced Ag-specific, proliferative T cells. Proliferative T cells expressed CD_4 Ag and were of the Th1 type.[4] Enhanced cell-mediated tumor killing was observed in patients immunized with human monoclonal anti-id Ab 105AD7. The author suggested that anti-Id Ab might stimulate CD_4 which facilitate activation of preexisting and silent antitumor cytotoxic T-cells. Activated T cells could produce cytokines such as IFN-β, TNF-α and granulocyte-macrophage colony stimulating factor (GMCS-F).[5] In our phase I clinic study we observed the increasing of cytokine levels, suggesting Ab2 immunization in NPC patients might have activated T cell subsets. However, more studies should be done to prove this point, and if so, the target specificities and phenotypes of the induced T cells should be determined. This is of potential interest for future research.

Although we have observed the final clinical outcome of all 2A9-immunized patients, yet because of parallel of radiotherapy, shortness of observation period and scanty of patient number, the clinic efficacy related to Ab2 immunization alone is hard to assess. It is inadequate to draw a definite statistical result from data we presented, but we think except for the recognized effect of radiotherapy to NPC patients, Ab2 might as well underlie the improvement of patients' general conditions of immune system and/or benefit in preventing further metastasis or early relapse. Anyhow the phase I clinical trials gave us following impressions: 1) Mouse-origined anti-Id monoclonal antibody was safe for clinic active immunotherapy; 2) It might enhance humoral and/or cellular immunity of NPC patients receiving radiotherapy. Further clinical studies of Ab2 active immunotherapy are justified, without the interference of other treatment strategy, and with emphasis to analyze the development of cell-mediated immunity and its potential role in the clinical course of the disease.

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