Immunogenicity of Multi–epitope DNA Vaccine of Mycobacterium Tuberculosis and Combined Therapeutic Effects with Chemotherapy in Mouse Model

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ABSTRCT **Objective** To study and compare the immunogenicity of multi–epitope (Hsp70, Ag85A, ESAT–6) DNA vaccine and BCG of Mycobacterium tuberculosis and therapeutic effects of the vaccines combined with chemotherapy in a mouse model infected with multi–drug resistant (MDR) Mycobacterium tuberculosis. **Methods** BALB / c mice were divided into PBS negative control group, Multi–epitope DNA vaccine group and BCG positive control group ,and all mice received three immunizations at intervals of once every 2 weeks. Specific IgG antibody in serum of mice was determined with indirect ELISA in 4, 6 ,8 weeks respectively after final vaccination. The splenic lymphocytes of mice were separated and stimulated with TB–PPD to measure their proliferation by MTT method, and to evaluate the production of interferon–γ (IFN–γ) and IL–4 in cell suspensions of spleen cells by ELISA. To study the therapeutic effects of the vaccine, BALB / c mice were infected by intravenous injection in a tail vein with Mycobacterium tuberculosis clinical isolate HB240 that is resistant to Isoniazid (INH) and Rifampin (RFP) for 4 weeks , and then were randomly divided into three groups. The mice in group E were treated with RFP and INH for 12 weeks. The mice in group F were treated by Multi–epitope DNA vaccine combined with INH and RFP for 12 weeks. DNA vaccines were injected intramuscularly 5 times at 3 weeks intervals. The lungs, livers and spleens were taken and observed their pathological changes, weighted and performed mycobacteria cul tures at 4 or 8 weeks after terminative treatment. **Results** Specific IgG responses in Multi–epitope DNA vaccine and BCG groups, average result is 1: 160 and 1:120 respectively. The antibody of the Multi–epitope DNA vaccine group are obviously higher than group BCG;.The splenic lymphocyte proliferation reactions and IFN–γ were detectable in Multi–epitope DNA vaccine and BCG groups and Multi–epitope DNA vaccine group induced significant higher production. At four and eight weeks after terminative treatment , the body weights of mice in group E were lower than that in group F , but it had no significant difference. At four weeks after terminative treatment, indexes of the lungs and spleens from the mice in group F were lower than that in group E. At eight weeks after terminative treatment , indexes of the lungs and livers from the mice in group F were lower than that in group E, but indexes of the spleens from the mice in group F were significantly lower than that in group E. **Conclusion** Multi–epitope (Hsp70, Ag85A, ESAT–6)DNA vaccine of Mycobacterium tuberculosis induce stronger induction specific immunoreaction in the body of mouse, produce high–level specific IgG antibody, induce specific lymphocyte hyperplasia and IFN – γ to secrete. The therapeutic efficacy of Multi–epitope DNA vaccine combined with chemotherapy is stronger significantly than that of chemotherapy alone in the mouse model of MDR tuberculosis.

Key words Mycobacterium tuberculosis; Multi–epitope DNA vaccine; immunogenicity; therapeutic

Tuberculosis ranked the first in death causes of infectious diseases .The incidence and mortality were increased year by year with the population flow and HIV infectors increased as same as the multiple antibiotic resistant stains appeared . As the traditional anti–tuberculosis vaccine, Bacillus calmette –guerin (BCG) had low protective effect and its protective efficacy was uncertain .That forced us to study a more suitable vaccine and more effective method of immunization. So , study a more newer and safer anti–tuberculosis vaccine becomes the primary task of tuberculosis control nowadays .The anti–tuberculosis immune reaction was mainly the cellular immunity. The results showed immunotherapy can not only increase body immunity and chemotherapeutic effect but shorten the treatment pro-

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cess .The DNA vaccine of tubercle bacillus protective immunity antigen gene had been the more researched new vaccine in resent years .We used tubercle bacillus protective immunity antigen gene Hsp70, Ag85A and ESAT-6 to construct multi-epitope DNA vaccine and discussed its immune effect .We observed the efficacy through the animal experiment by combining it with chemotherapeutic drugs to find an effective way used to prevent and treat drug-resistant tuberculosis .

MATERIALS and METHODS

Experimental Animals

Inbred strain BALB / c mice (30mice, 6~8weeks old,weight 18-20g)were provided by our experimental animal center .

Multiple-epitope DNA Vaccine and Strains

Mycobacterium tuberculosis Hsp70、Ag85A、ESAT-6 DNA vaccine were provided by Prince Henry Institute of Medical center of Australia Monash University . Batch number :0712;Content:2.1g/L

Mycobacterium tuberculosis HB240 were clinical isolated drug resistant strains .BCG were provided by institute of Chengdu Biological Products and routine cultured by our laboratory.

Reagent and Kits

Mouse interferon-γ (IFN-γ) and interleukin 4 (IL-4),cytokine enzyme-linked immunosorbent assay (ELISA) detection kits were purchased from Bio-source corporation . Sheep anti mouse IgG labeled by HRP, O-phenylene diamine (OPD) were purchased from Zhongshan Biotechnology Company of Beijing ;MTT was purchased from Santa Cruz Biological Company.

Drugs

Isoniazid was purchased from Yunpeng Pharmaceutical Limited Company of Shanxi Linfen ,batch number 20070306 ,specification 0.1g ; Rifampicin was purchased from Hongqi Pharmaceutical Limited Company of Shenyang, batch number 20070712 , specification 0.15g.

Process of Multi-epitope DNA vaccine

Detected the A260 /280 ratio by ultraviolet spectrophotometer (Bio-Rad) and quantitated ,then adjusted the density to 1 g/L by PBS(pH7.4)for use .

Preparation of BCG

Took the two weeks well-growth BCG culture on medium , weight wet 10mg strains ,then added 3ml PBST solutions (contained 0.5 g/L Tween80),next grinded to uniform suspensions by mortar ,finally stored at -70℃. Thawed and misced before injection ,then diluted to 1 mg/3 ml by physiological saline and subcutaneous injected 0.3 ml /mouse ,that is wet bacterium 0.1 mg/mouse (contained viable organism 106 CFU /ml).

Animal immunity

BALB / c mice were divided into three groups: A. PBS negative control, B. Multi-epitope DNA vaccine and C.BCG positive control group ,and each group had 10 mice .All mice received three immunizations in 4, 6 ,8 weeks by PBS , Multi-epitope DNA vaccine and BCG respectively. Immunizing dose :injected 7.5 g /L mixture contained bupivacaine and Multi-epitope DNA vaccine (1:4, 100μL)into each mouse anterior tibial muscle in B group while 0.3ml BCG suspensions in C group (that is wet bacterium 0.1 mg/mouse ,contained viable organism 106 CFU /ml).

Detection of Specific IgG antibody

Specific IgG antibody in serum of mice was determined with indirect ELISA in 4, 6, 8 weeks respectively after final vaccination. Antibody titer:the maximum serum diluted multiple when experimental group A490 /negative control group A490 ⩾ 2.0

The proliferation of splenic lymphocytes

Took the spleens under aseptic conditions in 4, 6,8 weeks respectively after final vaccination ,then added PBS and grinded ,finally adjusted the cell density to 4×10^8/L with RPMI1640 media contained 100 ml/L solcoseryl. Measured by MTT method when the viable cells were more than 90% .The result was demonstrated by stimulation index (SI),and SI =A experimental numerus /A control numerus

Evaluate the induction and production of IFN－γ and IL－4

Adjusted the cell density to 4 ×10^8/L with RPMI1640 media contained 100 ml /L solcoseryl while the viable cells were more than 90%.Next added 200 μl to each hole and 50 μL PPD （1 mg/L）to stimulate , then cultivated 72 hours at 37℃ ,50 ml /L CO2 incubator .Finally ,centrifugated 5min at 5 000 r/min and subpackaged the supernatant 250μL/tube ,then stored at －20℃. Used ELISA method to detect IFN－γ and IL－4.Drawed the standard curve based on IFN－γ and IL－4 standard substance and count the content of IFN－γ and IL－4

The preparation of the Multi－drug resistant mycobacterium tuberculosis model

Took 0.4ml HB240 suspensions contained 5 ×10^4 cfu and injected into 60 female 8 weeks old BALB/ c mice through caudal vein.Randomly divided into three groups infected one month later and each group had 20 mice. The mice in group D were treated with physiological saline as the negative control while group E were treated with RFP and INH. The mice in group F were treated with Multi－epitope DNA vaccine combined with INH and RFP. Weight once before the treatment and weight one time each week after treatment .

Chemotherapy

The group E and F were treated by RFP (0.02mg/(g·d) and INH (0.01mg/ g·d) through oral medication for 12 weeks from infected one month later.

Multi－epitope DNA Vaccine Therapy

The mice in group F were treated by 100μg Ag85A/ ESAT－6 Multi－epitope DNA vaccine combined with INH and RFP for 12 weeks. DNA vaccines were injected intramuscularly 5 times at 3 weeks intervals.

Observed the pathological changes and weight of the lungs, livers and spleens

Killed half mice of each group in 4, 8 weeks after treatment .Observed the edema, atrophy, lesion , pathological change degree and range of the lungs ,livers and spleens in each group .Weight the lungs ,livers and spleens with electronic balance and counted the lungs , livers and spleens index.

The lung and spleen bacterial colony count

Took the whole lungs,livers and spleens and grinded, then culture on medium, counted the bacterial colony after cultured 4 weeks at 37 ℃

Statistics analysis

Experimental data was demonstrated as average ± standard deviation. Used SAS 6112 software to analysis the data. Used the t test to analyze the normal distribution while used signed rank sum test to analyze abnormal distribution .

RESULTS

Detection of the Specific IgG Antibody

Specific IgG antibody in serum of mice was determined with indirect ELISA in 4, 6 ,8 weeks respectively after final vaccination. The ratio between Multi－epitope DNA vaccine group, BCG group and PBS group were all above 2 .The result all showed positive and antibody titer was raised up gradually .It reached the higher level 8 weeks later. Multi－epitope DNA vaccine group was obviously higher than the BCG group($P < 0.05$) .The average titer of three times were 1∶20、1∶160、1∶80 respectively .

Results of the specific lymphocyte proliferation test

The mice splenic lymphocyte were stimulated by PPD in vitro in 4、6、8 weeks after immunized. The SI of lymphocyte proliferation both in Multi－epitope DNA vaccine group and in BCG group during three phases were all higher than in PBS group ,while Multi－epitope DNA vaccine group were higher than in BCG group. ($P<0.05$)(as table 1)

IFN－γ level

Lymphocyte were stimulated by PPD in vitro in 4, 6, 8 weeks after immunized ,then detected the IFN－γ

Table 1 Lymphocyte proliferation stimulated by PPD ($x\pm s$,n=10)

Group	4 week	6 week	8 week
A	1.03±0.02	1.13±0.02	1.13±0.06
B	1.26±0.03*	1.39±0.03*	1.42±0.02*
C	1.16±0.06**	1.28±0.05**	1.33±0.03**

* vs A and C group $P < 0.05$; ** vs A group $P < 0.05$

Table 2 PPD specific IFN−γ level in culture supernatant of splenic lymphocytes of mice immunized by DNA vaccine($x\pm s$,n=10)

Group	4 week	6 week	8 week
A	12.62 ±0.06	13.32 ±0.03	13.69 ±0.09
B	19.53 ±0.10*	21.32 ±0.08*	32.54 ±0.18*
C	15.35 ±0.23**	18.63 ±0.33**	26.36 ±0. 09**

* vs A and C group $P < 0.05$; ** vs A group $P < 0.05$

Table 3 The change of the body weights of mice ($x\pm s$, n = 10)

Group	4 week	8 week
D	15.60±1.15	18.98±1.05
E	20.37±1.43**	20.70±1.64**
F	20.60±2.35*	20.98±1.95*

* vs D group $P < 0.05$, vs F group $P>0.05$,; ** vs D group $P < 0.05$

Table 4 The indexes of the lungs livers and spleens ($x\pm s$,n=10)

Group	4 week			8 week		
	Lungs	Livers	Spleens	Lungs	Livers	Spleens
D	0.033±0.007	0.017±0.012	0.032±0.004	0.032±0.005	0.071±0.006	0.021±0.004
E	0.180±0.007**	0.050±0.012**	0.022±0.004**	0.022±0.005**	0.051±0.006**	0.011±0.004**
F	0.017±0.006*	0.048±0.006*#	0.012±0.003*#	0.021±0.008*	0.037±0.004*#	0.008±0.003*#

* P < 0.05 vs D group , *# P < 0.05 vs E group; ** P < 0.05 vs D group

in supernatant .There was significant difference between the Multi−epitope DNA vaccine group ,BCG group and PBS group(P<0.01) .There was also significant difference between the Multi −epitope DNA vaccine group and the BCG group(P<0.05) (as table 2)

IL −4 level

The IL −4 level was low after lymphocyte were stimulated by PPD in vitro .There was no obviously changes in the Multi−epitope DNA vaccine group and BCG group in 6, 8 weeks compared to in 4 weeks while there was no significant difference between them(P>0.05) .

Mice weight changes

At four and eight weeks after terminative treatment , the body weights of mice in group E were lower than that in group F , but it had no significant difference(P> 0.05)(as table 3).

Weight and indexes of the lungs, livers and spleens

At four weeks after terminative treatment, indexes of the lungs and spleens (0.017 ,0.011) from the mice in group F were lower than that in group E (0.020 ,0.012) . At eight weeks after terminative treatment , indexes of the lungs and livers from the mice in group F (0.021 , 0.047)were lower than that in group E (0.022 ,0.048) (

$P>0.05$) ,but indexes of the spleens from the mice in group F (0.008) were significantly lower than that in group E (0.012) ($P<0.05$) and there is significant difference between them($P<0.05$)(as table 4).

Pathological changes of lungs ,livers and spleens

At four weeks after terminative treatment ,the lungs showed granular degeneration ,necrosis .The pathological changes in group F was lighter than group E. The spleen showed swelling,slightly swelling . Six in tenth was no abnormal in group F while one in tenth in group E. At eight weeks, the lungs showed granular degeneration,necrosis ,atrophy and hilar lymph node swelling . There was one hilar lymph node swelling in group F while there was three in group E. The spleen showed swelling,slightly swelling . Five in eighth was no abnormal in group F while two in eighth in group E.

Colony count of the lung and spleen

The colony count of the lunge was 9×103cfu in group F. It decreased 60 % than in group E.The colony count of the spleen was 4×103cfu in group F. It decreased 67 % than in group E.

DISCUSSION

Mycobacterium hsp70, Ag85A, ESAT-6 all have strong cellular immunocompetence and stimulated T cell proliferation in peripheral blood monouclear cell , promoted IFN-γ releasing. We can regard it as the candidate molecule because it played an important role in anti-MTB infection[1-3].

Hsp70, Ag85A, ESAT-6DNA Multi-epitope DNA vaccine were constructed by author during studying in Prince Henry Institute of Medical Center of Australia Monash University .This study proved its singular immunity effect .It can obviously increase the production of the specific IgG antibody,strengthen the proliferation of splenic lymphatic cell ,improve the IFN-γ excretion [4,5].Its immunity effect was obviously better than traditional GCB[6]. In these studies we discussed the immunity effect of tubercle bacillus Ag85A/ ESAT26 chimeric plasmids DNA vaccine and used it combined with chemotherapeutics (RFP, INH) to treat mice drug-resistant tuberculosis .We researched its effect by studying weight,organ index ,histic pathology and organ capacity of the bacterial. The results showed that the mice weight ,lungs and spleens index, lymphadenectasis lungs and spleens pathological changes and colony count in group E were better than chemotherapy group alone at 4 and 8 weeks after terminative treatment .The spleen index was obviously lower than group F in 8 weeks . Colony count in group F decreased 70% and 80% than in chemotherapy alone group at 4 and 8 weeks after terminative treatment.The results showed that Hsp70, Ag85A, ESAT26 Multi-epitope DNA vaccine combined with drugs is better than chemotherapy alone in treating mice drug-resistant tuberculosis. Hsp70,Ag85A, ESAT26 Multi-epitope DNA vaccine may be a new immune pharmaceutics.It combined with chemotherapeutics will provided an new pathway in treating drug-resistant tuberculosis.

REFERENCES

1. Wolff JA, Malone RW, Williams P, et al. Direct gene transfer in to mouse muscle in vivo. Science, 1990,247:1465-1468.

2. Yi Pan, Hong Cai, Shuxia Li, et al. Immune effect of the Mycobacterium tuberculosis combined DNA vaccine.biochemistry and biophysics journey,2003,35:71-76.

3.Cohen EP.DNA based vaccines for the treatment of cancer an experimental model. Trends Mol Med,2001,7:175-179.

4. BL Freidag, G B Melton, F Collins, et al. CpG oligodeoxynucleotides and interleukin_12 improve the efficacy of mycobacterium bovis BCG vaccination in mice challenged with M. tuberculosis. Infection and Immunity,2002,68:2948-2953.

5. Martin E, Roche PW, Triccas JA ,et al. DNA encoding a single mycobacterial antigen protects against leprosy infection.Vaccine,2001,19:1391-1396.

6.Nuermberger E , Tyagi S , Williams K N , et al . Rifapentine , moxi2 floxacin , or DNA vaccine improves treatment of latent tuberculosis in a mouse model. Am J Respir Crit Care Med ,2005 , 172: 1452 -1456.