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IMMUNE RESPONSES OF IMMUNIZAED BALB/C MICE WITH AVIAN REO VIRUS

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SUMMARY

As prelimenary procedure for production of monclonal antibodies against avian reovirus, cellular and humoral immunity were measured for immunized female balb/c mice after continuous exposure to purified avian reovirus 17.33 . Immunoglobulin concentrations of IgG, IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, IgA were measured in sera of mice at different intervals post inoculation with one, two, three , four doses of purified avian reovirus 1733, using Radial immunodiffusion test. At the time antibody titers were measured in sera of immunized mice by enzyme immunosorbent assay using single dilution of sera. Immunolgobulin secreting cells were counted in spleen of immunized mice, one week post second, third and fourth doses of avian reovirus using solid phase immunoenzymatic technique. The results indicated that there was no relation between hyperimmunization of mice and optimum time to obtain mature, large differentiated specific-B-lymphocyte

INTRODUCTION

Kohler and Milstein (1975) showed that it was possible to obtain continuous cell lines making homogenous antibody by fusing mouse myeloma cells to spleen cells. Avian reovirus has been suspected as the aetiologic agent of several disease conditions, among which malabsorption syndrome (Page et al., 1982), avian arthritis (Jones et al., 1975). The virus is endemic in most poultry farms and has become of significant economic importance which led to the development of sveral vaccines in the world. Schnitzer (1985) reported the polymorphism of the RNA genomes in avian reovirus. So, production of monoclonal antibodies against avian reovirus strain is essential for differentiation of different isolates, Kazuaki et al., (1987) described the characterization of monoclonal antibodies prepared against avian reovirus . Kohler and Milstein (1975) mentioned that appropriate immunization of mice increased the freaction of the spleen cells which are able to fuse with the myeloma ells and producing specific secreting hybiodom. Islam and Jones (1988) measured antibody titres against avian reovirus using a single dilution of serum. Jonathon and Patrick (1986) reported assay system for the detection of antibody-secreting cells based upon Eliza. Leonore and Herzenberg (1978) determined eight known H-chain classes in the mouse. Each associated with particular biological activities.

In this study we evaluated the immune response of immunized mice with purified avian reovirus for fusion with mouse myeloma to produce a stable source of monoclonal antibodies. Solid-phase immunoenzymatic technique was done to enumerate reovirus antigen specific immunoglobulin secreting cells, antibody Eliza titers and immunoglobulin concentration in sera were determined.

MATERIAL AND METHODS

Preparation of purified avian reovirus:

This procedure was done in Auburn University, USA, according to that described by Kazuaki et al., (1987). Reovirus 1733 was obtained from SPAFAS Co, passaged twice in primary chicken kidney cell, the infected culture collected, harvested by freezing and thawing three times and plaque purified three times using modified agar overlay method (Schnizer et al., 1982). Virus stocks were prepared in 850 cm roller bottles of vero cell culture from the plaque purified virus. Harvested culture fluids were centrifuged at 2000 rpm for 15 minutes. 30% (NH_4) SO_2 (w/v) were added to the supernatant and the mixer was stirred with a magnetic stirrer for 4 hr at 4°C. The fluid was then centrifuges at 3000 rpm for 40 min., and the resultant precipitate was resuspended in 1/50 the original volume of phosphate-Buffered saline (PBS) and dialyzed overnight against PBS. The fluid was extracted with one-quarter volume fluorocarbon and the aqueous phase was mixed with 40% sucrose and centrifuged at 28,000 rpm for 90 minutes. The virus pellet was resuspended overnight in a small amount of PBS., layered in CScl gradient 45%, 40%, 35%, and 35% sucrose and centrifuged in Backman ultracentrifuge tube. The complete virus was collected from the lower band at densities of 1.36 gm/ml, centrifuged with PBS at 28,000 rpm for 90 minutes. The virus pellet was resuspended in a small amount of PBS, stored at-80°C until used . Its titer was $3x10^{10}$ plaque-forming units (PFU/ml) and its protein conentration was 800 ug/ml by the Lowry method (Kuchler 1977) using the BCA protein assay reagent product No.2325x.

Immunization of mice:

Twenty, 6-week old female balb/c mice, obtained from Namero Lab., were used 15 mice were injected intraperioneally and in food pad, with 100 ug of purified avian reovirus 1733 mixed in 0.1 ml of complete adjuvant per mouse. Five mice were left uninoculated as negative control. Blood samples were collected at three days, and at weekly intervals for twelve weeks before inoculation of the second dose of avian reovrus 1733 which was mixed with incomplete freundas adjuvant 0.1/ mouse via the intraperitoneal route and in foot pad. Blood samples were collected at weekly intervals for six weeks before inoculation of mice with the third dose of purified avian reovirus 1733 in 0.2 ml./ mouse intraperitoneally and in foot pad.

Blood samples were collected one day, one week and eight weeks post inoculation of mice with the third dose of avian reovirus, Mice wee inoculated with the fourth dose of purified avian reovirus 0.2ml/ mouse, intrapritoneally and in the foot pad. Blood samples were collected one week post inoculation. Sera were separated , inactivated at 56°C for 1/2 hour and stored until used for radiaol immunodiffusion and Eliza tests.

Preparation of spleen cell suspension:

Mice were sacrified by cervical dislocation one week post second, third , fourth dose of inoculation with purified avian reovirus. Cells were released from spleen in RPMI media in Petri dish using two tuberculin syringes. After centrifugation at 900 rpm for 10 minutes, the pelleted cells were gentely resuspended and exposed for 5 min, to tris-buffered 0.83% ammonium chloride in order to lyse erythrocytes. The suspension was then under layered with 1 ml., FCS and spun at 1500 rpm for 5 min. After two washings in RPMI media, the mononuclear cells were suspended in 1 ml, of RPMI media containing 1% nonessential amino acids, 1% Na pyrovate, 1% L-glutamine and 10% FCS, viability was tested by tryban blue dye exclusion test and the cell adjusted to 10^6 cells/ml, RPMI media.

Solid-phase enzyme-linked immunosorbent assay (Elisa) for enumeration of antireo antibody secreting cell:

This test was adopted according to the procedure of Jonathon and Patrick (1986), twenty four tisue culture plate was coated by the addition of 0.5ml of avian reovirus 1733 / well. Wells blocked with bovine serum albumin, one ml, of cell suspension containing 10^6 of spleen cells was then added, followed by guinea pig antimice Ig, gutinea pig anti IgG horserdish peroxidase conjugate was added followed by ABTS substrate. Wells were washed twice after reagent by washing solution of KPL kit, Immunoglobulin Ig antiavian reovirus 1733 secreting sells were enumerated using RID Reader.

Radial immunodiffusion test:

The procedure was done as mentioned by Mancini et al. (1965) and binding Site Comp. Kits were used, for determination of immunoglobulin concentration of IgG., IgG1, IgG2a, IgG_2b, IgG_2, IgM, and IgA.

Eliza test:

The technique for one dilution of serum in Eliza test was done according to the procedure of Islam and Jones (1988) with KPL reovirus Eliza Kits.

RESULTS

Evaluation of responses for immunized mice with avian reovrus 1733 was determined by measurement of concentration of immunoglobulin classes and subclasses at different inervals post inoculation with the first, second, third and fourth dose of purified avian reovirus 1733. As shown in Table 1, the immunoglobulin IgG was 16.25 mg/ml , one week post inoculation of mice with avian reovirus. This concentration was elevated to 19.3, 22.4, 21.25, and 18.8 mg/ml one , two four and six weeks post inoculation with two doses of avian reovirus as compared to 11.7 mg/ml in sera of nonimmunized control mice. Concentration of IgG measured 6.59 mg.ml, 19.7 mg/ml, and 15.6 mg/ml, one day one week, and two months in sera of mice post inoculation of three doses of avian reovirus. Concentration of IgG1 was 4.6 at one week post inoculation of the fourth dose.

Concentration IgG_2a measured 5.045, 5.45, 5.524, 8.415, 8.195, 4.83, 5.445, 4.56 , at 3,6,7,9,10,11 and 12 weeks post inoculation of mice with first dose of avian reovirus compared to 2.56 mg/ml in sera of nonimmunized control mice. Concentrations of IgG_2a were 8.25, 6.48, 7.09 and 7.9 at one week, 2 week, 4 weeks and 6 weeks post inoculation of mice with two doses of avian reovirus. Concentration of IgG_2a measured 2.22 mg/ml, and 8.2 in sera of mice at one day, and one weeks post inoculation of three doses of avian reovirus, while it was 4.41 mg/ml, two months post inoculation of the third dose of avian reovirus. It measured 3.05 , one week post inoculation of four doses of virus , compared to 3.31 in nonimmunized mice with avian reovirus. Concentration of imunoglobulin IgG_2b in sera of immunized mice with avian reovirus was 0.845, three days post inoculation of one dose avian reovirus compared to 0.317 in nonimmunized control mice. Concentration of IgG_3 was 0.871 and 2.24 at three days and three weeks post inoculation of one dose of avian eovirus compared to 0.167 in control nonvaccinated mice. Concentration of IgG_3 measured 1.44 and 0.842, one day and one week post inoculation of mice with purified avian reovirus (three doses) compared to 0.276 mg/ml, in nonimmunized control ones.

Concentration of IgM was 0.289 mg/ml, three days post inoculation of one dose, 0.273,7 days post two doses of avian reovirus, 0.168 and 0.308 one day one week post inoculation of mice witl

Table (1): Immunoglobulin concentration in sera of mice post inoculation with avian reovirus using RID technique

Time of collection	Immunoglobulin concentration mg/ml						
	IgG	IgG1	IgG2a	IgG2b	IgG3	IgM	IgA
First dose							
Three days	11.4	2.44	1.195	0.845	0.871	0.289	3.16
Seven days	16.25	3.83	1.795	0.309	1.625	0.259	2.835
Two weeks	13.35	2.96	3.515	0.309	1.3	0.236	2.035
Three weeks	12.9	4.53	5.045	0.411	2.24	0.375	2.035
Six weeks	14.05	3.98	5.524	0.775	1.33	0.355	1.63
Seven weeks	13.05	3.89	8.415	0.641	1.515	0.345	1.59
Nine weeks	14.9	3.595	8.195	0.498	1.45	0.343	2.595
Ten weeks	12.58	3.125	4.83	0.419	1.575	0.289	2.39
Eleven weeks	12.73	2.86	5.445	0.669	1.3	0.299	2.69
Twelve weeks	12.96	3.26	4.56	0.854	1.365	0.258	2.245
Sec. dose							
One week	19.3	4.372	8.25	0.473	1.43	0.273	1.895
Two weeks	22.4	4.6	6.48	0.510	1.66	0.374	1.83
Four weeks	21.25	4.69	7.09	0.445	0.854	0.306	2.46
Six weeks	18.8	3.8	7.9	0.270	0.942	0.291	2.290
Third dose							
one day	6.59	1.65	2.22	0.651	1.44	0.168	3.77
One week	19.7	4.0	8.2	0.28	0.842	0.308	2.87
Two months	15.6	3.97	4.41	0.61	0.972	0.23	-
Fourth dose							
one week	13.9	4.6	3.05	0.418	0.316	0.13	2.11
Control							
Young mice	8.59	2.21	2.56	0.317	0.167	0.941	2.68
old mice	11.7	3.23	3.31	0.473	0.276	0.13	2.68

Table (2): Antibody titers for sera of inoculated mice with avian reovirus using Elisa test.

Period post inoculation	Antibody titer
Three days	497
Seven days	258
Two weeks	701.5
Three weeks	3967
Six weeks	1493
Seven weeks	3967
Nine weeks	1000.5
Ten weeks	1028.5
Eleven weeks	923
Twelve weeks	219
One week post second dose	4648
Two weeks	4786
Four weeks	5697.5
Six weeks	1202
One day post third dose	194
One week post third dose	3872
Two months post third dose	61
One week post fourth dose	154
Control of young mice sera	-ve
Control of old mice sera	-ve

Table (3): Number of specific immunolobulin secreting cells/10^6 spleenocytes of inoculated mice

Period post inoculation time of collection	No. and size of secreting cells
One week post second dose	180 large
One week post third dose	190 large
One week post fourth dose	191 large

three doses of avian reovirus. It measured 0.130 mg/ml one week post four doses of virus compared to 0.941 and 0.130 in control ones. Concentration of IgA in sera of inoculated mice was 3.16 mg/ml, three days post one dose compared to 2.68 in control ones and it was 3.77 and 2.87, one day and one week post three doses. It measured 2.11 one week post four doses while it was 2.68 in control ones.

Immune responses of inoculated mice were measured in sera as shown in table 2 Antibody titers after one dose of avian reovirus inoculation were 497, 701.5, 3967, 1493,3967,1000.5, 1028.5, 923, 219 at three days, two three , six , seven , nine, ten, eleven and twelve weeks. Antibody titers after two doses of avian reovirus measured 4648, 4786, 5697.5, 1202, at one , two , four , and six weeks of inoculation. The antibody titers post inoculation with three doses of avian reovirus gave 194 at one day, while it was 61 at two months post inoculation . One week post inoculation of mice with four doses of avian reovirus it measured 154.

The immunoglobulin secreting cells reached 180/10^6 spleen ocytes, one week post inoculation of mice with two doses of avian reovirus, while they were 190, and 191, one week post inoculation of mice with three and four doses of avian reovirus.

DISCUSSION

Hybridoma technology allows in principle, the production of antibodies against antigen and the technique applied for preparation of monoclonal antibodies against avian reovirus by Kazuaki Takehara et al., (1087). The only thing is to properly immunize mouse and use spleen cells which contain specific immuoglobulin secreting cells at the optimum stage of differentiation . The immune response of inoculated mice with purified avian reovirus was measured after several times of injection to determine the role of continuous exposure to antigen, as shown in table (1) concentration of immunoglobulin IgG, IgG_1, IgG_2a, IgG_2b, IgG_3, IgG_3 Igm, increased 7.4, 1.142, 4.94 , 1.154, 0.143 respectively, one week post inoculation of mice with two doses of purified avian recovirus . The antibody titers measured 4648, one week post inoculation of mice with second dose of avian reovirus table (2). The imunoglobulin secreting cells enumerated 180 cells/ 10^6 of spleen cell of inoculated mice one week post second dose of inoculation.

The previous results indicated that mice highly responded to the inoculated two doses of avian reovirus. The inoculated mice with three and four doses measured an immune response optimally and specific immunoglobulin secretory cells/ 10^6 splee cells enumerated 191, one week post fourth dose of inoculation, while the antibody activity was 154. Also the secreting cells enumerated 190, one week post third dose of inoculation and their size large as these enumerated post second and fourth doses of inoculation of mice with purified avian reovirus. The results indicated that multiple antigen injection failed to yield a high number of specific immunoglobulin secreting cells and hyperimmunization of mice failed to obtain a high number of monoclonal antibodies. This may be due to the indication of a state of tolerance or production of suppressor cells in hyperimmunized mice.

It is concluded that there has been so far no definite method to identify the appropriate maturation stage and optimum time for differentiation to B-lymphocyte that has to be caught during the fusion process to obtain a large quantity of antibody producing hyperidoma to reach its maximum size. Further detaled experiments have to be performed to explain the role of accessory cells (helper and suppressor T-cells, macrophages) in facilitating or inhibiting

the growth of specific immunoglobulin secreting cells against avian reovirus.

REFERENCES:

Islam, M.R. and Jones, R.C., (1988): An enzyme-linked immunosorbent assay for measuring antibody titre against avian Reovirus using a single dilution of serum, Avian Pathology, 17: 411-425.

Jonathon, D. Sedgwick and Patrick, G. Holt (1986): The Elisa Plaque assay for the detection and enumeration of antibody-secreting cells. Journal of Immunological Methods, 87, 37-44.

Jones , R.C., Jordan, F.T.W., and Lioupis, S. (1975): Characteristics of reovirus isolated from ruptured gastrocenemius tendons of chickenes, Vet. rec. 96, 153-154.

Kazuaki Takehara., Yoshiniko Kimura., Yoshio Tanaka, and Msao Yoshimura (1986): Preparation and characterization of monoclonal antibodies against an avian reovirus. Avian Dis., 31: 730-734.

Kohler, G. and Milstein, C., (1975): Continuous cultures of fused cells secreting antibody of predefined specificity. Nature, 256, 495-497.

Kuchler, R.J. (1977): Biochemical methods in cell culture and Virology , Dowden Hutchinson and Ross. Inc Stroudsburg, P 99.

Leonore, A. Herzenberg and L.A. Herzenberg, (1978): Mouse immunoglobulin allotype, description and special methology in; Hand-book of Experimenta Immunology. Edited by D.M. Weir. Third Edition.

Mancini, G., Carbonara, A.O. and Heremans, J.F. (1965): Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry, 2, 235.

Page, R.K. Fletcher, O.J., Rowland, G.N. Gaudry, D., and Villegas, P. (1982): Malabsorption syndrome in broiler chicken. Avian Dis., 26. 618-624.

Schnizewr, T.J. (1985): Protein coding assignment of the a genes of the avian reovirus 1133. Virology, 141, 167-170.

Schnitzer, T.J., Ramos, T., and Gouvea, V.S. (1982): Avian reovirus polypeptides analysis of intracellular virus-specified products , virons, top component and cores. J. Virol. 43, 1006-1014.