Rev. Inst. Med. trop. S. Paulo
40 (5): 291-294, September-October, 1998.

OBTENTION OF RABIES ANTIGEN THROUGH BHK_{21} CELLS ADHERED TO MICROCARRIERS

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SUMMARY

Four rabies antigen batches were produced from virus suspensions resulting from BHK_{21} cells adhered to microcarriers (Cytodex 1), inoculated and cultured in a bioreactor. In parallel the methodology of production of rabies virus through cultures of BHK_{21} cells in monolayers in bottles was used. The results obtained showed that infecting titles were $10^{6.69}$ DL_{50}/mL and $10^{7.28}$ DL_{50}/mL for suspensions cultured in bottles and in the bioreactor, respectively. The viral suspension volumes collected were on average 11,900 per batch from the bioreactor and 800mL per bottle. Ten horses were immunized with the antigen produced in the bioreactor. The means of antirabies antibody titers found were 240 and 212 IU/mL after the initial and the first booster doses, respectively. Rabies antigen with satisfactory infecting titers can be obtained on a large scale by culturing in a bioreactor inoculated BHK_{21} cells adhered to microcarriers.

KEYWORDS: Rabies antigen; Microcarriers.

INTRODUCTION

Since the 50's, the Butantan Institute (BI) has been producing heterologous anti-rabies serum by hyperimmunization of horses with rabies antigen. The antigen utilized until 1986 was the Pasteur fixed rabies virus (PV) suspension cultured in rabbit brains[11].

In 1987 the production of this antigen suffered an alteration being carried out with cell lines[2]. The antigen was obtained by inoculating BHK_{21} cell monolayers in bottles using the Pasteur fixed adapted to these cells.

In 1992, with the nationally increasing demand for antirabies serum, the BI tried to obtain a maximal yield in the production of this immunobiological reagent. To attain this goal, the number of horses to be immunized and, consequently, also the volume of antigen used increased.

Of the methodologies for large scale production of rabies virus[5,9,10], the one chosen was that in which the virus was obtained in BHK_{21} cells adhered to microcarriers and cultured in a bioreactor[4,6,8,14,15].

MATERIALS AND METHODS

BHK_{21} cells

The BHK_{21} cell line (clone 13), passage 54, from the Vallée Nordeste Laboratory (Brazil) was the cell utilized. After serial passages, a stock of seed cells was formed which was then kept in liquid nitrogen in vials containing BHK_{21} cells, passage 58, at a concentration of 4.8×10^6 cells per vial. This stock was tested for fungal, bacterial and mycoplasma contamination as well as regarding cellular aspects.

Culture medium

In order to maintain the BHK_{21} cell line, Leibovitz (L_{15}) medium was employed with the addition of 10% fetal bovine serum for noninoculated cultures and 0.3% bovine albumin fraction V, for those infected with rabies virus. The culture medium also contained 25µg/mL neomycin sulfate.

Rabies virus

The rabies virus employed to inoculate BHK_{21} cell cultures was the Pasteur fixed virus adapted to BHK_{21} cells, from the Pasteur Institute, Paris, with passages carried out by the Technology Institute of Paraná, Brazil. Five passages of this virus were carried out in BHK_{21} cells at the BI. The virus infecting titer obtained after these passages was $10^{6.32}$ DL_{50}/mL. Virus titration was carried out in mice weighting 11-14g.

Seed virus batch

Using a bioreactor, the seed virus was obtained by infecting BHK_{21} cells adhered to microcarriers (Cytodex 1-type) with the PV rabies virus adapted to BHK_{21} cells.

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Virus suspension production in the bioreactor

The virus suspensions were produced according to the following steps:

Cell seeding: To five Thompson bottles containing confluent BHK_{21} cells trypsin was added to remove the cells from the walls. The content was then suspended in 500mL of L_{15} medium with 10% fetal bovine serum and neomycin sulfate. The cell suspension (6.8 x 10^8 cells) was seeded in the 5L vat of the fermenting Celligen-type vessel (New Brunswick) containing 7.5g Cytodex 1 and 300mL fetal bovine serum. After adhesion to the microcarriers for 3 hours at 36.5°C, L_{15} medium was added to a final volume of 3,700mL. The culture was maintained for 3-4 days with a rotation of 60rpm. The pH and dissolved oxygen (DO) were automatically adjusted by addition gases (CO_2, N_2 and O_2). Samples were drawn daily to check cellular aspect and growth.

Inoculating with virus: was carried out when cellular growth had reached total coverage of 40% of the vat beads, that is 1-5 x 10^9cells.

After discarding approximately 80% of the culture medium, the beads with the adhered cells in the remaining 20% of the L_{15} medium were inoculated with the seed virus (MOI = 0.1 - 0.8).

The suspension was kept for 2 hours at 34°C to allow absorption of the virus by the cells. L_{15} medium with 0.3% bovine albumin was then added to a final volume of 3,700mL. During this time automatic control of DO and pH continued.

Harvesting of virus suspensions: Vat culture medium was harvested at 72, 96, 144 and 168 hours after virus inoculation. For each harvest 2,900mL of the supernatant were taken and an equal volume of bovine albumin in L_{15} medium was added. The harvested supernatants were clarified by filtration through flat membranes using a microfiber filter and 0.45µ porosity membrane.

In bioreactor batches 3 and 4 the harvests were also carried out at 240 hours after inoculation because in these cycles the cells continued to present a satisfactory aspect.

Samples from each clarified harvest were taken for virus titration and fungal and bacterial sterility tests.

Virus suspension in bottles

In order to evaluate the efficiency of the method of production of rabies virus suspensions in the bioreactor, three lots of three 350cm² bottles each were made in parallel, using BHK_{21} cells. In these experiments the seed virus used was the same as that utilized in the bioreactor.

Preparing antigen batches

Antigen batches with infecting titers of $10^{6.5}$ to $10^{7.0}$ DL_{50}/mL were prepared from the virus suspensions harvested in the bioreactor. The batches were tested for control of infecting virus titer, fungal and bacterial sterility and identity of the rabies virus.

Inactivation of the antigen batches was carried out with betapropiolactone at a rate of 1:4,000[13].

Control tests

Infecting titers of rabies virus suspensions: was determinated through tests carried out in mice weighing 11-14g[12].

Testing for rabies virus identity: a 1:1,000 dilution of the virus suspension was incubated at 37°C for 90 minutes with 0.5mL of antirabies serum(200IU/mL). After this period, 0.03mL of the virus-serum mixture were intracerebrally inoculated in 12 mice, weighing 11-14g. A group of mice inoculated with 0.03mL of a mixture of virus with normal equine serum was used as control. The animals were observed for 14 days with 100% of them remaining healthy while all the animals of the control group presented symptoms of rabies.

Mycoplasma detection: was used the direct method with specific liquid and solid media for the detection of mycoplasma[7].

Safety test: was determined through tests carried out in mice weighting 18-20g and rabbits weighing 1.5 - 2.5kg[3].

Developing antirabies antibodies in equines

Ten horses were immunized following a scheme of 4 subcutaneous doses of inactivated antigen (5, 10, 15 and 15mL) and 3 of noninactivated rabies virus (5, 10 and 15mL). After 40 days, a booster comprising 3 doses of the noninactivated virus (5, 10 and 15mL) was administered. The antigen batch utilized presented an infecting virus titer of $10^{6.65}$ DL_{50}/mL. Samples of the sera of the immunized animals, collected after the initial and booster doses, were titrated for antirabies antibodies by a serum neutralization method, in mice[1].

RESULTS

BHK_{21} cells adhered to microcarriers and cultured in a bioreactor

BHK_{21} cells cultured in a Celligen-type bioreactor presented a satisfactory cell growth in 3-4 days (cell density: 1.0 - 5.0 x 10^9 cells). The viral suspensions harvested at 72, 96, 144, 168 and 240 hours after PV rabies virus inoculation presented mean titers between $10^{6.07}$ and $10^{7.28}$ DL_{50}/mL, the viral multiplication peak occurring 96 hours after virus inoculation (Table 1). The volumes of the rabies virus suspensions obtained in the 4 bioreactor cycles were 8,300, 10,800, 14,000 and 14,500mL, respectively. The volume was greater in the two last cycles since, the cellular aspect being still satisfactory, a harvest was made 240 hours after inoculation. All control tests to which the cell samples were submitted presented satisfactory results.

TABLE 1
Infecting titers of rabies virus suspensions harvested from the bioreactor and determined by titration tests in mice

Virus titer: log DL_{50}/mL					
	Harvests (h)				
Lot	72	96	144	168	240
1	7.35	7.35	6.57	-	-
2	6.17	7.15	6.39	5.80	-
3	7.19	7.35	6.78	6.09	6.20
4	7.22	7.29	7.05	6.65	5.95
Mean	6.98	7.28	6.69	6.18	6.07

TABLE 2
Infecting titers of rabies virus suspensions obtained in virus infected BHK_{21} cells in bottles

Virus titer: log DL_{50}/mL				
	Harvests (h)			
Lot	72	96	144	168
1	5.96	6.01	6.93	7.14
2	5.55	6.48	7.09	7.40
3	5.58	6.13	6.07	5.29
Mean	5.69	6.20	6.69	6.61

BHK_{21} cells cultured in Thompson bottles

BHK_{21} cells cultured in Thompson bottles were harvested at 72, 96, 144 and 168 hours after virus inoculation. Mean titers of harvested rabies virus suspensions were between $10^{5.69}$ and $10^{6.69}$ DL_{50}/mL with a viral multiplication peak after 144 hours of inoculation (Table 2). The volume of the harvested viral suspension was 1,600mL (four 400mL harvests).

Immunization of horses with antigen obtained in the bioreactor

Serum samples of the 10 horses immunized with the rabies antigen were titrated according to the serum neutralization method in mice and presented mean titers of 240 IU/mL after initial immunization doses and 212 IU/mL after the booster doses (Table 3).

DISCUSSION

Data of Tables 1 and 2 show that the infecting virus titers are similar, independent of the methodology used. Regarding produced volumes, the superior yield of the bioreactor culture can be noted, since to obtain a viral suspension volume similar to one bioreactor cycle, 15 bottles would be needed.

Comparing the production peaks of both methodologies, it can be seen that it occurs much rapidly in the bioreactor (96 hours after inoculation) than in the bottles (144 hours after inoculation).

Results shown in Table 3 indicate that rabies antigen batches resulting from viral suspensions produced in the bioreactor presented a good immunogenic power.

In conclusion, the utilization of BHK_{21} cells adhered to microcarriers and cultured in a bioreactor showed to be an efficient process for the large scale production of rabies antigen, saving time, costs and personnel.

TABLE 3
Titration of 20 samples of antibodies serum of horses hyperimmunized with rabies antigen produced in the bioreactor

Titer of anti-rabies antibodies IU/mL											
	Horses										
Titration	1	2	3	4	5	6	7	8	9	10	Mean
After initial doses	181	398	225	161	203	522	101	216	195	203	240
After booster	101	320	123	196	246	456	103	218	201	161	212

RESUMO

Obtenção de antígeno rábico através de células BHK21 aderidas a microcarregadores

Foram produzidos quatro lotes de antígeno rábico a partir de suspensões de vírus resultantes de células BHK_{21} infectadas, aderidas a microcarregadores do tipo Cytodex 1 e cultivadas em biorreator. Em paralelo foi utilizada a metodologia de produção de vírus rábico com células BHK_{21} em monocamadas, contidas em garrafas de 350cm^2. Os resultados encontrados demonstraram que os títulos infectantes foram de $10^{6.69}$ DL_{50}/mL para as suspensões virais obtidas em garrafas e $10^{7.28}$ DL_{50}/mL para as do biorreator. Os volumes das suspensões virais colhidas foram, em média de 11.900mL por lote do biorreator e 800mL por garrafa . Com o antígeno produzido no biorreator foram imunizados 10 cavalos. As médias dos títulos de anticorpos anti-rábicos encontrados no soros destes animais foram de 240 e 212 UI/mL, respectivamente após a base e o primeiro reforço. Através da infecção de células BHK_{21} aderidas a microcarregadores e cultivadas em biorreator, pode-se obter antígeno rábico em larga escala e com títulos infectantes satisfatórios.

ACKNOWLEDGMENTS

We thank M. Luci Nunes da Silva and Ivete Teixeira for their technical assistance.

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Received: 7 March 1997.
Accepted: 21 August 1998.