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USE OF BETAPROPIONOLACTONE INACTIVATED EBOLA, MARBURG AND LASSA INTRACELLULAR ANTIGENS IN IMMUNOFLUORESCENT ANTIBODY ASSAY

by

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Summary — A method is described for preparation of inactivated antigens for screening for immunofluorescent antibodies to Ebola, Marburg, and Lassa viruses. The method uses betapropionolactone-inactivated, infected Vero cells placed on coated multi-spot slides. It was found to be as sensitive as the use of ultraviolet and gamma irradiated antigens for quantitation of antibodies to these hazardous viruses.

KEYWORDS : Indirect Immunofluorescence; Betapropionolactone; Lassa, Marburg, Ebola Viruses.

Introduction

Indirect immunofluorescence, using inactivated Vero cells infected with Lassa (LAS), Marburg (MBG) and Ebola (EBO) fixed to multi-spot microscope slides, is frequently used in antibody surveys of these viruses (van der Groen, 1979; Heymann, 1980; Johnson, 1981).

The antigen slides used so far, were inactivated by ultraviolet light and gamma irradiation (Johnson, 1981), so that they could be used by other investigators outside a maximum security laboratory. This inactivation procedure should only be performed where gamma irradiation facilities are available. It occurred to us that an alternative inactivation method might avoid the use of gamma irradiation, which is expensive and technically difficult to perform nearby a maximum security laboratory (MSL).

Materials and methods

Viruses used were from human origin and passaged only in Vero cells. Lassa virus was strain Josiah, CDC No 800789 passage 1. Ebola virus was strain Ecran Louise from Zaire 1976, passage 11, 12 and 13. Marburg virus was strain Voege, passage 2. All work with infectious virus was carried out in a maximum security laboratory equipped with flexible film type of isolators (van der Groen *et al.*, 1980).

Cell cultures. Vero cells, kindly provided by the Center of Disease Control were grown as monolayers in medium 199 (Wellcome) with 7 per

cent aseptic calf serum (Gibco). Vero cells were maintained in a medium without serum described by Plaisner *et al.* (1974).

Indirect immunofluorescence test (IIFT) was performed as described by Johnson *et al.* (1981). Antisera used for testing the slides were obtained from humans convalescing from the respective viral diseases and kindly provided to us by the Center for Disease Control (CDC), Atlanta, U. S. A. The sera used as positive controls were : human anti-Lassa serum DC 095312, human anti-Ebola serum DC 096029, human anti-Marburg serum CDC 700808.

Fluorescein-labeled, sheep antihuman IgG (Wellcome) Lot No K 8855 diluted 1 : 80 in phosphate buffer saline (PBS) pH 7.2 containing Evans blue at a 0.1 per cent final concentration was used.

Preparation of inactivated antigens. Vero cell monolayers (20×10^6) grown in plastic 800-ml flasks (Nunc) were inoculated respectively with 50 ml of 1/10 diluted EBO and MBG virus in Plaisner medium with initial titers of 10^6 and 10^7 50 per cent tissue culture infective doses (TCID 50). After one hour absorption at 37 °C the cells were washed once and refreshed with 50 ml Plaisner medium. After 7 days the cells were rinsed with 100 ml Hanks solution, incubated with 0.5 per cent trypsin (1 : 250 Difco) in Hanks solution (2 min) and disodium salt of ethylendinitrotetra acetic acid (Titriplex[III] Merck) 0.02 per cent (w/v) in PBS (2 min) at 37 °C. After removal of trypsin and titriplex the cells were kept 20 min at 37 °C. A 10-ml volume of PBS containing 0.75 per cent (w/v) bovine serum albumin was added to each flask and the cells were pipetted frequently to homogenize the cell suspension and to break up clumps. The cells were counted and a part of the cell suspension was stored for titration of infectious virus.

The cells were next placed in conical centrifuge tubes and centrifuged at 1,300 g for 5 min. The cells were resuspended in freshly prepared beta-propionolactone (Fluka) (BPL) solution 0.2 per cent (v/v) in Plaisner medium pH 8, and kept in ice during the necessary time, with occasional shaking. Instead of using one long incubation time with the same BPL solution, we preferred short incubations with freshly prepared BPL solutions each time, to keep spontaneous hydrolysis of BPL to a minimum. After the last BPL incubation, cells were washed one time with 5 à 10 ml PBS bovine serum albumine (BSA) 0.75 per cent (w/v). Suspensions were mixed with freshly trypsinized non-infected Vero cells in a ratio 1 : 2 or 1 : 3 and adjusted with PBS containing BSA 0.75 per cent (w/v) to a final concentration of 10^6 cells per ml. Cell suspensions were thoroughly mixed with a plastic pipet. About 0.02 ml was then placed on each spot of coated, multi-spot microscope slides (Wellcome SM011 10 spots per slide) by using plastic fibrotips (Bio Mérieux, No 95689). Slides were air-dried, fixed in acetone for 10 min, placed in plastic slide boxes and stored at − 80 °C.

Indirect immunofluorescence tests on non-activated antigen slides were performed inside the isolator and readings of the fluorescence were performed outside the isolator as previously described (van der Groen, 1980).

The titers of reference positive EBO, LAS and MBG sera were compared on BPL-inactivated slides and Ultraviolet and gamma-irradiated, multi-spot antigen slides (Johnson, 1981), kindly provided by CDC. The following

CDC slides were used in the comparative test : mixed antigen slide EBO, MBG, LAS CDC 801486; MB antigen slide CDC 802682, 803241, 80286; LAS antigen slide CDC 801623; EBO antigen slide CDC 801375. Vero cell monolayers grown in 24 well (100,000 cells/well) plates (Nunc) were inoculated with 0.25 ml per well of 1/100 in Plaisner-diluted Lassa virus with a titer of 10^7 TCID50. Five days post inoculation, the supernatant was removed. To each well 0.2 ml PBS containing 0.75 per cent BSA was added, cells were scraped off with plastic fibrotips (Bio Mérieux, No 95689), pooled, counted, inactivated with BPL and distributed on multi-spotslides as described above.

Safety tests were performed to check for residual infectivity in BPL inactivated antigen preparations. The inactivated cell suspension was frozen and thawed once, diluted 1/10 in Plaisner medium and inoculated (0.25 ml per well) into 20 wells of a 24-well plate of Vero cells (100,000 cells/well). After one hour absorption at 37 °C, the monolayers were washed two times and refreshed with 1 ml Plaisner medium. Immediately after absorption, two wells of the inoculated plate were scraped off in PBS containing 0.75 per cent BSA and multi-spot slides prepared. Monolayers were checked during 12 days for occurence of cytopathic effect. At the 12th day, the supernatant was discarded, 0.2 ml PBS containing 0.75 per cent (w/v) BSA added to each well, cells scraped off, pooled and applied on multispot-slides as earlier described. Indirect immunofluorescence readings performed on cells, 12 days post inoculation, were compared with those one hour post inoculation. If the number of fluorescent foci after 12 days was the same as after one hour, the inoculated antigen was judged to be inactivated. The fluorescent foci one hour postinoculation were sometimes due to some nonlysed, antigen-containing cells of the inoculum, which were not washed away after one hour adsorption. A part of the scraped cells were stored at − 80 °C in order to perform a passage on Vero cells, even when after the first inoculation of the inactivated cell suspension, no infectivity could be demonstrated. To test the residual activity of the BPL-treated cells after 10 min fixation in acetone, the cells of 6 acetone-fixed slides were scraped off with a needle of a Icc tuberculine syringe after addition of 0.02 ml PBS containing 0.75 per cent BSA on each spot. The cells were pooled, freeze-thawed once and inoculated in Plaisner (0.25 ml/well) in a 24-well plate of Vero cells and processed as described above.

Results

It is clear that successful BPL inactivation of EBO infected Vero cells was strongly dependent on the cell concentration used (table 1). Only satisfactory results could be obtained when the cell concentration is lower than 2×10^6 cells per ml of BPL solution. Additional acetone fixation of 10 min was sometimes necessary for complete inactivation of the BPL-treated solution. The same is true for BPL inactivated MBG infected Vero cells.

Complete inactivation of LAS-infected Vero cells with BPL was easier to perform. Twenty minutes contact with 0.2 per cent BPL was enough to completely inactivate 4.1 TCID50 of intracellular Lassa virus (table 1).

TABLE 1

Betapropionolactone inactivation of EBO, MBG and LAS infected Vero cells. Evaluation of these antigens in the indirect immunofluorescent antibody test (IFA)

Exp. No.	Virus	Titer of intracellular virus (-log)	Inactivation conditions					Reciprocal antibody titer		
			Cells/ml × 10^6	BPL concentration	Contact time (min)	Temperature (°C)	10 min aceton fixation	Completely inactivated	BPL slides	CDC slides[2]
1	MBG	12	0.81	0.2 %	2 × 30[1]	0	−	no	32-128	32
			0.81	0.2 %	2 × 30	0	+	yes		
2			0.89	0.2 %	2 × 30	0	−	no	256	128
			0.89	0.2 %	2 × 30	0	+	yes		
3			0.89	0.1 %	2 × 60	0	−	no	NT[4]	NT
			0.89	0.1 %	2 × 60	0	+	yes		
4	EBO	5.6	1.3	0.1 %	60[3]	0	+	yes	0	NT
					120	37				
			1.3	0.2 %	60	0	+	yes	0	NT
					120	37				
5		5.1	8.5	0.2 %	6 × 10	0	−	no	NT	NT
			8.5	0.2 %	6 × 10	0	+	no		
6		5.7	7	0.1 %	4 × 10	0	−	no	128-256	256
			7	0.1 %	4 × 10	0	+	no		
7			2.45	0.1 %	4 × 10	0	−	no	256	256
			2.45	0.1 %	4 × 10	0	+	no		
8		5.4	1.8	0.1 %	4 × 10	0	−			
			1.8	0.1 %	4 × 10	0	+	yes		
9		6.1	0.9	0.2 %	2 × 30	0	−	yes	64	256
			0.9	0.2 %	2 × 30	0	+	yes		
10	LAS	4.1	± 0.8	0.2 %	60	0	−	yes	128	64
					120	37				
			± 0.8	0.2 %	60	0	+	yes		
					120	37				
11			± 0.8	0.2 %	2 × 10	0	−	yes	128	64
			± 0.8	0.2 %	2 × 10	0	+	yes		
12			± 0.8	0.2 %	4 × 10	0	−	yes	NT	NT
			± 0.8	0.2 %	4 × 10	0	+	yes		

[1] 2 × 30 min = after 30 min incubation cells are centrifuged, and resuspended for a second period of 30 min in freshly prepared betapropionolactone solution.

[2] CDC slides = ultraviolet and gamma-irradiated multi-spot slides with EBO, LAS and MBG infected Vero cells (Johnson, 1981).

[3] 60 0 = cells were incubated 60 min at 0 °C and subsequently 120 min at 37 °C.
120 37

[4] NT = not tested.

Homogenous distribution of cells per spot was not always obtained, due to clumps in the cell suspension.

When slides prepared according to the described procedure were reacted with specific antisera for LAS, MBG or EBO viruses, an equal bright fluorescence was detected as on ultraviolet and gamma irradiated slides. Negative control sera were included in all experiments. The characteristic pattern of the fluorescent inclusions in BPL-inactivated slides corresponded with those of the CDC slides — very fine granules in LAS and large characteristic inclusions in EBO and MBG infected cells. Ebola and Marburg-infected Vero cell suspensions contained more than 90 per cent infected cells, and were therefore mixed with normal Vero cells in a ratio 1 : 2 or 1 : 3 before they were applied on slides.

Antibody titers to reference sera were similar whether BPL and ultraviolet, gamma-irradiated antigens were used (see table 1). Subsequent incubation at 37 °C of a BPL-treated, infected cell-suspension destroyed the antigen.

Discussion

The 0.1 per cent and 0.2 per cent BPL concentrations used in these study, were the most frequently used in other virus inactivation studies (Lo Grippo, 1955; Dandawate, 1979). In those studies BPL was used to inactivate extracellular virus, whereas in our study intracellular virus had to be inactivated. The BPL concentrations used may have been just the limit of complete inactivation. In the second passage of the safety test performed on BPL treated EBO and MBG cellsuspensions, only one or two positive cells were detected by IIFT on one whole spot. This means that only a very weak infectivity was left after BPL treatment. Additional fixation with acetone was able to inactivate completely the small residual infectivity.

To avoid as much as possible the spontaneous hydrolysis of BPL (Lo Grippo, 1955) all experiments were performed at 0 °C with freshly prepared BPL solutions each time used for short incubation. For example, incubation of 10 min each with freshly prepared 0.2 per cent BPL solution were used instead of one incubation of 40 min with one and the same 0.2 per cent BPL solution.

In order to obtain complete inactivation of EBO and MBG-infected Vero cells with BPL, longer incubation times, and perhaps higher BPL concentrations are necessary. Better homogeneous distribution of the cells on the slides could eventually be obtained by resuspending the cells in isotonic saline containing 5 per cent fetal bovine serum, as was recently described by Johnson *et al.* (1981), instead of using the PBS solution containing 0.75 per cent bovine serum albumin.

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Het gebruik van beta-propionolactone geïnaktiveerd intracellulair Ebola, Marburg en Lassa virus antigeen in de immunofluorescentie test voor de bepaling van antistoffen.

Samenvatting — Betapropionolactone geïnaktiveerde verocellen geïnfekteerd met Ebola, Marburg en Lassa virus worden op multispot microscoopdraagglaasjes gebracht en gebruikt

in een indirekte immunofluorescentie test om antistoffen tegen hoger vernoemde virussen te bepalen. De alzo bereide antigenen geven hetzelfde resultaat als ultraviolet en gamma-bestraalde antigenen.

L'emploi des antigènes inactivés par le beta-propionolactone dans la méthode d'immunofluorescence indirecte pour la détermination des anticorps vis-à-vis des virus Lassa, Ebola et Marburg.

Résumé — Des cellules vero inoculées avec les virus Ebola, Marburg et Lassa, ont été inactivées par le beta-propionolactone, et ont été distribuées sur des lames microscopiques type « multispot ». Les antigènes ont été utilisés dans la méthode d'immunofluorescence indirecte pour la détermination des anticorps vis-à-vis des virus mentionnés. Pour la détermination quantitative des anticorps, les antigènes inactivés par le beta-proprionolactone ont donnés le même résultat que les antigènes inactivés par des rayons ultraviolet et gamma.

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