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COMPARATIVE STUDY OF ADENOVIRUSES WITH MONOCLONAL ANTIBODIES

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SUMMARY

The obtainment of monoclonal antibodies for adenovirus species 4(Ad4) is described.The specificities of selected monoclonal antibodies were determined by means of viral neutralization test in cell culture, immunofluorescence and Enzyme-Linked Immunosorbent Assay (ELISA), in the presence of the following species of human adenovirus: 1, 2, 5 (subgenus C), 4 (subgenus E), 7 and 16 (subgenus B) and 9 (subgenus D).

Two monoclonal antibodies species specific to adenovirus 4 (1CIII and 3DIII) and one monoclonal antibody that cross reacted with adenovirus species 4 and 7 (2HIII) were obtained.

KEY WORDS: Adenovirus, Species Specific, Monoclonal antibodies, Adenovirus 4, Antigenic Relationship.

INTRODUCTION

Since it was discovered by ROWE et alii [19], 42 species of human adenoviruses have been identified [24], which demonstrates the genetic variability of individual species within the Adenovirus Family. These viruses have been classified into subgenera based on different biological and molecular properties. ROSEN [18] and HIERHOLZER [8] proposed a classification scheme based on the differential hemagglutination properties presented by the adenoviruses. PINA and GREEN [16], HUEBNER [10] classified the adenoviruses according to their ability to induce tumors when inoculated into newborn hamsters. McALLISTER et alii [12] classified the adenoviruses on the basis of differences in the antigenicity of the T antigen. The SDS polyacrylamide gel electrophoresis technique developed by WADELL [23], provided a classification scheme based on the apparent molecular weight of internal polypeptides of adenovirus.

Lately, a classification based on the difference in the nucleotide sequence of genomes of serotypes of adenovirus has been proposed [4,5,6].

All the parameters mentioned above have been used to classify human adenovirus, into seven subgenera as follow: A, B, C, D, E, F, and G. Adenovirus species 4, which has been the aim of our studies, is the only species of the subgenus E.

The antigenic structure of the icosahedral capsid of the virion is composed of 252 capsomers (240 hexons and 12 pentons consisting of a base and a protruding fiber). The hexon is the major protein of the adenovirus, and displays antigenic determinants with genus, intersubgenus, intrasubgenus, and type (species) specificity. The immunological properties of antigenic determinants are the basis of human adenovirus recognition. The alpha antigenic determinant common to all human adenoviruses enables its identification as an Adenovirus Family member. The type (species) is defined by its immunological distinctness by quantitative neutralization using specific immune serum against the prototypes of the identified species. This forementioned immunological distinctness is based on an

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antigenic determinant called epsilon. The human adenoviruses are associated with respiratory and urinary tract infections, conjunctivitis, encephalitis, gastroenterits, and severe infections in therapeutically immunosupressed patients after kidney or bone marrow transplants. Adenovirus of different serotypes have been isolated from patients with AIDS. Recently five new serotypes which are candidates of subgenus D (Types 43-47) have been described [9]. Consequently, the species identification of the adenoviruses is important to elucidate the etiology of the different diseases caused by this virus. The use of monoclonal antibodies provided a rapid and specific approach to identify viruses. The aim of our investigation is the obtainment of monoclonal antibodies to species specific antigenic determinants of adenovirus 4 allowing its rapid and specific identification by immunofluorescence assay and ELISA, replacing the usual neutralization test. Once the adenovirus protein bears complex and different antigenic determinants [13,22,15], the purpose of the present study was to test the obtained monoclonal antibodies of different specificities to adenovirus 4 with adenoviruses of different subgenera, in order to investigate a possible antigenic relationship with different adenoviruses species.

MATERIALS AND METHODS

Adenoviruses

The adenovirus species 4, selected for immunization of the Balb/c mice was isolated from throat wash of a 44 years old patient (S) with pharyngoconjunctivits. The adenovirus chosen for the comparative study were Ad 1 isolated from conjunctiva of 42 years old woman (OBN), Ad 2 from nasal aspirate of a 18 months old child (N), Ad 7 of a throat swab of a 4 months old child (W), Ad 1 of stools of a 9 months old patient (AL), Ad 4 from conjunctiva of a 39 years old patient (F). All the adenovirus species including Ad 5 from throat swab of a child with bronchopneumonia and Ad 9 were isolated and identified in the Instituto Adolfo Lutz, with the exception of type 16 which was kindly supplied by Dr. Jussara Nascimento from Oswaldo Cruz Foundation, Rio de Janeiro, Brazil.

Viral Concentration

HEp-2 cell cultures, maintained in Minimum Essential Medium (MEM) supplemented with gentamicim sulphate (20 ug/ml) were grown in bulk for antigen production. After virus inoculation, cell cultures were maintained at 35°C until 100% cytopathic effect. The fluid containing harvested cells was clarified at 12,100 g during 30 min. Supernatant was centrifuged at 100,000 g during 3 hours and the pellet obtained was resuspended in a phosphate buffered saline (pH 7,2). Protein concentration was determined as described elsewhere (2).

Monoclonal antibody production

Four Balb/c mice were immunized intraperitoneally with 335 ug of intact viral particles in Freund complete adjuvant. Two months later, the mice received a second dose of 670 ug in phosphate buffered saline (PBS). After one month, the presence of antibody was tested by indirect immunofluorescense assay. The mouse which showed a good immunological response and no response at all to cell antigens was selected to receive a third dose of 335 ug of virus in PBS. Three days before the spleen removal, this mouse received a booster of 675 ug in PBS. Splenic cells were fused with SP2/O mouse myeloma line.

Hybridoma Cell Production

Hybridoma cell production was essentially made as described by PRICE [17]. Briefly, spleen cells from immunized Balb/c mouse were washed by centrifugation with serum - free media. Paralelly, the myeloma cells were spun out of their culture medium and washed with serum-free media. The spleen and myeloma cells were counted. Each kind of cell was resuspended at a concentration of 1×10^8 cells/ml, mixed at a ratio of 2:1, and were spun down together. The cell pellet was disrupted by gently tapping, and 1 ml of PEG 4000 (Sigma Chemical Co.) was added slowly to cells. After incubation for 45 seconds, 10 ml of serum-free media was added drop wise, slowly, the tube was then topped to 50 ml with the hybridoma growth media. After fusion, the suspension was centrifuged, the supernatant was decanted, and the cells were resuspended in the HAT selection media. A concentration of 5×10^5 myeloma cell/ml was obtained by adding 1 ml of this suspension in each well of a 24 well tissue culture plates. Hybridomas were screened for antibodies to Ad 4 by neutralization in cell culture, immunofluorescence and ELISA tests.

Hybridomas selected were cloned by the limiting dilution procedure, mouse exsudate cells were used as feeder cells (10^5/ml) in 96 - well plates. The supernatants of clones were tested according to the techniques described below.

Neutralization test

The neutralization test was carried out in 96 - well microtiter plates (Flow). In each well, 75 ul of HEp-2 cells suspension (concentration of 5 x 10^4 cells/ml) were added and incubated at 35°C. The monolayer was washed with Hank's solution and incubated with MEM. Equal volumes of virus containing 100 TCD_{50} and supernatant fluid were mixed and incubated at 37°C during 30 min. After incubation the antibody - virus mixture, virus and antibody controls were inoculated in each well to be tested. Cells were examined daily in order to detect the virus neutralization activity of monoclonal antibodies.

Indirect immunofluorescence

HEp-2 cell cultures were inoculated with the following adenovirus species: 1, 2, 4, 5, 7, 9, 16 and Herpes Simplex Virus (HSV) as a negative control. Twenty four hours after infection, the cells were harvested, washed with sterile PBS (pH 7,2), placed on glass coverslips and fixed 5 minutes with acetone. Uninfected cell cultures were used as controls.

Cells were incubated with 25 ul of the supernatant to be tested during 30 minutes at 37°C. After washing with PBS, 25 ul of fluorescein conjugated antimouse IgG were added, and incubated for another 30 min. After three washes with PBS, the reaction was monitored in a fluorescent microscope with epi-ilumination.

Enzyme-Linked Immunosorbent Assay (ELISA)

Adenovirus species 1, 2, 4, 5, 7, 9, 16 and HSV were inoculated in HEp-2 cell cultures. Uninfected cell cultures were used as control. The infected cell cultures were washed in PBS (pH 7,2), ressuspended in 500 ul of the same buffer and submitted to three freeze-thawing cycles followed by a centrifugation at 580 g during 10 minutes. The resulted supernatant was used as antigen to coat the plates. The indirect ELISA was performed as previously described [21]. Briefly, 100 ul of supernatant diluted in PBS containing 10% BSA/ 0,05% Tween 20 were added to the wells of microtiter plates previously adsorbed with 100 ul of optimal antigen dilution in $NaHCO_3$ (pH 9,0). After incubation at 37°C during 2 hours, the platers were washed 4 times with 0,9% balanced saline solution containing 0,05% Tween 20. A peroxidase labelled goat antimouse IgG was added to the wells and incubated at 37°C during 2 hours. The plates were washed 4 times with 0.9% saline solution containing 0,05% Tween 20. Substrate for peroxidase (ABTS -2,2 azino - bis (3 - ethybenzthiazoline sulfonic acid) 1 mg/ml in 0,1 M citrate phosphate solution plus 3% hydrogen peroxid was added. The visual reading was made after 30 minutes, when positive samples developed a green color.

Ascitic Production

Selected clones, at concentration of 1 - 10^6 hybridoma cells per mouse, were injected intraperitoneally into Balb/c mice which had been primed 10 days earlier with 0.5 ml Pristane (2, 6, 10, 14 - Tetramethylpentadecane, Sigma Chemical Co.) After 20 days the ascitic fluid was tapped from the abdomen, clarified by centrifugation and stored at -70°C.

RESULTS

Balb/c mice were immunized with human adenovirus species 4, and its spleen cells were fused with SP2/0 mieloma cells. The supernatant fluid of thirteen hybridomas were tested for reactivity with Ad 4. The secretory activity of 13 hybridomas obtained are demonstrated in Table 1.

The hybridomas (4C2 and 3D6) were selected for cloning through the limiting dilution. All clones obtained from 4C2 neutralized the viral infectivity in cell culture. On the other hand, of the 50 clones obtained from 3D6 only one (3DIII) neutralized the viral infectivity. Clones 1CIII from 4C2, 3DIII, and 2HIII, 5CIV, 5GII, 12HII, obtained from recloning of 4DV - originated from 3D6 - were chosen for further studies.

The results obtained by neutralization in HEp-2 cell culture, immunofluorescence and ELISA tests, with differents clones, are shown in Table 2.

Two monoclonal antibodies (1CIII and 3DIII)

species specific to Ad 4 were obtained. The species specificity of these antibodies was verified by their capacity to neutralize the infectivity of Ad 4 in HEp-2 cells. These monoclonal antibodies were not able to neutralize adenoviruses species 1, 2, 5, 7, 9 and 16.

Ascitic fluid antibodies titers of 1CIII and 3DIII were respectively 1:8,000 and 1:4,000, by neutralization tests, and 1:20,480 and 1:2,560 by immunofluorescence tests. Only ascitic fluid from 1CIII was tested by ELISA and the titer of 1:100 was obtained. The pattern of fluorescence developed by these monoclonal antibodies are shown in the figs 1a and 1b in which the bright fluorescence in the nucleus of the cells, may be observed.

Table 1
Secretory Hybrid Selection

Hybrid designation	Sorologic tests		
	NT	IF	E
1D1	-	+	-
1D5	-	+	+
1C2	-	-	+
3D6	-	+	+
4C5	-	-	+
4C2	+	+	+
4D6	-	-	+
5A3	-	-	+
5C3	-	-	+
6C3	-	-	+
3A3	-	-	+
2A3	-	-	+
5C5	-	-	+

NT = Neutralization Tests
IF = Immunofluorescence
E = Enzyme Linked Immunosorbent Assay

The antigenic relationship between the Ad 4 and Ad 7 species was confirmed in immunofluorescence assay and ELISA by monoclonal antibody 2HIII (Table 2). The pattern of fluorescence developed by this monoclonal antibody is shown in fig. 1d. Curiously this antibody stained only the cytoplasmatic membrane region. Ascitic fluid antibody titer of 2HIII was 1:5,120 and 1:10,000, respectively, by immunofluorescence and ELISA test. This antigenic relationship is illustrated in fig. 2.

An adenovirus species isolated from conjunctiva of a patient (F) during an acute hemorrhagic conjunctivitis in 1984, was identified by species specific monoclonal antibody, obtained in this experiment, which neutralized the infectivity of this adenovirus species in HEp-2 cells. The 5CIV, 5GII and 12HII antibodies, discriminated in figure 2, have the same specificity of 2HIII clone.

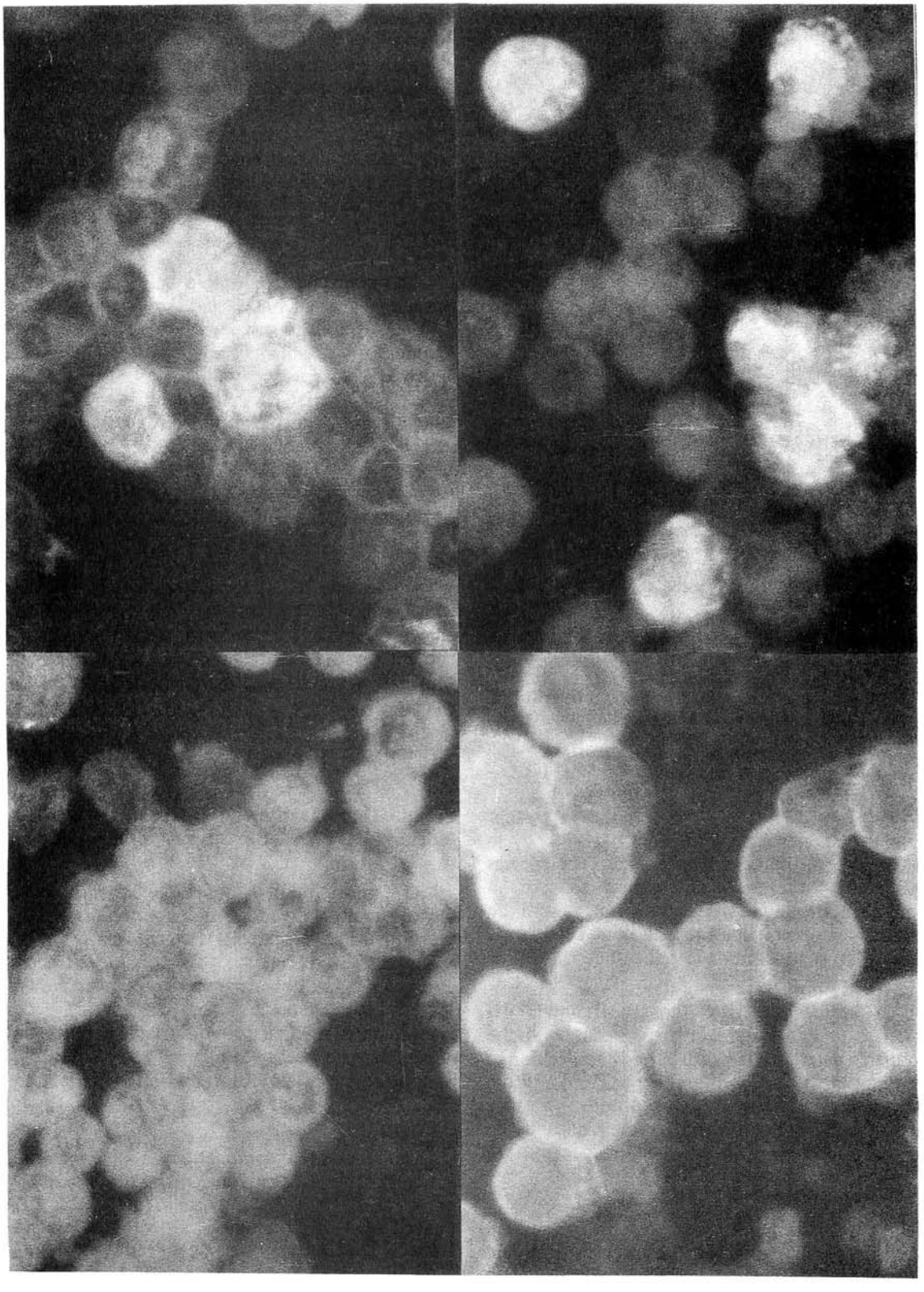
DISCUSSION

The results obtained when the clones 1CIII and 3DIII were tested in the presence of different adenovirus subgenera, clearly demonstrate that species specific monoclonal antibodies to Ad 4 were obtained. This fact was confirmed by three carried out for this purpose, neutralization in cell culture, immunofluorescence and ELISA. Although monoclonal antibodies species specific to Ad 40 and 41[7], Ad 35[1], Ad 3 and 7[11] have been

Table 2
Comparative study of adenovirus of different species by monoclonal antibodies

Original hybrids	Clone designation	Human adenovirus species according to subgenera																								
		E			B						C									D			HSV		
		4			7			16			1			2			5			9					
		NT	IF	E	NT	IF	E	NT	IF	E	NT	IF	E	NT	IF	E	NT	IF	E	NT	IF	E	NT	IF	E
4C2	1CIII	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3D6	3DIII	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3D6	2HIII	-	+	+	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-

NT = Neutralization Tests
IF = Immunofluorescence
E = Enzyme Linked Immunosorbent Assay
HSV = Herpes Simplex Virus
* = not perfomed



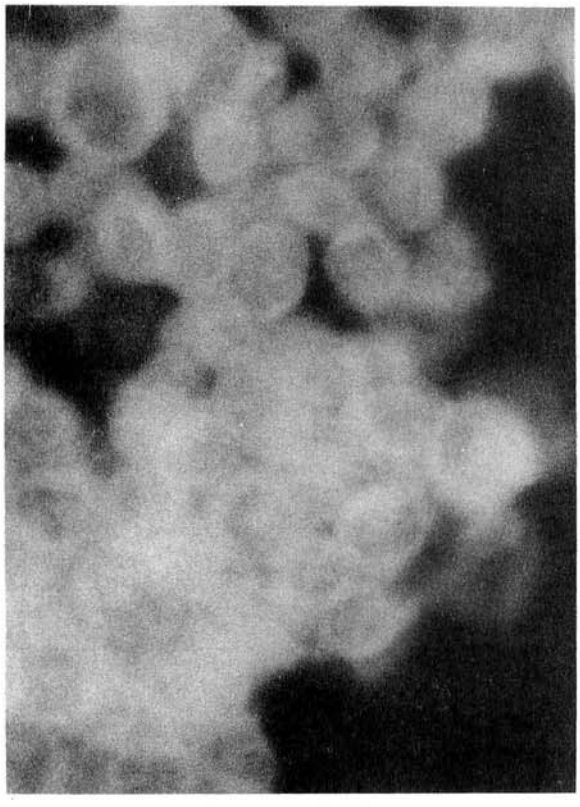


Fig. 1. Indirect immunofluorescence. HEp - 2 cell infected with Ad 4 tested by monoclonal antibodies from 1CIII fig (1a) and 3DIII fig. (1b) (640x); HEp - 2 cell control fig. (1c) (640x). HEp - 2 cell infected with Ad 4 tested with monoclonal antibody from 2HIII fig. (1d) (640x), HEp - 2 cell control fig. (1e) (350x).

obtained, species specific monoclonal antibody to Ad 4 has not been described so far. The species specific polyclonal antiserum to Ad 4 commercially available cross react with an antigenic determinant present in Ad 16, only detected by neutralization in cell culture[13].

It has been verified that 1CIII and 3DIII specific to Ad 4 did not cross react with the antigenic determinant of Ad 16 by neutralization in cell culture tests. The high specificity of the monoclonal antibodies obtained, in this experiment, was confirmed by their capacity in recognizing only the antigenic determinant responsible for species - specificity of Ad 4.

The obtainment of a species specific monoclonal antibody leads to an accurate etiologic diagnosis of infections caused by this adenovirus. Therefore, these results may contribute to clinic diagnosis of infections caused by this adenovirus and also epidemiologic studies which have been done by the time consuming neutralization test. Previous studies[13, 14, 25] suggested the localization of species specific determinant in both peripentonal and nonamer hexons, but their precise localization remains to be shown. The utilization of species specific monoclonal antibodies will also contribute to this study.

The antigenic relationship between the Ad 4 pertaining to subgenus E and Ad 7 pertaining to subgenus B was already described[13]. This antigenic relationship was also verified by epidemiologic studies[3]. Studies concerning structural analysis of E1 region of viral genome, which is responsible for morphological transformation in cell cultures, revealed 65% of homology in this region between species Ad 4 and Ad 7 [20]. It is necessary to emphasize that the genomic homology between the adenovirus species belonging to the same subgenus is higher than 90% and lower than 20% among the adenovirus species of the different subgenus[6]. It is suggestive that 2HIII monoclonal antibody reacts with vertex capsomers of the viral particle which are responsible for the antigenic relationship between the adenovirus species of subgenus B, which is in accord with the studies of WADELL and NORRBY[13].

RESUMO

Estudo Comparativo de Diferentes Tipos de Adenovirus Através de Anticorpos Monoclonais.

O estudo relata a obtenção de anticorpos monoclonais para o adenovírus tipo (espécie) 4. A especificidade dos anticorpos monoclonais, selecionados nesse experimento, foi determinada testando-os frente às diferentes espécies de adenovírus: 1, 2, 5 (subgênero C), 4 (subgênero E), 7 e 16 (subgênero B) e 9 (subgênero D), pelas técnicas de neutralização em cultura de células, imunofluorescência e ensaio imunoenzimático (ELISA).

Os resultados demonstram a obtenção de anticorpos monoclonais que reagiram de maneira específica para o adenovírus 4 (1CIII e 3DIII) e anticorpo monoclonal apresentando reação cruzada com as espécies de adenovírus 4 e 7 (2HIII).

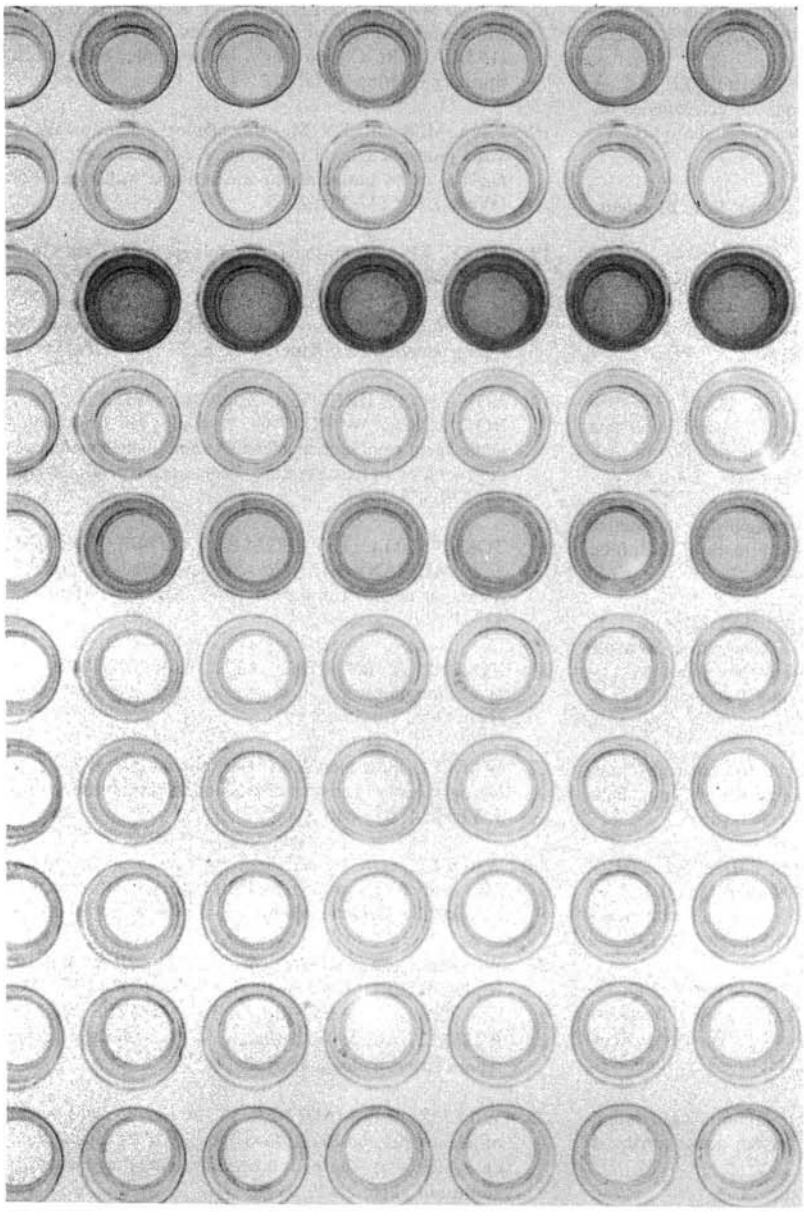


Fig. 2. Demonstration of the antigenic relationship between Ad 4 and Ad 7 species by ELISA test, utilising Mabs 5CIV, 5GII and 12HII, obtained from recloning of 4DV - originated from 3D6 - which react with vertex capsomers of the viral particle, responsible for the antigenic relationship between adenovirus species 4 and subgenus B species. The same antigenic relationship was demonstrated by 2HIII antibody. Line 1. Buffer Control (BC), lines 2 and 3 duplicate of monoclonal antibody (Mab) 12 HII: lines 4 and 5 duplicate of Mab 5GII: lines 6 and 7 duplicate of Mab 5CIV tested against the different adenovirus species, respectively; line 8 virus control (VC).
Columns: 2 Ad4 (S): 3 HSV(H); 4 Ad4 (F); 5 Ad1 (AL); 6 Ad7 (W); 7 Ad1 (OBN); 8 Ad9 (9); 9 Ad5 (5); 10 Ad2 (N), 11 Cell Control (CC).

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