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MONOCLONAL ANTIBODIES TO MONKEY POX VIRUS: PREPARATION AND APPLICATION

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Summary. — Two fusion experiments with NSO myeloma cells yielded 30 hybrid cell lines which continously and actively secreted monoclonal antibodies (MoAb) to monkey pox virus. Eight lines appeared to produce antibodies to specific antigenic determinants. The isotype and serologic reactivity of MoAb to monkey pox virus was characterized and the karyotype of several hybridomas determined. An enzyme labelled conjugate has been prepared from MoAb selectively reacting with monkey pox virus. This conjugate allowed the indentification of monkey pox virus in the materials from patients. It also helped to determine that an orthopoxvirus isolated from wild squirrel in the Republic of Zaire was monkey pox virus.

Key words: monoclonal antibodies; monkey pox virus; orthopoxviruses; species indentification; enzyme immunoassay

Introduction

The technique of monoclonal antibodies (MoAb) preparation has opened (Köhler, Milstein, 1975) new great perspectives. MoAb can be employed for studies on antigenic specificity of closely related viruses and for the development of improved methods of their differentiation and indentification. Polyclonal antibodies often appear inadequate for this purpose, which especially applies to orthopoxviruses. The available serologic methods of rapid diagnosis [immunofluorescence, passive haemagglutination (PHA) and others] are usually suitable only for attributing a virus to a certain group. Final species identification of an orthopoxvirus so far needs biologic tests.

The preparation of MoAb to a broad variety of viruses has been recently reported (Wiktor, Koprowski, 1978; Heinz *et al.*, 1982; Roehrig *et al.*, 1983; Ferms, Tedder, 1984; Novokhatsky *et al.*, 1984; Kushch *et al.*, 1985). However, we know of only two publications dealing with orthopoxviruses (Kitamoto *et al.*, 1984; Roumillet, 1984). The MoAb described by Kitamoto *et al.*, (1984) were not highly species-specific as they reacted with at least 2 another orthopoxviruses (Marennikova *et al.*, 1985). The purpose of the presented work was to prepare hybrid cell lines that would secrete MoAb to monkey pox

virus, to study their properties and to consider the possibility of their practical use. This task was necessitated by an WHO project carried out in Africa dealing with the studies on monkey pox in humans (improvement of methods of diagnostic and ecologic investigations).

Materials and Methods

Viruses. Monkey pox virus (MpV), strain Zaire-753 was isolated from a monkey pox patient in 1984. At the time of the experiment it underwent 3 passages in chick embryos. In some experiments vaccinia virus (VcV) strain L-IVP, and also the biovariant of cowpox virus isolated from predatory animals (strain Pumenok — Little puma) were used.

Virus cultures grown in diploid human embryo lung cells or in RK-13 cells were used for serologic tests.

Serologic tests. Neutralization test (NT) in chick embryos, enzyme immunoassay (EIA), haemagglutination inhibition (HI) and gel precipitation (GP) were carried out as described (Marennikova, Maltseva, 1961; Marennikova *et al.*, 1984). For PHA sheep erythrocytes sensibilized by vaccinia virus were used (Ertte *et al.*, 1973). For detection of antibodies by immunofluorescence (IF) the indirect procedure was performed using antispecies (antimouse) FITC labelled conjugate (Miles).

Animals. Balb/c mice weighing 8—10 g were immunized with monkey pox virus infected RK-13 cells in 3 repeated doses given by different administration routes (intranasally, intraperitoneally, intravenously).

Cell fusion. The fusion of splenocytes with mouse myeloma cells and cultivation of hybrid cells was carried out by conventional methods (Goging, 1983). Mouse NSO myeloma cells were kindly supplied by Dr. Novokhatsky. The NSO cells were grown in roller suspension in 2-liter roller flasks a with a fluid phase volume 500 ml. The rotation rate of the flasks was 10—14 rev/min and the cultivation temperature was 36.5 °C. NSO cells were grown in roller suspension conditions in a following fashion (unchanged throughout the experiment): every two days 300 ml of the cell culture medium was removed and replaced by the same volume of resh growth medium. The mean density of NSO cell cultures under described cultivation condition was $1.5—2.0 \times 10^6$ cells/ml at the plateau level. Three days before the fusion, NSO cells were maintained at the phase of logarithmic growth by daily reduction of the cell volume to the half and their replenishmet with an equal volume of a fresh growth medium.

Medium RPMI-1640 or DMEM (Flow) supplemented with 0.13% $NaHCO_3$ and 5 mmol/l of HEPES were used as a growth medium. The growth medium contained 5% of foetal calf serum (Flow or Byelorussian Research Institute of Epidemiology and Microbiology, Minsk), 5% of lambing ewe serum (experimental batches); 0.15 mg/ml oxalacetate, 0.05 mg/ml pyruvate, 0.2 units/ml of bovine insuline (Sigma) were added as growth factors. For preparation of selective medium the following components were added to the growth medium: 10^{-2} mol/l hypoxanthine, 4×10^{-5} mol/l aminopterine and 1.6×10^{-3} mol/l thymidine (Sigma). Finally 5×10^{-5} mol/l of 2-mercaptoethanol and 20 μg/ml of gentamycine were added to all the media directly prior to the work with mouse myeloma cells.

NSO cell suspension and immune splenocytes were preliminarily washed twice in serum-free medium and mixed in a 1 : 5 ratio. For the fusion 50% solution PEG-1000 was used (manufactured by Merck for gas chromatography). After treatment with fusogen, the mixture of fused cells (usually splenocytes from 2 mice were used in one experiment) was resuspended in the selective medium HAT and distributed into 96-well plates with a feeding layer of nonimmune splenocytes. The plates were cultivated for 20—25 days in the selective medium HAT changing the medium every 3 or 4 days. On cultivation days 10 to 14 the plates they were examined in the inverted microscope at a small magnification and the wells with singular hybrid cell colonies were registered. These hybrid cell colonies were then placed into 24-well plates with a feeding layer of nonimmune splenocytes in hybridoma medium HT for subsequent cultivation. The hybridoma medium consisted of the growth medium containing hypoxanthine and thymidine.

Hybrid cell clones were grown in 24-well plates for 10 to 14 days; and after formation of a continuous layer they were placed into another 24-well plates without feeder. When an almost continuous monolayer was formed, about 2 or 3 days prior to screening of the supernatants for

antibodies, the hybridoma medium HT containing 10% serum was replaced by the medium containing 2% of lambing ewe serum.

Antibody screening. The ability of clones to secrete antibodies was assessed in EIA with antigens of vaccinia virus and monkey pox virus after 2 passages of the selected cultures in a 24-well panel. The selected antibody-producing clones were grown in small flasks, and the activity and species-specificity of the MA were assessed. On the basis of these data the clones were selected for accumulation in the mass culture.

Identification of the MoAb isotype. The isotype of antibodies and the subtype of immunoglobulins were determined by the method of radioimmune adsorption inhibition (Kohlin *et al.*, 1983) in culture fluids (CF).

Karyologic analysis of hybrid cells lines were carried out according to a modification of the method described by Moorhead *et al.*, (1960).

Preparation of immune ascitic fluids (IAF). Female Balb/c mice aged 10—14 weeks were given intraperitoneally 0.5 ml of Pristane (Sigma); after 7 to 14 days the hybrid cells were inoculated at a concentration of $5.0 \times 10^6 - 1.0 \times 10^7$/ml. IAF were collected on days 10—21 and centrifuged at 4 °C for30 min before testing.

Preparation of the conjugate. Immunoglobulin was isolated by a twice-repeated precipitation with a saturated ammonium sulphate solution at room temperature followed by a removal of ammonium sulphate on Sephadex G-25. Conjugation of immunoglobulins with horseradish peroxidase was carried out according to the procedure of Wilson and Nakane (1978).

Results

Characterization of hybridomas

Fusion of splenocytes of mice immunized with monkey pox virus with mouse myeloma cells yielded (according to two fusion experiments) hybrid clones in an average of 63.5% of cases. A total of 612 clones has grown

Table 1. Grouping of prepared hybridomas according to specificity of produced antibodies

Group (conventional designation)	No. of clones tested	Reactivity with viruses in EIA		Titres in EIA			
				CF		IAF	
		monkey pox	vaccinia	min	max	min	max
1 MpV=VcV	11	+	+	9	2.4×10^3	3.9×10^5	6.6×10^5
		Titres with both viruses are equal					
2 VcV MpV	4	+	+	3	2.4×10^2	9×10^2	2.2×10^5
		Titre with vaccinia virus is higher than with monkey pox virus					
3 MpV VcV	7	+	+	7.3×10^2	2.4×10^3	6.5×10^5	1.8×10^6
		Titre with monkey pox virus is higher than with vaccinia virus					
4 MpV	8	+	—	9	2.4×10^3	9×10^4	2.2×10^5
		Only with monkey pox virus					

The titre with monkey pox virus is indicated.

Table 2. Characterization of some hybridomas according to serologic reactivity and antibody class

Group	Hybridoma designation	Antibody class	Tested material	Activity in tests		
				EIA	IF	HI
1	MpV = VcV-2	γ2Bκ	CF	$7.3 \times 10^2/7.3 \times 10^2$	10/10	16/256
			IAF	$3.9 \times 10^5/2.2 \times 10^5$	n.t.	—
	MpV = VcV-11	n.t.	CF	$7.3 \times 10^2/7.3 \times 10^2$	10/10	—
	VcV MpV-1	γ[1])	CF	$2.4 \times 10^2/2.4 \times 10^3$	10/10	—
			IAF	$2.2 \times 10^5/6.6 \times 10^5$	n.t.	—
2	VcV MpV-7	n.t.	CF	$2.4 \times 10^2/7.3 \times 10^2$	10/10	—
			IAF	$2.4 \times 10^3/6.6 \times 10^5$	n.t.	—
	MpV VcV-12	uκ	CF	$2.4 \times 10^2/7.3 \times 10^3$	10/10	—
			IAF	$1.8 \times 10^6/2.2 \times 10^5$	n.t.	—
	MpV VcV-266	γ2Bκ	CF	$7.3 \times 10^2/0.8 \times 10^2$	n.t.	—
			IAF	$6.5 \times 10^5/2.4 \times 10^4$	10/10	—
3	MpV VcV-7	n.t.	CF	$2.4 \times 10^3/2.4 \times 10^2$	10/10	—
	MpV-6	γ[1])	CF	$2.4 \times 10^3/3.0 \times 10^1$	10/10	—
			IAF	$1.8 \times 10^6/3.0 \times 10^2$	n.t.	—
4	MpV-3	γ[1])	CF	$2.4 \times 10^3/3.0 \times 10^1$	10/10	—
			IAF	$1.2 \times 10^6/3.0 \times 10^2$	n.t.	—

[1]) Numerator — titre with monkey pox virus, denominator — titre with vaccinia virus.
Notice: all clones tested in NT, HI and GP gave negative results except of MpV = VcV-2 in HI.

from 599 single colonies taken for cultivation (in 13 cases the wells with two cell colonies were used); from these 180 clones (29.4%) appeared to produce antibodies, and 30 hybridomas produced them actively and continuously.

The stability of antibody production was tested throughout 7 to 25 passages (observation time). The studies on serologic reactivity of MoAb produced by these 30 hybridomas indicated a wide variation in their ability to react with monkey pox virus and vaccinia virus. Four types of MoAb interaction

Table 3. Chromosome analysis of hybrid cell lines producing MoAb to monkey pox virus

Line designation	Variations of chromosome number	Modal class		Mean no. of chromosomes per cell
		number of chromosomes	percentage of cells	
MpV-3	80—190	89—93	50	90.64
MpV-6	73—170	88—96	50	89.69
MpV VcV-12	85—180	93—98	79	95.44
MpV VcV-266	79—99	94—98	58	94.01
MpV = VcV-2	83—185	89—94	70	91.21
MpV = VcV-9	81—180	88—93	72	91.24
VcV MpV-203	75—175	90—95	59	92.64
Parent myeloma cell line NSO	53—160	57—60	87	61.28

Table 4. Detection and adentification of monkey pox virus by EIA with conjugate based on highly-specific MoAb

Tested material	EIA with conjugate on the basis of					
	Polyclonal antibodies to vaccinia virus		MoAb to vaccinia virus		Highly specific MoAb to monkey pox virus	
	Titre	Inhibition with immune serum	Titre	Inhibition with immune serum	Titre	Inhibition with immune serum
Monkey pox virus, strain Zaire-753 (3 passages)	6600	9	2200	< 3	6600	< 3
Vaccinia virus culture	2200	< 3	2200	< 3	< 3	< 3
Carnivora pox virus culture	729	< 3	3	< 3	< 3	< 3
Content of skin lesions of a monkey pox patient M. A. (Zaire-3853)	2430	< 90	2430	< 90	7290	< 90
Contents of skin lesions of a monkey pox patient B. P. (Zaire-3699)	7290	< 90	2430	< 90	6600	< 90
Contents of skin lesions of a monkey pox patient W. A. (Zaire-3850)	810	< 90	270	< 90	2430	< 90
Isolate from squirrel (12 passages)	21870	< 90	7290	< 90	21870	< 90

with these viruses have been established, including a selective reaction with monkey pox virus only (Table 1). Group 1 was the most numerous. The hybridomas of this group produced MoAb to common antigenic determinants for monkey pox virus and vaccinia virus. The next group consisted of hybrid lines with species-specific MoAb selectively reacting only with monkey pox virus. Table 2 presents the data of studies on MoAb produced by different groups of clones.

MoAb variations in the class of immunoglobulins are noteworthy. For the present, the immunoglobulin class has been determined for 6 MoAb: 5 of then appeared to belong to isotype G (two MoAbs to slass G_{2B} and three MoAbs to G_1) and 1 — to isotope M. The clones producing MoAb with identical serologic characteristics (MpV VcV-12 and MpV VcV-266) belonged to different immunoglobulin classes. Like most our vaccinia monoclones prepared earlier the MoAb produced by hybridomas to monkey pox virus were found to be highly active in EIA only. A few of the MoAb produced by thirty selected hybridomas were also positive in IF (MoAb of clones MpV = = VcV-2, MpV = VcV-11, MpV = VcV-7, MpV-3, MpV-6) or in HI (MoAb of clone MpV = VcV-2).

Seven hybridoma lines belonging to different groups with respect to serologic activity underwent karyologic analysis at passage levels 10 to 15. It can be seen from Table 3 that in 58—79% of cases the modal class consisted of the cells with chromosome number 88—89.

Preparation and practical use of the monoclonal antibodies

Among the hybridomas described highly species-specific monoclones naturally attracted most attention, for they could be used for differentiation of orthopoxviruses and for identification of monkey pox virus. Taking this fact into account, we attempted to design a diagnostic system based on such MoAb. Considering that MoAb showed the highest activity in EIA we prepared an immunoenzyme conjugate for EIA. The conjugate was prepared from MoAb specific for monkey pox virus only (in EIA titre of 9×10^4). The immunoglobulin isolated from these MoAb was conjugated with horseradish peroxidase (protein concentration 8 mg/ml, peroxidase 4 mg) and tested in EIA. The testing has shown that the conjugate prepared from IAF was rather active (working dilution 1 : 700) and highly specific: it only reacted with monkey pox virus of all the orthopoxviruses tested (vaccinia virus, cowpox virus, monkey pox virus, Table 4). In addition to the testing with laboratory cultures of the above-listed viruses, the conjugate was also tested with field materials — skin lesions from monkey pox-infected patients supplied by W.H.O. from African countries. The clinical diagnosis was confirmed by virus isolation. Another two peroxidase conjugates were used for comparison: a conjugate prepared from polyclonal antibodies (from serum of rabbits hyperimmunized with vaccinia virus that is usually used for serologic identification) and a conjugate described in our previous paper prepared from MoAb to the same virus (Marennikova *et al.*, 1985).

It can be seen from Table 4 that the isolation and simultaneous identification of monkey pox virus was possible only by means of the conjugate from MoAb highly specific for monkey pox virus. The above-mentioned diagnostic conjugate from polyclonal antibodies to vaccinia virus could only provide genus indentification of the isolates and the conjugate from MoAb to the same virus could only rule out that the isolate was a cowpox virus.

These materials suggested that the conjugate based on MoAb to monkey pox virus could be employed for identification of an orthopoxvirus isolated in August 1985 from an infected squirrel caught in areas of smallpox circulation in Republic of Zaire. Laboratory studies with this isolate based on traditional biologic markers (morphology of pocks on chorio-allantoic membranes of chick embryos, skin response in rabbit and haemagglutinating activity) confirmed its identity with monkey pox virus. Considering, however, that the isolate was obtained from an unusual object and also, that it was the first instance of isolation of an orthopoxvirus similar to monkey pox virus from a wild living animal it seemed important to obtain additional evidence for the identity of these viruses. EIA test with the squirrel isolate against 3 conjugates mentioned above (Table 4) yielded the same results as

the laboratory strain of monkey pox virus and the virus-containing materials from patients.

Discussion

The studies on 30 hybridomas prepared to monkey pox virus have shown that only 8 of them (26.6%) secreted MoAb with high species specificity. Most hybridomas produced MoAb with antigen-binding sites to determinants that were common for the virus used for the preparation of monoclones and for vaccinia virus. All hybridomas were characterized by intensive antibody production both in vitro, and especially, in vivo. In the latter case, antibody titres determined in EIA reached in some clones $6.6 \times 10^5 - 1.8 \times 10^6$. Meanwhile, the activity of MoAb had a limited character: they only reacted in EIA. Only a few clones produced MoAb that could react not only in EIA but also by IF and HI in much lower titres. These results were of course determined by EIA screening.

Analogous data were obtained by Kitamoto *et al.* (1984). They selected 13 clones producing MoAb to cowpox and vaccinia viruses according to IF, and only one of these clones reacted in HI. Antibody-producing activity in the prepared clones continuously persisted in passages and was not lost after thawing. The evidence for the stability of the hybrid clones obtained was provided by the karyologic findings. Neither changes of basic karyologic indices nor chromosome losses have been observed in the 6 clones tested. It should be also noted that not all hybrid lines were stable, and several investigators who prepared MA to some other viruses observed chromosome loss in the course of cultivation of the hybrid cells (Goding, 1983).

In terms of practical use MoAb of high species-specificity deserves more attention. Our findings have shown that immunoenzyme conjugate prepared on the basis of such MoAb to monkey pox virus (unlike the polyclonal conjugate) could help not only detect the presence of orthopoxvirus antigen by EIA in the material from monkey pox patients, but also to identify it as monkey pox virus. In a similar fashion, with the help of this preparation we managed to obtain additional evidence for identification of an orthopoxvirus isolated from an infected wild squirrel as monkey pox virus.

Thus, the use of highly species-specific MoAb allowed to develope diagnostic preparations of a new generation providing rapid species identification of closely related orthopoxviruses. This, fact however, does not belittle the importance of the diagnostic preparations based on polyclonal antibodies, since group identification of any unknown isolate is a necessary step in the diagnosis.

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References

Ertte, A. P., Konikova, R. E., and Avdeenko, M. M. (1973): Indirect haemagglutination inhibition. 4. Employment for the assessment of immunologic potency of pox vaccines (in Russian). *Zh. Microb. Epidem.* (*Moscow*) **1973** (5), 110–114.

Ferms, R. B., and Tedder, R. S. (1984): Monoclonal antibodies to hepatitis Be antigen (HBe Ag) derived from hepatitis B core antigen (HBcAg): their use in characte ization and detection of HBeAg. *J. gen. Virol.* **65**, 899–988.

Goding, J. W. (1983): *Monoclonal Antibodies; Principles and Practice*, Acad. Press, New York.

Heinz, F. X., Berger, R., Majdic, O., Knapp, W. and Kunz, Ch. (1982): Monoclonal antibodies to the structural glycoprotein of tick-borne encephalitis virus. *Infect. Immun.* **57**, 869–874.

Kitamoto, N., Goto, E., and Tinimoto, S. (1984): Gross-reactivity between cowpox and vaccinia viruses with monoclonal antibodies. *Arch. Virol.* **82**, 129–136.

Köhler, G. and Milstein, C. (1975): Continuous cultures of fused cells secreting antibodies of predefined specificity. *Nature* (*Lond.*). **256**, 495–497.

Kushch, A. A., Tsoi, L. A. and Isaeva, E. I. (1985): Studies on hybridomas producing monoclonal antibodies to influenza virus (in Russian). *Vopr. Virusol.* **30** (1), 22–24.

Marennikova, S. S. and Maltseva, N. N. (1961): Employment of microprecipitation reaction on glass for laboratory diagnosis of smallpox (in Russian). *Vopr. Virusol.* **6** (2), 204–206.

Marennikova, S. S., Shelukhina, E. M., Maltseva, N. N., Efremova, E. V., Matsevich, G. R., Nikulina, V. G., Khabakhpasheva, N. A., Stepanova, L. G., Arita, I. and Gromyko, A. I. (1984): Data of serologic examination of the population of Republic of Kongo for antibodies to orthopoxviruses. I. Comparative assessment of different methodos and General summing-up (in Russian). *Zh Microb. Epidem.* (*Moscow*) **1984** (3), 95–100.

Marennikova, S. S., Shelukhina, E. M., Matsevich., GR., Maltseva, N. N., Khabakhpasheva, N. A., Novokhatsky, A. S. and Malakhova, I. V., pp133–136 (in Russian) (1985): Properties of monoclonal antibodies to vaccinia virus. In: *Monoclonal Antibodies in Microbiology and Virology*. Transactions of Mechnikov Central Research Institute of Vaccines and Sera, Moscow.

Moorhead, P. S., Nowell, P. S., and Mellman, W. J. (1960): Chromosome preparations of leukocytes cultures from human peripheral blood. *Exp. Cell Res.* **20**, 613–616.

Novokhatsky, A. S., Kushch, A. A., Malakhova, I. V., Gaidamovich, S. Ya., Melnikova, E. E., and Svehnikova, N. A. pp 42–43 (in Russian) (1984): Monoclonal antibodies to tick-borne encephalitis virus. In: *Arboviruses*, Tallinn.

Roehring, J. T., Mathews, J. H., and Trent, D. W. (1983):,Identification of epitopes on the glycoprotein of Saint Louis encephalitis virus using monoclonal antibodies. *Virology* **128**, 118–126.

Roumillat, L. F., Patten, J. L. and Davis, M. L. (1984): Monoclonal antibodies to monkey pox virus polypeptide determinant. *J. Virol.* **52**, 290–292.

Viktor, F. J., and Koprowski, H. (1978): Monoclonal antibodies against rabies virus produced by somatic cell hybridization: detection of antigenic variants. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3938–3947.

Wilson, M. B. and Nakane, P. K. (1978): Recent developments in the periodate method of conjugating horseradish peroxidase (HRPO) to antibodies, pp 215–224. In W. Knapp, K. Holubar, G. Wiek (Eds): *VI International Conference on Immunofluorescence and Related Staining Techniques*, Vienna.