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Preparation of monoclonal antibodies to a baculovirus

P.L. Roberts * and W. Naser

Institut für Virologie und Immunbiologie der Universität Würzburg, Versbacher Strasse 7, 8700 Würzburg, F.R.G.

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1. INTRODUCTION

The development of the mouse hybridoma technique for preparing monoclonal antibodies by Köhler and Milstein [1] has led to improved serological methods for the identification of animal viruses [2,3]. This technique, when combined with sensitive serological assays [4–7], should also prove useful for studying insect viruses. In the present study we have prepared monoclonal antibodies against a baculovirus [8], the *Autographa californica* nuclear polyhedrosis virus (AcNPV), and determined some of their properties. Part of this work was presented at the Fifth International Congress of Virology [9].

2. MATERIALS AND METHODS

Virus was released from insect-derived polyhedra, supplied by Dr. A. Röder of Hoechst AG, Frankfurt am Main, by alkali treatment [10] and purified on a 10 to 60% sucrose gradient made in 0.01 M Tris pH 7.4 containing 0.001 M EDTA (TE buffer). The multiple nucleocapsids were harvested and used as antigen.

Hybridomas were produced by i.p. injection of 7-week-old Balb/cJ mice with approx. 30 μg of virus, disrupted with 1% NP-40 in TE buffer, in an aluminium hydroxide precipitate using *B. pertussis* as adjuvant. An i.v. boost of disrupted virus only was given after 4 weeks and spleen lymphocytes ($1.5 \cdot 10^8$) were taken 4 days later and fused with P3-X63Ag8 myeloma cells ($1.5 \cdot 10^7$) using polyethylene glycol [11]. Cells were then seeded in 5 '96-well plates' (Costar), together with $2 \cdot 10^5$ spleen lymphocytes per well from nu/nu mice, and hybridomas selected using HAT medium [11].

2 weeks after fusion cultures which secreted antibody to AcNPV were identified by an indirect ELISA [12]. Micro ELISA plates (Dynatech) were coated with virus at 0.3 μg/well and bound mouse antibody detected using alkaline phosphatase conjugated rabbit antimouse immunoglobulin. Positive cultures were further cloned by limiting dilution in 200-μl wells of '96-well plates' or 10-μl wells of '60-well plates' (Nunc) together with $2 \cdot 10^5$ or $2 \cdot 10^4$, respectively, spleen lymphocytes from nu/nu mice per well. A plating efficiency of approx. 90% was obtained when cells were seeded at < 1 per well, however, when feeders were omitted this was reduced to almost zero. The hybridoma cells from wells which contained a single colony were grown up, recloned and/or approx. $2 \cdot 10^6$ cells injected i.p. into Balb/cJ mice, which had been pretreated with 0.5 ml pristane, to produce antibody in ascites form.

Immunodiffusion tests were carried out using glass plates coated with 1% agarose (Behringwerke AG) in 0.005 M barbital buffer pH 7.4 containing 0.2 M NaCl. After incubation at 4°C for 2 to

* Reprint requests to Dr. P.L. Roberts at his present address: Department of Microbiology, University of Leeds, Leeds LS2 9JT, U.K.

3 days, plates were washed and dried before being stained with Coomassie Brilliant Blue.

A plaque assay using the *Spodoptera littoralis* cell line HPB-SL [13] was used to test for virus neutralization. Samples were incubated at 37°C for 1 h with virus at approx. $0.7 \cdot 10^3$ pfu/ml and aliquots of 0.15 ml assayed on cells seeded at $0.4 \cdot 10^6$ per 35-mm well in '6-well plates' (Costar) 2 days previously. After 2 h, 1.5 ml of a 1% agarose (Behringwerke AG) overlay in Grace's medium was added, followed by 2.5 ml of medium. Plaques were counted 5 days later after staining with 0.5 ml of 0.13% iodonitrotetrazoliumchloride in PBS.

Immunofluorescence was carried out using cell cultures that had been infected with tissue culture-derived extracellular virus at 0.1–1.0 $TCLD_{50}$/cell. Cells were harvested after approx. 2 days when a severe CPE of cell rounding and death was seen and, after washing with PBS, air-dried on glass slides and fixed with ice-cold acetone. Mouse sera or ascites fluid, diluted 1 in 10 with 1% tryptic soybroth (Gibco) in PBS, was added and slides incubated at 37°C for 30 min. After washing, FITC-conjugated goat anti-mouse immunoglobulin (Meloy), diluted 1 in 30, was added and the slide reincubated. Finally slides were examined directly, or after mounting in PBS–glycerol (1:7), with a Leitz Orthoplan fluorescence microscope.

Extracts were also prepared from washed cells (approx. 10^7) by sonication in 3 ml PBS. After the removal of large debris by centrifugation (800 $\times g$, 10 min), serial dilutions of infected and control cell extracts were used to coat the wells of micro ELISA plates. An indirect ELISA [12] was then carried out using constant amounts of antiserum or ascites fluid.

3. RESULTS AND DISCUSSION

Each of the 480 wells seeded after fusion produced an average of 2 hybridoma colonies, i.e. 6 per 10^6 parent cells. Preliminary experiments, in which mice were boosted i.p. using adjuvant, produced only 26 hybridomas and only 3 hybridomas when combined with the use of the myeloma cell line P3-X63Ag8.655 [14] which does not produce immunoglobulins.

40, i.e. 4%, of the hybridomas were found to produce antibodies to AcNPV. Frequencies of ap-

Table 1

Properties of monoclonal antibodies to AcNPV

Serum [a]	Antibody class	Reaction with virus			Reaction with infected cells [d]
		ELISA [b]	Neutralization [c]	Immunodiffusion	
3D10	IgG1+2	5.7	5	−	+++
3F3	IgG1	3.2	<5	−	±
5E9	IgG1	2.7	<5	−	+
3G4	IgG1+2	2.0	n.d. [e]	−	−
Mixture [f]	IgG1+2	n.d.	5	−	+++
−Clone	IgG1	1.0	5	−	−
−Mu	IgG, M, A	1.0	5	−	−
+Mu	IgG, M, A	3.6	40	+	++
−Rb	IgG, M, A	n.d.	20	−	n.d.
+Rb	IgG, M, A	n.d.	130	+	n.d.

[a] Monoclonal antibody and pre-immune (−) or immune (+) serum from mouse (Mu) or rabbit (Rb).
[b] Reciprocal of $\log_{10}$ dilution end-point using antigen at 0.3 μg/well.
[c] Reciprocal of dilution giving 50% reduction in plaque number.
[d] By immunofluorescence or ELISA. Strength of reaction from − to +++.
[e] Not done.
[f] Equal volums of 3D10, 3D3 and 5E9.

prox. 1% have been reported for herpes simplex [15] and measles [16], while those for rabies and influenza can be $> 50\%$ [2].

5 of the positive cultures were selected for further study and cloned in an attempt to stabilize antibody production. Although the dilution end-point titre of the positive hybridoma supernatants in ELISA was $< 10^{1.0}$, ascites fluid had a titre of $10^{2.7}$–$10^{5.7}$ (Table 1) and was therefore used in all subsequent studies. The activity of the antibody produced by clone 3G4 was very low and that from another clone had lost its ability to react and was considered negative. The activity of the antibody produced by the different clones varied (Table 1), with that from 3D10 giving the strongest reaction, although they all, including the negative clone, contained similar levels of immunoglobulin (Fig. 1). The variation may be because each is directed against a different antigenic determinant, which may be present in different amounts, or because the monoclonal antibodies react with different affinities.

The class of antibody produced by the hybridoma clones was determined by immunodiffusion using type-specific antisera. Preliminary experiments showed that they were not IgM, however in further studies they were all found to contain IgG1 (Fig. 1a). The IgG1 produced by the 'negative clone' probably represents 'non-specific' antibody that is derived from the myeloma parent [11]. Clones 3D10 and 3G4 also produced IgG2 (Fig. 1b), which must be derived from the lymphocyte parent [11], and this, therefore, further confirms that the hybridomas were fusions of lymphocyte and myeloma cells.

The ability of the monoclonal antibodies to react in other serological tests was determined and the results have been summarized in Table 1. They did not react, when used singly or in combination, with virus to form precipitation bands in immunodiffusion tests. In tests for virus neutralization it was found essential to heat-inactivate samples because pre-immune mouse serum had a high non-specific ability to neutralize virus of $> 1/200$ which could be reduced to $< 1/20$ by this treatment. Virus released from polyhedra was used as antigen because we found that an antiserum to this form of the virus did not neutralize tissue culture-derived extracellular virus as efficiently as the homologous virus (unpublished data). The monoclonal antibodies, when used singly or in combination, could not neutralize virus (Table 1). However, as neutralization has been obtained in other systems [2,16], this is probably because we have not studied antibodies that are directed against the appropriate viral antigen.

Further evidence that the monoclonal antibodies are directed against viral proteins comes from their ability to distinguish between healthy and infected cells by immunofluorescence (Fig. 2, a–f, Table 1). Although reports on the use of this and similar techniques for studying baculoviruses in

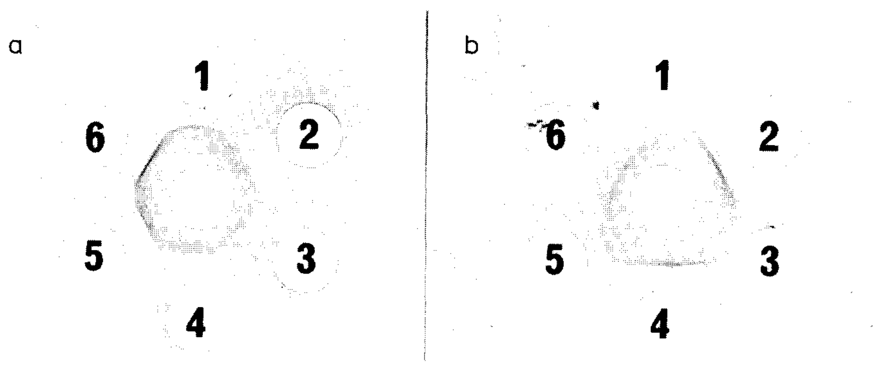

Fig. 1. Determination of monoclonal antibody class by immunodiffusion. Goat antibody to mouse IgG1 (a) or IgG2 (b) (Meloy) was placed in the centre well. Antigens were antibodies from: 'negative clone' (1), 3D10 (2), 3F3 (3), 3G4 (4), 5E9 (5) and mouse immunoglobulin reference standard (6) (Meloy).

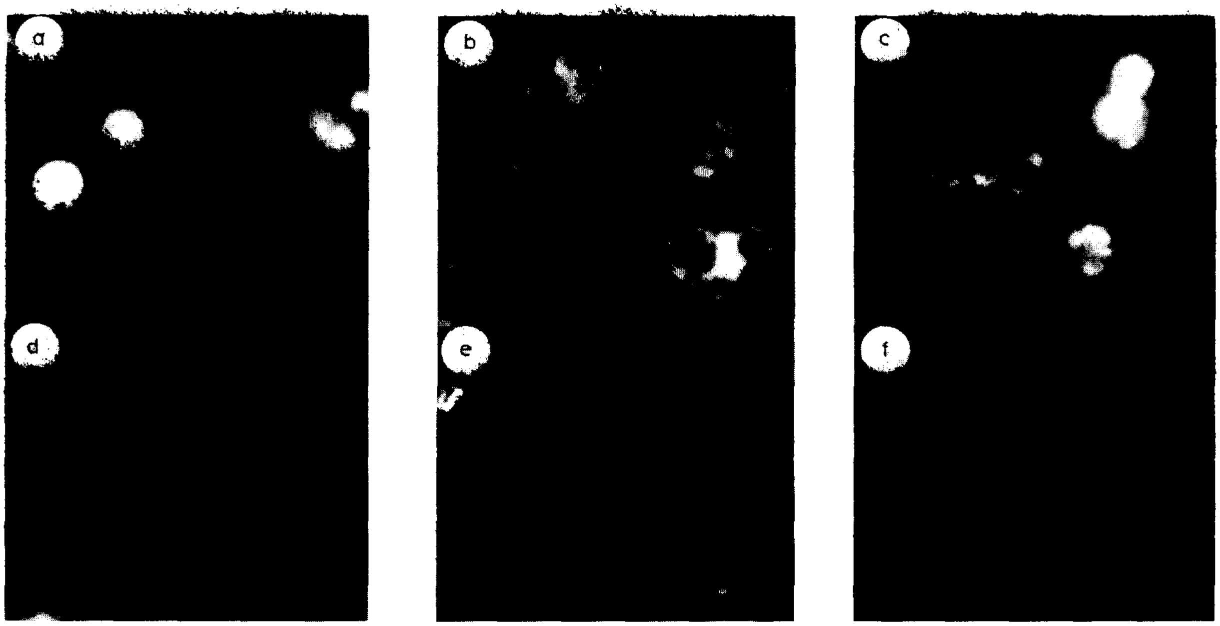

Fig. 2. Reaction of monoclonal antibodies with infected cells by indirect immunofluorescence. Immune (a) or pre-immune (d) mouse serum with infected cells. Monoclonal antibodies from clone 5E9 with healthy (e) or infected (b) cells, and from clone 3D10 with healthy (f) or infected (c) cells. Note that (d) has been overexposed (×7) relative to (a), and that (e) has been overexposed (×5) relative to (b).

cell culture are limited [17,18], immunofluorescence proved very useful for identifying infected cells. The antibody produced by clone 3D10 again gave the strongest reaction, with infected cells showing uniform fluorescence or a patchy distribution near the cell edge (Fig. 2c) similar to that found in the early stages of AcNPV infection of *Trichoplusia ni* cells using an antiserum to virus [18]. The monoclonal antibody produced by clone 5E9 (Fig. 2b) and clone 3F3 (not shown) produced a weaker reaction with infected cells. In all cases controls, e.g. pre-immune serum with infected cells (Fig. 2d) or monoclonal antibodies with healthy cells (Fig. 2, e and f), showed only very weak fluorescence which was confined to the cytoplasm.

Healthy and infected cell cultures could also be distinguished when cell extracts were compared by ELISA. The dilution end-points of infected cell extracts with pre-immune or immune mouse serum and with the most strongly reacting monoclonal antibody, i.e. 3D10, were 1 in 2, 1 in 10 and 1 in 250, respectively.

Further work has suggested that the monoclonal antibodies produced by clones 3F3 and 5E9 are directed against polyhedron protein in contrast to that from clone 3D10 which is directed against a major viral polypeptide of M_r 42000 (unpublished data).

As the monoclonal antibodies we have prepared to AcNPV may be used in sensitive and specific serological tests such as ELISA and immunofluorescence, further studies are aimed at assessing their usefulness in insect virus identification.

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