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MONOCLONAL ANTIBODIES TO *TREPONEMA PALLIDUM*

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ABSTRACT

Three successive fusions of mouse myeloma cells and spleen lymphocytes of a mouse immunized with *Treponema Pallidum* resulted in one hybridoma producing anti *T. pallidum* antibodies for each fusion. The mice were immunized with live pallidum cells respectively 1, 3 and 5 months before fusion and with antigen purified on density gradients 4, 3 and 2 days before fusion. Hybridomas cultures were tested for antibody production with an Enzyme Linked Immunosorbent Assay (ELISA) and a Western blotting technique. Two of the three anti *T. pallidum* antibody producing hybridomas were found with the ELISA, the third was found with a Western blotting technique. These hybridomas were also tested for the production of antibodies to rabbit antigens and *T. phagedenis* antigens in the ELISA : none appeared to be positive.

Two of the hybridomas produce antibodies to a *T. pallidum* protein antigen of a molecular weight of 46 000 : one hybridoma produces antibodies to a *T. pallidum* protein antigen of a molecular weight of 44 000 as determined by the Western blotting. Antibodies against these antigens are found during almost all stages of syphilis in man.

One of the hybridomas produces monoclonal antibodies that react with treponemal antigen from E. coli cells, prepared by recombinant DNA technology as appeared in the Western blotting technique and this antibody will be used for purification of the 44 000 protein.

INTRODUCTION

The in vitro cultivation of *Treponema pallidum* has not been very successful up till now (2) and the in vivo cultivation does not allow the production of large batches.

However, serodiagnosis of syphilis cannot be done without either complete *T. pallidum* cells or its antigens.

To estimate interbatch variations of diagnostic tests, well documented antibody references are needed. The establishment of hybridomas, producing antibody against *T. pallidum* of unique specificity will contribute to the standardization of the diagnosis of syphilis.

Moreover, monoclonal antibodies can be used for the purification of *T. pallidum* antigen produced by *Escherichia coli* carrying *T. pallidum* recombinant DNA.

MATERIALS AND METHODS

Preparation of hybridomas

Hybridoma cell lines were produced according to techniques applied by other authors (3). Balb/c mice (CPN-TNO, Zeist, The Netherlands) were inoculated intraperitoneally with 10^7 *T. pallidum* cells, either 1 (fusion 81/47), 3 (fusion 81/41) or 5 (fusion 82/19) months before the fusion and a booster with 2 x 10^7 urografin gradient purified *T. pallidum* cells were given

intravenously 4, 3 and 2 days before fusion. Spleen cells were isolated and a single cell suspension was prepared. The erythrocytes were lysed by incubation with 0.83% NH_4Cl (w/v) for 1 min at 25°C. For each fusion 2.5 x 10^7 myeloma cells (P3/X63-Ag8 Balb/c mouse myeloma cell line (obtained from Dr. T. Witker, Wistar Institute, Philadelphia, Pa) and 10^8 spleen cells of an immunized mouse were mixed and washed thrice in Dulbecco's Modified Eagle's Medium containing hypoxanthine, thymidine, penicillin and streptomycin (DMEM). The pellet was resuspended in 1 ml 50% (w/v) polyethylene glycol (Baker Chemicals bv. Deventer, The Netherlands) in DMEM containing 10% dimethylsulfoxide (BDH Chemicals Ltd., Poole, England). After respectively 1, 3 and 5.5 min at 37°C 1, 2 and 5 ml DMEM were added. After 1.5 min the suspension was centrifuged for 8 min at 2500 g. The pellet was resuspended in DMEM containing 20% heat-inactivated fetal calf serum (DMEM-20). 150 ml DMEM-20 and 10^8 rat thymocytes were added as feeder cells. This suspension was distributed into six 24-well cell-clone plates (Griener art. 704160) and incubated at 37°C in a CO_2 incubator for 24 h. The wells were subsequently filled up with DMEM-20 containing aminopterin. Culture media were partially (about 60%) refreshed every 3 or 4 days with aminopterin containing DMEM-20. After 7 to 14 days hybridoma colonies were microscopically visible and the fluid was screened with Enzyme Linked Immuno Sorbent Assay (ELISA). Antibody producing clones were partially stored in liquid nitrogen. The rest was passaged into tissue culture flasks. If ELISA positive, they were further cloned by terminal dilutions in microtiter trays and were subsequently passaged in tissue culture flasks and screened with ELISA. The hybridoma cells were always grown in the presence 10^8 feeder cells/ml. These cloning procedures including screenings were performed thrice. Finally the Ig subclass was assayed with an ELISA.

Screening techniques

Blotting

The blotting method as developed by one worker (5) and modified by another (1) was used in this investigation. In short the crude *T. pallidum* lysate were subjected to one dimensional PAGE and subsequently the proteins were transferred to nitrocellulose sheets by electrophoresis. The blots were each incubated with 30 ul of the hybridoma culture medium under investigation in 3 ml 0.01 M phosphate + 0.15 M NaCl, pH = 7.2 (PBS) + 0.05% Tween 20 (PBS-T5) for 1 h. The blots were washed thrice by incubating with 3 ml PBS-T5 for 20 min and incubated overnight with 3 ml PBS-T5 and again washed twice. The blots were then incubated with rabbit anti-mouse total immunoglobulin horseradish peroxidase conjugate (RAMIgG(H+L)/PO ; (Nordic, Tilburg, The Netherlands) diluted 1 :1000 in PBS-T5 + 5% fetal calf serum (FCS) and were washed four times for 15 min in PBS-T5. The blots were then laid down on a gel containing 450 mg agarose, 24 mg tetramethylbenzidine, 80 mg dioctylsodium sulphate and 20 ul 30% H_2O_2. The reaction was stopped in water. The whole procedure was performed at room temperature.

ELISA

Screening of hybridoma culture media was performed with a micro ELISA at room temperature. Purified *T. pallidum* antigen was prepared by sonification of freshly isolated density gradient purified *T. pallidum* cells (10^8/ml). The plate (polystyrene, micro ELISA titer plates, Dynatech) was coated with 0.15 ml purified *T. pallidum* antigen or likewise prepared testicle antigen of non-infected rabbits (both pH = 8.0) 1 :75 overnight, rinsed thrice with PBS + 0.01% Tween 20 (PBS-T) and incubated with 0.15 ml DMEM-20 for one hour. Successively the plate was rinsed thrice with PBS-T and incubated for one hour with 0.1 ml of the samples under investigation, 0.1 ml of a hybridoma fluid (81/41 3-22D6F7) as a reference or 0.1 ml PBS. After rinsing thrice with PBS-T the plate was incubated for one hour with 0.1 ml RAMIgG(H+L)/PO 1 :500, rinsed thrice with PBS-T and incubated with substrate including tetramethylbenzidine (0.12 mg/0.1ml), H_2O_2 (0.003%), sodium acetate (6.8%) and citric acid (1.4%) for 10 min. The reaction was stopped by adding 0.05 ml of 2N sulphuric acid. The extinctions of the contents of the cups were measured with a Multiscan (Titertek) at 450 nm. The results are presented as relative extinctions (the extinction of the sample is divided by the extinction of the reference).

RESULTS

Fusions of mouse cells and splenocytes of mice previously immunized with *T. pallidum* resulted in three hybridoma cell lines, which produce antibodies against *T. pallidum.*

The first column of Table I shows the relative extinctions of culture media of two of these hybridomas in the ELISA. The next columns show the extinction after preincubation with serum of healthy volunteers and pooled syphilitic serum, respectively. The reaction of the monoclonal antibodies is partially inhibited by the pooled syphilitic serum.

The hybridomas do not produce antibodies against rabbit antigen nor against *T. phagedenis* as measured by ELISA in which a coat was used of antigen of rabbit testicles or *T. phagedenis.* These monoclonal antibodies were investigated with the Western blotting (Fig. 1). The clones 81/47 1-2C_3E_5 and 81/41 3-22D_6F_7 both produced antibodies that only reacted with the antigen fraction corresponding with a molecular weight of 46 kd in the blotting (two right lanes). The clone 82/19 1-14 M_1 which was negative in the ELISA but accidently spotted in the blotting, produces antibodies that react with antigen with a molecular weight of 44 kd (second lane from the left), but also with components having molecular weights of 41 kd, 4 kd and 35 kd in the blotting (Fig. 2, left lane).

All the three monoclonal antibodies belong to the IgG 2b class as appeared from an ELISA using conjugated specific antiserum.

Some of the *T. pallidum* antigens can now be prepared by expression of *T. pallidum* DNA in *E. coli* K12 (1).

Antibodies produced by hybridomas 82/19 1-14M_1 also bind the 44 kd polypeptide antigen produced by *E. coli* K12 carrying the recombinant DNA plasmid pRIT4694. Various deletion derivates of this plasmid have been constructed and some of these do not produce the 44 kd polypeptide. These deleted plasmids do not express any antigen as measured by Western blotting adding syphilitic serum. Concomitantly, no binding of monoclonal antibodies was observed with lysates of strains carrying these deleted plasmids.

Table I. Inhibition of monoclonal antibody-binding to total *T. pallidum* antigen by human syphilitic serum

dilution of monoclonal antibodies	monoclonal 81/41 3-22D6F7 preincubation with			monoclonal 81/47 1-2C3E5 preincubation with		
	PBS	normal serum	syphilitic serum	PBS	normal serum	syphilitic serum
5^0	1.00 [1)]	.95	.66	1.03	1.07	.56
5^{-1}	1.16	1.01	.67	1.12	1.04	.57
5^{-2}	1.04	1.04	.75	.94	.94	.38
5^{-3}	.89	.95	.63	.48	.50	.22
5^{-4}	.88	.88	.50	.24	.23	.13
5^{-5}	.58	.55	.31	.12	.12	.11
5^{-6}	.25	.26	.20	.08	.10	.11
5^{-7}	.11	.13	.10	.05	.07	.09

ELISA were done as described in Materials and Methods, except that before the incubation with the hybridoma fluids, the wells were filled either with PBS, normal serum (1 :50 diluted in PBS-T) or pooled syphilitic serum (1 :50 diluted in PBS-T) for one hr at 37°C and then washed with PBS-T buffer.

1) relative extinction

DISCUSSION

The more intensive immunization scheme of Robertson et al. (first injection 2×10^8 *T. pallidum* cells with Freund adjuvant i.p., successively three injections of 2×10^7 cells and finally a booster partially i.p. and i.v.) resulted in more hybridomas producing antibodies to *T. pallidum*. Unfortunately others (4) did not perform

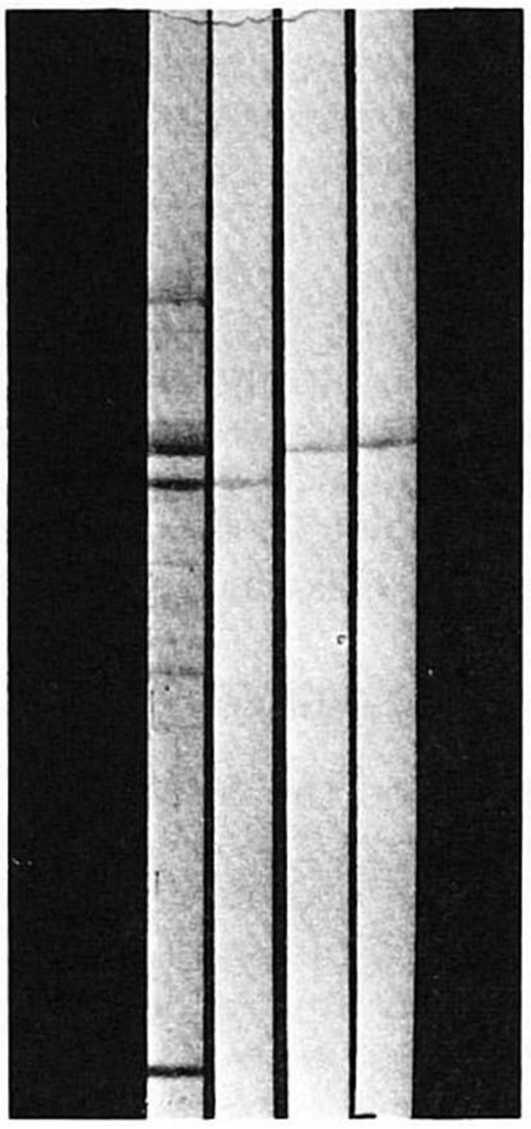

Fig. 1. Western blotting, incubated with from left to right pooled syphilitic serum, monoclonal antibodies 82/19 1-14M_1 (44 kd), 81/47 1-2C_3E_5 (46 kd) and 81/41 3-22D_6F_7 (46 kd).

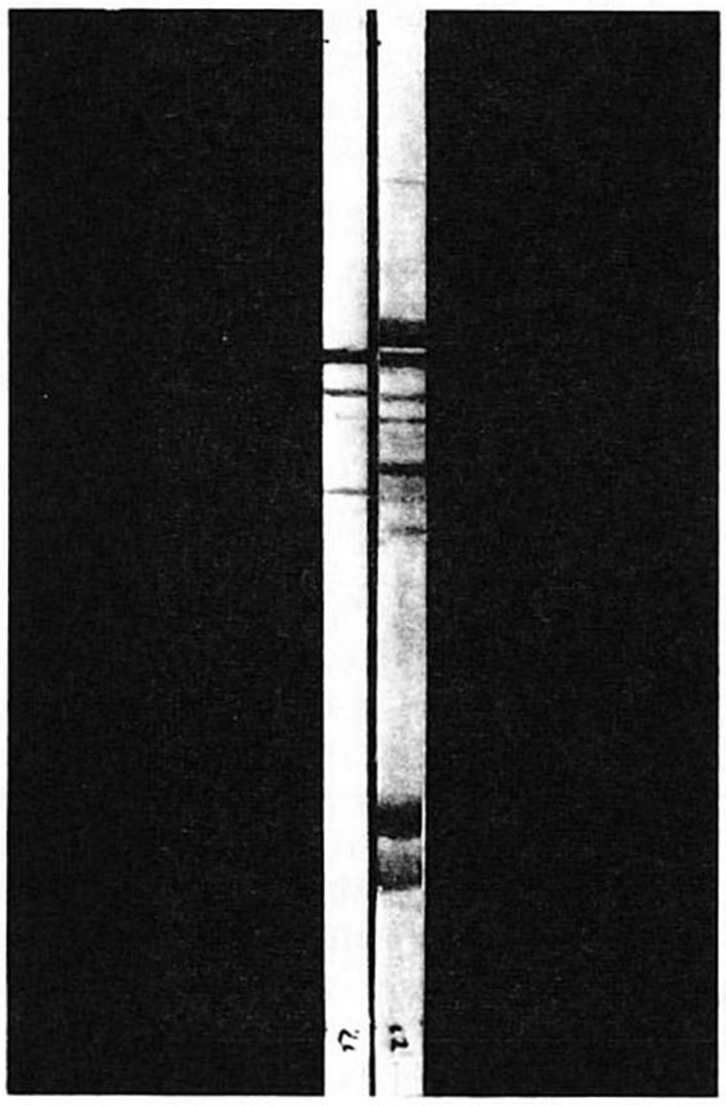

Fig. 2. Western blotting, incubated with from left to right monoclonal antibody 82/19 1-14M_1 (downwards 44, 41, 40 and 35 kd) and pooled syphilitic serum.

Western blotting so the hybridomas cannot be compared, moreover some of these hybridomas (4) might produce identical antibodies. We have just recently performed two fusions after an immunization scheme comparable to that described (4), which resulted so far in a hybridoma producing an antibody reacting with antigens of molecular weights of 73, 58 and 44 kd.

The specificity of the three monoclonal antibodies have been analysed in two ways.

Firstly the binding of two monoclonal antibodies that react in the ELISA can be partially inhibited by pooled syphilitic serum as shown by Figure 1. The blocking is not complete as there will be some competition between the monoclonal antibodies and the human antibodies and as the conjugate used appeared not to be absolutely murine specific.

Secondly the monoclonal antibodies of hybridoma 82/19 1-14M_1, did bind to the 44 kd polypeptide in lysates of only those *E. coli* strains, which expressed this polypeptide antigen as measured by the binding of polyclonal antibodies from syphilitic sera. Antigen-negative deletion mutants bind neither to polyclonal nor to monoclonal antibodies. This genetic evidence demonstrates the specificity of the monoclonal antibody.

The monoclonal antibody 82/19 1-14M_1 reacted sometimes with fragments smaller than 44 kd (Fig. 1 and 2) dependent on the antigen preparation used in the blotting. This may be explained by non-reproducible disrupture of the antigen proteins.

The monoclonal antibodies will be used to purify the E. coli lysates by affinity chromatography.

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