DIAGNOSTIC MICROBIOLOGY

Development, characterisation and diagnostic application of monoclonal antibodies against *Yersinia pestis* fibrinolysin and coagulase

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A library of monoclonal antibodies (MAbs) which recognised different epitopes of *Yersinia pestis* fibrinolysin (Fib) was developed. These MAbs were species-specific and demonstrated no cross-reaction in indirect immunofluorescence tests (IIFT) with other gram-negative bacteria possessing plasminogen activator activity. All the MAbs provided equally high levels of immunofluorescence with pPst⁺ *Y. pestis* strains cultivated at 37°C and at 28°C. In all cases, the MAbs inhibited both fibrinolytic and coagulase (Coag) activities of *Y. pestis* in Fib-activity inhibition and coagulase-activity inhibition reactions, and reacted with 35- and 37-kDa proteins of *Y. pestis* in immunoblotting, demonstrating bifunctional activity possibly similar to the properties of MAbs produced by hybrid hybridomas. On the basis of these and earlier studies, the immunochemical identity of Fib and Coag, two distinct subunits of a bifunctional fusion protein whose specific functional activity depends upon the temperature factor, was established. A new rapid, cheap, strictly specific and safe dot-ELISA based on the use of MAb against *Y. pestis* Fib (MAb-Fib) for reliable identification of *Y. pestis* strains was developed. This technique has great advantages over monoclonal diagnostic kits based on the use of MAb against *Y. pestis* fraction I (FI) because it allows detection of plague bacilli grown at 37°C as well as at 28°C. This dot-ELISA will be valuable as a clinical diagnostic tool and might be applicable to field studies and plague surveillance.

Introduction

Yersinia pestis possesses fibrinolytic (plasminogen activator) and coagulase activities that play a significant role in the pathogenesis of plague [1–3]. These activities are encoded by the same gene, *pla*, which has been cloned and sequenced and is present in a 9.5-kb plasmid designated pPst, or pPCP1 [2, 4–7]. The modulation between coagulase and fibrinolytic activities is temperature-dependent. Fibrinolytic activity is most evident at temperatures above 30°C, but coagulase activity increases with lower temperatures (< 30°C) of *Y. pestis* incubation [2, 5, 6]. These activities are determined by two products of *pla* gene – peptides of 37 and 35 kDa [4–6] – but it is still unclear how the biochemical activities are distributed between the proteins.

Although *pla*-related genes of several other pathogenic bacteria are chromosomal, in contrast to the location of *pla* on a plasmid in *Y. pestis*, their respective genes exhibit a striking degree of homology with that of the plague agent [7] and it is not clear if the *pla*-encoded products of *Y. pestis* differ from those of other bacteria in functional activity and antigenic specificity. The difficulties of purification [8] have led to loss of fibrinolysin (Fib) enzyme activity or to preparations contaminated with carbohydrate, which limits characterisation with homologous antisera.

In this study, several monoclonal antibodies (MAbs) specific for different epitopes of the Fib molecule were characterised. A special system of screening of MAbs based on different properties of the antigen was used to examine the MAbs activity against both peptides encoded by the *pla* gene. One of the MAbs was used to develop a new rapid, strictly specific, sensitive and safe dot-ELISA and the properties of this MAb (–Fib) were compared with those of MAb against fraction I, FI (MAb-FI) for the recognition of *Y. pestis* strains cultivated at 28°C as well as at 37°C. The possibility of

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its further application to systems for monitoring plague infection in natural foci is discussed.

Materials and methods

Bacterial strains

The strains examined included 110 *Y. pestis* isolates from natural plague foci of the Community of Independent States (CIS), Mongolia, China, Vietnam, Pakistan, India, Africa and South America; 15 *Y. pestis* strains constructed by genetic modification and harbouring different combinations of their innate plasmids including isogenic variants of *Y. pestis* EV (*Y. pestis* EV) (pPst⁺; pCad⁺; pFra⁺), *Y. pestis* KM-216 (pPst⁺; pCad⁻; pFra⁻), *Y. pestis* KM-218 (pPst⁻; pCad⁻; pFra⁻) [9]; 65 *Y. pseudotuberculosis* strains (serovars I–VI); 12 *Y. enterocolitica* strains; 10 *Escherichia coli* strains; 10 *Salmonella* strains; eight *Shigella* strains and five *Proteus* strains. The *Y. pestis* strains were cultured on Hottinger agar (pH 7.2–7.4) incubated at 28°C for 48 h. For some experiments, several *Y. pestis* strains were grown at 28°C for 24 h and then incubated at 37°C for 24 h. The remaining bacteria were grown on Hottinger agar (pH 7.2–7.4) at 37°C for 48 h. *Y. pestis* strain 231 cultivated on Hottinger agar (pH 7.2–7.4) at 28°C for 24 h was used for acute challenge of mice.

Antigens

Fib was extracted from *E. coli* strain K802(pEK4) which had been transformed by insertion of the unique *Y. pestis* pla gene, and purified as described previously [6, 8]. Chemical analysis showed that, in addition to proteins, the preparation contained (13.0 ± 3.0)% of carbohydrate and (6.0 ± 1.5)% of lipids.

Lipopolysaccharides (LPS) from *Y. pestis* EV, *Y. pseudotuberculosis* serovar I strains and *E. coli* strain K802 were purified by the Westphal [10] or the Galanos [11] procedures. LPS from *E. coli* O111:B4 (Sigma) was also used. Pure lipid A from *Y. pestis* EV, *E. coli* K802 and *E. coli* O111:B4 were produced according to Galanos [12].

Preparation of hybridomas producing MAbs

Female BALB/c mice, 6–10 weeks old and weighing 18–20 g, were given three intraperitoneal doses of Fib at 2-week intervals, the first injection comprised 25 µg of Fib emulsified in complete Freund's adjuvant (Sigma) and the remaining injections comprised 50 µg of the antigen. The sensitised spleen cells were fused with a mouse myeloma cell line Sp2/O-Ag 14 according to the method of Galfre et al. [13]. The hybridoma cells obtained were cloned by limiting dilution. MAbs produced by hybridomas were screened by an indirect immunofluorescence test (IIFT), ELISA and reaction of Fib-activity inhibition (RFai).

MAbs

Mab-FI, produced by hybridoma Yp.FI.B₂.D₃.Sp [14] and Mab-Fib (Mab-A) were obtained from the ascitic fluids of BALB/c mice inoculated intraperitoneally with hybridoma cells and purified by affinity chromatography as described previously [15].

Determination of MAbs class and subclass

The class and subclass of all MAbs were determined by immunodiffusion by the method of Ouchterlony with agarose (Difco) 1% in 10 mM PBS (pH 7.2–7.4) and lyophilised antisera to mouse immunoglobulin M (IgM), IgG, IgG₁, IgG_{2a}, IgG_{2b} and IgG₃ (Sigma) [16].

IIFT

The following bacterial strains were used for the IIFT: (i) for initial screening, *Y. pestis* EV; (ii) after cloning, three *Y. pestis* strains with different plasmid profiles – *Y. pestis* EV, KM-216, KM-218; (iii) for studying specific properties, 139 different strains of closely related bacteria. Slide smears were prepared from a suspension of live bacteria containing 10⁷–10⁸ cells/ml. All the smears were air-dried and fixed with ethyl alcohol 96% and incubated with cultural fluids of tested hybridomas of MAbs. The smears were stained with FITC-labelled rabbit anti-mouse immunoglobulins for 20 min, washed in phosphate-buffered saline (PBS; pH 7.2) and air-dried. Then, 0.05 ml of glycerol in PBS was placed on each smear and covered with a coverslip. The intensity of immunofluorescence was estimated visually with a luminescent microscope ML-2 (Lomo, Russia). RPMI-1640 medium (Flow Laboratories) and culture supernate of myeloma cells maintained in RPMI-1640 medium were used as negative controls.

IIFT was also used for comparison of specificity of Mab-FI and Mab-Fib by the procedure described above. Ascitic fluids of intact BALB/c mice inoculated intraperitoneally with 1.5 × 10⁶ Sp2/O-Ag 14 myeloma cells were used as a negative control.

ELISA

At different stages of the investigation several modifications of ELISA were used. At the initial screening, Mab production was measured by ELISA performed in 96-well microtitration plates (VNIIMedpolymer, Russia). Briefly, 100 µl of cell culture supernate were added to wells previously coated with purified Fib at 10 µg of protein/ml in PBS. Then, 100 µl of horse-radish peroxidase-labelled rabbit anti-mouse immunoglobulins (Sigma) were added to each well. As substrate, 100 µl of 2,2'-azinobis(3-ethylbenzthiazoline sulphonic acid) 22.3 mg/ml in 0.05 mM citrate buffer (pH 4.0) with H₂O₂ 0.0003% was used. The OD₄₀₅ was measured in a Titertec Multiscan spectrophotometer.

(Flow Laboratories). An OD value of 0.2 above background was considered a positive result. RPMI-1640 medium was used as a negative control.

Indirect ELISA was also used for studying the ability of MAbs to cross-react with various bacterial LPS and lipid A. The plates were sensitised with 100 μ l/well of the antigen samples in PBS at a concentration of 10 μ g/ml. Then, 100 μ l of culture fluid containing MAbs was added to each well. Peroxidase-labelled anti-mouse IgG was employed as conjugate.

Indirect dot-ELISA. The MAbs were tested for their substrate specificity, i.e., their ability to react with protein or carbohydrate fractions of Fib preparations in native and protein-free samples of the antigen as recommended by Magnani [17]. For protein digestion, the preparation was treated with proteinase K (PK) (Calbiochem) according to Hitchcock and Brown [18]. Several drops of each sample (native and PK-digested) were placed on to a nitrocellulose membrane (NCM) pore size 22 μ m (Schleicher-Schuell) and fixed with ethyl alcohol 96% for 20 min. After being washed three times in PBS and blocked with skimmed milk 3% for 15 min, the NCM was incubated for 15 min with the appropriate MAbs. Peroxidase-labelled anti-mouse IgG were employed as a conjugate. O-Dianisidine (Sigma) was used as a substrate. Finally, the NCM was dried and observed for colour differentiation.

Direct dot-ELISA. This was used to compare specific activity of MAb-Fib and MAb-FI and to detect plague bacilli in suspensions of organs of infected mice. Briefly, bacterial suspensions were prepared with doubling dilutions (from an initial dilution of 1×10^{12} to a final dilution of 1×10 microbial cells/ml) in PBS. A drop (c. 2 μ l) of each dilution was placed on to a NCM and fixed with ethyl alcohol 96% for 20 min. After being washed three times in PBS and blocked with skimmed milk 3% for 15 min, the NCM was incubated for 15 min with the relevant MAbs, peroxidase-labelled according to Nakane and Kawaoi [19]. O-Dianisidine (Sigma) was used as a substrate. The dried NCM was observed for colour differentiation.

Suspensions of organs were dotted on to the NCM at one single dilution. Dot-ELISA was performed as above. Bovine serum albumin (BSA; Sigma) 1% in PBS was used as a negative control in both cases.

Specimens of murine organs for dot-ELISA

Twenty BALB/c mice (8–12 weeks old, male and female) were infected subcutaneously with 1×10^2 microbial cells of virulent *Y. pestis* strain 231 suspended in 0.2 ml of 0.15 M NaCl. The deaths associated with *Y. pestis* inoculation were observed not later than the 6th day after challenge. Regional lymph nodes, liver and spleen were recovered and a

suspension of each organ in 0.15 M NaCl was prepared as described by Naumov and Samoilova [20].

RFAI

Plates containing human fibrinogen were prepared as described by Beesley et al. [3]; 10 μ l of hybridoma cell culture supernate with 5 μ l of live *Y. pestis* KM-216 (pPst⁺) suspension (10^8 – 10^9 cells/ml in 0.15 M NaCl solution) were added to the wells of the plates and incubated for 18 h at 37°C. The ability of MAbs to inhibit fibrinolysis was estimated by the absence of a zone of lysis around wells. *Y. pestis* KM-216 (pPst⁺) cells plus equine antiplague antiserum containing anti-Fib antibodies at dilutions of 1 in 1 to 1 in 128, were used as a positive control (absence of a zone of lysis). *Y. pestis* KM-216 (pPst⁺) (which has fibrinolytic activity) was used as a negative control (presence of a zone of lysis).

Reaction of Coag-activity inhibition (RCAI)

The micromethod described by Naumov and Samoilova [20] was performed to determine the ability of MAbs to inhibit coagulase activity. Briefly, 10 μ l of MAbs with 5 μ l of live *Y. pestis* KM-216 (pPst⁺) suspension (10^8 – 10^9 cells/ml in 0.15 M NaCl solution) were added to 100 μ l of rabbit plasma containing sodium citrate 1% and incubated for 2–3 h at 28°C. The reaction was estimated by the formation of a clot. *Y. pestis* KM-216 (which has coagulase activity) was used as a positive control (presence of a clot). Equine antiplague antiserum containing anti-Coag antibodies, *Y. pseudotuberculosis* strain 66 or pure rabbit plasma were used as negative controls (absence of a clot).

SDS-PAGE

Fib and whole-cell lysates of *Y. pestis* EV and *Y. pseudotuberculosis*, both cultivated at 28°C or 37°C, were subjected to SDS-PAGE according to Laemmli [21], with a 4% stacking gel and 12.5% separating gel. Electrophoresis was at 35 mA constant current in Tris-glycine (pH 8.3) plus SDS 0.1% buffer for c. 2.5 h. A set of low-mol. wt markers (Sigma) was used. Gels were counterstained with Coomassie Brilliant Blue R 250 (Sigma) 0.2% w/v in ethanol 25% v/v, acetic acid 7% v/v.

Immunoblotting

The method of Towbin et al. [22] was used. Protein bands in the polyacrylamide gel were blotted electrophoretically on to a NCM, pore size 22 μ m (Schleicher-Schuell), for 1 h at 25°C. After being blocked with skimmed milk 3% in PBS and washed, the membrane was incubated with the MAbs for 12 h at 4°C. Protein bands were visualised with peroxidase-conjugated rabbit anti-mouse IgG (Sigma). O-Dianisidine (Sigma) was used as a substrate.

Results

Selection and testing of MAb

In all, 3% of the MABs reacted with *Y. pestis* EV cells in IIFT whereas 23% of the MABs were positive in ELISA with the Fib antigen itself. The same 26% of MABs inhibited fibrinolytic activity of *Y. pestis* when tested in RFAI.

After cloning, 36 clones of hybridomas were selected by IIFT with whole cells of *Y. pestis* with different plasmid profiles. Of the hybridomas, 27 produced MABs that were positive in RFAI. The remaining nine hybridomas produced RFAI-negative MABs. All the MABs were divided into five groups (A–E) according to their specific activity (Table 1). Within each group, MABs reacted with the same epitope and this was confirmed by an epitope analysis (data not shown). Thus, MABs directed against five different epitopes of the Fib-antigen were obtained. A single MAB from each of the five groups was chosen and designated MAB-A, MAB-B, MAB-C, MAB-D and MAB-E, respectively. All the MABs were of the immunoglobulin G₁ subclass.

When tested in dot-ELISA with native and PK-digested Fib, MABs A–D appeared to react with only the native preparation. No reaction with PK-digested antigen was observed. MAB-E reacted with both native and PK-digested Fib.

In indirect ELISA with various LPS and lipid A preparations, only MAB-E reacted with *Y. pestis* LPS whereas other MABs demonstrated no cross-reaction with the above-mentioned antigens.

Specificity of MABs

In IIFT, MAB-A detected all pPst⁺ strains of *Y. pestis* independently of the presence of other plasmids. MAB-B and MAB-C were shown to give fewer ($p < 0.05$) positive reactions with *Y. pestis* harbouring pPst. No positive reactions with *Y. pestis* lacking pPst and other gram-negative bacteria were observed. MAB-D gave a negative reaction with all the strains used. The temperature of cultivation did not influence the results of IIFT. MAB-E reacted with all *Y. pestis* strains,

including those lacking pPst, and with *Y. pseudotuberculosis* strains (Table 2).

Effect of MABs on coagulase activity

All 27 MABs that were positive in RFAI (groups A–D) inhibited the coagulase activity of *Y. pestis* but nine RFAI-negative strains did not.

Band patterns revealed by MABs on immunoblotting

Whole-cell lysates of *Y. pestis* EV and *Y. pseudotuberculosis* cultivated at 28°C and 37°C and Fib preparation were separated from the gel on to nitrocellulose and incubated with MAB-A. As shown in Fig. 1, MAB-A gave strong positive immunoblotting with two peptides of mol. wts 35 and 37 kDa in the fractionated Fib and *Y. pestis* EV, independently of the temperature of cultivation. Both bands were of the same intensity. There was no cross-reaction with *Y. pseudotuberculosis*. Immunoblotting with MAB-B and MAB-C gave the same results.

Detection of *Y. pestis* by MAB-FI and MAB-Fib (MAB-A)

Two MABs – MAB-FI, obtained and characterised in our laboratory previously [14] and MAB-Fib (MAB-A) – were tested in IIFT. Both MABs gave positive results at similar working (optimal) dilutions (1 in 2500) and were species-specific, i.e., recognised only *Y. pestis* strains and not other gram-negative bacteria used in the current investigation. However, some differences in the reaction of MABs with *Y. pestis* strains cultivated at 28°C and 37°C were observed (Table 3). MAB-FI demonstrated a positive reaction only with *Y. pestis* strains cultivated at 37°C, whereas MAB-Fib reacted with *Y. pestis* strains independently of the cultivation temperature. Similar results were obtained in the dot-ELISA. Both MABs detected *Y. pestis* strains at a concentration of 10^3 – 10^5 microbial cells/ml. In suspensions of organs of plague-infected mice *Y. pestis* strain 231 was detected in dot-ELISA by both MAB-Fib and MAB-FI.

Table 1. Specific activity of RFAI-positive MABs produced by recloned hybridomas in IIFT

Groups of hybridomas	Number of hybridomas/ group	Strains of <i>Y. pestis</i> and plasmid profiles			Percentage of positive reactions
		EV	KM-216	KM-218	
		(pPst ⁺ ; pCad ⁺ ; pFra ⁺)	(pPst ⁺ ; pCad ⁺ ; pFra ⁺)	(pPst ⁺ ; pCad ⁺ ; pFra ⁺)	
A	15	+	+	–	55.5
B	8	–	+	–	29.6
C	2	+	–	–	7.4
D	3	–	–	–	11.1

Table 2. Comparative characteristics of specificity of different MAb groups

Bacterial species and plasmid profile	Number of strains	Cultivation temperature (°C)	Percentage of positive reactions with MAb groups				
			A	B	C	D	E
<i>Y. pestis</i>							
pPst ⁺ ; pCad ⁺ ; pFra ⁺	44	28	100	84	100	0	100
		37	100	84	100	0	100
pPst ⁺ ; pCad ⁺ ; pFra ⁻	8	28	100	75	100	0	100
		37	100	75	100	0	100
pPst ⁺ ; pCad ⁻ ; pFra ⁻	3	28	100	100	33.3	0	100
		37	100	100	33.3	0	100
pPst ⁺ ; pCad ⁻ ; pFra ⁺	4	28	100	50	75	0	100
		37	100	50	75	0	100
pPst ⁻ ; pCad ⁺ ; pFra ⁺	19	28	0	0	0	0	100
		37	0	0	0	0	100
pPst ⁻ ; pCad ⁺ ; pFra ⁻	2	28	0	0	0	0	100
		37	0	0	0	0	100
pPst ⁻ ; pCad ⁻ ; pFra ⁺	6	28	0	0	0	0	100
		37	0	0	0	0	100
pPst ⁻ ; pCad ⁻ ; pFra ⁻	6	28	0	0	0	0	100
		37	0	0	0	0	100
<i>Y. pseudotuberculosis</i>	28	28	0	0	0	0	0
		37	0	0	0	0	0
<i>Y. enterocolitica</i>	6	28	0	0	0	0	0
		37	0	0	0	0	0
Other micro-organisms*	13	28	0	0	0	0	0
		37	0	0	0	0	0

*See Materials and methods.

kDa

69 -

46 -

43 -

30 -

A B C D E

Fig. 1. Immunoblotting of Fib preparation and whole-cell lysates of *Y. pestis* and *Y. pseudotuberculosis* with MAb-A. Lane A, Fib preparation; B, *Y. pestis* EV cultivated at 28°C; C, *Y. pestis* EV cultivated at 37°C; D, *Y. pseudotuberculosis* strain 66 cultivated at 28°C; E, *Y. pseudotuberculosis* strain 66 cultivated at 37°C. Numbers on the right indicate molecular ratios ($\times 10^3$) of the standard proteins employed.

Discussion

The contribution of plasminogen activators to bacterial virulence is well established [1-3, 5, 6]. Although the striking degree of homology between the known

plasminogen activators [7] supposes the same mechanism of fibrinolysis [3], *Y. pestis* Fib contributes to the extreme virulence of the plague agent and to the highly invasive, fulminant character of plague infection [1-3, 6]. In contrast to most gram-negative bacteria whose *pla* genes are chromosomal, the *Y. pestis* *pla* gene is located on a unique plasmid - pPst [3-6]. This gene has been cloned, sequenced and shown to have incomplete homology with other plasminogen activators [4, 7], but the peculiarities of functional activity and antigenic specificity of its product, Fib, remain unclear. Being an outer-membrane protein (OMP) [4, 6, 8], Fib is closely bound to other OMPs and LPS [8]. Attempts at purification of the protein by different methods met with limited success [8] and produced highly hydrophobic preparations containing carbohydrate. The use of such a preparation for immunisation induces anti-LPS antibodies which predominate over antibody to protein antigen. The presence of LPS epitopes in the *Y. pestis* Fib preparation causes extensive cross-reactions with closely related bacteria [8]. At the same time, Fib lacking LPS loses its enzymic activity [8] which indicates significant alteration of native structures of the antigen. With hybridoma technology, it is possible to obtain a panel of MAbs directed against distinct protein epitopes of any complicated antigen containing carbohydrate with no necessity to purify it to a very high standard. In the current study, MAbs were obtained through the development of a new system of hybridoma screening which was based, on the one hand, on the use of pPst⁺ and pPst⁻ *Y. pestis* strains constructed by genetic modification [9] for selection of MAbs specific for *pla*-products encoded by pPst in IIFT, and on the other hand, on testing of MAbs for their ability to influence Fib functional activity (RFAI). This original system of

Table 3. Comparison of specific properties of MAb-FI and MAb-Fib in IIFT

Strains and plasmids	Number of strains	Cultivation temperature (°C)	Specific reaction with	
			MAb-FI	MAb-Fib
<i>Y. pestis</i>	44	28	—	+
pPst ⁺ ; pFra ⁺ ; pCad ⁺	44	37	+	+
pPst ⁺ ; pFra ⁺ ; pCad ⁻	8	28	—	+
	8	37	+	+
pPst ⁺ ; pFra ⁻ ; pCad ⁺	4	28	—	+
	4	37	—	+
pPst ⁺ ; pFra ⁻ ; pCad ⁻	6	28	—	+
	6	37	—	+
<i>Y. pseudotuberculosis</i>	28	28	—	—
	28	37	—	—
<i>Y. enterocolitica</i>	13	28	—	—
	13	37	—	—

screening appeared to be optimal and the data generated coincided with the combined results of the ELISA and IIFT. It should be mentioned that, in IIFT, *Y. pestis* cells cultivated at 28°C were used because, at 37°C but not at 28°C, the organism forms a large capsule or envelope [2, 23] which covers other surface antigenic determinants of *Y. pestis*, including Fib [23]. The difference between the ELISA and IIFT data could be explained by: (i) decreased production of Fib when bacteria were grown at 28°C; (ii) inaccessibility of Fib epitopes for MAbs because of cell wall LPS; (iii) heterogeneity of Fib preparation used for coating ELISA plates; (iv) as an enzyme, Fib can be inactivated easily under the influence of various factors. Modification of a native epitope of *Y. pestis* Fib in the process of fixation with ethyl alcohol or on treatment with a detergent at different steps of the assays (dot-ELISA, ELISA, immunoblot, etc.) probably led to the loss of the antigen-antibody interaction.

ELISA and IIFT were the methods which allowed selection of MAbs to surface determinants of the microbial cell wall (both Fib and LPS). As the original Fib preparation contained a large amount of LPS, the selection of several hybridomas producing anti-LPS MAbs was expected and found; nine hybridomas positive in IIFT and ELISA were RFAI-negative (group E). On the one hand, MAbs produced by group E hybridomas could recognise an epitope responsible for the serological properties of Fib but not for its functional activity. On the other hand, they could be directed against *Y. pestis* cell-wall structures. Although Fib has been shown previously to be a pure protein [4–6], the difficulties of obtaining Fib preparations devoid of polysaccharide led to the supposition that this is a complex antigen-glycopeptide and that polysaccharide was a subunit of a complex Fib molecule [8]. Studies of the properties of MAb-E helped to solve this problem. This MAb possessed the following characteristics: (i) it was not able to inhibit fibrinolytic activity (i.e., was RFAI-negative); (ii) it reacted with both native and PK-digested Fib preparation; (iii) it reacted with *Y. pestis* LPS; (iv) it reacted with *Y. pestis* strains

regardless of plasmid profiles, including strains devoid of plasmids, i.e., recognised determinants encoded by chromosomal genes; (v) it gave no reaction with other Enterobacteriaceae (Table 2). Interestingly, all MAbs produced by the group E hybridomas demonstrated the same properties; as they were of IgG₁ subclass they did not give non-specific cross-reactions. This suggests that the carbohydrate part of Fib was a *Y. pestis* cell-wall fragment, but not a subunit of a complex Fib molecule.

In contrast, 27 RFAI-positive MAbs A–C reacted only with native Fib preparations, but not with the PK-digested antigen. This strongly indicated that the epitopes recognised had a protein nature.

Study of the spectrum of specific activities of MAbs A–C in IIFT with *Y. pestis* containing different plasmids showed that only MAb-A detected all pPst⁺ *Y. pestis* strains. As for MAb-B and MAb-C, they provided fewer positive reactions with the same strains (Table 2). As all pPst⁺ strains used in the assay produced lysis of fibrin, this observation could be explained by different expression of epitopes which were recognised by both MAbs, B and C. Different expression of Fib epitopes could be connected with variable antigenic structure of *Y. pestis* strains from different natural foci [24]. The data obtained led to the conclusion that the epitopes recognised by these MAbs are encoded by pPst alone.

One of the most significant properties of the MAbs obtained was their ability to detect only *Y. pestis* strains. The pla protein was reported previously to share homology with other plasminogen activators [7]. Thus, sequencing of the pla gene revealed a high level of coincidence of its DNA sequence with one of gene E of *S. typhimurium* (69%) and ompT of *E. coli* (59%). This homology could be expected to predict similar properties of these gene products, including plasminogen activator activity. However, MAbs gave no cross-reaction with other closely related bacteria, as well as the above-mentioned *S. typhimurium* and *E. coli* strains. It appears that MAbs A–C recognised the

species-specific epitopes of *Y. pestis*. These data agree with the fact that the *pla* gene does not possess complete homology with known plasminogen activators of bacterial (streptokinase and staphylokinase) or eukaryotic (urokinase and tissue plasminogen activator) origin [7]. The absence of homology between *Y. pestis* *pla* gene and genomes of a great number of gram-negative bacteria was confirmed by an examination of the relevant DNA sequences by the P1 probe, constructed on the basis of the site of the *Y. pestis* *pla* gene encoding Fib [6]. All these data strongly indicated a specific mechanism of *Y. pestis* fibrinolysis despite the number of observations [3–5] on the similar activity of other plasminogen activators.

The most striking property of MAbs A–C was their ability to detect *Y. pestis* strains cultivated at 37°C as well as at 28°C, providing equally high levels of immunofluorescence in IIFT (Table 2). Cavanaugh and McDonough reported that the fibrinolytic activity is temperature-dependent, being higher at 37°C than at 28°C [5]. Thus, it could be expected that the specific activity of MAbs should follow the same pattern. However, MAbs appeared to be capable of detecting plague bacilli cultivated at 28°C with the same frequency as those cultivated at higher temperatures. This observation prompted the investigation as to whether MAbs reacted with both Fib and Coag; thus, RCAI was used to answer this question. All RFAI-positive MAbs appeared to inhibit the coagulase activity of *Y. pestis*. This was an indication that the MAbs recognised epitopes common to Fib and Coag. The results obtained were confirmed by immunoblotting analysis (Fig. 1). The bands visualised corresponded exactly to these two peptides of 37 and 35 kDa previously identified as *Y. pestis* Fib and Coag [4–6, 8]. The reaction of the MAbs with both Fib and Coag corroborated the immunochemical identity of two distinct subunits of a bifunctional peptide whose functional activity depends upon the temperature factor.

Existence of Fib and Coag in the form of a bifunctional protein is not a unique phenomenon. Several bacteria also have polyfunctional proteins composed of two and more distinct subunits involved in different biological reactions, e.g., anthranilate synthetase from *Neurospora crassa* [25], aspartokinase I-homoserine dehydrogenase I from *E. coli* [26], protein E of *S. typhimurium* [7]. *Y. pestis* itself has another polyfunctional peptide, 'murine' toxin, which demonstrates phospholipase D, kinase, phosphatase, phosphodiesterase, amylase and NAD-glycohydrolase activities [27, 28]. Another *Y. pestis* peptide possessing bifunctional activity is V-antigen, whose dual role in virulence and in the regulation of the LCRS in *Y. pestis* has been shown by Skrzypek and Straley [29]. The probable explanation of the phenomenon of the existence of Fib and Coag in the form of a two-subunit bifunctional protein is through fusion of two independent genes in the process

of evolution. As a result, the bacterium synthesises a so-called fusion peptide – one long protein chain instead of two. This is consistent with the experimental data of Truffa-Bachi et al. [30] which showed that two consequent frame-shift mutations on the sequence of two distinct genes of histidine biosynthesis of *S. typhimurium* caused translation of a single product. Further evidence of the Fib and Coag existence in the form of a bifunctional peptide is the impossibility of separating them by chromatographic methods [6, 8, 23], including high-performance liquid chromatography [6]. In addition, both peptides, Fib and Coag, showed only one protein when subjected to continuous free-flow electrophoresis and PAGE without SDS [8].

It should be emphasised also that the MAbs obtained demonstrated the properties similar to those inherent in bi-specific MAbs produced by hybrid hybridomas. These MAbs (i) inhibited fibrinolytic activity of *Y. pestis* as well as coagulase, (ii) provided an equally high level of immunofluorescence in IIFT with *Y. pestis* cells cultivated at optimal conditions for synthesis of Fib (37°C) and Coag (28°C); and (iii) reacted with both 35- and 37-kDa peptide bands in immunoblotting analysis. Thus, another fusion was not required to produce MAbs with bifunctional activity because of the properties of the *Y. pestis* Fib.

MAb-D lacked the ability to interact with *Y. pestis* strains in IIFT. This MAb was probably directed against an intracellular precursor of two *pla* forms (Fib and Coag) as described by Sodeinde and Goguen [4] and could not react with cell-surface structures.

The majority of *Y. pestis* strains harbour three plasmids, pPst, pCad and pFra [2, 24]. Two plasmids, pPst and pFra, have been detected only in *Y. pestis* [2]. So, the products encoded by these plasmids, especially Fib and FI, being species-specific, serve as a basis for development of diagnostic preparations. However, Fib has advantages over FI (Table 3). Thus, MAb-Fib detected *Y. pestis* strains cultivated at 28°C as well as at 37°C, whereas MAb-FI reacted only with *Y. pestis* strains cultivated at 37°C. This phenomenon is possibly connected with the unique structure of *Y. pestis* Fib, immunochemically identical to *Y. pestis* coagulase. As coagulase activity of *Y. pestis* is observed at temperatures lower than 28°C [3, 5, 6], the MAb-Fib capable of recognising the relevant antigen can be used for the detection of *Y. pestis* strains in different specimens, especially in plague vectors. Moreover, there are some *Y. pestis* strains that lack FI antigen or produce it only at 37°C [2, 14, 23, 24] and cannot be identified by the present serological methods. Furthermore, some data suggesting a non-specific character of several FI epitopes have been obtained [31, 32].

The use of MAb-Fib but not MAb-FI provided detection of *Y. pestis* pPst⁺ strains which, being probably more virulent, are also the most dangerous

epidemiologically, as strains that have lost pPst exhibit a marked increase in LD50 [2]. Although there are observations that pPst⁻ mutants injected intravenously are fully virulent [2], there is no evidence for the absence of expression of antigens encoded by pPst in these *Y. pestis* strains. As this plasmid replicon is very likely capable of integration into the bacterial chromosome as well as pFra [33], expression of Fib in pPst⁻ strains can be explained by residence of the *pla* gene within the bacterial chromosome [7].

Being strictly specific, the MAb-Fib could be useful for differentiation between *Y. pestis* and *Y. pseudotuberculosis* – whose high level of genome homology has been shown previously [34]. Furthermore, *Y. pseudotuberculosis* was reported to form a capsule and it is unclear whether both *Yersinia* spp. have identical epitopes associated with the microbial capsule [31]. At the same time, *Y. pseudotuberculosis* exhibits neither fibrinolytic nor coagulase activity [1, 2, 35]. This marks Fib but not FI as the most diagnostically important *Y. pestis* antigen. It was easy to find plague bacilli in specimens of organs of mice after an acute challenge with virulent *Y. pestis* strain 231, by direct dot-ELISA. These data are corroborated by the data of McDonough et al [35], who used a fragment from *pla* region to specifically detect *Y. pestis* in fleas.

Titenko et al. [23] postulated that specific fluorescence of capsulate and non-capsulate plague bacilli stained with plague polyclonal luminescent immunoglobulins is provided by different antigens (FI and Fib, respectively) emphasising that specific fluorescence of capsulate plague bacilli is due only to FI. The MAb-Fib produced specific fluorescence of *Y. pestis* strains cultivated at both 37°C (capsulate) and at 28°C (non-capsulate) in IIFT. This means that surface Fib epitopes, being available for specific MAb, are not covered with FI antigenic determinants.

In summary, this study used a complicated screening assay procedure for testing specific and functional activities of MAbs. This approach provided an opportunity to select MAbs with different properties, the most important of which was inhibition of two distinct activities of *Y. pestis*. These MAbs recognised different epitopes of Fib which correlated with the reports of a polydeterminant structure for this antigen [4, 6, 8]. The use of the library of MAbs made it possible to get more precise information about the *Y. pestis* Fib antigenic molecule. A new technique was developed for reliable detection and serological identification of *Y. pestis* based on the use of strictly specific MAb-Fib. Further studies concerning the possible application of the MAbs obtained for improvement of the serological diagnosis of plague and extension of knowledge of the plague pathogen are in progress.

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