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Characterisation of shrimp haemocytes and plasma components by monoclonal antibodies

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SUMMARY

Various haemolymph components of the shrimp *Penaeus japonicus* were identified and characterised by monoclonal antibodies. Three groups of monoclonal antibodies were raised. Their reactivity to haemocyte types and/or secreted molecules was determined by immunofluorescence and the molecular masses of the antigens were analysed by western-blotting. A 170 kDa protein, in reducing conditions, was recognized by four panhaemocytic monoclonal antibodies from group 1. This protein was present both in the plasma and in the haemocytes from which it appears to be secreted. The shrimp haemocytes were separated by isopycnic centrifugation on a Percoll gradient and the different subpopulations were antigenically analysed using the two monoclonal antibodies, 40E2-2A and 40E10-2B, from group 2. The granular cells were labelled by 40E2-2A which was specific for a protein of 142 kDa also present in plasma. By comparison, the 40E10-2B monoclonal antibody was assumed to be the marker for small hyaline and semi-granular cells since the granular ones were not labelled. Moreover, the antigen immunoprecipitated by this monoclonal antibody was shown to have different molecular masses of 250, 150, 66 and 27 kDa under nonreducing conditions. It appeared to be secreted by the haemocytes. Some plasma proteins were recognized by the third group of monoclonal antibodies. The antibodies, designated 41D11-3A, 42C11-3B and 42E8-3C, all immunoprecipitated a protein with an apparent molecular mass of 180 kDa under reduced conditions. The 44E6-3D antibody was specific for a 75 kDa protein under reduced conditions and was shown to be immunoreactive against *P. japonicus* haemocyanin extract. An antigenic relationship was studied for the haemolymph components of *P. japonicus* and two other shrimp species, *P. vannamei* and *P. indicus.* Hypotheses about the possible nature of the proteins, antigenically identified, are advanced.

Key words: crustacea, shrimp haemolymph, monoclonal antibody, haemocyte subpopulation, plasma protein, haemocyanin, immunity

INTRODUCTION

Research in crustacean immunology has been essentially focused on crayfish, and in particular those processes known to be mediated by haemocytes: phagocytosis, encapsulation, melanisation and coagulation. The immune reactions are beginning to be understood, especially those linked to the prophenol oxidase activating system and associated recognition factors, through the isolation and characterisation of numerous components of this system (Söderhäll and Cerenius, 1992).

In comparison, the present knowledge of the immune system of penaeid crustaceans is limited, but research is currently expanding because of the economic importance of shrimp aquaculture throughout the world and the significant impact of infectious diseases (Lightner, 1983). Noninfectious diseases are also now being studied in relation to marine pollution caused by pesticides. It has become crucial to better understand the immune processes, in terms of basic research and also for disease prevention. Diseases could be prevented by regularly monitoring the immune state of the shrimps in the farms to detect as early as possible any abnormal conditions, by performing immunostimulation to prevent infection, or by selecting shrimp strains with increased immune capability. For these purposes, however, it is first necessary to characterise immune cells and secreted molecules, and then to study the immune mechanisms at the cellular and molecular levels.

The haemocytes of *Penaeus japonicus* have been characterised on the basis of ultrastructural features (Tsing et al., 1989). The following three cell types have been recognized: haemocytes with small granules, haemocytes with large granules and undifferenciated haemocytes. By combining the results from morphological and cytochemical analyses, two cell lineages have been determined for the haemocytes of *Sicyonia ingentis*. One lineage is characterised by granular deposited material and corresponds to agranular and striated granular haemocytes that are associated with haemolymph coagulation. The other lineage corresponds to granular cells that are related to the prophenol oxidase system (Hose et al., 1987; Hose and Martin, 1989). Concerning humoral factors, a lectin, monodin, and an agglutinin have been purified and characterised, respectively, from the haemolymph of *P. monodon* and *P. californiensis* (Ratanapo and Chulavatnatol, 1990; Vargas-Albores et al., 1993).

In order to study further immune cellular and humoral effectors in shrimp, monoclonal antibodies (mAbs) have been prepared for *P. japonicus* using hybridoma technology (Kölher and Milstein, 1975). The paper describes the production and the use of a panel of ten cloned haemolymph-specific hybridomas. Various components of haemolymph were discriminated by these mAbs which were first analysed by immunofluorescence on haemolymph samples. Then primary cultures were prepared in several ways in order to determine the mAb reactivity against haemocyte types and/or secreted molecules and their ability to discriminate haemocyte subpopulations. The molecular masses of the antigens were determined by western blot analyses or by electrophoresis of immunoprecipitates. The antigenic relationship between the haemolymphs of *P. japonicus* and of two other economically important shrimp species, *P. vannamei* and *P. indicus*, was then investigated.

MATERIALS AND METHODS

Animals and haemolymph collection

For all experiments, juvenile male and female shrimps were used from the species *Penaeus japonicus*, the Kuruma prawn, *P. indicus*, the Indian white prawn and *P. vannamei*, the white leg shrimp.

The *P. japonicus* and *P. indicus* shrimps were maintained in the laboratory in 500-l tanks (17°C) equiped with air-lift circulating sea water. *P. vannamei* haemolymph samples were prepared in Ecuador (CENAIM, ESPOL, San Pedro). Only animals in the intermoult stage, at about 15-20 g, were used in the experiments.

Haemolymph was obtained from the ventral sinus located at the base of the first abdominal segment. The samples were collected using a 23G gauge syringe and immediately half diluted with 1 ml of precooled (4°C) modified Alsever solution (AS) (27 mM Na citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7) used as anticoagulant, which also prevents melanization and keeps haemocytes in a quiescent state.

Haemocyte preparations

The different haemocyte preparations were either used for immunocytochemical labelling or stained with Hemacolor kit (Merck).

Adherent primary cultures

The haemolymph collected in AS, pH 7, was used for primary cultures, either directly or after washing from plasma components by centrifugation (700 ***g***, 10 minutes, 4°C) with AS, pH 7 or pH 4.6. To avoid cell disruption and degranulation (Söderhäll and Smith, 1983), the cells were washed with AS, pH 4.6. To restore haemocyte adherence and spreading capability, haemocytes were resuspended in sterile saline solution (SSS) (15 mM $CaCl_2$, 7 mM KCl, 12.5 mM $MgCl_2{\cdot}6\,H_2O$, 7.4 mM $NaHCO_3$, 336 mM $NaCl_2$, pH 7; La Peyre and Chu, 1990) and 50 µl suspension drops were layered on microprint slides. Haemocytes were allowed to adhere to the surface for 2 hours before gentle washing with SSS.

Primary cultures in suspension

After washing, haemocytes were resuspended and incubated in microtubes for 1 or 2 hours in SSS then used for preparing haemocyte extracts.

Cytocentrifugation

Haemocytes collected in AS were fixed by adding 4% formalin (30 minutes), spun, washed twice and resuspended in AS. Approximately $2{\times}10^5$ haemocytes were then cytocentrifuged (Cytotek-centrifuge, Miles) per microscope slide (200 ***g***, 5 minutes).

Haemocyte separation procedure

Shrimp haemocyte subpopulations were separated by isopycnic centrifugation in a discontinuous Percoll gradient according to the protocol used for molluscan haemocytes (Bachère et al., 1988). Briefly, the haemocyte suspension in AS, pH 4.6, was layered onto Percoll gradient consisting of 1.5 ml fractions of 10%, 20%, 30%, 40%, 50%, 60% and 70% Percoll (Pharmacia) in AS, pH 7. After 30 minutes centrifugation at 200 ***g*** (4°C), the cells banding at the different interfaces were collected, washed from Percoll by centrifugation through a 10% sucrose cushion (500 ***g***, 15 minutes, 4°C) then resuspended in AS, pH 4.6. The haemocytes were then prepared either for primary cultures or cytocentrifugation.

Production of monoclonal antibodies

For immunization, the haemolymph/AS mixtures were made isoosmotic to mice serum by dilution with sterile distilled water and then subjected to a freeze-thaw cycle. Two Balb/c mice were immunized by intraperitoneal injection of 500 µl antigen suspension ($2{\times}10^6$ haemocytes), followed by two subsequent injections at weekly intervals. Serum titers were estimated 13 days after the last booster by indirect immunofluorescence assay (IIFA) as described below. The selected mouse presenting the highest antibody titer was reinjected 3 days before lymphocyte hybridization.

Spleen from selected mice was dissected and fused with P3-X63-Ag8-653 myeloma using polyethylene glycol as fusogen, as described by French et al. (1986). Hybridomas were selected in RPMI 1640 medium (Gibco Ltd) supplemented with 15% foetal calf serum, 2 mM L-glutamine, 0.1 mM hypoxanthine, $0.4{\times}10^{-3}$ mM aminopterine and 0.016 mM thymidine. Screening of hybridomas secreting specific antibodies was performed by indirect immunofluorescence assay (IIFA) on haemocyte primary cultures prepared with whole haemolymph in microplates. The cells were formalin fixed. Hybridomas giving positive results were subcloned by limited dilution with 0.3 cells distributed per well, then tested again for their specificity by IIFA.

Indirect immunofluorescence assay (IIFA)

The haemocyte preparations, either primary cultures or cytocentrifugated cells, were fixed and permeabilized with acetone for 5 minutes. Slides were overlaid with 50 µl specific antibody hybridoma culture medium and incubated for 30 minutes at room temperature (RT) in a moisture chamber. After three washings with immunofluorescence buffer (IF; Diagnostics Pasteur), the cells were incubated for 30 minutes (at RT) in the dark with FITC-conjugated goat anti-mouse Ig serum diluted at 1:1000 in IF buffer containing 1% Evans Blue. Finally, the slides were washed three times and mounted in buffered glycerin for observation by epifluorescence.

Isotyping of monoclonal antibodies

The monoclonal antibodies were isotyped using the Ouchterlony immunodiffusion method with a ‘Mouse monoclonal typing kit’ (ICN ImmunoBiologicals) and following the manufacturer’s instructions.

Dot blotting and western blotting

The library of monoclonal antibodies was first assayed by dot blots against plasma and haemocytic extract samples compared under conditions where proteins were either native or reduced in the presence of 2% SDS and 100 mM dithiothreitol. The mAbs were also tested against samples of haemocyanin. The respiratory pigment purified from *P. japonicus* haemolymph was kindly supplied by Dr Yen-Lin Song (Taïwan).

Haemolymph was collected in AS, pH 7; the haemocytes were pelleted and the supernatant was kept at –20°C as plasma. The haemocytes were washed twice with AS, pH 4.6, and finally resuspended in single detergent lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.02% $NaNO_3$, 100 μg/ml PMSF, 1 μg/ml aprotinin, 1% Triton X-100) according to Sambrook et al. (1989). After 30 minutes of incubation on ice, cell debris was removed by centrifugation (9000 ***g***, 5 minutes) and the supernatant kept at −20°C until used.

The different samples of plasma and haemocyte lysate, both native and denaturated, were applied to Hybond nitrocellulose paper (Amersham International Ltd.) using a dot-blot apparatus (Biodot, Bio-Rad). For western blotting, the samples were run on SDS-polyacrylamide gels (SDS-PAGE) using the PhastSystem apparatus (Pharmacia) under reducing and non reducing conditions. The proteins were then electrotransferred onto nitrocellulose Hybond or nylon membrane (Millipore) at 5 V/h for 15 minutes in 25 mM Tris-HCl buffer containing 192 mM glycine and 20% methanol, pH 8.3.

For labelling with antibodies, dot-blot or western blot membranes were incubated for 1 hour at RT, in blocking solution consisting of 20% gelatine hydrolysate in PBS. After three washings in PBS with 1% Tween-20, the hybridoma culture media were incubated overnight at 4°C with agitation. The membranes were washed three times in PBS-1% Tween-20 then incubated (1 hour, RT) with goat anti-mouse whole IgG molecules conjugated to alkaline phosphatase (Biosys) prepared in PBS with 5% gelatine hydrolysate. After three washes, the membranes were then incubated in substrate solution (0.016% (w/v) 5-bromo-4-chloro-3-indolyl phosphate, 0.033% (w/v) Nitro Blue Tetrazolium in 100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5) for 10 minutes at RT. Reactions were stopped with 2 mM EDTA in PBS.

Immunoprecipitations

Plasma or haemocyte lysate samples were dialysed or diluted with buffer (NET gel buffer: 0.1% Triton X-100, 150 mM NaCl, 5 mM EDTA and 50 mM Tris-HCl, pH 7). The antibodies, either purified or as hybridoma culture medium, were added and incubated under agitation for 1 hour at 4°C. Then, 30 μl Protein A-Sepharose CL-4B beads (Pharmacia) were added and incubated for 1 hour under agitation at 4°C. The immune complexes were pelleted and washed twice with NET buffer. Finally, the beads were resuspended and boiled in denaturating 2% SDS electrophoretic sample buffer. After electrophoresis on homogeneous 4.5% gels, the samples were silver-nitrate stained (PhastSystem, Pharmacia).

Antigenic cross-reactivities

The different monoclonal antibodies were assayed using IIF immunocytochemistry and dot-blotting techniques, respectively, against the haemocytes and the plasma of the other shrimp species, *P. indicus* and *P. vannamei*.

RESULTS

Selection of anti-haemolymph hybridomas and mAb immunofluorescent patterns

The reactivity of antibodies produced by precloned hybridomas was first analysed by an indirect immunofluorescence assay performed on haemolymph primary cultures. These cultures were directly prepared from haemolymph collected in modified Alsever solution at pH 7 then diluted in SSS, so that haemocytes were adherent to the bottom of plastic microplate wells, were secreting, and that plasma proteins were present. From 440 analysed hybridomas, 72 were shown to be reacting against haemolymph primary cultures. Ten cloned hybridomas were selected and classified according to the mAb immunofluorescent patterns. For this purpose, mAb reactivities were determined, on the one hand, towards cytocentrifuged suspensions of washed and fixed haemocytes, and on the other hand, towards primary cultures of haemocytes previously washed in modified Alsever solution, either at pH 7, where haemocytes recover their ability to adhere and to secrete, or at pH 4.6, where haemocytes are quiescent. Subsequently, it was possible to determine the specificity of mAbs for haemocyte types and/or secreted molecules.

The mAbs were classified into 3 groups (Table 1). Group 1 was composed of 35D6-1A, 40G8-1B, 40G11-1C and 41B12-1D cloned hybridomas which secrete mAbs reacting similarly against a common antigen present on all the haemocyte types and in plasma (Fig. 1). Group 2 was composed of 40E2-2A and 40E10-2B cloned hybridomas which secrete mAbs reacting against different haemocyte subpopulations (Fig. 2A and B). Group 3 was composed of 41D11-3A, 42C11-3B, 42E8-3C and 44E6-3D cloned hybridomas which produce mAbs strictly reacting with epitopes present only in the plasma (Fig. 3).

Characterisation of haemocytes and related plasma protein using Group 1 mAbs

At the light microscopy level, it was possible to localize the

Table 1. Immunostaining in indirect immunofluorescence assay (IIFA) of *P. japonicus* haemolymph by specific monoclonal antibodies

Group	MAb clone no.	Ig isotype	IIFA on total haemolymph: Haemocyte	IIFA on total haemolymph: Plasma	AS pH 7 or 4.6 washed haemocyte primary cultures (%)		Cytocentrifuged, AS pH 4.6 washed haemocytes (%)	
1	35D6-1A	IgG1	+++	++	+++	100	+++	100
	40G8-1B	IgG1	+++	++	+++	100	+++	100
	40G11-1C	ND	+++	++	+++	100	+++	100
	41B12-1D	IgG1	+++	++	+++	100	+++	100
2	40E2-2A	IgG1	++	++	++	54	+	9
	40E10-2B	IgG2b	++	++	++	40	+	16
3	41D11-3A	IgG1	–	++	–		–	
	42C11-3B	IgG1	–	+	–		–	
	42E8-3C	IgG1	–	+	–		–	
	44E6-3D	IgG1	–	++	–		–	

mAb Ig isotypes as indicated.
AS, modified Alsever solution.
+, positive staining of haemocytes or extracellular compounds (number of + refers to the intensity of staining); –, no staining; ND, not determined.

epitopes on the cell membranes of all the haemocytes (Fig. 1), whatever the type of haemolymph preparation. Epitopes were also identified in large vesicular inclusions of about 30% of haemocytes, only when they were maintained in conditions of activity, suggesting that the antigen was secreted by haemocytes.

To determine the molecular masses of antigens, the stability of the epitopes was previously verified with regard to the respective conditions of the western blot assay. For this purpose, plasma and haemocyte extracts were assayed in their native and denatured reduced forms by dot-blot analysis. The molecular mass of this antigen was determined by western blot since no epitope was shown to be sensitive to denaturation (Table 2). A protein of 170 kDa, in reduced conditions, was recognized by all the mAbs of Group 1 and was found in plasma and in extracts of haemocytes previously washed to eliminate absorbed plasma proteins (Fig. 4).

Characterisation of haemocyte subpopulation products using group 2 mAbs

Based on IIFA patterns, two mAbs were shown to be specific

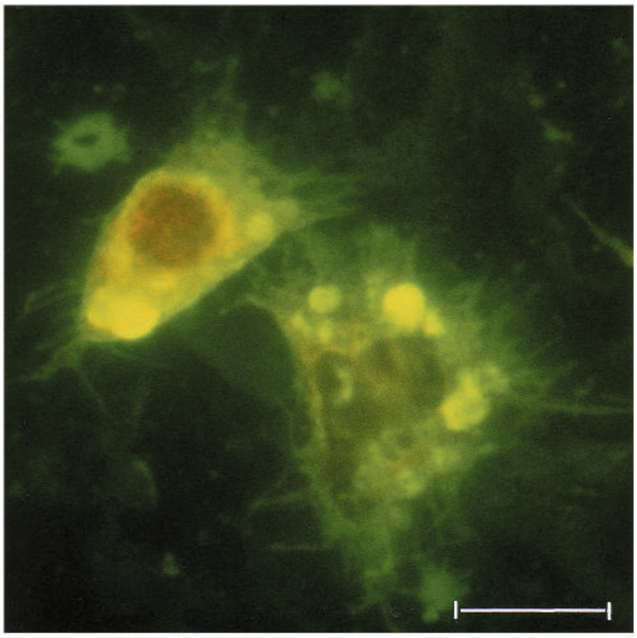

Fig. 1. Immunofluorescent pattern of Group 1 monoclonal antibodies on *P. japonicus* haemocytes. The pan-haemocytic antibody 41B12-1D also reacted against secreted plasma component as shown by the staining of the slide background. Primary culture prepared with AS washed haemocytes (One hour incubation). Note large vesicular inclusions, strongly labelled. Native fluorescence appears red whereas the specific fluorescent labelling appears in yellow (strong reaction) or green. Bar, 10 µm.

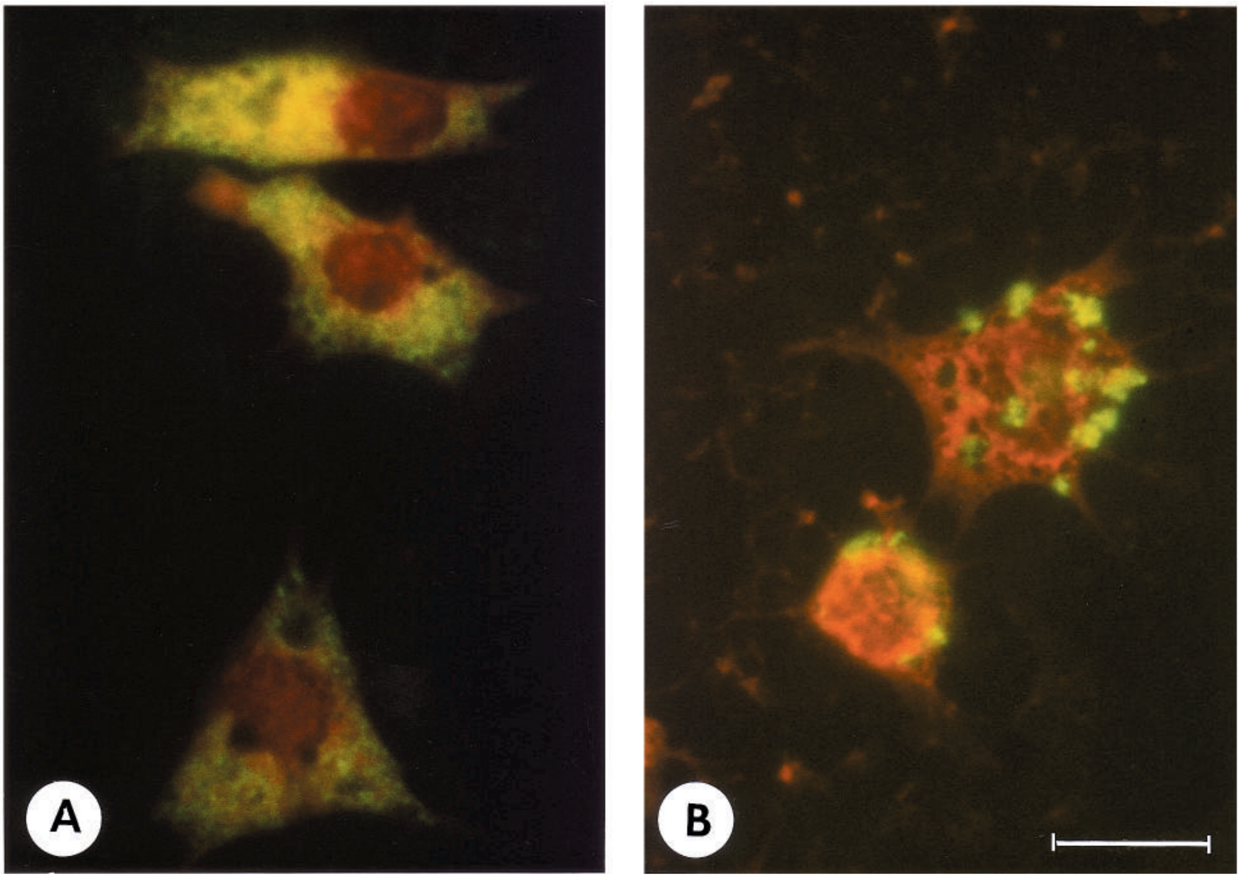

Fig. 2. Immunofluorescent pattern of Group 2 monoclonal antibodies on *P. japonicus* haemocytes. Primary culture of AS pH 7 washed haemocytes: (A) the 40E2-2A antibody reacted with cytoplasmic epitope (×1500); (B) the 40E10-2B antibody stained peripheral cytoplasmic vesicles. Bar, 10 µm.

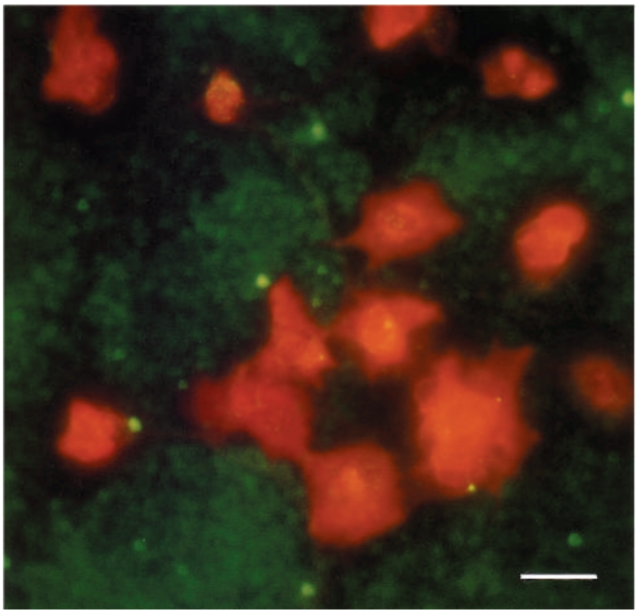

Fig. 3. Reactivity of monoclonal antibody from group 3 against *P. japonicus* haemocyte primary culture prepared with total haemolymph; plasma component was labelled on the slide background when the haemocytes were negative. Bar, 10 µm.

Table 2. Determination of molecular masses of relevant antigens to the mAbs from groups 1, 2 and 3 by western blot or immunoprecipitation assays

		Molecular mass (kDa)	
Groups	mAbs	Plasma	Haemocyte extract
1	35D6-1A	170 (Rd)	170 (Rd)
	4OG8-1B	170 (Rd)	170 (Rd)
	40G1-1C	170 (Rd)	170 (Rd)
	41B12-1D	170 (Rd)	170 (Rd)
2	40E2-2A	142 (Rd)	ND
	40E10-2B	250, 150, 66, 27 (noRd) 27 (Rd)	150, 66, 27 (noRd) 27 (Rd)
3	41D11-3A	180 (Rd)	No relevant antigen in haemocytes
	42C11-3B	180 (Rd)	
	42E8-3C	180 (Rd)	
	44E6-3D	75 (Rd)	

(Rd) reduced conditions; (noRd) nonreduced conditions.

for epitopes present in some of the haemocytes and in the medium when haemocytes were cultivated under conditions permitting their secretion activity.

mAb 40E2-2A showed a strong reactivity with cytoplasmic epitopes exhibited by about 54% of the haemocytes in primary cultures established after washing with Alsever at pH 7 (Fig. 2A), whereas only 9% of haemocytes were immunorecognized when previously washed at pH 4.6.

mAb 40E10-2B recognized, respectively, 40% and 16% of the haemocytes previously washed with Alsever at pH 7 and pH 4.6. The epitope was localized inside cytoplasmic vesicles (Fig. 2B).

It was possible to separate four subpopulations of quiescent haemocytes from *P. japonicus* by isopycnic centrifugation on a discontinuous Percoll density gradient. Each subpopulation was

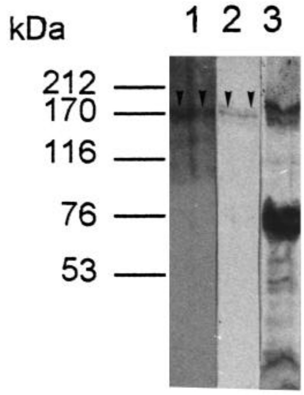

Fig. 4. Western blots of protein extracts prepared from the haemocytes (lane 1) and the plasma (lane 2) of *P. japonicus* with 41B12-1D mAb; lane 3, crude plasma, silver stained.

Table 3. Antigenic characterisation of *P. japonicus* haemocyte subpopulations separated by isopycnic centrifugation on a discontinuous Percoll gradient with monoclonal antibodies from Group 2, 40E2-2A and 40E10-2B

% Percoll	Separated subpopulations	mAb 40E2-2A (%)	mAb 40E10-2B (%)
20-30	small hyalines	0	100
30-40	hyaline/ semi-granular cells	0	85
40-50	granular/semi-granular cells	85	49
50-60	granular cells	100	0

Values are % of positive haemocytes.

collected and then cytocentrifuged to determine in parallel the morphologic and antigenic types of the haemocytes (Table 3).

Hyaline haemocytes

A few small hyaline haemocytes with high nucleo-cytoplasmic ratios were collected at the 20-30% interface of the Percoll gradient. All these cells were recognized by mAb 40E10-2B.

Hyaline and semi-granular haemocytes

At the 30-40% interface, we found a mixture of hyaline haemocytes and large haemocytes with dense and basophilic cytoplasm, constituting about 70% of the cells, which were considered to be semi-granular haemocytes. These separated haemocytes, when reinstated in SSS for primary cultures, displayed quick and intense adherence and spreading. About 85% of the haemocytes of this interface were recognized by 40E10-2B but no haemocytes were labelled by mAb 40E2-2A.

Semi-granular and granular haemocytes

At the 40-50% interface, semi-granular haemocytes were obtained, mixed with the major population of granular haemocytes. About 49% of the haemocytes of this interface were stained by the mAb 40E10-2B while 85% were labelled by the mAb 40E2-2A.

Granular haemocytes

After isopycnic centrifugation of haemolymph on a Percoll gradient, no semi-granular cells were collected at the 50-60%

interface while granular haemocytes strictly banded at the higher densities in the gradient. They were recognizable on cytocentrifuged preparations by their big cytoplasmic granules. Prepared in primary cultures, the granular haemocytes displayed adherence and moderate spreading with formation of pseudopodia emission.

The 50-60% interface corresponded to a homogeneous population of granular haemocytes on the basis of their morphology as well as antigenic features, since 100% of the haemocytes were recognized by 40E2-2A. No staining with mAb 40E10-2B was obtained.

Both mAbs of Group 2, 40E2-2A and 40E10-2B, reacted only against native proteins, which required that we determine their molecular mass after immunoprecipitation.

As described above, the antigen recognized by mAb 40E2-2A was regularly distributed in granular cell cytoplasm and was present in the plasma. The molecular mass of the protein immunoprecipitated from the plasma was estimated at 142 kDa, in SDS-PAGE run under reduced conditions (Fig. 5A; Table 2).

According to dot-blot assay, the antigen recognized by mAb 40E10-2B was present both in plasma and in haemocyte extract. Moreover, it was demonstrated that the protein was secreted by haemocytes since dot-blots were negative for medium from haemocyte-fresh primary cultures and positive for medium from 1 hour-old primary cultures. From haemocyte extract, it was established that the protein recognized by mAb 40E10 is present in different forms with molecular masses of 150 kDa, 66 kDa and 27 kDa. From plasma, a supplementary form was identified with a molecular mass of 250 kDa (Fig. 5A). After denaturation, the epitope was found to be associated with the 27 kDa form (Table 2).

Characterisation of plasma proteins by Group 3 mAbs

All the mAbs of Group 3 were reactive only against native proteins of plasma non represented inside the haemocytes. mAb 44E6-3D recognized a protein of 75 kDa under reducing conditions (Fig. 5A) and cross-reacted against haemocyanin extract.

The three other mAbs immunoprecipitated a protein with an apparent molecular mass of 180 kDa under reduced conditions (Fig. 5B; Table 3).

Cross-reactivity studies against haemolymph of *P. japonicus*, *P. vannamei* and *P. indicus*

The reactivity of mAbs against haemolymph of *P. vannamei* and *P. indicus* was investigated by IIFA on haemocyte preparations and/or by dot-blotting on plasma, according to the nature of the antigen determined for *P. japonicus* (Table 4).

All the mAbs of Group 1 reacted against haemocytes of both species, but the immunofluorescent patterns were less intense in comparison to those of *P. japonicus*.

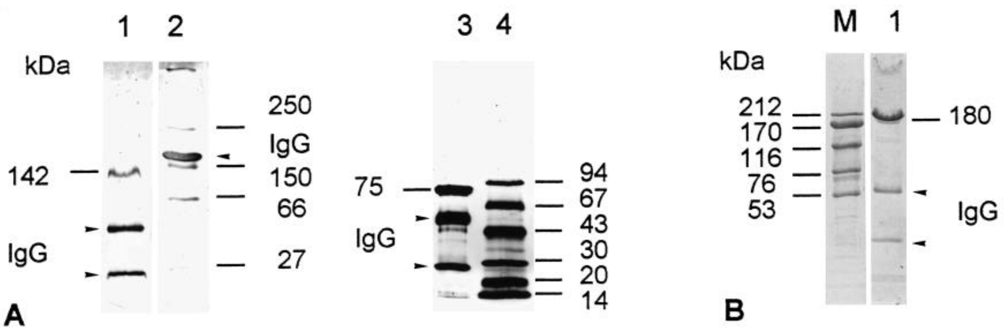

Fig. 5. Electrophoresis patterns of proteins immunoprecipitated by different mAbs. (A) (Phast-gel 4-15%, Pharmacia) lane 1, plasma protein precipitated with 40E2-2A, reduced conditions; lane 2, plasma protein precipitated with 40E10-2B, unreduced conditions; lane 3, plasma protein precipitated with 44E6-3D, reduced conditions; lane 4, molecular mass markers. (B) (12% gel) lane M, molecular mass markers; lane 1, plasma protein precipitated with 42E8-3C, reduced conditions. The gels were stained with Coomassie Brilliant Blue.

Table 4. Antigenic relationship between haemocytes and plasma of *P. japonicus* with those of *P. vannamei* and *P. indicus*

		P. japonicus		*P. vannamei*		*P. indicus*	
Groups	mAbs	Haemocytes	Plasma	Haemocytes	Plasma	Haemocytes	Plasma
1	35D6-1A	+++	++	+	ND	+	+
	40G8-1B	+++	++	+	ND	+	+
	40G11-1C	+++	++	+	ND	+	+
	41B12-1	+++	++	+	ND	+++	++
2	40E2-2A	++	++	++	++	+	+
	40E10-2B	++	+	+	ND	++	+
3	41D11-3A	−	++	−	±	−	ND
	42C11-3B	−	+	−	±	−	+
	42E8-3C	−	+	−	±	−	ND
	44E6-3D	−	++	−	−	−	−

Reactivity against haemocytes performed by IIFA and reactivity against plasma performed by dot-blot; number of + refers to the intensity of reactivity. ND, not determined.

Similar results were obtained with mAbs of Group 2 for haemolymph samples from the different shrimp species.

The three mAbs of Group 3, specific for a 180 kDa plasma protein of *P. japonicus,* cross-reacted with plasma of *P. vannamei*, but to a lesser extent. Only mAb 44E6-3D, which recognizes the haemocyanin of *P. japonicus*, was found to be specific.

DISCUSSION

Although monoclonal antibodies have been proved essential as reagents for characterising immune cells and humoral factors in vertebrates, few studies have produced and used these antibodies in invertebrate immunology. Using hybridoma technology, monoclonal antibodies have been recently prepared for the haemocytes of ascidians (Schlumpberger et al., 1984), annelids (Porchet-Hennere, 1990), gastropods (Yoshino and Granath, 1983; Dikkeboom et al., 1985), marine bivalves (Noël et al., 1991), and insects (Trenczek, 1991; Mullett et al., 1993). These antibodies have been primarily used to recognize haemocyte subpopulations (Noël et al., 1994); they can also permit the study of ontogeny and transformation processes.

This paper, as far we are aware, reports for the first time on the production and the used of monoclonal antibodies to characterize shrimp haemolymph components.

From a technical point of view, it appeared that the use of haemolymph primary cultures prepared in microplate wells was very suitable for screening hybridomas by IIFA. First, this screening led the selection of hybridomas specific for haemocytes and/or plasma components. Moreover, this assay permitted the quick screening of a large number of hybridoma supernatants by taking advantage of the ease of performing all the steps of IIFA in microplates and the observation with microscope at high magnification of the primary cultures after inverting the microplates (Stitz et al., 1988).

The four mAbs of Group 1 are pan-haemocytic markers that may be useful for investigations on hematopoiesis and for histological studies of the immune process because these mAbs recognize any cell irrespective of haemocyte lineage. All these mAbs recognize a 170 kDa protein secreted in the plasma. Recently, a protein with a monomeric form of 190 kDa was purified from the plasma of crayfish (Hergenhahn et al., 1988) and identified as an α-macroglobulin. The four mAbs of Group 1 reacted with plasma of other shrimp species. Preliminary results of positive cross-reactions with human and crayfish α-macroglobulin (K. Soderhäll, personal communication) suggest that the 170 kDa protein could be the monomeric form of shrimp α-macroglobulin. The purification of this molecule will be facilitated by the availability of mAbs, either for affinity chromatography or for following the molecule during the purification process according to any standard technique. It must be noted that α-macroglobulin of invertebrates has similarities with the C3 and C4 proteins of the complement system (Sottrup-Jensen et al., 1990). In the limule, *Limulus polyphemus*, it was shown that α-macroglobulin is involved with a C-reactive protein in cytolytic processes (Armstrong et al., 1993).

The shrimp haemocyte types which can be morphologically identified as hyalin and semi-granular cells or as granular cells, correspond to two distinct antigenic populations, respectively, labelled by the mAbs 40E10-2B and 40E2-2A. However, unfortunately, the epitopes are not associated with the cell membranes, and thus these mAbs cannot be used as reagents for specifically purifying the haemocyte subpopulations by immunoaffinity, as is done with vertebrate blood cells (Naume et al., 1991).

Because the antigens recognized by these mAbs are secreted by haemocytes, their purification could also be greatly facilitated for *P. japonicus*, as well as for *P. vannamei* and *P. indicus* since the mAbs cross-reacted.

The hyaline and semi-granular haemocytes, stained by the mAb 40E10-2B, seem to secrete a protein with 27 kDa sub-units, which could be organized in polymeric forms or complexed with other proteins. These characteristics may relate the *P. japonicus* 27 kDa protein with lectins purified in other crustacean species. In the balane, *Balanus balanoides*, a glycoprotein of 330 kDa has been purified and shown to be constituted by sub-units of 70 kDa and 26 kDa (Ogata et al., 1983). A hemagglutinin of 420 kDa, which is constituted with 80 and 65 kDa sub-units, has been purified in the crayfish *Pacifastacus leniusculus* (Kopacek et al., 1993a). In the shrimp *P. monodon*, the monodin is a lectin of 420 kDa constituted with 27 kDa sub-units (Ratanapo and Chulavatnatol, 1990).

The granulocytes are characterised by a protein of 142 kDa which is found in the plasma and also strongly present in the cell cytoplasm as shown by the specific marker, mAb 40E2-2A. First of all, this mAb will be of great use to study the phylogenetic relationship between granular cells and the other haemocyte types morphologically identified as hyalines and semi-granular cells. The nature of the reactive 142 kDa protein is unknown but the specific mAb will be important in further investigating its role and consequently in the understanding of shrimp granular cell functions.

The mAbs of Group 3 are specific for plasma proteins, which may mean that they are not secreted by haemocytes or that they are antigenically modified after their secretion by haemocytes. The protein recognized by mAb 44E6-3D corresponds to haemocyanin, the respiratory protein which is the major constituent since it represents about 60% of the haemolymph proteins. From the immunostaining pattern of the haemolymph, it was confirmed that in penaeids the haemocyanin is not present inside the haemocytes, the haemocyanin synthesis site in other decapods having been localised in the hepatopancreas (Gellisen et al., 1991). Beside the haemocyanin, the most abundant haemolymph protein is the coagulogen, also designated as a clotting protein. The three other mAbs from Group 3 probably recognize the shrimp coagulogen, antigenically detected as a 180 kDa monomeric form. This protein is highly represented in shrimp plasma and disappeared from the haemolymph after coagulation as demonstrated by SDS-PAGE (data not shown). In crustaceans, a plasma clotting protein consisting of two disulfide-linked 210 kDa subunits was shown to be involved in clotting reactions (Durliat, 1985). In crayfish *P. leniuscus*, the purified clotting protein monomer has been shown to have a molecular mass of 180 kDa (Kopacek et al., 1993b). The coagulation, by preventing blood loss but also by entrapping pathogenic microorganisms, is considered in crustaceans as an important defense system (Söderhäll and Cerenius, 1992).

In conclusion, a variety of proteins possibly involved in the shrimp immune responses have been identified and detected by specific mAbs. The availability of two mAbs, specific markers of distinct haemocyte types morphologically identified as semi-granular and granular haemocytes, will now permit us to develop studies on haemocyte lineage, and also on hemogram composition. Indeed, the antigenic characterisation of hemograms constitutes an essential component in clinical studies of the shrimp immune state and of any modification revealing immunodeficiency. Furthermore, on the one hand, the proteins identified in this study can be purified by immuno-affinity. On the other hand, the mAbs will also be very useful for characterising the corresponding genes by their use as specific probes for screening cDNA libraries cloned in expression vectors.

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