

## Characterisation of monoclonal antibodies to haemocyte subpopulations of tiger shrimp (*Penaeus monodon*): immunochemical differentiation of three major haemocyte types

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In order to study immune cellular effectors in tiger shrimp (*Penaeus monodon*), monoclonal antibodies (mAbs) raised from haemocytes could be a potential tool for separating and identifying haemocyte subpopulations. In this study, four mAbs – Z5E10 (IgG2a), Z6A5 (IgG1), Z6A6 (IgG1), and Z6H8 (IgG2b) – were produced by immunising balb/c mouse with a high-density granular cell (GC) suspension prepared from tiger shrimp through negative selection with concanavalin A-linked beads. Haemocyte reactivities were determined by means of immunoenzyme staining, and the molecular masses of antigens were analysed by Western blotting. Z5E10 mAb showed reactivity with both GCs and semigranular cells (SGCs) and recognised a 29 kDa antigen. Z6A6 specifically bound to SGCs while recognising a 163 kDa antigen. The other two mAbs (Z6A5 and Z6H8) separately reacted with SGCs and were able to recognise two antigens which were larger than 205 kDa under non-reducing conditions but could not recognise them under reducing conditions. Data from a periodate oxidation assay revealed that Z6A6 antibody was specific for one glycoprotein, while the antigens recognised by the other three mAbs consisted of protein molecules. According to these results, it was concluded that the Z5E10 antigen and the Z6A6 antigen were different not only from each other, but also from those recognised by the other two mAbs. Furthermore, additivity test results showed that the additivity index value for the mAb pairs Z6H8-Z6A6 (29%) was higher than that of Z6A5-A6A6 (0%). It is suggested that Z6A5 and Z6H8 mAbs could be specific for non-identical epitopes. Following the negative selection with mAb-linked beads, an increase in hyaline cell density in haemocyte suspension was observed which suggests that the epitopes recognised by the four mAbs were located on the surface of haemocytes and that these mAbs may be useful when they are employed to label and separate haemocyte subpopulations for further study of haemocyte functions.

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**Key words:** crustacea, monoclonal antibody, shrimp haemocyte, granulocyte, semigranulocyte, hyaline cell.

### I. Introduction

Haemocytes play an essential role in protecting crustacean, insects and other invertebrates from parasitic and pathogenic infections. Their primary

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response mechanisms include phagocytosis, nodule formation, encapsulation, and cytotoxicity (for a review, see Söderhäll & Cerenius, 1992). Several types of haemocytes have been identified based on light and electron microscopy. However, identification by morphology alone presents some experimental difficulties because the morphologies of granular cells (GCs) and semigranular cells (SGCs) in particular are variable. Thus, an accurate means of identifying biochemical markers on the cell surface for haemocyte types would allow identification based on molecular rather than morphological criteria and provide researchers with a useful analytical tool. Limited success in this area has been achieved through the use of lectins which bind to cell surface determinants (Nappi, 1973; Nappi & Christensen, 1986; Rizki *et al.*, 1983; Richards *et al.*, 1989). A more promising approach appears to be the use of monoclonal antibodies (mAbs) to classify cells according to antigenic differences. A similar approach has been successfully used to identify differences among surface antigens in mammalian lymphocytes (for examples, see Milstein & Lennox, 1980; Sayers *et al.*, 1993). However, the use of such markers in recent crustacean research has only appeared on the studies of shrimp haemocytes and plasma components by Rodriguez *et al.* (1995).

A number of shrimp diseases are associated with mariculture, mostly caused by viral or bacterial etiologies. In Taiwan, significantly higher mortality rates due to these pathogens have been reported since 1987, leading to a marked increase in researches on the immune mechanisms of shrimp and other economically significant species. In previous studies it has been shown that tiger shrimp (*Penaeus monodon*) responses to the invasion of foreign materials (e.g. yeast  $\beta$ -glucan, lipopolysaccharide and *Vibrio* cells) include phagocytosis, nodule formation, activation of prophenoloxidase system, and production of superoxide anion and hydrogen peroxide (Sung & Song, 1996; Sung *et al.*, 1996; Song & Hsieh, 1994). Previous studies have noted varying percentages of each haemocyte type in the haemolymph of individual shrimp. The observation is very important for determining the role of each type of haemocyte in shrimp defence reactions.

Three haemocyte types have been identified from microscopical observations of tiger shrimp haemolymph: (1) GCs, with a large number of large granules and a low nuclear-to-plasma ratio; (2) SGCs, in much smaller quantities, and (3) hyaline cells (HCs), without granules but with a high nuclear-to-plasma ratio (Sung *et al.*, 1996; Song *et al.*, 1998). It has also been shown that either concanavalin A (ConA) or peanut agglutinin (PNA) is capable of binding to most tiger shrimp HCs and a few SGCs, but not to their GCs. These haemocytes can be separated into three subpopulations, with approximately 95% of the HCs obtainable through a combination of lectin staining and percoll gradient centrifugation (Song *et al.*, 1998). In order to study immune cellular effectors in shrimp, mAbs have been developed for *Penaeus monodon* according to hybridoma technology described by Köhler and Milstein (1975).

Here the production and characterisation of a set of four mAbs which may be used for the antibody-based identification of GC, SGC, and HC haemocyte types in tiger shrimp is reported. These mAbs also have the potential to be used as tools for separating haemocyte subpopulations or for identifying and

isolating molecules that are important in the defence mechanisms of shrimp haemocytes. If successful, this procedure will help to define the roles of different haemocyte types in tiger shrimp immune functions.

## II. Materials and Methods

### ANIMALS

Apparently healthy tiger shrimp (*Penaeus monodon*) weighing approximately 25 to 30 g each were purchased from a local market and acclimated in a 120-L plastic container of pond water with  $2.5\% \pm 0.3\%$  salinity at  $25^\circ\text{C}$  for a minimum 2 days prior to bleeding. Stock density was maintained at 10 individuals  $\text{L}^{-1}$ .

### MONOCLONAL ANTIBODIES (mAbs)

Three mAbs were employed as both negative and positive controls: Anti-15.1 mAb raised from a N-terminal domain of the 135 kDa lymphoid of the human 4.1 protein (Tang *et al.*, 1990) (a gift from Dr Steve Roffler, Institute of Biomedical Science, Academia Sinica, Taipei, Taiwan) failed to recognise tiger shrimp haemocytes, while T5D4 and Y1E9 raised from whole haemocytes from tiger shrimp (gifts from Mr Pei-Hsin Hsu, Department of Zoology, National Taiwan University) were capable of recognising all tiger shrimp haemocytes.

### HAEMOCYTE PREPARATION

Using a 23-gauge hypodermic needle on a 2.5-mL syringe, approximately 1–2 mL of haemolymph was collected from individual shrimp; the syringe contained 0.5 mL of 10% neutral buffered formalin (NBF) solution (10 mL of 37% formaldehyde solution in 0.01 M PBS, pH 6.9). Following a procedure described in Song and Hsieh (1994), these haemolymph samples were centrifuged at  $300 \times g$  for 10 min at  $4^\circ\text{C}$  and washed twice with modified complete Hank's balanced salt solution (MCHBSS) (10 mM  $\text{CaCl}_2$ , 3 mM  $\text{MgCl}_2$ , 5 mM  $\text{MgSO}_4$ , 24 mg  $\text{mL}^{-1}$  HBSS (Gibco). Cell pellets were resuspended in MCHBSS containing 10% DMSO, and adjusted to a concentration of  $10^7$  cells  $\text{mL}^{-1}$ , then stored at  $-70^\circ\text{C}$ , prior to examination.

To ensure the establishment of a high-density granular cell (GC) suspension as an antigen source, most hyaline cells were removed from the haemocyte samples via negative selection with ConA-linked beads (That is, non-ConA-bound haemocytes were collected following bead removal) which were prepared as described by Song *et al.* (1998). In test tubes, ConA-linked beads were mixed with the haemocyte suspension to a ratio of 40–70 beads to 1 cell; following agitation in an end-over-end rotator at  $10 \text{ rpm min}^{-1}$  for 30 min at  $4^\circ\text{C}$ , the test tube were placed in a concentrator for 2 min. Supernatant containing non-ConA-bound haemocytes was collected and centrifuged at  $300 \times g$  for 10 min and washed twice with 0.01 M PBS. The non-ConA-bound haemocytes which were used as an immunogen were then suspended to  $0.5 - 1 \times 10^7$  cells  $\text{mL}^{-1}$  of 0.01 M PBS.

## IMMUNISATION AND PREPARATION OF HYBRIDOMAS

Eight-week-old Balb/c mice were immunised via intraperitoneal injection of 0.5 mL haemocyte suspension ( $1 \times 10^7$  cells  $\text{mL}^{-1}$ ) consisted of 78% GCs, 13% semigranular cells (SGCs) and 9% hyaline cells (HCs); three concurrent booster injections (intraperitoneal at  $5 \times 10^6$  cells  $\text{mL}^{-1}$  and intravenous at  $1 \times 10^6$  cells  $\text{mL}^{-1}$ ) were given at 2 week intervals. Three days following the final injection, primed spleen cells were prepared and fused with FO myeloma cells using polyethylene glycol (PEG 1500, 50%) (Köhler & Milstein, 1975; Lane, 1985).

Antibody producing hybridomas were pre-screened for specificity by enzyme-linked immunosorbent assay (EIA). To increase the haemocyte adsorption, EIA plate wells were treated with 50  $\mu\text{L}$  of 1% poly-L-lysine solution at room temperature (RT) for 30 min. After removal of this solution, each well was coated with 50  $\mu\text{L}$  fixed haemocyte suspension ( $5 \times 10^6$  cells  $\text{mL}^{-1}$ ); plates were centrifuged at  $300 \times g$  for 10 min and the supernatant was removed. After blocking each well with a solution of 7.5% glycine and 1% BSA in 0.01 M PBS for 30 min at RT and three additional washes with 0.01 M PBS, the wells were blocked with 300  $\mu\text{L}$  of 1% skim milk-PBS at 4° C for overnight. Another set of three washes was then followed by the addition of 50  $\mu\text{L}$  of hybridoma solution, after which plates were incubated at 37° C for 1 h. Three washes with PBST (PBS containing 0.05% Tween 20) were followed by a single washing with PBS before measuring reactivity with alkaline phosphatase-conjugated goat antimouse Ig antisera (AP-GAM Ig, Vector, 1:2000 dilution in PBST). After adding 200  $\mu\text{L}$  of substrate solution (10 mg of 4-Nitrophenylphosphate in 10 mL of a solution containing 0.01%  $\text{MgCl}_2$  and 0.106% diethanolamine), the optical density of each well was recorded at 405 nm with an EIA reader (Emax, Precision microplate reader, Molecular Devices). Selected hybridomas were cloned three times via limited dilution to ensure the establishment of a single colony per well. mAbs were then isotyped with a haemagglutination test kit (Serotec) for heavy chains and with a sandwich ELISA using peroxidase-conjugated goat anti-mouse light chain ( $\kappa$  and  $\lambda$ ) antisera (Serotec) for light chains.

## IMMUNOENZYME STAIN

Two haemocyte samples were prepared: formalin-fixed haemocytes were prepared using the procedure for haemocyte preparation described above; and unfixed haemocytes were collected from shrimp haemolymph with a 2.5 mL syringe containing anticoagulant buffer (0.01 M Tris-HCl, 0.25 M sucrose, 0.1 M tri-sodium citrate; pH 7.6) (Sung *et al.*, 1994). One hundred microlitres of fixed and unfixed haemocyte suspension ( $10^8$  cells  $\text{mL}^{-1}$  of PBSB, consisting of 0.01 M PBS with 0.1% bovine serum albumin added) respectively were placed on microslides and air-dried. After adding 1 mL of hybridoma solution, slides were incubated at RT for 1 h, rinsed three times with PBSB, and incubated a second time for 1 h with 0.5 mL horseradish peroxidase-GAM Ig diluted to 1:2000 in PBSB. Following the addition of colour developer (5 g of 3,3'-diaminobenzidine and 10  $\mu\text{L}$  of 30% hydrogen peroxide in 10 mL of 0.2 M Tris-HCl), slides were blotted dry and counterstained with Evan's blue prior to

observation with a light microscope (BH-2, Olympus). In this experiment, mAb T5D4 and Y1E9 which replaced hybridoma solution were used as a mAb positive control, and preimmune serum was used as a negative control.

#### ADDITIVITY TEST

The indirect EIA additivity test described by *Friguet et al. (1983)* was used to determine whether the studied mAbs were able to recognise their corresponding epitopes. After haemocytes were coated onto 96-well microplates, a pair of excess mAbs (for example, Z6A5 and Z6A6) were added in combination to additive group wells and individually to non-additive group wells. The additivity of enzyme activity is observable when mAbs bind to different epitopes. An additivity index (AI) (*Friguet et al., 1983*) was used to quantify antibody pairs as follows:

$$AI = [(2A_{1+2}/A_1 + A_2) - 1] \times 100\%$$

where  $A_1$ ,  $A_2$  and  $A_{1+2}$  are the optical densities at 405 nm reached during the EIA test for the first mAb, second mAb, and the two mAbs combined, respectively. If two mAbs randomly bind at the same site – that is, share the same specificity –  $A_{1+2}$  should be equal to the mean value of  $A_1$  and  $A_2$ , leading to an AI value of zero. However, if the two mAbs bind at independent sites,  $A_{1+2}$  should be equal to the sum of  $A_1$  and  $A_2$ , thus creating an AI of 100%. Mouse polyclonal anti-haemocyte serum was used as a positive control throughout the additivity test.

#### WESTERN BLOTTING

Both fixed and unfixed haemocytes ( $2 \times 10^7$  cells) were suspended separately in 100  $\mu$ L lysis buffer consisting of two solutions: solution A with 1% (v/v) Nonidet P-40, 0.15 M NaCl, 25 mL of 0.2 M Tris-HCl (pH 8.0), and 0.186 g EDTA, and solution B with 0.17 g phenylmethylsulfonyl-fluoride and 2 mL methanol. A mixture of 1 mL of solution A and 20  $\mu$ L of solution B was made just before use. Following 1 h incubation on ice, cell debris was removed by centrifugation at  $9,000 \times g$  for 5 min. Supernatant was kept at  $-70^\circ \text{C}$  until use.

Lysis samples containing extracted membrane-proteins were run on 4–15% SDS-polyacrylamide gels (Bio-Rad Mini PII ready gel) under both reducing (with the presence of 2% (w/v) SDS and 0.71 M  $\beta$ -mercaptoethanol) and non-reducing (with the presence of 2% SDS) conditions. Proteins were electrotransferred onto a polyvinylidene difluoride membrane (PVDF, Millipore) at  $5 \text{V h}^{-1}$  for 20 min in a transfer buffer consisting of 25 mM Tris (pH 8.3), 192 mM glycine, and 10% methanol.

For antibody labelling, the PVDF sheets were washed in 0.01 M PBS and blocked with 3% gelatin (Difco) in Tris-buffered saline (TBS) (10 mM Tris base at pH 7.5, 0.85% NaCl) at  $37^\circ \text{C}$  for 30 min. The hybridoma culture media were allowed to react with the blots for 1 h at RT under gentle agitation. Membranes were washed three times in TTBS, then incubated with AP-GAM Ig at RT for 1 h. After three additional washes, the membranes were 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<sub>2</sub>, 100 mM Tris-HCl, pH 9.5) at RT until bands appeared. Reactions were stopped with 2 mM EDTA in PBS. Developed blots were washed in deionized water, air-dried, and stored in darkness.

#### PERIODATE OXIDATION

The periodate oxidation procedure, which was used to oxidise the sialic acid residues of carbohydrate, as described by Woodward *et al.* (1985) was employed to determine whether or not the epitopes recognised by the mAbs used in these experiments were glycosylated. The haemocyte-coated 96-well microplates described above were washed with 50 mM sodium acetate buffer (pH 4.5). Following incubation with 20 mM periodate acid in sodium acetate buffer for 1 h in the dark, samples were washed with sodium acetate buffer before incubation with 50 mM sodium borohydrite in 0.01 M PBS at RT for 30 min. After three washes, the hybridoma culture media were added and the resulting mixture incubated at 37° C for 1 h. Finally, samples were assayed by EIA. Here, mAb T5D4 and Y1E9 were used in replacing the hybridoma solution as control.

#### HAEMOCYTE SEPARATION BY NEGATIVE SELECTION

All separation steps were performed at 4° C. Haemocyte suspensions ( $1 \times 10^7$  cells mL<sup>-1</sup>) were incubated with an equal volume of hybridoma culture media for 30 min, then haemocytes were pelleted and washed with 0.1% (w/v) BSA in PBS. The mAb-labelled haemocytes ( $5 \times 10^5$  cells) were incubated with 50  $\mu$ L of beads coated with GAM Ig-conjugate (Dynabeads M-450, A04810) for 30 min under gentle agitation, the test tube were placed in a concentrator for 2 min. Supernatant containing non-mAb-bound haemocytes were collected following bead removal, and their morphology studied with a light microscope. Percentages of each haemocyte type were then determined and recorded.

### III. Results

#### SCREENING AND CHARACTERISATION OF MONOCLONAL ANTIBODIES

For this study, four hybridomas capable of secreting antibodies to one or two classes of haemocytes were isolated. The four mAbs were designated Z5E10, Z6A5, Z6A6, and Z6H8, and were isotyped as IgG2a, IgG1, IgG1, and IgG2b heavy chains, respectively; the four mAbs were additionally isotyped as kappa light chains. Using immunoenzyme staining, it was found that the Z5E10 mAb recognised both fixed GCs and SGCs (Fig. 1a), however, the Z6A5, Z6A6, and Z6H8 mAbs only labeled fixed SGCs specifically (Fig. 1b-d). As for Y1E9, which was used as a positive control, it reacted with whole haemocytes from tiger shrimp (Fig. 1e). The results from unfixed haemocytes using an identical procedure showed the same staining characteristics. An additivity test was performed to determine whether the epitopes recognised by the latter three mAbs were identical. Test results showed that the optical densities ( $A_1 + A_2$ ) of the additive group wells (Z6A5-Z6A6 or Z6A5-Z6H8) in which Z6A5

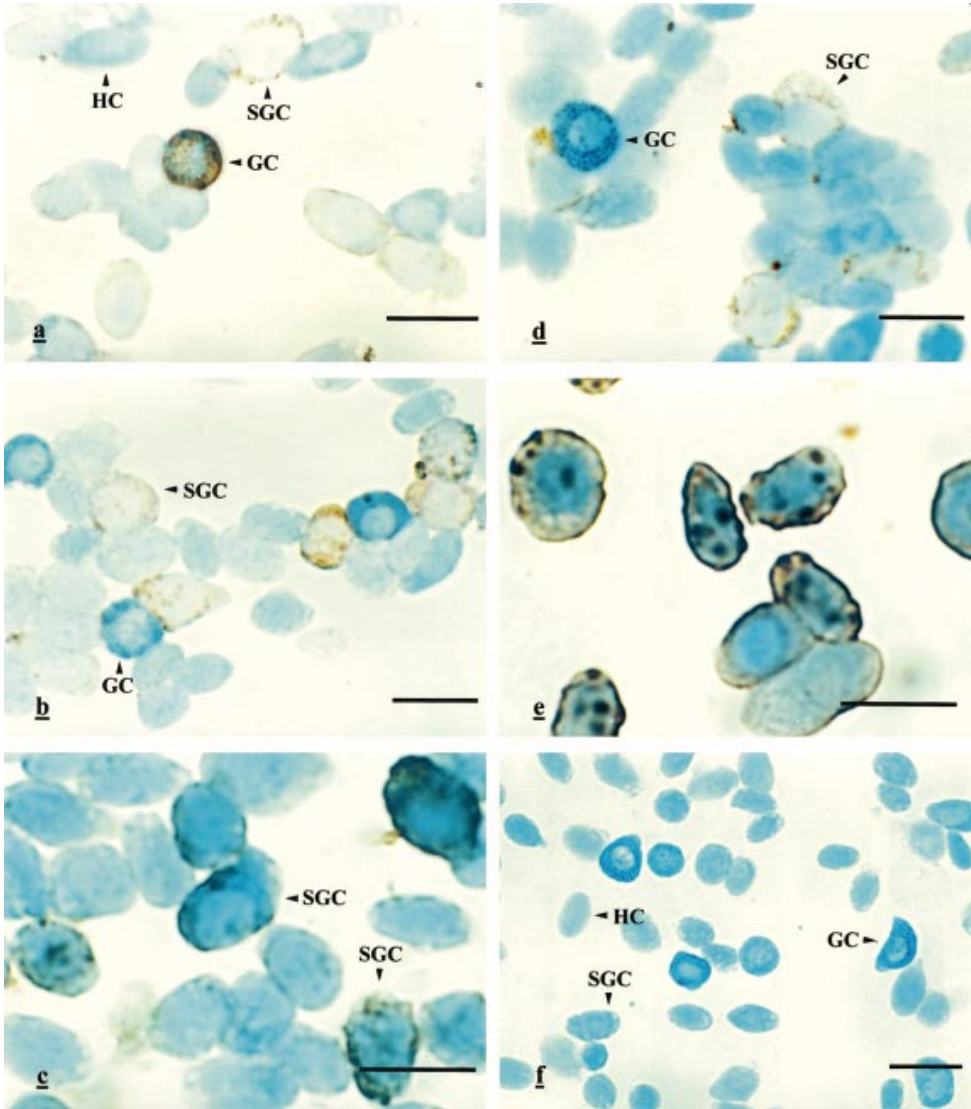


Fig. 1. Light micrographs of fixed haemocytes from *Penaeus monodon* labelled with (a) Z5E10; (b) Z6A5; (c) Z6A6; (d) Z6H8; (e) Y1E9; or (f) mouse preimmune serum from immunoenzyme staining and counterstaining with Evan's blue. Brown indicates mAb-labelled haemocytes. GC, granular cell; SGC, semigranular cell; HC, hyaline cell. Bar=10  $\mu$ m.

was the first mAb were identical to the optical densities ( $A_t$ ) for Z6A5 alone (Fig. 2), and that the additivity indexes (AIs) for Z6A5-Z6A6 and Z6A5-Z6H8 pairs were 0% and 1%, respectively. The optical densities for the mAb pairs Z6A6-Z6A5 and Z6A6-Z6H8 were higher than that for Z6A6 alone, with estimated AIs at 48% and 44%, respectively. Additivity index results when Z6H8 was the first mAb were -23% for Z6H8-Z6A5 pair and 29% for the Z6H8-Z6A6 pair.

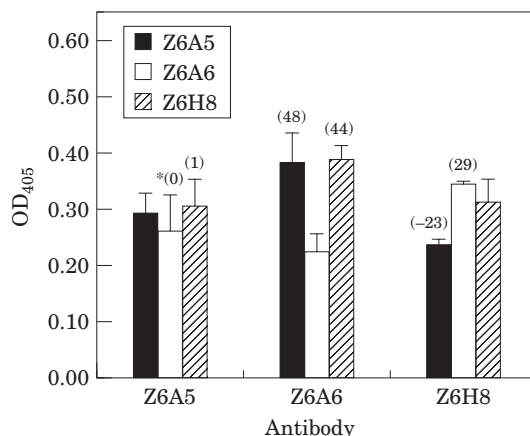


Fig. 2. Epitope analysis data from indirect EIA additivity test. \*, Additivity index (AI).

#### EPITOPES RECOGNISED BY mAbs

Results from the western blot analysis showed the approximate molecular masses of the relevant antigens to which the mAbs bound. Z5E10 and Z6A6 recognised the antigens prepared from either fixed or unfixed haemocytes in both non-reduced and reduced forms, and the respective masses of antigens were 29 kDa and 163 kDa under reducing condition. However, the antigens recognised by the other two mAbs were not detectable under reducing conditions, and their masses were larger than 205 kDa when run under non-reducing conditions (Fig. 3).

Haemocyte samples were treated with periodate acid to determine whether or not the mAbs reacted with glycosylated epitopes. Data from EIA showed that the optical densities for the reactivities of Z6A6, T5D4 or Y1E9 to treated haemocytes were lower than those to untreated haemocytes. It represents that the configuration of epitopes recognised by these three mAbs were destroyed by periodate treatment. On the other hand, no differences were found in the optical densities for reactivities of Z5E10, Z6A5 or Z6H8 to either treated or untreated haemocytes (Fig. 4).

#### HAEMOCYTE SEPARATION

The densities of three subpopulations in the haemocyte suspensions were clearly altered by a process of negative selection using mAb-coated beads (Table 1). From an original HC figure of 73%, negative selection with Z5E10, Z6A5, Z6A6 and Z6H8 antibodies produced HC increased to 87%, 82%, 80% and 87%, respectively, at the same time, the original SGCs figure of 9% dropped to 6.5%, 0.6%, 0% and 0%, also respectively. A decrease in GC percentage from 17% to 6.5% was observed after haemocyte samples were treated with Z5E10-coated beads. Measurements of all three haemocyte subpopulations revealed no differences between negative mAb-treated and untreated haemocyte samples.

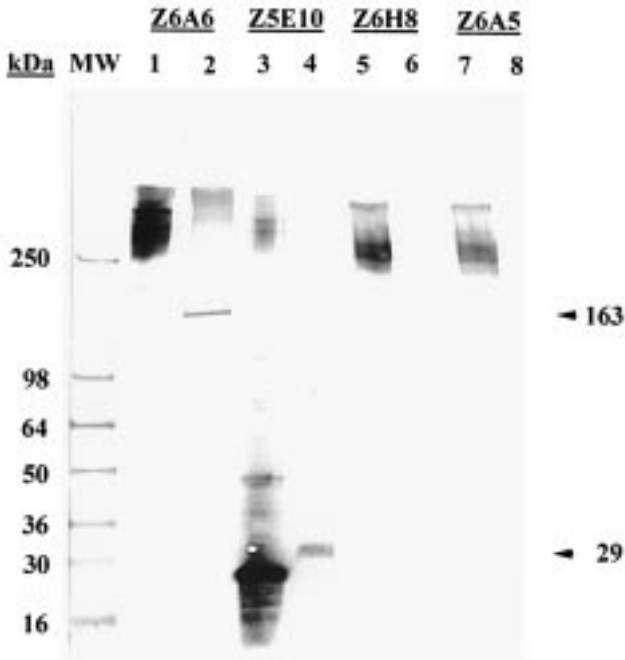
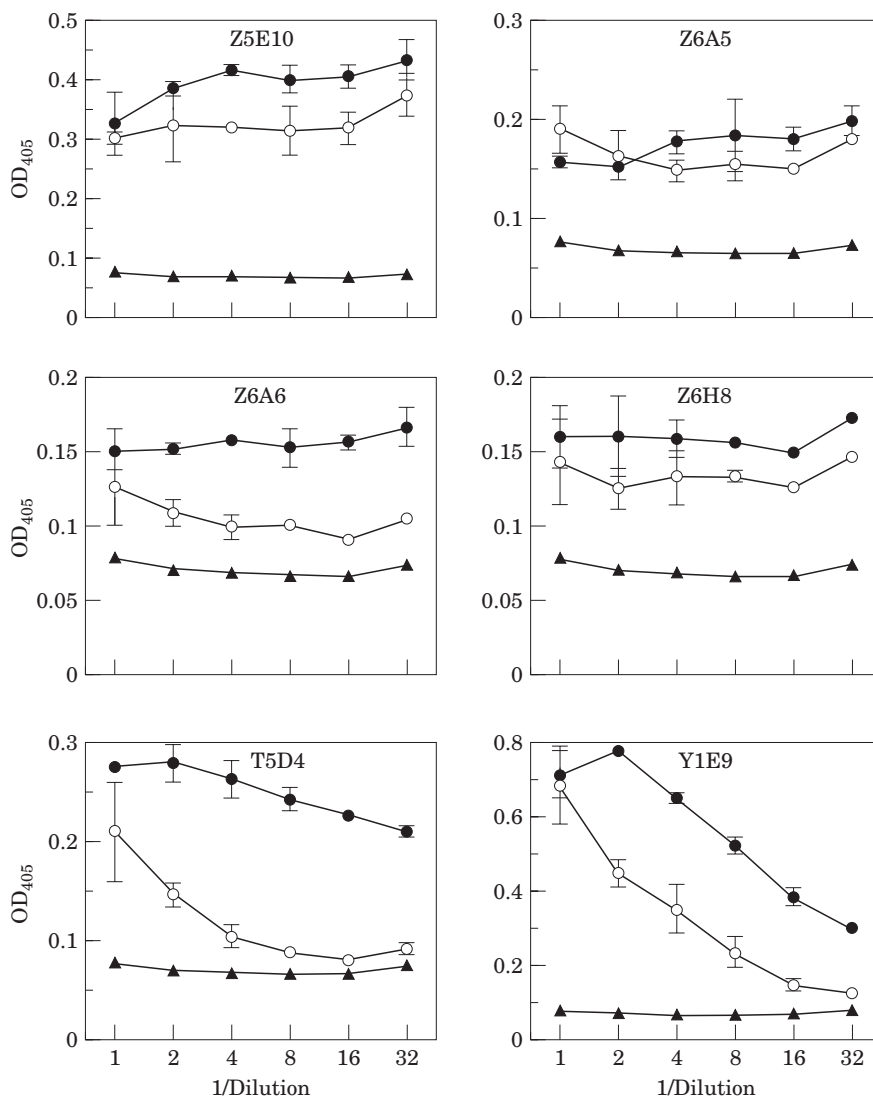


Fig. 3. Western blots of membrane proteins extracted from live haemocytes stained with Z6A6 (lane 1, 2), Z5E10 (lane 3, 4), Z6H8 (lane 5, 6), or Z6A5 (lane 7, 8). Odd-numbered lanes represent non-reducing conditions, and even-numbered lanes represent reducing conditions. Numbers to the left of lane 1 indicate size in kDa of standards.

#### IV. Discussion

As with other invertebrates, shrimp haemocytes can break down easily, change shape, and degranulate during *in vitro* culturing or following centrifugation (Martin & Graves, 1985; Söderhäll & Smith, 1983; Söderhäll & Cerenius, 1992). In the present study, formaldehyde-fixed haemocytes were used separately as immunogen for mAb development and as haemocyte samples for most assays. They were prepared from cultured shrimp and stored in MCHBSS containing 10% DMSO at  $-70^{\circ}\text{C}$  prior to examination. Willott *et al.* (1994) obtained one mAb raised from haemocytes of *Manduca sexta* which can recognise fixed oenocytoids, but did not recognise live cells. This indicated that, following formaldehyde-fixed treatment, some protein conformations can be altered. In this study, both fixed- and unfixed-haemocyte samples were analysed by immunoenzyme stain and western blotting. Results from the two assays were not different between fixed and unfixed haemocytes.

To date, few mAbs have been produced which react to invertebrate haemocytes; the number is even smaller for those which react to crustaceans. Rodriguez *et al.* (1995) established a set of ten mAbs from immunised mice with whole haemolymph containing both haemocytes and plasma from kuruma prawn (*P. japonicus*). They were classified into three groups: those reacting with a common antigen present on all the haemocyte types and in



*Fig. 4.* Glycosylation analysis of epitopes recognised by different mAbs via periodation oxidation. Haemocyte samples were treated with periodate acid in sodium acetate buffer (●), or sodium acetate buffer alone (○). Background optical values (▲).

plasma, those reacting with different haemocyte subpopulations, and those reacting with plasma antigens. Using whole haemocytes from tiger shrimp as an immunogen, Hsu (1991) cloned seven hybridomas which secreted mAbs capable of labelling all haemocytes. For the present study, shrimp haemocytes were separated by negative selection using ConA-coated beads to obtain high GC densities before using the haemocyte sample as an immunogen. As a consequence one mAb (Z5E10) was found to be specific for a combination of two haemocyte types (GC and SGC), while the other three were specific for a single type only (SGC). These results suggest that the specific reactivity of

*Table 1.* Densities of haemocyte subpopulations following treatment with monoclonal antibody-bound beads

mAbs Types	% of haemocytes					
	Z5E10	Z6A5	Z6A6	Z6H8	Neg. Ab <sup>a</sup>	before selection
GC	6.5	17.0	20.0	12.9	18.6	17.1
SGC	6.5	0.6	0.0	0.0	8.7	9
HC	87.0	82.4	80.0	87.1	72.0	73.7

<sup>a</sup>mAb anti-15.1 which did not react with any tiger shrimp haemocyte, was used as a negative control.

*Table 2.* A summary of the properties of four monoclonal antibodies which recognise tiger shrimp haemocytes

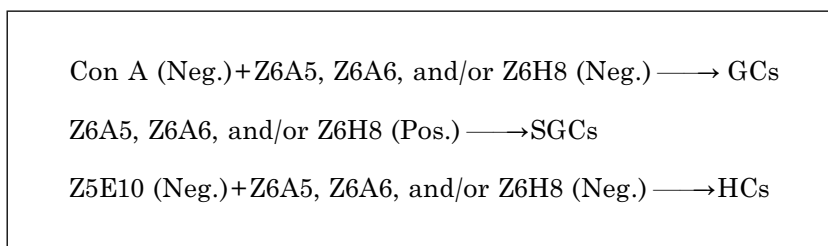
mAb	Isotype	Target cell	M. W. of Ag (kDa) <sup>a</sup>	Glycosylation <sup>b</sup>
Z5E10	IgG2a ( $\kappa$ )	GC & SGC	29	—
Z6A5	IgG1 ( $\kappa$ )	SGC	>205	—
Z6A6	IgG1 ( $\kappa$ )	SGC	163	+
Z6H8	IgG2b ( $\kappa$ )	SGC	>205	—

<sup>a</sup>Mass weight of antigen recognised by monoclonal antibody.

<sup>b</sup>Chemical structure of antigen recognised by monoclonal antibody.

lectin to various cells should be incorporated as part of an initial step of cell separation to enhance success in development of mAbs which can recognise specific haemocyte subpopulations.

Data from periodate oxidation analysed by using EIA showed that the optical density for the reactivity of Z6A6 to treated haemocytes was lower than that to untreated haemocytes since the epitope structure recognised by Z6A6 was destroyed following periodate treatment. It is suggested that the epitope specifically recognised by Z6A6 was glycosylated. On the other hand, the epitopes recognised by Z5E10, Z6A6 or Z6H8 were not glycosylated since, following reaction with the specific mAb, the optical densities were not different between treated and untreated haemocytes. It was also noted that mAb Z5E10 recognised an antigen of approximately 29 kDa when run under reducing conditions. Since the secondary or tertiary structure of a protein can be denatured to form a primary structure under reducing conditions, this indicates that the epitope present in the 29 kDa antigen recognised by Z5E10 may be linear. Many bands were present under non-reducing conditions, suggesting that identical epitopes may be present on a number of different membrane proteins. Similar results were produced when Z6A6 was characterised; in addition to recognising an 163 kDa glycoprotein, it was also found to react with a linear epitope. It was concluded that the Z5E10- and Z6A6-specific epitopes differed not only from each other, but also from those recognised by Z6A5 and Z6H8. Since the Z6A5 and Z6H8 antigens present in the SGCs had similar molecular masses, and in both cases their epitopes only reacted with



*Fig. 5.* Flow chart showing the process of separating tiger shrimp haemocytes into three subpopulations by using the four monoclonal antibody bound-beads and/or ConA-coated beads via negative (Neg.) and positive (Pos.) selection.

mAbs under non-reducing conditions, it was necessary to determine whether the two mAbs reacted with an identical epitope by using an additivity test. Optical density measurements and AI calculations showed an absence of additivity between Z6A5 and Z6H8. However, since the AI value for the mAb pair Z6H8-Z6A6 (29%) was higher than that for Z6A5-Z6A6 (0%), it is suggested that the Z6A5 and Z6H8 epitopes were not identical, and that the distance between them may be close enough to cause interference in the form of stereoconformation. Furthermore, the additivities of the combined mAbs were only detectable for pairs (Z6A6-Z6A5 and A6A6-A6H8) in which Z6A6 was the first antibody. Such data support the contention that the Z6A6 epitope differs from those recognised by Z6A5 and Z6H8.

The increase in HC densities produced by negative selection (Table 1) suggests that all of the epitopes recognised by the four mAbs were surface epitopes. These specific mAb properties are summarized in Table 2. From the present results, the binding specificities of the four mAbs and ConA to haemocytes showed no difference which suggests that shrimp haemocytes can be separated into at least three subpopulations (HC, GC and SGC) by either negative or positive selection (that is, mAb-bound haemocytes were collected following haemocytes dissociated from beads) (Fig. 5), and that the four mAbs will be useful in studying the roles of individual haemocyte types in tiger shrimp defences in the future.

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