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# A comparative characterization of dipentameric $(IgM)_2$ and pentameric IgM species present in preparations of a monoclonal IgM for therapeutic use

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ABSTRACT

IgM aggregates in biotechnologically produced preparations have been reported, however, *in vitro* characteristics and *in vivo* activity of IgM aggregates have not been well studied. We separated two species of the human monoclonal IgM antibody KBPA-101 by size-exclusion chromatography. Molecular weight determination indicated the presence of dipentameric and pentameric forms. We present here the results of a comparative characterization of these IgM species, including *in vitro* and *in vivo* effector function against *Pseudomonas aeruginosa*. Dipentameric $(IgM)_2$ species were observed to dissociate into pentameric IgM at 37 °C, suggesting a dynamic equilibrium, in which the pentameric species is the predominant form. *In vitro* antigen binding (*P. aeruginosa* LPS) and IgM-mediated complement-dependent phagocytosis of labeled bacterial cells did not differ significantly between the dipentameric $(IgM)_2$ and pentameric IgM species. Furthermore, the *in vivo* efficacy of dipentameric and pentameric IgM in protecting mice from a lethal dose of *P. aeruginosa* through passive immunization was nearly equivalent. In conclusion, low concentrations of dipentameric $(IgM)_2$ may contain an additional but equally active component of the principal biological form. The data presented in this work support the conclusion that the pentameric form of IgM directed against the O-polysaccharide moiety of *P. aeruginosa* serotype IATS-O11 and dipentameric $(IgM)_2$ are functionally equivalent.



## 1. Introduction

The role of recombinant monoclonal antibodies (mAbs) as targeted therapies in different therapeutic areas such as oncology, rheumatology and infectious disease is increasingly important. Antibodies or antibody derivatives are estimated to represent 20% of biopharmaceutical products currently being developed [1]. Furthermore, the rise in the number of antibody-based therapies approved by the FDA since 2007 has surpassed all other classes of biopharmaceuticals [2].

Some reasons for the growing interest in mAbs as therapeutics include the specificity of pharmacological action, which results in better tolerability, as well as the ability to combine mAbs with other drugs, or even to conjugate mAbs with other therapeutic entities and use them as target specific carriers (e.g., to target cytotoxic substances to cancer cells). Furthermore, new technology has facilitated the introduction of fully human monoclonal antibodies which are less immunogenic than humanized antibodies derived from murine mAbs [1].

However, the development and production of therapeutic antibodies has not been without challenges. Antibodies, like other proteins, are prone to physical instability and chemical degradation [3,4]. Physical instability occurs by two major pathways; denaturation and aggregation, the latter being the more common type [1,3,4]. Aggregation of antibody molecules is a concern since aggregation often results in reduced efficacy and can induce strong immunogenic reactions [3]. In addition, immunoglobulin aggregates have been shown to cause nephropathy in patients with highly elevated serum IgM concentrations [5].

Monoclonal antibodies usually are of the IgG isotype and the knowledge relating to antibody aggregates is largely restricted to this isotype. Nevertheless, monoclonal IgM antibodies are increasingly of interest for the treatment of various diseases [6]. IgM is the first class of antibodies produced during a primary antibody response [7] and this, along with its high potential for complement activation and opsonization, makes it the primary defense against bacterial infections. In circulation, serum IgM can exist in different forms. The pentameric form, which has a molecular weight of roughly 950 kDa, normally comprises approximately 95% of the total circulating IgM. It is widely held that

*Abbreviations:* LPS, lipopolysaccharide; IATS, International Antigenic Typing Scheme; O-PS, O-polysaccharide; EBV, Epstein–Barr virus.

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a hexameric form, with a molecular weight of 1150 kDa, comprises the majority of the remaining 5% [8]. Interestingly, there is no J-chain incorporated in the hexameric form, which seems to activate complement more effectively than the pentameric form [9–11].

We have recently purified a human monoclonal IgM antibody with activity directed against LPS of *P. aeruginosa* serotype IATS-O11. This bacterium is a versatile, potentially lethal pathogen and one of the leading pathogens responsible for nosocomial infections [12,13]. Of the 20 serotypes of *P. aeruginosa*, the O11 serotype is one of the most frequently observed serotypes, accounting for 18–21% of all *P. aeruginosa* infections [14]. The fully human IgM monoclonal antibody (termed KBPA-101) directed against the LPS O-polysaccharide of serotype O11 was developed by generating a human hybridoma cell line derived from human B-lymphocytes isolated from an actively immunized donor [15]. The resulting hybridoma consistently secreted high levels of human monoclonal IgM KBPA-101 in its pentameric structure. KBPA-101 demonstrated high specificity and efficacy *in vitro* and *in vivo* and has been tested in a clinical phase 1 study [16]. Nevertheless, repeated production of KBPA-101 antibody in a serum-free perfusion cell culture system under GMP guidelines resulted in highly purified preparations of approximately 95% IgM plus about 3–4% of a side-product, which was assumed to represent a dipentameric $(IgM)_2$ species made up of two non-covalently associated pentameric IgM molecules.

IgM aggregates in biotechnologically produced preparations are known and very common [17]. However, the *in vitro* characteristics and the *in vivo* activity of these aggregates have not been well studied. Presented here are the results of our characterization of the dipentameric species of IgM and its functional activity, both *in vitro* and *in vivo*, in relation to the corresponding activity of the pentameric IgM molecule.

## 2. Materials and methods

### 2.1. Materials

*N*,*N*-Dimethylformamide and o-phenylene-diamine (OPD) was purchased from Sigma–Aldrich GmbH, Switzerland. 5-(6-)carboxyfluorescein succinimidylester (FAM-SE), an isomer mixture, was purchased from Invitrogen, Switzerland. 2-Chloro-1,1,2-trifluoroethyl-difluoromethy-ether (Ethrane) was purchased from Abbott Laboratories, Chicago, IL, USA. Unlabeled and horseradish peroxidase-labeled goat anti-human IgM antibodies were purchased from Zymed Laboratories, USA. Lipopolysaccharide (LPS) IATS-O11 was purchased from Berna Biotech AG, Switzerland. Superose 6 prep grade resin was purchased from GE Healthcare. HPLC was performed using a Dionex P580 pump, UVD340U Detector and a Gina50 autosampler (Dionex AG, Switzerland). Nunc MaxiSorp flat bottom microtiter plates were purchased from Milian SA, Switzerland. A SpectraMax (Molecular Devices, USA) or Synergy HT microtiter plate reader (Biotek AG, Switzerland) were used for absorbance measurements.

*Bacteria/cell lines*: *P. aeruginosa* strain FT-2 serotype IATS-O11 and HL-60 cell line were purchased from the American Type Culture Collection (ATCC #27313 and ATCC # CCL240). *P. aeruginosa* strain 2310.55 was isolated from a urinary tract infection (University Hospital Bern, Switzerland) and serotyped by agglutination using a mouse MAb kit (Erfa Biotech, Westmount, Quebec, Canada) and by PCR, using serotype-specific primers as described by Raymond et al. [18]. The O-serotype designation used is according to the International Antigenic Typing System (IATS). The strain was stored in 20% glycerol at −80 °C. Bacteria were grown in Luria Broth (LB) medium at 37 °C.

### 2.2. Methods

#### 2.2.1. Source of KBPA-101

Cells expressing the human monoclonal antibody KBPA-101 were generated by the following procedure: isolated lymphocytes from a healthy individual actively immunized with a *P. aeruginosa* O-PS-toxin A conjugate vaccine were immortalized by EBV transformation and subsequently fused with the mouse–human hetero-myeloma cell line LA55. The selected hybridoma clone 1BO11 was obtained after several rounds of limiting dilutions. Cells were grown under GMP conditions in a perfusion reactor of 20 l volume with a BioSep cell retention system in a mixture of IMDM/Ham's F12 media (1:1, v/v) for 57 days with a medium exchange rate of 1.5–2 volumes per day for 44 days. The IgM, KBPA-101, was purified from clarified supernatant using anion exchange chromatography as capturing step and ceramic hydroxyapatite chromatography as polishing step. Purified KBPA-101 was stored at 2–8 °C as a sterile, non-pyrogenic, phosphate-buffered solution. The primary amino acid sequence of the mAb was confirmed by peptide mapping with LC–MS/MS after digestions of the protein with trypsin and Glu-C protease (data not shown). Antibody preparations were stored at 2–8 °C during the time between all experiments.

#### 2.2.2. Isolation of KBPA-101 by size-exclusion HPLC

IgM species in KBPA-101 batch 06221KB were separated on Superose 6 prep grade resin using a XK26/70 custom-packed column. The flow rate was 2 mL/min and UV absorbance was monitored at 280 nm and/or 210 nm. Sample loading volume per run was 9 mL and four runs were performed in total. PBS (pH 7.2–7.4) was used as the mobile phase. For analytical purposes, samples were analyzed using a Superose 6 10/300 GL column with a flow rate of 0.25 mL/min (mobile phase PBS) and detection at 280 and/or 210 nm.

#### 2.2.3. Asymmetrical flow field-flow fractionation (AF4)

Asymmetrical flow field-flow fractionation coupled with a multi-angle laser light scattering (MALS) detector was performed for the determination of the molecular weight of the IgM species [19,20].

#### 2.2.4. Quantification of KBPA-101

The concentrations of the IgM species were determined by indirect ELISA. Pooled fractions from the Superose 6 column were assayed using twofold serial dilutions (pentameric IgM 1:200 to 1:25,600, dipentameric IgM 1:50 to 1:6400) by incubation in microtiter plates coated with goat anti-human IgM. Bound IgM was detected by addition of horseradish peroxidase-labeled goat anti-human IgM and OPD followed by measuring absorbance at 490 nm. Quantification was achieved using reference KBPA-101 samples.

#### 2.2.5. Avidity determination by inhibition ELISA

The avidity constant of the IgM species for P. aeruginosa LPS serotype O11 was measured by inhibition ELISA as described by Bruderer et al. [21] and was defined as the reciprocal antigen concentration (in moles per liter) resulting in 50% inhibition of antibody binding.

#### 2.2.6. Stability of dipentameric $(IgM)_2$ at physiological temperature

45 μg of dipentameric $(IgM)_2$ in 0.5 mL PBS were incubated at 37 °C in a ventilated incubation room in screw-cap vials. At predetermined time points, samples were analyzed by size-exclusion chromatography.

*2.2.7. Binding of KBPA-101 to P. aeruginosa LPS*

ELISA assay was performed as previously described [22]. In short: microtiter plates were coated overnight with 0.5 μg of IATS-O11 LPS and 0.5 μg methylated human serum albumin in 0.1 mL PBS per well. Subsequently IgM species were added to an initial concentration of 500 ng/mL and serially diluted down to 3.9 ng/mL. Bound IgM was detected by addition of horseradish peroxidase-labeled goat anti-human IgM and OPD followed by measuring absorbance at 490 nm.

*2.2.8. Opsonophagocytosis assay*

HL-60 cells were cultured in RPMI containing 10% FCS. For use in the opsonophagocytosis assay, HL-60 cells were differentiated by exposure to 100 mM dimethylformamide for 3 days. *P. aeruginosa* strain FT-2 was stained with FAM-SE as described by Jansen et al. [23], and $1.7 \times 10^6$ bacteria per tube were incubated with serially diluted antibody as well as baby rabbit complement or heat-inactivated baby rabbit complement (Charles River Laboratories, Germany) for 30 min. Thereafter, $2.4 \times 10^5$ differentiated HL-60 cells were added and incubated for 90 min. Opsonophagocytosis was determined by analyzing the green fluorescence of the HL-60 cells in comparison to background staining by flow cytometry (FACSCalibur; BD Biosciences, Allschwil, Switzerland) using CellQuest Pro for primary data analysis. Gates were set to identify HL-60 cells and determine the percentage of green fluorescent cells based on signal intensity in the FL-1 channel. Background staining was determined by incubating FAM-SE labeled bacteria in the presence of complement and HL-60 cells but in the absence of antibody.

*2.2.9. Murine burn-wound model*

The murine burn-wound model was performed as described earlier [24] upon approval by the Tierschutzkommission of the Canton of Bern, Switzerland. Temgesic® (buprenorphine; Essex Pharma GmbH, München, Germany) was injected twice daily s.c. for the entire duration of the experiment, starting immediately before the challenge.

Four groups of female NMRI-Mice (Charles River Laboratories, L'Arbresle, France) were injected i.v. with 0.2 or 0.04 mg of antibody/kg body weight 2 h prior to challenge. A fifth group of mice served as control group and was given 0.1 mL PBS i.v. For the challenge, groups of 10 mice were anesthetized by i.p. injection of Ketamin/Xylasol. In addition, immediately before inflicting the burn wound, mice were placed in an atmosphere of Ethrane for approximately 30 s. Thereafter, mice were subjected to a 10 s ethanol burn covering approximately 2 $cm^2$ of area on the back. $1.2 \times 10^6$ colony forming units (CFU) of *P. aeruginosa* strain 2310.55, suspended in 0.5 mL PBS, were immediately injected subcutaneously beneath the burned area. Mice were monitored three times per day for a total duration of 100 h after challenge. Dead animals were removed and moribund animals were euthanized.

*2.2.10. Data analysis*

Data was analyzed using MS Excel for primary data analysis and Graphpad Prism (Version 4.03, January 2005, GraphPad Software Inc., San Diego, CA, USA) for curve fitting and statistical analysis.

## 3. Results

### 3.1. Separation of dipentameric $(IgM)_2$ and pentameric IgM

Analytical size-exclusion chromatography of a KBPA-101 batch (stored frozen over 2 years) revealed an impurity of high-molecular weight (approximately twice the mass of pentameric IgM) comprising approximately 10% of the total material. We successfully separated this impurity by size-exclusion chromatography for further analysis *in vitro* and *in vivo*.

Molecular weight determination of the impurity and the principal form of IgM by asymmetrical flow field-flow fractionation analysis indicated weights of approximately 1700 kDa and 900 kDa, respectively (Fig. 1). One-thousand seven hundred kilodaltons is consistent with approximately double the mass of a pentameric IgM. Thus we hypothesized the impurity to be a dipentameric aggregate species of normal pentameric IgM, hereafter referred to as dipentameric $(IgM)_2$.

### 3.2. In vitro characterization: dissociation, antigen binding, opsonophagocytosis

In order to verify above assumption, we investigated the dissociation of dipentameric $(IgM)_2$ by placing samples at 37 °C and analyzing them by size-exclusion chromatography at vari-

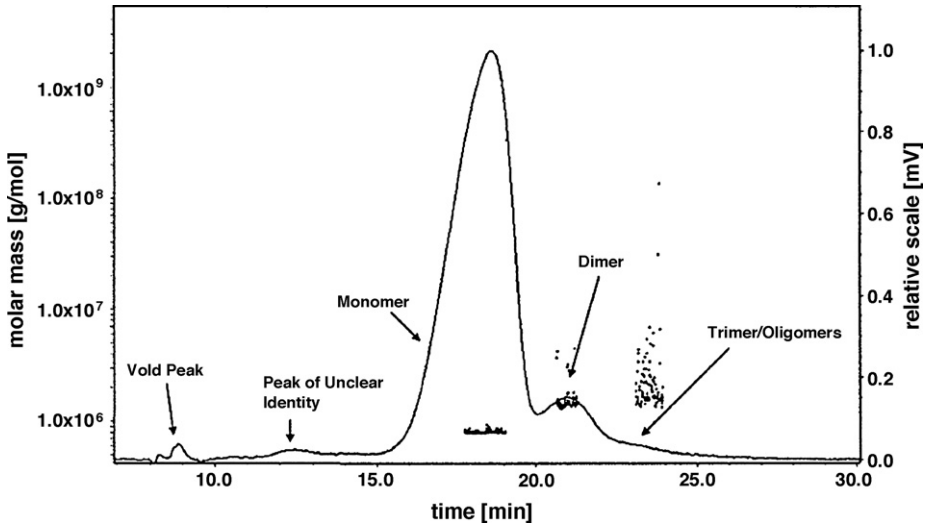


**Fig. 1.** Asymmetrical flow field-flow fractionation trace of IgM KBPA-101 batch 06221KB. Molecular weights of dipentameric $(IgM)_2$ (10% of total area) and pentameric IgM (85% of total area) species were determined to be 1700 kDa and 900 kDa, respectively. The MALS detector signal is shown as a distribution of dots with the scale on the left hand side ($1.0 \times 10^6$ to $1.0 \times 10^9$ g/mol).

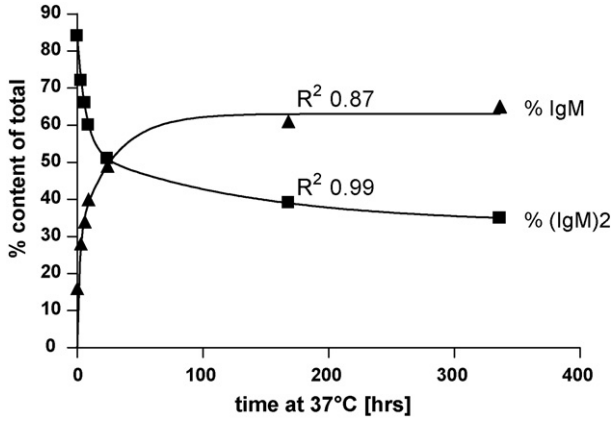


**Fig. 2.** Dissociation of dipentameric $(IgM)_2$ to pentameric IgM at physiological temperature over time. Purified dipentameric $(IgM)_2$ was incubated at 37 °C without shaking, and relative amounts (%) of pentameric IgM or dipentameric $(IgM)_2$ were extracted from size-exclusion HPLC data performed at different time points. A two-phase exponential decay model for dipentameric $(IgM)_2$, or a two-phase exponential model for pentameric IgM was used.

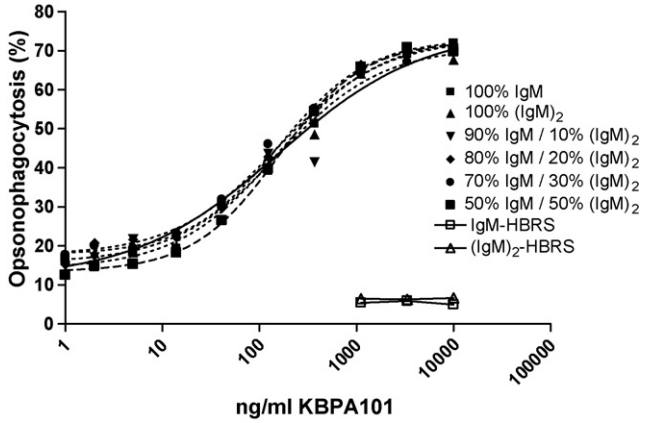


**Fig. 4.** Opsonophagocytosis of labeled *P. aeruginosa* bacteria in the presence of specific IgM antibody by differentiated human phagocytes. The opsonophagocytic activity $EC_{50}$ of the investigated test samples (dipentamer, pentamer and mixtures thereof) was calculated from a sigmoidal dose response curve with variable slope. Statistical analysis by *F*-test revealed no significant differences between the calculated $EC_{50}$ values. Experimental controls utilizing heat-inactivated baby rabbit serum (HBRS) as a complement source for IgM or $(IgM)_2$ did not show any opsonophagocytosis.

ous intervals. Aggregated dipentameric species were observed to dissociate into pentameric IgM whereby the proportion of dipentameric $(IgM)_2$ decreased from 84% to 60% within 9 h. The observed change in distribution could best be described by a curve fitting using a two-phase exponential decay model (Fig. 2). The half-life of the dipentameric IgM was calculated as a decay half-life 1 of 4.8 h and a half-life 2 of 90.4 h. Thus, the conclusion was that the impurity consisted of a dipentameric species of IgM comprising two non-covalently associated pentameric IgM molecules.

Comparability of the two species was assessed by evaluating the *in vitro* binding behavior of the dipentameric and pentameric IgM species to LPS. Binding of the two species to the antigen was not significantly different (Fig. 3). In addition, the original preparation from which the two species were purified displayed binding activity that was virtually indistinguishable from that of the individual species. A curve fitting using a model based on sigmoidal dose response with variable curve slope identified very similar $EC_{50}$ values for all three preparations ($EC_{50}$ of 492, 729 and 490 ng/mL for batch 06221KB, pentamer and dipentamer, respectively). Statistical analysis of the curve fitting models revealed no significant differences between the calculated $EC_{50}$ values (with a *p*-value of 0.85 for comparison by *F*-test among the three curves). In addition, an inhibition ELISA revealed identical avidity constants for the interaction of dipentameric and pentameric IgM with *P. aeruginosa* LPS serotype IATS-O11 ($5.24 \times 10^8\ M^{-1}$ and $5.55 \times 10^8\ M^{-1}$, respectively).

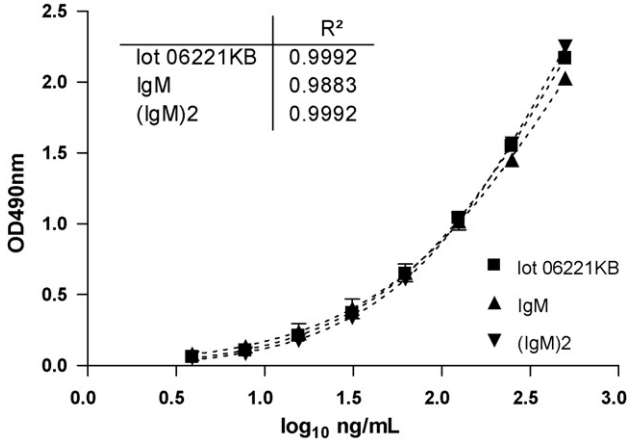


**Fig. 3.** Binding of pentameric IgM or dipentameric $(IgM)_2$ to LPS-IATS-O11 serotype in comparison to the original non-purified batch 06221KB. Absorbance values at 490 nm were plotted against the concentration covering the range from 500 to 3.9 ng/mL of the respective species (logarithmic scale). Curve fitting was done using a sigmoidal dose response curve with variable slope. Statistical analysis by *F*-test did not reveal significant differences for the calculated $EC_{50}$ values.

Since antigen recognition was equal for the two species, we intended to verify *in vitro* effector function for both species in an opsonophagocytosis assay. For this purpose, samples containing dipentameric $(IgM)_2$ and pentameric IgM in various ratios were used to assess the ability of dipentameric and pentameric IgM species to stimulate complement-dependent phagocytosis. IgM mediated phagocytosis of fluorescein-labeled bacterial cells by differentiated HL-60 cells occurred in a dose-dependent manner at all ratios of pentameric and dipentameric IgM tested (Fig. 4). When heat-inactivated complement (HBRS) was used instead of untreated serum, almost no phagocytosis was observed. The opsonophagocytotic activity ($EC_{50}$), defined as the concentration resulting in the half-maximal percentage of FAM-SE positive HL-60 cells, was calculated for all experiments through a curve fitting procedure that provided a sigmoidal dose response curve with variable slope for all samples tested (Table 1). The various mixtures of dipentameric and pentameric IgM produced very similar curves as well as $EC_{50}$ values, with no statistically significant differences ($p = 0.98$ by *F*-test comparing the individual curves). The averaged

**Table 1**
Opsonophagocytosis of labeled *P. aeruginosa* serotype IATS-O11 cells in the presence of KBPA-101 by differentiated human phagocytes (HL-60 cell line).

| Pentamer:dipentamer ratio | $EC_{50} \pm$ Std. Error (95% CI)[a] | Fit $R^2$ |
|---|---|---|
| 100% IgM | 181.5 ± 1.18 (120.4–273.7) | 0.9951 |
| 90% IgM/10% $(IgM)_2$ | 269.3 ± 2.06 (46.01–1577) | 0.9714 |
| 80% IgM/20% $(IgM)_2$ | 154.2 ± 1.15 (110.5–215.2) | 0.9957 |
| 70% IgM/30% $(IgM)_2$ | 118.5 ± 1.12 (90.76–154.7) | 0.9975 |
| 50% IgM/50% $(IgM)_2$ | 147.8 ± 1.07 (123.9–176.4) | 0.9987 |
| 100% $(IgM)_2$ | 150.7 ± 1.27 (84.71–268.1) | 0.9903 |

[a] Opsonophagocytotic activity ($EC_{50}$) of the investigated test samples (dipentamer, pentamer and mixtures thereof), defined as the concentration resulting in the half-maximal percentage of FAM-SE positive HL-60 cells, was calculated for all experiments through a curve fitting procedure (sigmoidal dose response curve with variable slope).

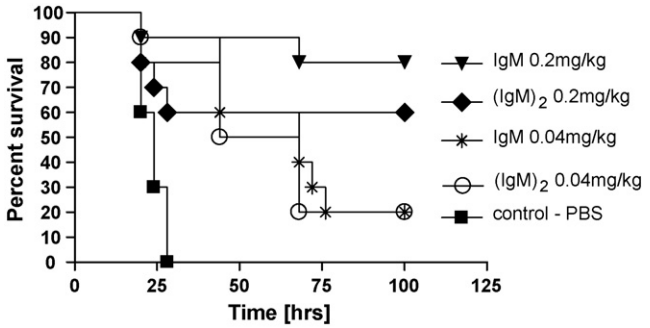


**Fig. 5.** Survival curves from burn-wound challenge assay. Animals were treated with pentameric IgM or dipentameric $(IgM)_2$ prior to challenge with a lethal dose of *P. aeruginosa* bacteria. Animals in the control group (■) died very rapidly compared to treated animals. Statistical analysis by log rank test showed no significant difference between the survival curves of animals that received corresponding doses of pentameric IgM or dipentameric $(IgM)_2$. However, statistically significant differences were observed between treated animals versus control animals ($p = 0.001$ for 0.04 mg/kg and $p = 0.0001$ for 0.2 mg/kg).

$EC_{50}$ value for all of the assessed mixtures was 170 pg/mL. Therefore, it can reasonably be accepted that there is no change in effector function of KBPA-101 due to the presence of the dipentameric $(IgM)_2$.

### 3.3. *In vivo protection efficiency*

The efficacy of dipentameric $(IgM)_2$ or pentameric IgM in protecting animals from a challenge with a lethal dose of *P. aeruginosa* serotype IATS-O11 through passive immunization was determined by a mouse burn-wound challenge assay [24] (Fig. 5). The disseminated infection induced by the *P. aeruginosa* challenge in the burn-wound model generally leads to death of the animals within 48 h post-inoculation, whereas passive immunization with protective doses of KBPA-101 antibody efficiently protects animals from death [15]. The same effect was observed in this study when animals were passively immunized with either dipentameric $(IgM)_2$ or pentameric IgM. Yet all 10 animals in the control group died within 20–28 h after challenge with a lethal dose of *P. aeruginosa* (Fig. 5). When low doses (0.04 mg/kg body weight) of dipentameric $(IgM)_2$ or pentameric IgM were used for preventive treatment, 8 out of 10 animals died within 76 h in both groups. However, when a higher dose of 0.2 mg dipentameric $(IgM)_2$ or pentameric IgM/kg body weight was injected, the rate of survival was substantially higher: 8 out of 10 animals in the pentameric IgM group and 6 out of 10 animals in the dipentameric $(IgM)_2$ group survived the challenge. Log rank analysis showed that the difference in survival rate observed between the dipentameric $(IgM)_2$ and pentameric IgM groups was not statistically significant ($p = 0.85$ and 0.31, by log rank test for 0.2 mg/kg and 0.4 mg/kg treatment groups, respectively). Nevertheless, treatment with both dipentameric $(IgM)_2$ and pentameric IgM resulted in significantly extended survival times ($p = 0.0001$ and 0.001 by log rank test for 0.2 mg/kg and 0.4 mg/kg) compared to the control group.

In addition, dipentameric $(IgM)_2$ treated animals did not show any signs of increased toxicity, organ dysfunction or increased weight loss compared to IgM treated animals. No signs of increased toxicity were observed by macroscopic evaluation of internal organs at the end of the observation period (100 h) for $(IgM)_2$ versus IgM treated animals. Thus, it appears that the use of dipentameric $(IgM)_2$ for treatment has the same beneficial effect as pentameric IgM, and that the dipentameric $(IgM)_2$ has no detrimental effect on the well-being of the animals.

## 4. Discussion

Under physiological conditions, IgM exists in different forms. The well known pentameric form comprises approximately 95% of the total circulating IgM and a hexameric form makes up the majority of the remaining 5% [8]. However, it may be that other species of IgM are also present, especially in biotechnologically produced, highly purified preparations stored at higher concentrations. Therefore, we analyzed a frozen batch of KBPA-101, a human monoclonal IgM antibody against *P. aeruginosa* serotype O11, for the distribution of pentameric and aggregated forms of IgM. Initial analyses revealed that a high-molecular weight species of IgM comprised approximately 10% of the total IgM, whereas freshly prepared batches of KBPA-101 normally contain 3–4% of the high-molecular weight species. The higher than normal proportion of the high-molecular weight species in the batch that we analyzed could be due to unfavorable freezing conditions used at that time. Our hypothesis was that this species represents a dipentameric form of the IgM molecule, whereby two pentameric IgM are non-covalently associated with each other. Therefore, we separated the batch components to obtain pure fractions of pentameric IgM and the higher molecular weight fraction suspected to be dipentameric $(IgM)_2$.

This hypothesis was verified by the determination of the mass of the high-molecular weight species of IgM by asymmetrical flow field-flow fractionation analysis (AF4). The results indicated approximately double the mass of a normal pentameric IgM, consistent with our hypothesis. In general, mass determination of high-molecular weight molecules poses a significant problem when using standard methods, such as HPLC. Molecular weight determination by AF4 enables circumvention of some disadvantages of size-exclusion HPLC such as shear stress and adsorption phenomena associated with the stationary phase. Moreover, AF4 is very well suited to separate macromolecules under very mild experimental conditions. Thus, there is good evidence that the observed mass accurately reflects the actual molecular weight of a dipentameric $(IgM)_2$ molecule.

We showed that purified dipentameric $(IgM)_2$ in solution dissociates into the pentameric species following incubation at physiological temperature, with a decay pattern that could best be described as biphasic decay. This suggests that, at least at physiological temperature, the dipentameric $(IgM)_2$ and pentameric IgM species are in a dynamic equilibrium with one another in which the pentameric species is the predominant form. During the observation period, the ratio of dipentameric $(IgM)_2$ did not go down to the expected level of approximately 90% pentameric versus 10% dipentameric $(IgM)_2$, as in the original batch. It should be noted that the observed ratio in the original batch was always determined at 4 °C and no changes in the ratio were observed over long periods of time (>12 months). Thus, it could be possible that the thermodynamic equilibrium is not yet reached at 4 °C and that the association of pentameric to dipentameric $(IgM)_2$ is slower at lower temperatures. Second, pentameric IgM degrades readily at 37 °C to lower molecular weight fragments within a week, and it could be possible that the dipentameric $(IgM)_2$ is less prone to degradation at 37 °C, thus shifting the equilibrium towards the dipentameric $(IgM)_2$. Such a phenomenon might also explain the biphasic decay pattern observed in our experimental setting. Nevertheless, currently we do not have a solid explanation for the observed discrepancies.

Other *in vitro* analytical tests revealed that the dipentameric $(IgM)_2$ peak was not a contaminating entity (e.g. host cell proteins not eliminated during isolation and purification of the monoclonal IgM) since the high-molecular weight fraction could be identified by dot blot as consisting of μ-heavy chain and κ-light chains (data not shown). Besides, the high-molecular weight fraction bound with the same specificity to the relevant antigen, and free LPS could

inhibit the interaction with the antigen. The calculated avidity of the high-molecular weight fraction for the LPS-IATS-O11 antigen was identical to that measured for pentameric IgM. This result suggests either that both species have the same avidity for *P. aeruginosa* LPS or, the dipentameric species rapidly dissociates into the pentameric species, leading to a result indicative of equal binding avidity. However, based on the time scale of dipentameric $(IgM)_2$ dissociation, the former explanation is more likely. Thus, *in vitro* analysis supported the hypothesis that the high-molecular weight fraction consists of a dipentameric species of the IgM KBPA-101.

In line with the *in vitro* data, the *in vivo* efficacy of the dipentameric and pentameric IgM species were not significantly different in a mouse burn-wound model in which animals were challenged with a lethal dose of *P. aeruginosa*. The outcome in complement-dependent eradication of *P. aeruginosa* was virtually indistinguishable between dipentameric and pentameric IgM. Given the time scale of dissociation of the dipentameric form into the pentameric form discussed above, this supports the conclusion that the dipentameric and pentameric species of IgM stimulate complement mediated phagocytosis to a similar extent and with similar kinetics. As such, it is evident that the efficacy associated with the treatment is not dependent on the species of IgM used for immunization of the animals. It should be noted that administration of non-specific monoclonal IgM antibodies would not prevent the death of the animals. In addition, using a different serotype of *P. aeruginosa* for introducing an infection will lead to a rapid death of the animals even if treated with KBPA-101 (data not shown).

During the *in vivo* testing in the animal model, there was no indication of excess complement activation in dipentameric $(IgM)_2$ treated animals and no infusion related side effects could be observed, such as signs of shock or delayed recovery from the procedures. Furthermore, in a phase I clinical study KPBA-101 with a dipentameric $(IgM)_2$ content of about 5–8%, was shown to be safe [16] and no spontaneous activation of total complement was observed. Therefore, the conclusion can be drawn that dipentameric and pentameric IgM species have similar biological activities and that dipentameric $(IgM)_2$ should not pose a safety risk.

Hence, based on the performed experiments as well as on the findings from other stability, toxicology and clinical studies, we are confident to propose a changed release specification for the molecular size distribution of our IgM preparation. Long-term stability studies over 30 months at 2–8 °C demonstrated that the content of dipentamers never significantly exceeds the 5% threshold. We do foresee to switch from a release specification based on the amount of pentameric IgM for the therapeutic drug product to a combined specification allowing explicitly a defined amount of dipentameric $(IgM)_2$ in there. A future release specification could simply comprise a defined content of total IgM in solution, including not more than a well defined amount of dipentameric $(IgM)_2$. However, the issue has to be carefully discussed with regulatory authorities.

## 5. Conclusions

Low concentrations of dipentameric $(IgM)_2$ may comprise an additional but equally active component of the principal biological form. The data presented in this work support the conclusion that the pentameric form of IgM directed against the O-polysaccharide moiety of *P. aeruginosa* serotype O11 and dipentameric $(IgM)_2$ are functionally equivalent. Therefore, we conclude that the total content of intact IgM molecules in a solution is most likely the relevant measure for assessing the potency of a drug in solution rather than the content of pentameric versus dipentameric $(IgM)_2$. This should be reflected in changed release specifications for our IgM preparation, and these suggestions need to be openly discussed with regulatory authorities.

## Disclosure statement

The authors disclose that they are employees of Kenta Biotech Ltd.

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