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Chicken scFvs and bivalent scFv-C_H fusions directed against HSP65 of *Mycobacterium bovis*

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ABSTRACT

Two chicken single-chain variable region antibody fragments (scFvs) that recognised the 65 kDa heat-shock protein (HSP65) of *Mycobacterium bovis* were selected from a large semi-synthetic phage displayed library. Both recognised HSP65 in indirect enzyme-linked immunosorbent assay (ELISA) and immunoblots and retained their activity during storage. Neither, however, could function as the capture reagent in a sandwich ELISA when immobilised on polystyrene. To establish whether they could be engineered for general use in immunotests, the genes coding for these scFvs were subcloned in expression vectors that contained sequences encoding chicken IgY heavy-chain constant region domains. This resulted in larger bivalent constructs which more closely resembled IgY molecules. The engineered fragments were evaluated in ELISAs and gold-conjugated immunochromatographic tests (ICTs). In contrast to their previous behaviour as scFvs, the modified fragments (designated "gallibodies") could be used for immunocapture in ELISA and could be readily conjugated to colloidal gold nanoparticles. A sandwich ICT that could detect recombinant HSP65 was also devised. Although converting the recombinant single-chain monomeric antibody fragments to bivalent immunoglobulin-like molecules did not entirely 'standardise' the behaviour of the scFvs, this approach remains potentially useful for developing practical, robust, immunodiagnostic reagents.

1. Introduction

Bovine tuberculosis (BTB) is caused by the acid-fast, Gram-positive bacillus *Mycobacterium bovis* [1,2]. Although mainly a disease of cattle, BTB also affects wildlife [3] and humans [4]. The 65 kDa heat-shock protein (HSP65) from Mycobacterium is highly immunoreactive and since it is conserved in the tubercle bacilli, immunoassays targeting this antigen can be useful for the general diagnosis of mycobacterial infections [5]. For large scale screening (e.g. herds) and penside use, rapid and convenient assays such as enzyme-linked immunosorbent assays (ELISA) and lateral-flow immunochromatographic tests (ICTs) are regarded as being potentially more practical than the standard intradermal tuberculin test [6].

Recombinant antibody technology [7,8] allows immunoreagents to be derived, engineered and optimised using standard molecular biological methods. For immunodiagnostic applications, tests based on antibodies of which the encoding sequences are known can be more easily standardised than those derived from immune serum or a potentially unstable hybridoma. Single-chain variable fragments (scFvs) based on chicken immunoglobulins are particularly suitable as a basis for developing immunotests, not only because they are serologically distinct from mammalian immunoglobulins, but because the avian antibody repertoire can be accessed [9–11] more readily than that of any mammal other than the camelids [12]. Recombinant chicken antibodies derived either from large "universal" or dedicated immune repertoires have been shown to be eminently useful in a variety of immunotests aimed at detecting either antigens or antibodies [13–19]. This suggests that if judiciously applied, they could also play a role in the diagnosis of tuberculosis. While ELISAs are likely to be useful in the laboratory, for use in the field, ICTs [20–23] offer the advantages of economy and rapidity. So far, however, the use of chicken-derived antibody fragments in gold-conjugated ICTs has not been widely reported.

With the objective of evaluating a possible role for recombinant chicken antibodies in developing immunodiagnostic tests for bovine tuberculosis, we describe the derivation, modification and use of scFvs directed against dimeric recombinant HSP65 of *M. bovis*. Three different scFvs were obtained by screening a large semi-synthetic antibody library based on chicken immunoglobulin genes [11]. Two of these were converted into bivalent IgY-like molecules [24]. Originally called IgY-C_{H2-4} constructs because the first heavy-chain constant domain (C_{H1}) is omitted, for convenience

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we designated these modified antibody fragments as 'gallibodies', a word derived from the Latin binomial nomenclature for the domestic chicken, *Gallus gallus domesticus*. Their behaviour in various ELISA formats and in colloidal gold-based ICTs was evaluated. Depending upon the particular scFv and the system in which it was being used, converting it into a gallibody could markedly affect its suitability as an immunoreagent.

2. Materials and methods

2.1. Single-chain Fvs from the Nkuku® library

Immuno™ Tubes (Nunc Maxisorp, Roskilde, Denmark) were coated by overnight incubation at 4 °C with 10 μg/mL recombinant HSP65 from *M. bovis* BCG dissolved in phosphate-buffered saline (PBS). Single-chain Fvs were selected by panning a phage displayed repertoire derived from the immunoglobulin genes of the chicken (*Nkuku*® library) using methods described previously [11]. Phages displaying antibody fragments that bound to HSP65 were released at high pH and used to reinfect exponentially growing TG1 host cells. After overnight growth, the bacteria were collected by centrifugation and phage particles were rescued using M13KO7 helper phage. These phages were used as input for the next round of selection. For the first two rounds, the Immuno™ Tubes were washed 10 times. After three rounds of selection, single bacterial clones were selected, phage were rescued and tested in indirect ELISA to identify those that produced binders. These were then induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) to obtain soluble scFvs. DNA coding for the binders was sequenced to categorise individual clones. The deduced amino acid sequences were aligned [25] and numbered according to Kabat using published scFv sequences as a guideline [26]. For subsequent use, scFvs were expressed and immunoaffinity-purified via their c-myc tags by Ms J. Frischmuth (National Bioproducts Institute, Pinetown, South Africa).

2.2. Adding truncated C_H-region sequences to scFv genes

The scFv genes were recovered from the vector pHEN [27] by PCR amplification using primers to introduce a BsiW cleavage site (5′ GATCCGTACGGCCGTGACGTTGGACG 3′) and an AscI site (5′ GATCGGCGCGCCACCTAGGACGGTCAGGG 3′) (Inqaba Biotech, Pretoria, South Africa). The scFv-encoding inserts were then subcloned in the mammalian expression vector scFvIgY(C_{H2-4})His [24] at these restriction endonuclease sites. Ultracompetent M15 *Escherichia coli* (QIAGEN®, Hilden, Germany) were transformed to ampicillin resistance with the two constructs and 10 transformants from each subcloning experiment were selected and subjected to PCR using primers specific for the 5′ (5′ TAATACGACTCACTATAGGG 3′) and 3′ (5′ AGGAGGAGGGGTGGAGGACC 3′) ends of the scFv genes, to check for the presence of inserts. Plasmid DNA from clones bearing inserts of approximately 800 bp was sequenced and compared to the original template.

2.3. Gallibody expression and purification

After overnight growth in Luria-Bertani (LB) medium, plasmid DNA was extracted using the QIAfilter™ Plasmid Midi Kit (QIAGEN®, Hilden, Germany). Human embryonic kidney (HEK) 293-H cells (Invitrogen™, Carlsbad, USA) grown to 50–80% confluence in Dulbecco's Modified Eagle Medium (DMEM) (Cat. No. 31966-021, Gibco™, Carlsbad, USA), supplemented with 10% (v/v) foetal bovine serum (FBS) (Gibco™, Carlsbad, USA) were transfected with plasmid DNA using GeneJuice® Transfection Reagent (Merck, Darmstadt, Germany) or TransIT®-293 Transfection Reagent (Mirus Bio Products, Madison, USA). The cultures were grown at 37 °C in 5 % CO_2. Individual foci were expanded in fresh tissue culture plates containing DMEM, 10 % (v/v) FBS and Zeocin™ (Invitrogen™, Carlsbad, USA). Secreted gallibodies were purified using nickel-nitrilotriacetic acid (Ni–NTA) agarose according to the manufacturer's (QIAGEN®, Hilden, Germany) instructions and purity was verified on a 4–12 % SDS-PAGE gel (Criterion™ XT, Bio-Rad Laboratories, Hercules, USA) stained first with Coomassie blue, then destained overnight with 4 % (v/v) acetic acid. Specificity of the gallibodies for the target protein (HSP65) was confirmed by an immunoblot using these antibodies in cell culture medium for detection.

2.4. Stability studies of scFvs and gallibodies

Recombinant antibody samples were subjected to consecutive freeze–thaw cycles while suspended in LB medium (for scFvs) and DMEM (for gallibodies). After each cycle, an aliquot was removed and tested for its capacity to recognise the target antigen in indirect ELISA.

To determine the threshold temperature at which the recombinant antibodies lose binding activity, 12 aliquots (100 μL) of each of the scFvs and the gallibodies were incubated at different temperatures for 16 h using the temperature gradient programme on the Mastercycler® ep gradient S (Eppendorf, Hamburg, Germany) thermocycler. The heat-treated samples were tested in duplicate in indirect ELISA. To ascertain their long-term storage ability under various conditions, one millilitre samples were stored at −70 °C, −20 °C, room temperature (RT) and 4 °C for four weeks before testing in indirect ELISA. The storage media were supplemented with tetracycline to prevent microbial growth. To evaluate the propensity of the scFvs and gallibodies to refold after denaturation, recombinant antibodies were each exposed to 6 M guanidinium chloride (GdmHCl) overnight. Prior to testing in an indirect ELISA, the denaturant was removed by ultrafiltration (Vivaspin, Sartorius, Goettingen, Germany) replaced with three volumes of PBS and then concentrated to the original starting volume of approximately three millilitres.

2.5. ELISAs

For indirect ELISAs, Immuno™ Plate (Poly- or Maxisorp, Nunc™, Roskilde, Denmark) wells were coated overnight at 4 °C with 50 μL volumes of 10 μg/mL of HSP65 in single-strength PBS. Unoccupied binding sites were blocked for 1 h at 37 °C with 300 μL volumes of 2 % (w/v) milk powder dissolved in single-strength PBS (MPPBS). Recombinant antibodies in culture medium were then added (50 μL/well) and incubated for 1 h at 37 °C. Wells were then washed three times with PBS containing 0.05 % (v/v) Tween 20. Single-chain antibody fragments were detected by adding the anti-c-*myc* monoclonal antibody 9E10 [28] (produced in-house by Mr. W. van Wyngaardt, Onderstepoort Veterinary Institute, South Africa) diluted 1:1 with 4 % (w/v) MPPBS followed by rabbit anti-mouse IgG conjugated to horseradish peroxidise (HRP) (DakoCytomation, Glostrup, Denmark) diluted 1:1000 (v/v) in 2 % (w/v) MPPBS; while gallibodies were detected by adding HRP-conjugated anti-IgY Fc-region antibody (Rockland Immunochemicals, Gilbertsville, USA) diluted 1:5000 (v/v) in 2 % (w/v) MPPBS to the washed wells. To detect recombinant HSP65 directly, polyclonal rabbit anti-M. bovis antibody (DakoCytomation, Glostrup, Denmark) diluted 1:1000 (v/v) with 2 % (w/v) MPPBS was used, followed by polyclonal anti-rabbit antibody conjugated to HRP (DakoCytomation, Glostrup, Denmark) diluted 1:1000 (v/v) in 2 % (w/v) MPPBS. In all cases, after a final wash, 50 μL of 5 mg *o*-phenylenediamine (OPD) dissolved in 5 mL 0.1 M citrate buffer (pH 4.5) containing 2.5 μL 30 % (v/v) H_2O_2 was added to each well. The colour reaction was stopped using