Use of Chimeric Antibodies as Positive Controls in an Enzyme-Linked Immunosorbent Assay for Diagnosis of Scrub Typhus (Infection by Orientia tsutsugamushi)\(^V\)

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The use of human sera collected from individuals of known infected and noninfected status is necessary for the validation of diagnostic assays and for the determination of cutoff values. However, the routine inclusion of pooled human sera from infected individuals for use as positive controls in commercial assay kits has many disadvantages. Sufficient quantities of sera can be difficult to obtain, and there are ethical and safety issues to be considered. Additionally, each batch of control material requires standardization, as each will differ in antibody titer. We have genetically engineered chimeric immunoglobulin G (IgG), IgM, and IgA antibodies consisting of mouse-derived variable regions and human constant regions derived from peripheral blood lymphocytes. The chimeric nature of these antibodies allows the desired antigen specificity created through mouse immunization and hybridoma technology while retaining a human constant region required for recognition by the enzyme-conjugated antihuman signal antibody. We have investigated the potential use of chimeric IgG with specificity for the major surface antigen of Orientia tsutsugamushi as an alternative positive control for inclusion in a commercial enzyme-linked immunosorbent assay kit for the diagnosis of rickettsia scrub typhus (caused by infection with O. tsutsugamushi). Chimeric IgG was expressed in stably transfected CHO cells, allowing production of unlimited quantities. The purified protein was found to have a much greater specificity for the scrub typhus antigen than the serum-derived controls. The methods described could be applied to other assay kits for the detection of antibodies against infectious agents.

Enzyme-linked immunosorbent assay (ELISA) kits for the detection of antibodies against infectious agents use pooled human sera as reference material to establish cutoff values and to routinely confirm test integrity (3). The sera are then included in commercial kits as positive and cutoff calibrator controls. Sera are collected from individuals with confirmed cases of specific infectious diseases and pooled, but the routine use of this material has several disadvantages. Material for rare or exotic diseases can be difficult to obtain, particularly if immunoglobulin M (IgM) is required, as high titers occur only early in disease progression, often before diagnosis. Ethical problems arise from the collection of blood from individuals who are unwell, from children, or in societies where blood collection is unacceptable. There are safety concerns dealing with potentially infectious human sera, and each finite batch of material collected requires standardization due to differences in antibody titer (2).

An alternative, non-serum-derived positive control that overcomes these disadvantages would be greatly beneficial for routine inclusion in commercial ELISA kits. Such a reagent must fulfill two essential criteria: it must specifically bind to a particular antigen, and it must contain epitopes recognized by the enzyme-conjugated antihuman antibody used to generate the assay end point.

Chimeric antibodies containing variable regions cloned from specific mouse hybridomas and constant regions obtained from human peripheral blood lymphocytes satisfy these requirements. Chimeric antibodies were shown to be useful positive controls in an ELISA for Toxoplasma gondii (2). We have furthered this concept using our chimerization techniques reported previously (4) and show here its application in an ELISA for the diagnosis of rickettsia scrub typhus.

Rickettsia scrub typhus results from infection with Orientia tsutsugamushi, a bacterium transmitted to humans by bites from Leptotrombidium mite larvae. Patients with this infection present with the nonspecific symptoms of fever, headache, muscle aches, and rash, while the characteristic eschars (scabbing) are not seen in all patients (7). Thus, the disease is often misdiagnosed in patients with diseases with similar symptoms and is incorrectly treated with penicillin, to which O. tsutsugamushi is resistant, allowing progression of the disease to more serious conditions. Therefore, assays which specifically diagnose scrub typhus are very useful, and an antibody ELISA is a practical format for use in regions where facilities and trained personnel may be limited.

O. tsutsugamushi expresses a 56-kDa protein in its cell envelope which is recognized by antibodies present in the serum of patients and of experimentally immunized animals (6). A recombinant version of this protein (r56) (1) is used as the capture antigen in the commercially available rickettsia scrub typhus group IgG and group IgM ELISA kits (Panbio, Brisbane, Australia). Therefore, the chimeric antibody that we have tested in this assay contains r56-specific mouse-derived
variable regions and human constant regions for recognition by the enzyme-conjugated signal antibody.

MATERIALS AND METHODS

DNA constructs for chimeric antibodies. A mouse-human chimeric IgG construct was produced as described previously (4). Briefly, variable heavy and kappa chain cDNAs were amplified by reverse transcription-PCR from RNA isolated from mouse hybridoma cells secreting antibody against r56. Similarly, human constant-region heavy and kappa chain cDNAs were amplified from peripheral blood lymphocyte mRNA isolated from a human blood sample. The mouse and human fragments were chimerized by splice overlap extension PCR (4) and were cloned into a bicistronic expression vector, pBudCE4.1 (Invitrogen, Melbourne, Australia). This vector was transfected into CHO-S cells, a derivative of the CHO cell line adapted to serum-free growth (Invitrogen). A stable cell line secreting chimeric IgG was produced by selection with 400 μg/ml phleomycin (Zeocin, Invitrogen).

Purification of chimeric IgG. To harvest chimeric IgG for purification, four flasks of 30-ml stable cell line cultures at 2 × 10^5 cells/ml were incubated with shaking for 7 days at 37°C. The supernatants were collected after centrifugation at 250 × g for 3 min at ambient temperature and were then passed through a 0.45-μm-pore-size filter. Purification of chimeric IgG was performed with a 1-ml HiTrap protein G Sepharose column (Amersham Biosciences, Sydney, Australia), according to the manufacturer's instructions.

Quantitation of chimeric IgG. The purified IgG preparation was quantitated by an in-house ELISA. Microwell plates coated with a goat anti-human IgG antibody were obtained from a dengue virus capture IgG ELISA kit (Panbio). A standard curve was produced for each assay run by using known concentrations of purified IgG from human serum (Sigma, St. Louis, MO) as the standard. Standards and samples were detected by using horseradish peroxidase-conjugated sheep anti-human IgG and tetramethylbenzidine substrate (both obtained from the rickettsia scrub typhus group IgG ELISA kit [Panbio]). After quantitation, the purified preparation was diluted twofold in control serum diluent (CSD; Panbio) to enhance its stability.

Titration of chimeric IgG. The chimeric IgG preparation was diluted in CSD to produce a series of concentrations down to 0.1 μg/ml. These were assayed, following the manufacturer’s instructions, by using a rickettsia scrub typhus group IgG ELISA kit (Panbio), which uses immobilized r56 antigen to capture anti-r56 antibodies. Detection uses horseradish peroxidase-conjugated sheep anti-human IgG and tetramethylbenzidine. The assay results were compared with those obtained with the kit’s positive and cutoff calibrator controls. The purpose was to obtain preparations of the chimeric IgG which return results equivalent to those for the serum-derived controls, which could then be used as alternative controls.

Serial dilutions of both the serum-derived controls and the alternative chimeric controls were then prepared in CSD and assayed by using the rickettsia scrub typhus group IgG ELISA kit to determine if the dilutions of a non-serum-based preparation behave the same as dilutions of the serum reagents in the assay.

RESULTS

Purification and titration of chimeric IgG. Purification of chimeric IgG from culture supernatants yielded 0.5 ml of 50.8 μg/ml antibody in the peak fraction. This was diluted to 25.4 μg/ml in CSD. Serial dilutions of the purified chimeric IgG were then used for subsequent experiments.

Figure 2 shows the parallelism between the dilution curves for the chimeric IgG controls (positive at 22.8 μg/ml and the cutoff reactivity at 5.0-μg/ml starting concentrations) and the serum-derived controls by the rickettsia scrub typhus group IgG ELISA. The dilution curves were each fitted to a sigmoidal dose-response curve by using Prism (version 3.00) software for Windows (GraphPad Software, San Diego, CA) (Fig. 1). The equation for the curve was used to determine that 22.8 μg/ml and 5.0 μg/ml of the purified chimeric IgG gave absorbance results equivalent to those for the serum-derived positive and cutoff calibrator controls, respectively. These concentrations of chimeric IgG were then used for subsequent experiments.

Cross-reactivity of serum standards and chimeric IgG. The cross-reactivity of the rickettsia scrub typhus group IgG ELISA results for the current serum-derived kit controls and the alternative chimeric IgG controls. The chimeric IgG was prepared at initial concentrations which return ELISA results equivalent to those for the serum-derived positive and cutoff calibrator controls.

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which cross-reactivity has resulted in equivocal or positive results. Rickettsia scrub typhus antigen plate results are also shown in boldface. As negative results, those between 0.9 and 1.1 are classified as equivocal results, and those above 1.1 are classified as positive results. Boldface indicates instances in which cross-reactivity has resulted in equivocal or positive results. Rickettsia scrub typhus antigen plate results are also shown in boldface.

Barr virus, human herpesvirus 6, Leptospira, varicella-zoster virus, and West Nile virus, such that the \( A_{450} \) result was greater than or close to the result for the specific cutoff calibrator control, resulting in equivocal or positive ratios. For all antigens tested (except r56), the chimeric anti-r56 IgG showed no cross-reactivity.

**DISCUSSION**

Serum-derived reference material must be used during the development and validation of a diagnostic assay for an infectious disease. Reference material is required to determine cutoff values for the assay which will optimize the balance between assay sensitivity and specificity (3). Serum-derived material is also necessary for the continued monitoring of the assay performance. For example, the cutoff values may need to be optimized for different populations or may drift with time. However, the controls included in the assay kit for distribution to the end user need not be serum derived, as long as the alternative performs equivalently in the assay.

We have determined the concentrations of chimeric mouse-human anti-r56 IgG which gave assay results equivalent to those for the serum-derived positive control and the cutoff calibrator and have shown that dilutions of these preparations made in CSD behave identically to the serum-derived reagents in the ELISA. This validates the use of purified recombinant chimeric antibodies as alternative positive control reagents, even though the matrix is not serum and is therefore different from the sample matrix. Additionally, we have studied the degradation of the chimeric IgG at elevated temperatures and predict that the preparation should remain stable for the shelf-life of an ELISA kit (nominally 15 months at 4°C for the rickettsia scrub typhus group IgG ELISA) (data not shown) (5).

Recombinant chimeric antibodies would be superior alternatives to serum-derived positive and cutoff calibrator controls for routine inclusion in ELISA kits, as they eliminate many of the disadvantages associated with serum-derived controls. The supply of serum-derived controls is finite, and so new batches must be obtained regularly from regions of the world where the disease is endemic. The chimeric antibodies, however, are expressed from a stable cell line, allowing consistent and continuous production. This also avoids the ethical problems associated with the collection of serum from individuals who are unwell and safety issues involving the handling of potentially infectious material.

The serum-derived controls from the rickettsia scrub typhus group IgG ELISA kit were found to cross-react with antigens from several other infectious agents. The serum is collected from several individuals with known *O. tsutsugamushi* infection and pooled; therefore, the batch contains a mixed population of antibodies reflecting the immune status of each individual. Therefore, it is conceivable that the positive control serum contains antibodies against many other antigens. This cross-reactivity will differ between each batch of control material collected. This is not a problem for the performance of each individual assay, as the controls are intended for use only with the corresponding kit. However, in a clinical laboratory that is processing several different assays at high throughput, the potential use of the incorrect standard may not be detected if it cross-reacts with the antigen. This will cause errors in the assay cutoff values, potentially leading to false-positive or false-negative results. The chimeric anti-r56 IgG is a monoclonal preparation which showed no cross-reactivity with the other antigens tested. Therefore, the chimeric antibody is a more reliable, safe, and consistent positive control.

By using the same principles described here, chimeric antibodies with specificity toward many different antigens can be created, making the procedures described in this study applicable to the production of alternative controls for other commercial ELISA kits. The human constant regions are cloned...
and ready for chimerization to specific variable-region cDNA, which can be isolated from hybridomas secreting specific antibodies, if they are available. The manufacturers of diagnostic kits may wish to outsource the creation of chimeric antibody DNA constructs and stable cell lines. The chimeric antibodies can then be expressed and purified in-house, providing a constant supply of consistent control reagents. Thus, the initial high cost of creating the antibodies will be offset by eliminating the high costs and risks associated with sourcing, transporting, and standardizing serum-derived reagents.

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REFERENCES