BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **218,** 260–266 (1996)
Article No. 0046

Expression of the Carbohydrate Recognition Domain of Bovine Conglutinin and Demonstration of Its Binding to iC3b and Yeast Mannan

Boon-Leong Lim*,[1] and Uffe Holmskov†

*Department of Zoology, The University of Hong Kong, Pokfulam, Hong Kong; and †Institute of Medical Biology, Department of Microbiology, Odense University, Winsløwsparken 19 DK-5000, Denmark

Received November 27, 1995

Bovine conglutinin is a collagenous C-type lectin (collectin) that is found in bovine serum. A recombinant protein, composed of the neck-region and the carbohydrate binding domain of bovine conglutinin, has been overexpressed in *E. coli*. The recombinant protein has been successfully renatured and showed the same sugar binding specificity as the native protein and was able to bind to yeast mannan and complement-activated immune complexes. The binding was calcium-dependent and was inhibited by N-acetylglucosamine. The concentration of N-acetylglucosamine required for 50% inhibition of binding to mannan was 1.77 mM for recombinant conglutinin and 0.71 mM for native conglutinin, respectively. The recombinant conglutinin should be useful in the assay and purification of circulating immune complexes and for therapeutic purposes involving the removal of immune complexes from patient's plasma.

Bovine conglutinin (BK) was the first vertebrate C-type lectin to be described (1). It was found to be able to agglutinate complement-reacted erythrocytes (2). This function is mediated by the ability of conglutinin to bind to the carbohydrate moiety on the α-chain of iC3b (3), with a dissociation constant (Kd) of 1.3×10^8M (4). Conglutinin is thus able to enhance the clearance of bacteria from the circulation by promoting contact between iC3b-coated bacteria and effector cells (5). It has been shown to exhibit a complement-dependent enhancement of the respiratory burst of phagocytes stimulated by *E. coli* (6). Moreover, conglutinin has been shown to be one of the β-inhibitors of influenza virus in bovine serum, which binds the high mannose type oligosaccharide side chain of the viral hemagglutinin and thus inhibits the infectivity and hemagglutinating activity of influenza A viruses of the H1 and H3 subtypes (7). Electron microscopy image of BK showed that it is a tetramer composed of four "lollipop"-shaped structures radiating from a central hub (8). Each subunit of the tetramer has a rod-length of 38 nm and is composed of 3 identical polypeptide chains. Each polypeptide chain has been shown, by cDNA cloning (9), to have an N-terminal, cysteine containing region of 25 residues-long followed by a 171 residues-long collagenous region containing 55 Gly-Xaa-Yaa repeats with one interruption, a neck region of 28 residues and a 127 residues-long, C-terminal, C-type lectin domain. Three polypeptide chains from a triple-helical structure via their collagenous region so that the individually folded CRDs of each chain are clustered together, and the N-terminal regions of the subunits are associated by disulphide bonding, as well as by non-covalent bonding, into a tetrameric structure.

In the present studies, the neck region and the CRD of BK has been overexpressed as a His-tag fusion protein in bacteria and purified. The recombinant protein, which lacked the N-terminal region and the collagen region of conglutinin, is considered to form a trimer of a polypeptide

[1] To whom correspondence should be addressed. Department of Zoology, The University of Hong Kong, Pokfulam, Hong Kong. Fax: 852-28574672.

Abbreviations: BCIP, 5-bromo-4-chloro-3-indoyl phosphate; BK, bovine conglutinin; BSA, bovine serum albumin; CRD, carbohydrate recognition domain; ELISA, enzyme-linked immunosorbent assay; GalNAc, N-acetyl-D-galactosamine; GluNAc, N-acetyl-D-glucosamine; IPTG, isopropyl β-D-thiogalactoside; Kd, dissociation constant; NBT, nitro blue tetrazolum; PCR, Polymerase chain reaction; PNPP, 4-nitrophenylphosphate disodium salt; rBK, recombinant bovine conglutinin; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SP-D, lung surfactant protein D.

0006-291X/96 $12.00

composed of a His-tag at the N-terminus and followed by the neck region (residues 197–224) and the CRD (residues 225–351) of conglutinin (9). The neck region on its own is known to form a homotrimer composed of an α-helical bundle held together by extremely strong hydrophobic interactions (10). The recombinant protein showed the same sugar binding specificity as the native protein and was able to bind to the yeast mannan and complement-activated immune complexes. The binding was calcium-dependent and was inhibited by N-acetylglucosamine.

MATERIALS AND METHODS

Chemicals and reagents. The following chemicals and reagents were purchased from the sources indicated: ampicillin, bovine serum albumin (BSA), 5-Bromo-4-chloro-3-indoyl phosphate (BCIP), divinylsulfon, EDTA, ethanolamine, extravidin-alkaline phosphatase conjugate, goat anti-rabbit IgG-alkaline phosphatase conjugates, nitro blue tetrazolum (NBT), sodium azide, thrombin, Tris-HCl, Triton X-100, yeast mannan (Sigma); N-hydroxyl succinomidobiotin (BNHS) (Pierce); isopropyl β-D-thiogalactoside (IPTG) (Novabiochem); His-Band affinity matrix (Qiagen), microtitre plate (Polysorb) (Nunc, Denmark). pBluescript plasmid, pfu DNA polymerase (Strategene); pRsetA plasmid (Invitrogen); TSK beads (Fractogel HW75F, Merck).

Buffers. Denaturing buffer (1 × TBS, 6M Urea, 0.05% (v/v) Tween-20, 20 mM $CaCl_2$); *E. coli* lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 0.05% (w/v) NaN_3, 1 mM PMSF, 10 mM EDTA, 0.05%(v/v) Tween-20, pH 7.5); ELISA coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.05%(w/v) NaN_3, pH 9.6); ELISA developing buffer (100 mM Tris-HCl, pH 9.6, 100 mM NaCl, 5 mM $MgCl_2$); Elution buffer (0.3 M imidazole, 0.15M NaCl, 6M Urea and 20 mM Tris-HCl, pH 7.5); TBS (20 mM Tris-HCl, 140 mM NaCl, 0.05% (w/v) NaN_3, pH 7.4); TBS/EDTA (TBS with 10 mM EDTA, 0.05%(v/v) Tween-20, pH 7.4); TBS/NTC (TBS with 5 mM $CaCl_2$, 0.05%(v/v) Tween-20, pH 7.4); TBS/NTCB (TBS/NTC with 1 mg/ml BSA).

Construction of the conglutinin expression plasmids. An expression plasmid had been constructed from the pRSetA plasmid (11) as described below. pRSet A plasmid was digested by Bam HI and Eco RI, dephosphorylated by alkaline phosphatase and purified by agarose electrophoresis. In order to create a Bam HI site on the bovine conglutinin cDNA insert for subcloning, a PCR primer (BK-1, 5′-ATTAGGATCCGCAGAGGCCAATGCT CTCA-3′) was synthesised. A 50 μl PCR reaction was carried out using BK-1 and T7P (a pBluescript primer) as primers and pBluescript containing the conglutinin cDNA as a template (9). pfu DNA polymerase was used to secure a high fidelity. The amplified PCR product was then precipitated and digested by Bam HI and Eco RI. The digested insert was purified by gel electrophoresis and subcloned into the digested, desphosphorylated and purified pGex-2T. Plasmids that contained the right inserts were transformed into the *E. coli.* strain BL 21 (DE3) (12).

Overexpression of the recombinant conglutinin in E. coli. A colony carrying the expression plasmid was inoculated into 20 ml of LA (Luria-Bertani medium + 100 mg/L ampicillin) and grown for 5 hours at 37°C. After keeping the bacterial culture at 4°C overnight, the cells were sedimented by centrifugation and inoculated into 1 litre LA. After 2.5 hours at 37°C, protein expression was induced by 0.1 mM IPTG and the culture was allowed to grow for 2 hours at 37°C. The cells were harvested by centrifugation at 5000g for 15 minutes. The cells were resuspended by *E. coli* lysis buffer (15ml/litre culture) and lysed by sonication (3 × 1 min). The pellet, after centrifugation at 12000g for 15 minutes, contained the recombinant product. After washing twice in TBS, the pellet was dissolved in denaturing buffer, by gentle shaking at room temperature. Undissolved particulates were removed by centrifugation at 12000g and the supernatant was passed through a His-Band affinity column (3 cm × 10 mm) which had been equilibrated in the denaturing buffer. Non-binding proteins were washed away with the denaturing buffer and the fusion protein was eluted by elution buffer. For refolding, the protein was diluted 6 fold (final urea concentration = 1M) and 2 fold (final urea concentration = 0.5M) by TBS/NTC containing 20 mM $CaCl_2$, in a two step manner. Each step was carried out overnight at 4°C. The refolded material was then dialysed against 3 changes of one litre TBS/NTC before passing through a N-acetylglucosamine-TSK column (16 mm × 150 mm). N-acetylglucosamine was coupled to TSK beads as described (13). After washing with TBS/NTC, the bound protein was eluted by TBS/EDTA. The concentration of the eluted fusion protein was determined by its absorbance at 280 nm using extinction coefficient $E_{1\%,\ 1\ cm,\ 280nm}$ of 11.0. The identities of the peaks were confirmed by Western blotting.

Isolation of native bovine conglutinin. Native conglutinin was isolated from bovine serum by the method of Holmskov et al. (13).

Preparation of complement-activated immune complexes. Rabbit IgG (10mg/ml) was heat-aggregated at 63°C for 30 minutes. The aggregated IgG was centrifuged at 10000g for 10 minutes and the top two-third supernatant containing small heat-aggregated complexes was retained. The concentration of the complexes was determined spectrometrically by dissolving an aliquot of the complexes in 0.1N NaOH. The complexes was reacted with complement by incubating with three volume of human serum for 30 minutes at 37°C. The concentration of the complement-activated complexes was adjusted to 2 mg/ml and stored at −70°C.

ELISA. Microtitre plates were coated with mannan in various concentrations in 100 μl coating buffer at 4°C overnight. The plates were washed 3 times with TBS/NTC after each subsequent step. After coating, the plate was blocked with 1% (w/v) BSA in TBS/NTC for 1 hour at room temperature. Recombinant or native conglutinin (100 μl, 0–1000 ng/ml in

TBS/NTCB, with or without N-acetylglucosamine), rabbit α-conglutinin serum (100 μl of a 1 in 1000 dilution in TBS/NTCB), goat α-rabbit IgG alkaline phosphatase conjugate (100 μl of a 1 in 10000 dilution in TBS/NTCB) were added sequentially with washing between each step. All incubation steps were carried out at room temperature for 2 hours. Finally, 100 μl alkaline phosphatase substrate (PNPP, 1mg/ml) in developing solution was applied to each well. Reactions were developed at 37°C and the OD 405nm was read in an ELISA plate reader. For sugar inhibition assays, microtitre plates were coated with yeast mannan (100 μl, 10 μg/ml) and the biotinylated lectins was incubated in TBS/NTCB with increasing concentrations of sugars. The concentration of sugar that gave 50% inhibition of binding was defined as IC50.

RESULTS

Overexpression of the recombinant protein in E. coli. The cDNA of conglutinin encodes a polypeptide of 351 residues with a 20 residue-long leader peptide (9). Using the primers BK-1 and T7P, an insert of approximately 550 bp was amplified from the cDNA, by PCR reaction, and then subsequently cloned into the expression vectors pRsetA. The expressed fusion proteins, which had a histidine-rich peptide chain fused to the N-terminus, carried residues 197 to 351 of the mature conglutinin protein, corresponding to the neck region (residues 197–224) and the carbohydrate recognition domain (residues 225–351) of conglutinin (9). Purification of this His-tag fusion protein is shown in Figure 1. The His-tag fusion protein was insoluble in *E. coli* lysate buffer (lane 5). The polypeptide representing the neck region and CRD of conglutinin should be 17.4 kDa in length, which is linked to a N-terminal histidine-rich peptide of 3.8 kDa. Hence, the fusion protein should have a molecular weight of approximately 21.2 kDa. The insoluble protein was solubilized by denaturing buffer containing 6M urea. Since the CRD of conglutinin required calcium ions for sugar binding, 20 mM calcium chloride was included in the denaturing buffer and refolding buffer. Even in the present of 6M urea, the His-tag fusion protein was capable of binding to the His-band affinity column. After washing away the impurities with the denaturing buffer, the recombinant protein was eluted by imidazole-containing buffer (lane 7). The recombinant protein was then refolded by two-step dilution in TBS/NTC containing 20 mM calcium chloride (lane 8). In order to remove incorrectly folded protein, a sugar affinity column was used (GlcNAc-TSK). Most of the refolded protein was retained on the column (80%, determined by OD absorbance), as shown in lane 9, indicating that the refolding process is efficient. The final yield of the protein was 3 mg per litre cell culture. By cross-linking studies, the recombinant protein was shown to be a trimer of three polypeptides (unpublished data).

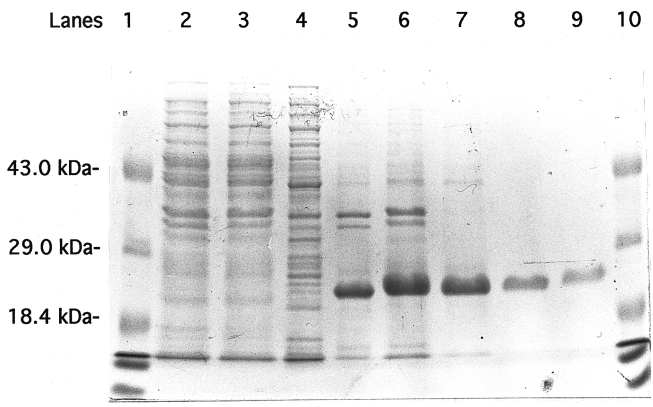

FIG. 1. Purification of the recombinant protein composed of His-tag plus the neck region and the CRD domain of conglutinin. Lanes 1 and 10 contain molecular weight standards; from top to bottom these are 43.0 kDa, 29.0 kDa and 18.4 kDa. Lanes 2 to 9 show various steps in the purification : total cell lysate without IPTG induction (2); total cell lysate after IPTG induction (3); soluble fraction after sonication (4); insoluble fraction after sonication (5); proteins soluble in denaturing buffer, after centrifugation (6); proteins retained on the His-band affinity column (7); soluble proteins after refolding (8); protein retained on the N-acetylglucosamine-TSK affinity column (9). All the samples were run on 15% (w/v) SDS-PAGE under reducing conditions.

Binding of the recombinant protein and native bovine conglutinin to yeast mannan. The abilities of the recombinant conglutinin protein to bind to yeast mannan, under a variety of different conditions, were tested by ELISA. As seen in Figure 2, the recombinant protein was able to bind to the yeast mannan in a sugar-dependent manner (inhibited by N-acetylglucosamine and maltose), indicating that the binding was mediated through the C-type lectin activity of the protein. The calcium dependence of the binding was also confirmed by replacing calcium chloride with EDTA in the binding buffer, which abolished the binding. Since the positive signals (rBK in TBS/NTC) increased with the amount of yeast mannan coated on the plate, the signals were not due to non-specific binding of recombinant protein to the ELISA plate. In addition, controls which lacked recombinant conglutinin gave zero values which proved that the positive signals were not derived from anti-mannan antibodies in the primary or secondary antisera. The sugar specificities of the recombinant protein were examined by sugar inhibition assays. Fixed amount of biotinylated recombinant conglutinin or native conglutinin, diluted in buffers containing various concentration of sugars, was applied to microtiter plates coated with yeast mannan. As shown in Figure 3, N-acetylglucosamine was the most potent sugar in inhibiting the binding of both recombinant conglutinin and native conglutinin to yeast mannan. The sugar specificities of the recombinant protein are represented by IC50 (Table I), which is defined as the concentration of the sugar required for 50% inhibition of binding. In general, comparing to the native protein, the recombinant protein have slightly lower affinities towards the sugar tested.

Binding of the recombinant protein to complement-activated immune complexes. The abilities of the recombinant conglutinin protein to bind to iC3b-containing immune complexes were tested by ELISA. As seen in Figure 4, the complement-activated immune complexes were able to bind to the recombinant conglutinin in a sugar-dependent manner (N-acetylglucosamine), indicating that the binding was mediated through the C-type lectin activity of the protein. The calcium dependence of the binding was also confirmed by replacing calcium chloride with EDTA in the binding buffer, which abolished the binding. Since the positive signals (immune complexes in TBS/NTC) increased with the amount of the recombinant protein coated on the plate, the signals were not due to non-specific binding of immune complexes to the ELISA plate.

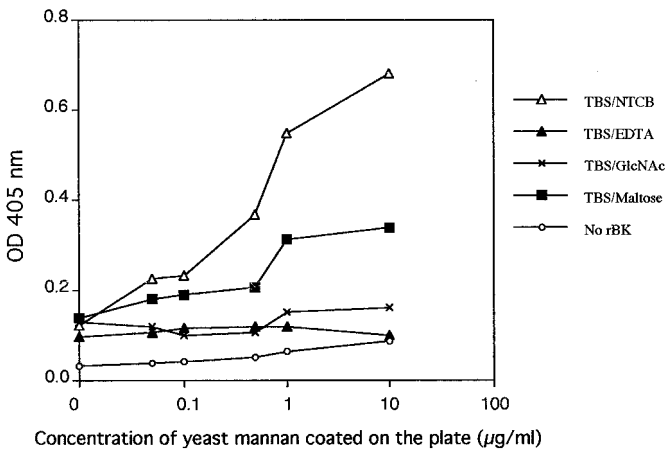

FIG. 2. 100 μl (0.01–10 μg/ml) yeast mannan were coated on the polysorb ELISA plate. After blocking, 100 μl recombinant protein (5 μg/ml) diluted in TBS/NTCB, in TBS/EDTA, or in TBS/NTCB with 50 mM N-acetylglucosamine or maltose were added onto the plates. α-BK antiserum and alkaline phosphatase-secondary antibody conjugates were diluted 5000 fold or 10000 fold respectively in TBS/NTCB. OD reading was taken at 405 nm. (△, recombinant protein in TBS/NTCB; ▲, recombinant protein in TBS/EDTA; x, recombinant protein in TBS/NTCB with 50 mM N-acetylglucosamine; ■, recombinant protein in TBS/NTCB with 50 mM maltose; ○, TBS/NTCB only).

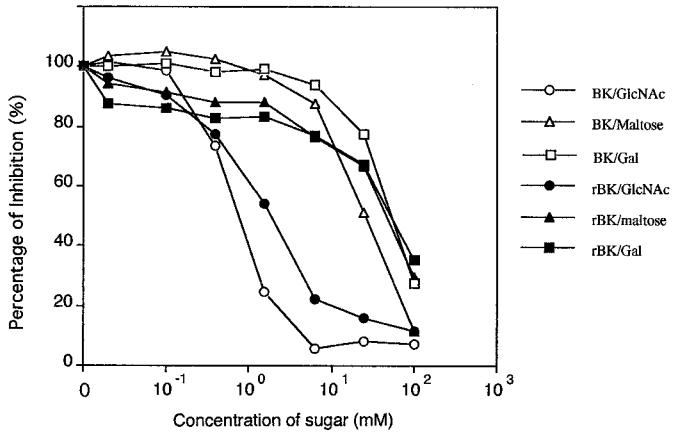

FIG. 3. Yeast mannan (100 μl of 10 μg/ml) was coated on the plates. After blocking, 100 μl biotinylated native conglutinin (BK, 1.9 μg/ml) or biotinylated recombinant conglutinin (rBK, 1.3 μg/ml) were added in TBS/NTCB or TBS/NTCB with various concentrations (0, 0.02, 0.1, 0.39, 1.56, 6.25, 25, 100 mM) of sugars (○, ●, N-acetylglucosamine; △, ▲, maltose; □, ■, galactose). Alkaline phosphatase-conjugated avidin were diluted 2000 fold in TBS/NTCB and the colour was developed with PNPP. OD reading was taken at 405 nm.

DISCUSSION

The fusion protein, which contained the neck region (aa. 197–224) and CRD (aa. 225–351) of conglutinin, was insoluble. In order to produce a functional protein, refolding studies have been carried out. The insoluble fraction was washed three times in TBS to remove most of the soluble proteins before solubilized by a buffer containing 6M urea. Two methods of refolding have been tried, dialysis or rapid dilution. In the dialysis method, the denatured recombinant protein was dialyzed against TBS containing 20 mM $CaCl_2$ and 0.05%(v/v) Tween-20. Almost all of the protein precipitated out during dialysis, leaving only low levels of soluble protein. Other attempts, using rapid dilution, has shown that: 1. Refolding at 4°C gives a much better yield than at room temperature. 2. It is advantageous to include non-ionic detergent, such as Tween-20, in the buffer. 3. Calcium ions help the refolding of conglutinin, since it is a calcium-binding protein. 4. Two steps of dilution give a better yield than one step of dilution. Correctly refolded protein was purified from incorrectly folded protein by the G1cNAc-TSK affinity column, which only retained the correctly

TABLE 1
Sugar Specificities of Recombinant CRD of Conglutinin* and Native Conglutinin

Sugar Inhibitors	Recombinant CRD of conglutinin	Native conglutinin
	I50 (mM)**	I50 (mM)
Maltose	44.6	25.7
Glucose	>100	34.7
Galactose	57.5	57.5
L-fucose	70.8	20.0
GluNAc	1.77	0.71
GalNAc	>100	>100
Lactose	>100	>100

* The recombinant CRD of bovine conglutinin is contained as a homotrimer of a polypeptide chain composed of: His tag-the neck region of BK-the CRD of BK.

** The values given are I50, the concentration required for 50% inhibition of binding.

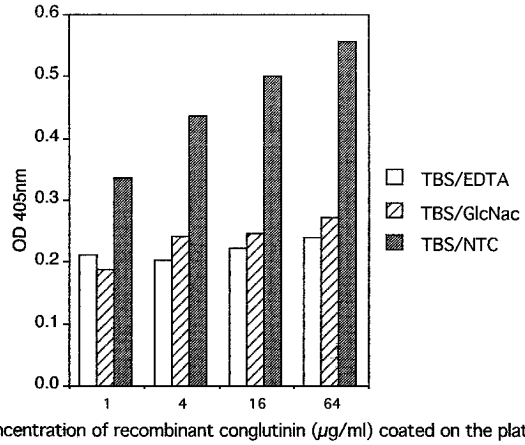

FIG. 4. Recombinant conglutinin (100 μl of 1, 4, 16, 64 μg/ml) was coated on the microtitre plate. After blocking with BSA, 100 μl complement-activated immune complexes (10 μg/ml) was added in TBS/NTCB, TBS/EDTA or TBS/NTCB with 50mM GlcNAc. Alkaline phosphatase-conjugated, goat anti-rabbit IgG antibody diluted 5000 fold in TBS/NTCB was used as secondary antibodies and the colour was developed with PNPP. OD reading was taken at 405 nm.

folded protein. The procedure that resulted in the best yield (up to 80% of protein refolded) was described in the materials and method section. The recombinant protein has a sugar specificity similar to the native protein, in which the affinity is lower than the native protein (Table 1). It may be due to the fact that the native conglutinin is a tetramer composed of four trimeric CRDs, which greatly enhance the avidity of the protein. Comparing to the native protein, recombinant conglutinin is only composed of one trimer subunit.

The recombinant protein is able to capture complement-activated, iC3b coated immune complexes. This properties of the recombinant protein may be exploited in the isolation of immune complexes. Elevated circulating immune complexes (CIC) have been associated with many diseases, including systemic lupus erythematosus (14), rheumatoid arthritis, Alzheimer's disease (15), Hodgkin's disease (16), leprosy (17), chronic inflammatory bowel disease (18), uveoretinitis (19), Sjogren's syndrome (20) and various cancers (21,22). Currently, the C1q binding (Fc) (23) and the conglutinin binding (iC3b) (17) assays are the most reliable and clinically useful methods for measuring the levels of CIC in patient's serum. The major disadvantage of these two methods is the costly procedures required for the purification of native C1q and conglutinin. Recombinant C-type lectin domains of conglutinin can be used to replace the native conglutinin protein and can be employed in the development of standard clinical kits for the measurement of CIC. By using a construct of neck and CRD without the collagen part of the molecule, the influence of anti-collagen antibodies are avoided.

Another use of the recombinant protein is its application on the isolation of CIC. The present procedure for CIC isolation involves a multi-steps process. CIC are first precipitated from patients' serum by the addition of polyethylene glycol (PEG). The precipitated CIC are then further purified by binding to a protein A-sepharose affinity column and the bound CIC are subsequently eluted with low pH (2.8) buffer (17). This protocol have been successfully employed to identify several aetiological antigens in patients' serum that are complexed with CIC (17). The same approach has also been used to identify several tumour-associated antigens (18) and parasite antigen (24). However, this method has several disadvantages. Firstly, PEG can only precipitate large CIC and thus leaves small antigen-antibody complexes in solution, which may contain potential aetiological agents. Secondly, protein A does not bind human IgG3, IgM, IgD and IgA and therefore it is not able to capture CIC constituted by these antibodies subclasses. Thirdly, protein A not only binds CIC, but also binds monomeric IgG subclasses. Finally, the condition required for the elution of

CIC and the regeneration of the protein A column is very harsh and protein A is an expensive reagent. A one step method for CIC isolation can be developed by the use of recombinant conglutinin. CIC in patients' serum can be captured by a conglutinin-sepharose affinity column. All CIC capable of activating complement system through the classical pathway or the alternative pathway will be bound, independent of the subclasses of the antibodies. The bound CIC can then be simply eluted by EDTA-containing buffer at neutral pH and the conglutinin column can simply be regenerated by calcium-containing buffer. Thus the uses of recombinant conglutinin can resolve the problems associated with the PEG method.

The third potential clinical application of the recombinant conglutinin is its use for the extracorporeal immunoadsorption treatment of patients' plasma. Staphylococcal protein A column (PROSORBA columns, IMRE Corporation, Seattle, WA) has been used to remove immunoglobulin and CIC from patients' plasma in the treatment of malignant and autoimmune diseases (25). However, due to its high cost, its detrimental complement-activating effect, and the other disadvantages mentioned in the above paragraphs, protein A is not the ideal ligand for immunoadsorption treatment. Recombinant conglutinin might be a substitute for protein A in the preparation of CIC immunoadsorption column.

ACKNOWLEDGMENTS

We are grateful to Mr. J. K. Fung for his technical assistance. This project is supported by the Committee on Research and Conference Grants (337/026/0020).

REFERENCES

1. Bordet, J., and Gay, F. P. (1906) *Ann. Inst. Pasteur* **20,** 467–498.
2. Leon, M. A. (1957) *Proc. Soc. Exp. Biol. Med.* **96,** 202–204.
3. Hirani, S., Lambris, J. D., and Muller-Eberhard, H. J. (1985) *J. Immunol.* **134,** 1105–1109.
4. Brown, E. J., Gaither, T. A., Hammer, C. H., Hoseq, S. W., and Frank, M. M. (1982) *J.Immunol.* **128,** 860–865.
5. Friis Christiansen, P., Thiel, S., Svehag, S. E., Dessau, R., Svendsen, P., Andersen, O., Laursen, S. B., and Jensenius, J. C. (1990) *Scand. J. Immunol.* **31,** 453–460.
6. Friis, P., Svehag, S. E., Andersen, O., Gahrn-Hansen, B., and Leslie, R. G. (1991) *Immunology* **74,** 680–684.
7. Hartley, C. A., Jackson, D. C., and Anders, E. M. (1992) *J. Virol.* **66,** 4358–4363.
8. Strang, C. J., Slayter, H. S., Lachmann, P. J., and Davis, A. E. (1986) *Biochem. J.* **234,** 381–389.
9. Lu, J., Laursen, S. B., Thiel, S., Jensenius, J. C., and Reid, K. B. (1993) *Biochem. J.* **292,** 157–162.
10. Hoppe, H., Barlow, P. N., and Reid, K.B. M. (1994) *FEBS Lett.* **344,** 191–195.
11. Kroll, D. J., Abdel-Malek, A. H., Marcell, T., Simpson, S., Chen, C. Y., Gutierrez, H. A., Lustbader, J. W., and Hoeffler, J. P. (1993) *DNA Cell Biol.* **12,** 441–453.
12. Moore, J. T., Uppal, A., Maley, F., and Maley, G. F. (1993) *Protein Expr. Purif.* **4,** 160–163.
13. Holmskov, U., Teisner, B., Willis, A. C., Reid, K. B., and Jensenius, J. C. (1993) *J. Biol. Chem.* **268,** 1012010125.
14. Kabakov, A. E., Tertov, V. V., Saenko, V. A., Poverenny, A. M., and Orekhov, A. N. (1992) *Clin. Immunol. Immunopathol.* **63,** 214–220.
15. Heinonen, O., Syrjanen, S., Soininen, H., Talasniemi, S., Kaski, M., Mantyjarvi, R., Syrjanen, K., and Riekkinen, P. S. (1993) *Neurosci. Lett.* **149,** 67–70.
16. Nerurkar, A. V., Advani, S. H., and Gothoskar, B. P. (1993) *Neoplasma.* **40,** 87–91.
17. Corsico, B., Croce, M. V., Mukherjee, R., and Segal Eiras, A. (1994) *Clin. Immunol. Immunopathol.* **71,** 38–43.
18. Bartoloni, C., Guidi, L., Pili, R., Lewis, Z. A., Tricerri, A., Cursi, F., Gentiloni, N., Cortesi, E., and Gambassi, G. (1993) *Oncology.* **50,** 27–34.
19. Kasp, E., Stanford, M. R., Brown, E. A., Coombes, G., and Dumonde, D. C. (1992) *Clin. Exp. Immunol.* **88,** 307–312.
20. Bendaoud, B., Pennec, Y. L., Lelong, A., Le Noac'h, J. F., Magadur, G., Jouquan, J., and Youinou, P. (1991) *J. Autoimmun.* **4,** 177–184.
21. Aziz, M., Dass, T. K., and Rattan, A. (1992) *J. Trop. Pediatr.* **38,** 185–188.
22. Dass, T. K., Aziz, M., and Rattan, A. (1991) *Jpn. J. Cancer Res.* **82,** 1284–1291.
23. Devey, M. V., Taylor, J., and Steward, M. W. (1980) *J. Immunol. Methods* **34,** 191–203.
24. Thambiah, G., Whitworth, J., Hommel, M., and Devaney, E. (1992) *Trop. Med. Parasitol.* **43,** 271–276.
25. Snyder, H. W. J., Mittelman, A., Oral, A., Messerschmidt, G. L., Henry, D. H., Korec, S., Bertram, J. H., Guthrie, T. H. J., Ciavarella, D., Wuest, D., and et al. (1993) *Cancer* **71,** 1882–1892.