Antigen detection ELISAs: pretreatment of serum to reduce interference by specific host antibodies

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Abstract

The pretreatment of serum to reduce interference by specific host antibodies was investigated as a means of improving the sensitivity of antigen detection ELISAs whilst screening serum samples. Four antigen detection assays based on monoclonal antibodies directed against antigens of the bovine filariid *Onchocerca gibsoni* were used in this study and, of these, three assays suffered a dramatic drop in sensitivity when detecting male *O. gibsoni* antigen in the presence of bovine serum as compared with antigen in buffer. A number of methods for pretreating serum to eliminate the problem of antibody interference with antigen detection were attempted, including heat and alkali treatments, detergent treatment of heat treated samples and the use of a reducing agent. The pretreatment of serum by boiling for 5 minutes in the presence of an equal volume of 0.1 M Na_2EDTA pH 4.0 and recovery of the supernatant fluid following centrifugation at 16000 g was the most effective method of restoring the sensitivity of each of these three assays whilst screening bovine serum. Pretreatment of serum using this method produced up to a 512-fold increase in sensitivity compared with results obtained in assays with non-treated serum.

Introduction

There has been increasing interest over the past few years in application of antigen detection immunoassays to diagnosis of filarial infections because of their potential to detect cases that are prepatent, patent and occult.

Progress in the development of antigen detection assays has been hampered, however, by a significant reduction in the sensitivity of these tests in the presence of immune complexes and interfering host antibodies (Forsyth et al., 1985; Maizels et al., 1987). There are numerous reports of a drop in the amount of detectable antigen in serum accompanied by a rise in specific host antibody (Forsyth et al., 1985; Weiss et al., 1986; Lutsch et al., 1988; Maizels et al., 1988; Lal and Ottesen, 1989), and several studies have directly implicated the action of specific host antibodies as the cause of this problem (Hamilton and Scott, 1984; Maizels et al., 1987). Methods of dissociating immune complexes, thereby increasing sensitivity of the assay, first hypothesised by Hamilton et al. (1984), have been devised using increased incubation times (Forsyth, 1983), and the pretreatment of serum with heat (Weil et al., 1985; Santhanam et al., 1989) and acid conditions (Maizels et al., 1987).

Four monoclonal antibody-based ELISAs were recently developed to detect antigens of the bovine parasite *Onchocerca gibsoni* circulating in the serum of infected cattle. A significant reduction in the sensitivity of three of the four assays accompanied the testing of known amounts of crude male *O. gibsoni* in bovine serum compared with antigen in buffer. This paper describes investigations undertaken to develop a method of pretreating serum to overcome this problem.

Materials and methods

Monoclonal antibody-based antigen detection ELISAs

The four monoclonal antibodies used in this study were produced followed the fusion of NS-1 myeloma cells with spleen cells from mice immunised with a deoxycholate (1% [w/v]) soluble extract of male *O. gibsoni* (hereafter termed Og male Ag). Further details of the target antigen of each monoclonal antibody is provided elsewhere (More and Copeman, 1990, 1991). None of the monoclonal antibodies bound to host-derived connective tissue in either a direct ELISA or during immunoperoxidase staining of histological sections of *O. gibsoni* nodules. Antigen detection ELISAs were produced using methods previously described (More and Copeman, 1990); and each monoclonal antibody, used as the capturing antibody in one of the four assays, was coated as follows: Og4C3 and Og5A4 at 5 and 2 µg of protein/ml, respectively, of ascitic fluid precipitated using saturated ammonium sulphate, and Og5A6 and Og5C3 at 5 and 1 µg of protein/ml, respectively, of Protein A-purified supernatant fluid.

A known amount of Og male Ag was serially diluted into either buffer [PBS/T/cas; phosphate buffered saline with 0.05% (v/v) Tween 20 (Fisher Scientific Company, Fair Lawn, U.S.A.) and 0.5% (w/v) high nitrogen casein (United States Biochemical Corporation, Cleveland, U.S.A.)] or serum [collected from a 6 month old calf raised since 1 day old in insect and helminth-free conditions]. Samples were then pretreated using the methods described below before testing with each of the four antigen detection assays. A comparison was made between the minimum amount (with an absorbance equivalent to 3 × the mean background level) of antigen detectable in each sample following pretreatment, compared with the minimum amount detected using the same assay in an untreated sample of buffer containing parasite antigen.

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Table 1 The effect of different methods of sample pretreatment on the detection of *Onchocerca gibsoni* antigen in either buffer or serum, using four monoclonal antibody-based ELISAs. Results are expressed as the minimum amount (with an absorbance equivalent to 3× the mean background level) of antigen detectable in each sample following pretreatment, compared with the minimum amount detected in an untreated sample of buffer containing parasite antigen, which was defined as 1

Sample preparation	Minimum detectable antigen			
	Og4C3	Og5A4	Og5A6	Og5C3
BUFFER [1] containing *O. gibsoni* antigen				
Untreated sample	1	1	1	1
Treated sample				
a) Heat treatment (100 °C for 5 min, in equal volume of 0.1 M Na_2EDTA) at pH 7.5	4	1	2	1
b) Alkali treatment using:				
0.02 M NaOH	2	2	1	2
0.1 M NaOH	256	> 1024	2	32
0.5 M NaOH	> 256	> 1024	8	512
c) Reducing treatment with:				
0.01 M DTT [2]	0.5	> 64	1	8
0.05 M DTT	1	> 64	8	8
0.1 M DTT	1	> 64	8	16
SERUM [3] containing *O. gibsoni* antigen				
Untreated sample	256	128	256	0.125
Treated sample				
a) Heat treatment (100 °C for 5 min, in equal volume of 0.1 M Na_2EDTA) at:				
pH 7.5	4	2	1	8
pH 6.0	8	2	0.5	4
pH 5.0	8	2	1	4
pH 4.0	4	2	0.5	4
b) Detergent solubilisation following heat treatment at pH 4 using:				
0.2% DOC [4]	8	16	nd	nd
0.5% DOC	16	32	nd	4
1.0% DOC	32	16	nd	64
2.0% DOC	nd	nd	nd	64
c) Alkali treatment with 0.02 M NaOH	64	128	nd	nd
d) Reduction using 0.01 M DTT	16	> 128	nd	nd

Key: nd not done; 1 Phosphate buffered saline with 0.05% (v/v) Tween 20 and 0.5% (w/v) casein; 2 1,4-dithiothreitol; 3 Serum collected from a calf raised since birth in helminth and insect-free conditions; 4 Sodium deoxycholate

Sample pretreatment

Heat treatment. Using the method first described by Weil et al. (1985), test samples were added to an equal volume of 0.1 M Na_2EDTA, pH 7.5 (ethylenediaminetetra-acetic acid, disodium salt; Ajax Chemicals, Sydney, Australia) (and later this buffer at a pH of 4.0, 5.0 and 6.0) and boiled for 5 min. Each sample was centrifuged at 16,000 g for 7 min and the supernatant fluid recovered.

Detergent treatment of heat treated sample. The ability of detergent to free parasite antigen from the clot formed following heat treatment was investigated. After heating samples in the presence of 0.1 M Na_2EDTA pH 4, a volume (equal to the original sample volume) of 0.2, 0.5, 1.0 and 2.0% (w/v) sodium deoxycholate [BDH (Australia Pty) Ltd, Boronia, Australia] dissolved in PBS was added. Each sample was then vortexed, centrifuged at 16,000 g for 7 min and the supernatant fluid recovered.

Alkali treatment. Using the method described by Meckstroth et al. (1981), alkali was added to the test sample to give a final concentration of 0.05, 0.1 and 0.5 M NaOH. The solution was incubated at 56 °C for 2 h before neutralising with 1 M Tris-HCl, pH 8.0.

The use of a reducing agent. Using the method of Gunewardena and Cooke (1966), 1,4-dithiothreitol[(DTT) Cleland's reagent; Bio-Rad Laboratories, Richmond, U.S.A.] was added to each test sample to give a final concentration of 0.01, 0.05 and 0.1 M DTT. After incubating at room temperature for 30 min, iodoacetamide (Koch-Light Laboratories, Colnbrook, U.K.) in Tris-HCl, pH 8.0, to give a final concentration of 0.1 M, was added. The samples were then incubated at about 25 °C for 1 h.

Results

Changes in the sensitivity of each assay as a result of pretreatment of samples of buffer and serum, each containing known amounts of male *O. gibsoni* antigen, is shown in Table 1. In buffer, the target epitope of each monoclonal antibody was heat stable, unaltered by 0.02 M NaOH and, with the exception of Og5A4, was not greatly altered by reducing conditions. However, in the presence of untreated serum there was a dramatic drop in the sensitivity of all assays, except that based on Og5C3 which increased in sensitivity. Of all the pretreatment methods tested in this study, the pretreatment of serum samples with heat in the presence of 0.1 M Na_2EDTA resulted in the largest increase (×64512) in sensitivity of the assays based on Og4C3, Og5A4 and Og5A6. The effect was similar over the pH ranges tried (pH 4 – pH 7.5).

Discussion

The purpose of this study was to identify methods of breaking the antibody-antigen bond whilst leaving

the integrity of the antigen molecule undamaged. At the completion of an appropriate procedure, the sample solution needed to be restored to an environment suitable for such a bond to reform in the presence of the assay-based antibody.

A range of methods, both novel and previously described, were tested during this work including those directly causing antibody denaturation (heat and alkali treatment), disruption of the antigen-antibody bond (detergent treatment of heat treated samples) and a method causing damage to the tertiary conformation of the binding site on the antibody molecule (reducing treatment). Each method of sample pretreatment was first evaluated using parasite antigen in buffer but in the absence of bovine serum. This allowed for the identification of those procedures which caused little or no damage (at appropriate reagent dilutions) to the integrity of each target antigen. For example, the amount of antigen detected using all assays was not altered following the treatment of parasite antigen with 0.02 M NaOH and (with the exception of the assay based on Og5A4) with 0.01 M dithiothreitol. Those pretreatment methods identified with each assay as causing little damage to antigen in buffer were then evaluated further using parasite antigen in the presence of bovine serum. The same antigen preparation (Og male Ag) has been used throughout this study, both during the initial production of the monoclonal antibodies and also during the series of experiments described here, ensuring that any confounding effects due to alteration of antigen conformation as a result of the use of 1% (w/v) deoxycholate have been avoided.

In each of the three ELISAs where the presence of untreated bovine serum resulted in a dramatic reduction in sensitivity of the assay (that is, those assays based on Og4C3, Og5A4 and Og5A6) the use of heat in the presence of 0.1 M Na_2EDTA pH 4.0 proved the most suitable pretreatment method for serum. Indeed, following this treatment the sensitivity of these assays was only slightly less than those attained using each assay under optimal conditions. This method (at pH 7.5) has been previously described by Weil et al. (1985) and used extensively since that time (Weil et al., 1987; Weil and Liftis, 1987; Weil, 1988; Santhanam et al., 1989). However, EDTA is normally utilised for its properties as a strong chelator of divalent cations (Dawson et al., 1986) and its action in the present context is unclear, since the antibody-antigen bond is a result of multiple non-covalent [hydrogen bonds, electrostatic, Van der Waals and hydrophobic] forces between the antigen and the binding sites of the immunoglobulin molecule (Steward, 1989). All other pretreatment methods tested in this study proved inappropriate as approaches for reversing antigen-antibody complexes in bovine serum.

The presence of host antibodies in serum from a helminth-free calf able to interfere with the detection of helminth antigens is surprising. It is likely, however, that these antigens cross-react or are shared with antigens in other living organisms to which the calf was exposed. For example, the target antigen of Og5A4, phosphorylcholine, is widely distributed in helminths, bacteria, fungi and dermatophytes (Weiss, 1985; Briles et al., 1987) and therefore antibodies directed against phosphorylcholine are not unexpected in a calf raised in the absence of helminths or biting insects. In contrast, the detection of the target antigen of Og5C3, a sperm-associated protein of *O. gibsoni*, was not affected by a range of bovine sera, including serum collected from animals heavily infected with *O. gibsoni* (More and Copeman, in preparation). Thus, it appears that this protein does not normally contact the host immune system of animals infected with *O. gibsoni* and is probably not widely distributed in other infectious organisms.

In conclusion, the pretreatment of bovine serum with heat in the presence of 0.1 M Na_2EDTA pH 4.0 dramatically increased the sensitivity of 3 of the 4 antigen detection assays used in this work. The procedure is simple to use and has been successfully applied to human (More and Copeman, 1990) and bovine (More and Copeman, 1991) serum samples.

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