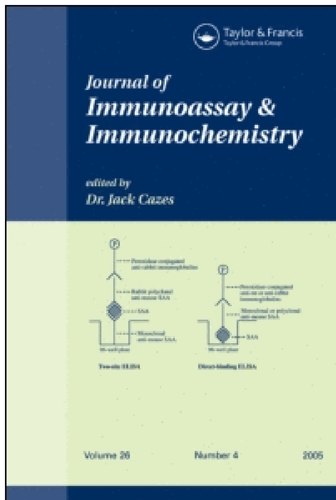
This article was downloaded by:
On: *16 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Antibody to *Aspergillus fumigatus* Antigens in Normal Sera: Influence on Positive-Negative Discrimination in ELISA

M. D. Richardson[a]; P. A. Llewellyn[a]; D. W. Warnock[a]
[a] Department of Microbiology, Bristol Royal Infirmary, Bristol, England

To cite this Article Richardson, M. D. , Llewellyn, P. A. and Warnock, D. W.(1984) 'Antibody to *Aspergillus fumigatus* Antigens in Normal Sera: Influence on Positive-Negative Discrimination in ELISA', Journal of Immunoassay and Immunochemistry, 5: 3, 205 — 220
To link to this Article: DOI: 10.1080/01971528408063008
URL: http://dx.doi.org/10.1080/01971528408063008

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

JOURNAL OF IMMUNOASSAY, 5(3&4), 205-220 (1984)

ANTIBODY TO ASPERGILLUS FUMIGATUS ANTIGENS IN NORMAL SERA: INFLUENCE ON POSITIVE-NEGATIVE DISCRIMINATION IN ELISA

M.D. Richardson, P.A. Llewellyn & D.W. Warnock
Department of Microbiology, Bristol Royal Infirmary, Bristol BS2 8HW, England

Downloaded At: 12:21 16 January 2011

ABSTRACT

The response of precipitin-negative sera from non-selected asthmatic patients against a range of somatic and culture filtrate antigens of *Aspergillus fumigatus* in ELISA for anti-*A. fumigatus* IgG is described. Antibody to the various antigens was widely distributed but the precise distribution was dependent upon antigen type. To determine how the selection of negative reference sera from precipitin-negative sera can influence the discrimination between precipitin-positive and -negative sera in ELISA, panels of 10 sera were chosen from the extremes of the frequency distribution and by random selection. The closest agreement between precipitin testing and ELISA was seen when randomly-selected negative reference sera were incorporated in the assay, although the degree of correlation was dependent upon the detector antigen. These findings demonstrate the requirement for careful selection of sera included as negative reference sera in ELISA for anti-*A. fumigatus* IgG.

(KEY WORDS: *Aspergillus fumigatus*, ELISA)

INTRODUCTION

The demonstration of antibody to *Aspergillus fumigatus* is widely used as an index of certain clinical forms of aspergillosis in man. The relatively high rate

Copyright © 1984 by Marcel Dekker, Inc.

0197-1522/84/0503-0205$3.50/0

of association with disease and the comparative ease with which most methods are carried out makes the detection of antibody a useful adjunct to microbiological methods for diagnosis of aspergillosis in both non-compromised and compromised patients (1).

Although a number of serological tests have been applied, agar gel double diffusion and counterimmunoelectrophoresis have been shown to be the simplest and most specific serological methods for establishing a diagnosis of the various forms of aspergillosis (2, 3, 4, 5). These procedures may be the most appropriate to use since they only detect extreme elevations of antibody. Furthermore, both the number and strength of precipitin reactions are significant for interpretation of the degree of involvement with _A. fumigatus_. However, these techniques are qualitative in nature and sometimes fail to differentiate between patients with colonization with Aspergillus and individuals with clinically active disease. The successful application of precipitin methods has not, however, inhibited the development of quantitative ELISA methods (6).

One area of concern with the ELISA technique is deciding exactly how to quantitate the level of antibody and report the values produced in the test (7). Useful information concerning the qualitative

Downloaded At: 12:21 16 January 2011

composition of a serum sample may be lost if the ELISA result is expressed as a single figure of antibody activity or concentration. However, for routine diagnostic applications, the interpretation of values for specific antibody can be given as positive or negative.

The method of designating ELISA values as either positive or negative, and the selection of appropriate control groups are fundamental aspects of ELISA design. Several methods have been described (7), but one approach is to set the minimum response value to 2 or 3 times the mean absorbance of a group of selected negative sera. It has been found, however, that the choice of negative reference sera can influence the threshold for classifying sera as either positive or negative, thus altering the incidence of 'false positive' and 'false negative' ELISA results (8). In previous preliminary evaluations of ELISA methods for the measurement of anti-A. fumigatus IgG using a limited number of antigens, inclusion of precipitin test-negative sera as a control group established the accuracy of the method when compared to agar gel double diffusion (9, 10).

We describe here the response of sera without detectable precipitins, against a range of A. fumigatus antigens in ELISA and the influence of selected sera on

Downloaded At: 12:21 16 January 2011

the correlation between precipitin test results and ELISA values.

MATERIALS AND METHODS

Antigens.

Antigens were obtained from Bencard (Brentford, Middlesex, England) and Mercia Brocades Limited (West Byfleet, Surrey, England). The Bencard antigens were labelled by the manufacturers as antigen 1 and antigen 2 (both 20 mg/ml). Antigen 1 was prepared from mycelium grown as a deep submerged culture for 72 hr in an organic medium. Antigen 2 was prepared from culture medium in which the fungus had been grown as a surface culture for about 6 weeks. The Mercia Brocades antigens were either somatic or culture filtrate preparations of *A. fumigatus* (both 20 mg/ml).

Downloaded At: 12:21 16 January 2011

Agar gel double diffusion (AGDD).

To detect precipitins against *A. fumigatus* the procedure described by Warnock (5) was used , incorporating the *A. fumigatus* antigens at a concentration of 20 mg/ml.

ELISA.

This was performed using the method described by Richardson *et al.* (10). Individual wells of 96-well microdilution plates (No. 1-220-29, Dynatech Laboratories) were first coated with 200 µl of a

solution containing 3.0 μg of A. fumigatus antigen diluted in sodium carbonate buffer, prewarmed to 37°C. The wells were coated for 10 min at 37°C with vigorous agitation and then washed with phosphate buffered saline-Tween 20 (PBS-T). Duplicate 200 μl volumes of sera diluted 1:100 with PBS-T were prewarmed to 37°C, added to the antigen coated wells and incubated with vigorous agitation for 10 min. The plates were then washed with PBS-T. Heavy chain-specific goat antiserum to human IgG labelled with alkaline phosphatase (Miles Laboratories) was diluted 1:2000 in PBS-T, prewarmed to 37°C, and 200 μl added to each antigen-coated well. The plates were incubated with vigorous agitation, washed with PBS-T and then 200 μl of prewarmed p-nitrophenyl phosphate in diethanolamine buffer was added to the washed wells and incubated with vigorous agitation for about 30 min at 37°C. Optical density determinations were carried out by using a Dynatech MR580 microELISA Autoreader operating at 405 and 650 nm.

Serum samples.

Serum samples used in this investigation were acquired from asthmatic patients with suspected allergic aspergillosis. These specimens were tested for A. fumigatus precipitins on receipt and were then stored for up to 12 months at -20°C until required for further tests. The positive reference serum used was obtained

Downloaded At: 12:21 16 January 2011

from a patient with the mycetomal form of aspergillosis. This serum contained a high titre (1:64) of _A. fumigatus_ precipitins when tested by AGDD.

Expression of ELISA data.

To minimize the effect of inter-test variation, the absorbance of individual sera (all 1:100 dilution) was expressed as ELISA units, i.e. as percentage absorbance of that given by the positive reference serum dilution (1:100) tested on the same occasion.

Discrimination between positive and negative sera was achieved by incorporating the positive reference serum and 3 panels of 10 negative reference sera selected from 24 sera as detailed below on each test plate. All sera from these three panels were negative when tested by AGDD for precipitins against the four _A. fumigatus_ antigens. The threshold value (ELISA factor) for discrimination between positive and negative sera was calculated as the mean of the ELISA-unit values of the negative reference sera plus twice the standard deviation. To avoid fine distinctions between negative and positive sera, the ELISA-unit values and ELISA factor were taken as integers. The ELISA-unit value of each test serum was then compared with the ELISA factor, equal or greater being scored as positive, and lower values as negative.

Downloaded At: 12:21 16 January 2011

RESULTS

Frequency distribution of anti-A. fumigatus IgG levels in ELISA.

The frequency distributions of anti-*A. fumigatus* IgG in ELISA when sera from 24 precipitin negative sera were tested with four *A. fumigatus* antigens are shown in Figure 1. All the distribution histograms show a divergence from a normal distribution, but differ in their precise appearance according to the antigen used in the test. Distribution frequencies for the Bencard antigens and the Mercia Brocades somatic extract are similar in appearance with the majority of sera exhibiting low absorbance in the assay. However, the distribution of ELISA units for anti-*A. fumigatus* IgG when the Mercia Brocades culture filtrate extract was used as the detector antigen approximates more closely to a normal distribution. The median, range and semi-interquartile range ELISA-unit values for each *A. fumigatus* antigen are given in Table 1. Here also, it can be seen that the Bencard preparations and the Mercia Brocades somatic extract behave similarly as detector antigens for anti-*A. fumigatus* IgG in precipitin-negative sera. The values given in Table 1 demonstrate further the greater number of sera exhibiting higher levels of specific antibody when tested against the Mercia Brocades culture filtrate antigen.

Downloaded At: 12:21 16 January 2011

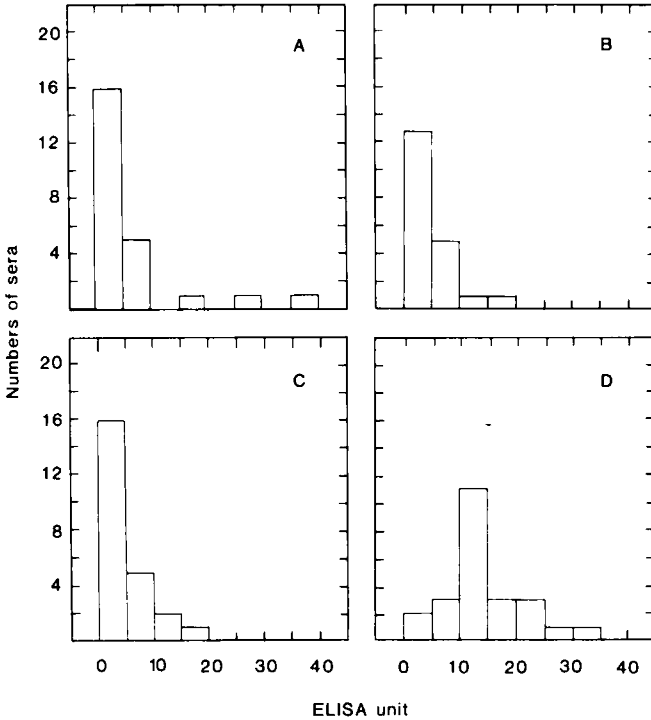


FIGURE 1. Distribution of anti-*Aspergillus fumigatus* IgG unit values in ELISA in sera from asthmatic patients. Detector antigen: A. Bencard 1, B. Bencard 2, C. Mercia Brocades somatic, D. Mercia Brocades culture filtrate.

Downloaded At: 12:21 16 January 2011

Effect of negative reference sera on positive-negative discrimination in ELISA.

To determine how the selection of negative reference sera from a series of precipitin-negative sera can effect the discrimination between positive and negative sera in ELISA, panels of 10 sera were chosen using one of three methods. The first negative reference panel was selected from those precipitin-negative sera

TABLE 1

Median, range and semi-interquartile range of ELISA units (OD%) for anti- A. fumigatus IgG levels in precipitin-negative sera in tests with four A. fumigatus antigens.

Antigen	Median (OD%)	Range (OD%)	Semi-interquartile range (OD%)
Bencard 1	4	2 - 41	3 - 6
Bencard 2	4	2 - 18	2 - 6
MB* Somatic	4	0 - 16	2 - 9
MB* Culture filtrate	12	3 - 31	11 - 17

*MB: Mercia Brocades Limited

Downloaded At: 12:21 16 January 2011

with the lowest ELISA values (panel 1). The second panel was comprised of 10 sera with the highest ELISA values (panel 2). To approximate closely a non-selective method of choosing negative reference sera, 10 sera were also selected on the basis of pseudo-random numbers supplied by a short BASIC microcomputer programme using the randomize function (panel 3).

The positive-negative status of 24 precipitin-negative sera and six sera positive for precipitins against all four A. fumigatus antigens, based on the ELISA factors created using the three panels of negative

reference sera, is shown in Table 2. It can be seen that the greatest number of false positive results in the ELISA when precipitin-negative sera were tested, was generated when the negative reference panel was selected from those sera with the lowest ELISA units. This was seen with all antigens except the Mercia Brocades culture filtrate extract. With this antigen the median and semi-interquartile range of the precipitin-negative sera were significantly higher that that recorded for the other three antigens (Table 1).

When the 24 precipitin-negative sera were categorised using the 10 randomly selected sera as the negative reference panel very few false positive results were recorded. The closest agreement between the precipitin test and ELISA was seen with the Mercia Brocades culture filtrate antigen. Likewise, when negative reference sera were selected from those sera with high absorbance in the ELISA, only one false positive was recorded against each of the Bencard antigens.

Sera positive for precipitins were generally recorded as positive in ELISA when the negative reference panel was selected from those sera with minimal ELISA-unit values. The ELISA factor calculated from the 10 randomly selected precipitin-negative sera produced the closest agreement with the reaction of the test sera in agar gel double diffusion. All six sera

Downloaded At: 12:21 16 January 2011

TABLE 2

Effect of choice of negative reference sera on the correlation between precipitin detection by AGDD and ELISA for IgG antibodies to four A. fumigatus antigens.

Antigen	Reaction in AGDD	Number of sera	Number of sera positive in ELISA subject to negative serum panel		
			Panel 1	Panel 2	Panel 3
Bencard 1	-	24	14	1	1
	+	6	6	4	2
Bencard 2	-	24	15	1	1
	+	6	6	6	4
MB* Somatic	-	24	10	2	0
	+	6	6	5	4
MB* Culture filtrate	-	24	1	0	0
	+	6	5	4	4

*MB: Mercia Brocades Limited

positive in agar gel double diffusion using the Bencard antigen 2 were classed as positive in the ELISA with this negative reference panel.

When the six precipitin-positive sera were tested with negative reference sera selected from those with high ELISA-unit values, four sera were classed as positive with the Bencard antigen 2 and the Mercia

Downloaded At: 12:21 16 January 2011

Brocades antigens, and two sera were recorded as positive in the ELISA when tested with the Bencard antigen 1.

DISCUSSION

Serodiagnosis of aspergillosis by detection of antibodies to either somatic or culture filtrate antigens has been extensively studied, most commonly by precipitin methods (1). However, problems with interlaboratory reproducibility, low sensitivity in immunocompromised patients and low specificity have encouraged the development of alternative approaches to serodiagnosis of the various forms of this disease (6). Additional major problems with precipitin methods are their lack of precise antibody quantitation and inadequate standardization. Standardization is essential in view of the observation that anti-*A. fumigatus* immunoglobulin levels are widely distributed (11).

These problems with precipitin tests have led to the development and evaluation of ELISA methods for the detection of anti-*A. fumigatus* immunoglobulins (9, 12, 13). The usefulness of such tests is not as yet resolved. In previous reports on the use of ELISA in the serodiagnosis of aspergillosis, the question of appropriate control groups has been rather neglected. All individuals appear to have some circulating antibody to Aspergillus (11) so the ability to quantitate

Downloaded At: 12:21 16 January 2011

specific serum immunoglobulin levels may assist in establishing a diagnosis and provide an index to judge the efficacy of therapy.

Quantitative expression of antibody activity determined by ELISA is difficult because of the lack of reference standards. We chose to standardize the ELISA by including positive and negative sera in each test run and expressing individual absorbance values as a percentage of the positive reference serum. This approach reduced inter-test variation and led to the establishment of criteria by which we could compare sera against different antigens. Furthermore, the shape of the dose-response curves for IgG antibodies to A. fumigatus using commercial antigen preparations, together with the requirement of a rapid screening test, suggested that the determination of antibody titres as opposed to testing a single serum dilution was not required (10).

To select the most appropriate negative control group from precipitin-negative sera in order to determine the accuracy of the assay compared to AGDD we first confirmed that antibody to various A. fumigatus antigens was present in a non-selected group of asthmatic patients and then showed that antibody activity was widely distributed. Analysis of the frequency distribution data demonstrated the range of ELISA-unit values in the non-selected group of

Downloaded At: 12:21 16 January 2011

precipitin-negative sera. Extreme values of anti-*A. fumigatus* IgG activity were recorded with the Bencard antigen 1 and the Mercia Brocades culture filtrate preparation. When less emphasis was placed on those sera with high ELISA-unit values, the semi-interquartile range demonstrated the similarity of antibody activity detected by the Bencard antigens and the Mercia Brocades somatic extract, and the greater number of sera with a significant antibody level detected by the Mercia Brocades culture filtrate antigen.

These findings emphasise the requirement for careful selection of sera to be included as negative reference sera in the assay. The random selection of negative reference sera closely approximates the ultimate use and design of such a test. This panel of negative sera provided the best correlation with precipitin test results although the number of sera categorized as positive in the ELISA was dependent upon the detector antigen. The number of precipitin-negative sera that were classed as positive in the ELISA when a panel of randomly selected negative sera was incorporated as an internal standard in the test was minimal and few precipitin-positive sera were recorded as negative with this panel. The selection of precipitin-negative sera from either extreme of the anti-*A. fumigatus* IgG frequency distribution histogram

Downloaded At: 12:21 16 January 2011

demonstrates further the need for careful selection of standard reference sera.

Our studies indicate that small numbers of reference sera selected from appropriate control groups can be used to validate the accuracy of ELISA compared to precipitin tests. With adequate test standardization, ELISA may be helpful in the diagnosis of the different forms of aspergillosis where significant elevations of serum anti-A. fumigatus IgE and IgM antibodies besides those of the IgG class may be present. The application of the rapid ELISA procedure to these antibodies awaits evaluation.

Downloaded At: 12:21 16 January 2011

REFERENCES

1. Richardson, M.D. and Warnock, D.W. Serological tests in the diagnosis and prognosis of fungal infection in the compromised patient. In: Warnock, D.W. and Richardson, M.D., eds. Fungal Infection in the Compromised Patient. Chichester: John Wiley & Sons Ltd., 1982: 229-50.

2. Coleman, R.M. and Kaufman, L. Use of the immunodiffusion test in the serodiagnosis of aspergillosis. Appl. Microbiol. 1972; 23: 301-8.

3. Dee, T.H. Detection of Aspergillus fumigatus serum precipitins by counter-immunoelectrophoresis. J. Clin. Microbiol. 1975; 2: 482-5.

4. Longbottom, J.L. and Pepys, J. Pulmonary aspergillosis: diagnostic and immunological significance of antigens and C-substance in Aspergillus fumigatus. J. Pathol. Bacteriol. 1964; 88: 141-51.

5. Warnock, D.W. Detection of Aspergillus fumigatus precipitins: a comparison of counter-immunoelectrophoresis and double diffusion. J. Clin. Pathol. 1977; 30: 388-9.

6. Richardson, M.D. and Warnock, D.W. Enzyme-linked immunosorbent assay and its application to the serological diagnosis of fungal infection. Sabouraudia. 1983; 21: 1-14.

7. De Savigny, D. and Voller, A. The communication of ELISA data from laboratory to clinician. J. Immunoassay. 1980; 1; 105-28.

8. Richardson, M.D., Smith, S. and Warnock, D.W. Further evaluation of factors affecting a rapid ELISA procedure for detection of IgG antibodies to Candida albicans. Mycopathologia. 1983; 82: 167-73.

9. Richardson, M.D., Stubbins, J.M. and Warnock, D.W. Rapid enzyme-linked immunosorbent assay (ELISA) for Aspergillus fumigatus antibodies. 1982; J. Clin. Pathol. 35: 1134-37.

10. Richardson, M.D., Turner, A., Warnock, D.W. and Llewellyn, P.A. Computer-assisted rapid enzyme-linked immunosorbent assay (ELISA) in the serological diagnosis of aspergillosis. J. Immunol. Meths. 1983; 56: 201-7.

11. Bardana, E.J., McClatchy, J.K., Farr, R.S. and Minden, P. The primary interaction of antibody to components of aspergilli. II. Antibodies in sera from normal persons and from patients with aspergillosis. J. Allergy Clin. Immunol. 1972; 50: 222-34.

12. Mishra, S.K., Falkenberg, S. and Masihi, K.N. Efficacy of enzyme-linked immunosorbent assay in serodiagnosis of aspergillosis. J. Clin. Microbiol. 1983; 17: 708-10.

13. Sepulveda, R., Longbottom, J.L. and Pepys, J. Enzyme-linked immunological assay (ELISA) for IgG and IgE antibodies to protein and polysaccharide antigens of Aspergillus fumigatus. Clin. Allergy. 1977; 9: 359-71.

Downloaded At: 12:21 16 January 2011