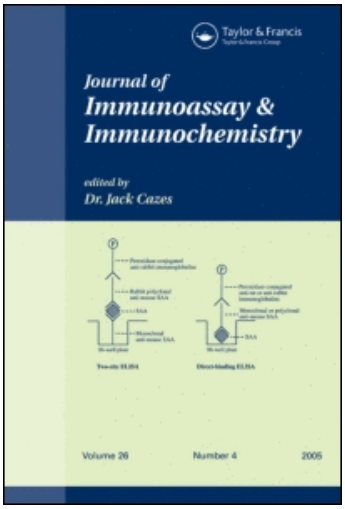

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A Method to Determine Significant Levels of Immunoglobulin G to *Aspergillus Fumigatus* Antigens in an ELISA System and a Comparison with Counterimmunoelectrophoresis and Double Diffusion Techniques

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A METHOD TO DETERMINE SIGNIFICANT LEVELS OF IMMUNOGLOBULIN G TO ASPERGILLUS FUMIGATUS ANTIGENS IN AN ELISA SYSTEM AND A COMPARISON WITH COUNTERIMMUNOELECTROPHORESIS AND DOUBLE DIFFUSION TECHNIQUES

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ABSTRACT

A method is described which assesses results obtained from an ELISA system for the determination of human serum levels of IgG class antibodies to Aspergillus fumigatus. The method is used to discriminate positive from negative samples, and significant antibody activity may be reported to the clinician, relative to a reference positive control serum monitored simultaneously under the same test conditions. Antibody content is expressed as the absorbance of a certain dilution of serum. Duplicate samples were analysed at a single serum dilution and their absorbtion values obtained from a semi-automated ELISA microplate reader. These were entered into a computer programmed to convert the data into units on a logarithmic scale.

In parallel experiments, ELISA results were compared with those obtained by the techniques of counterimmunoelectrophoresis and double diffusion which measure precipitating antibody of all classes. A relatively good degree of correlation between tests was found only among sera with a high level of antibody.

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INTRODUCTION

The use of an ELISA technique for the detection of A. fumigatus antibodies in serum, has been reported from several laboratories (1-8). Its further application as a diagnostic tool for large-scale screening of serum samples would be facilitated if results were expressed as absorbance (A) values at a single serum dilution (9). This demands considerable precision of the method, and while it is technically simple to perform, the multiple steps involved and the inherent sensitivity of ELISA requires stringent control of assay parameters (10, 11). These are most readily assessed by the routine inclusion of a number of known positive and negative reference sera in the test system (5, 9). The difference in A values between these 2 groups of sera gives an indication of the actual amounts of antibody present in the reference positive sera and it permits evaluation of the performance of the assay. Based on experimental data, a minimum difference between reference sera is required before the test may be used for assessment of an individual A value on a relative basis. The A values are fed into the computer where, by the application of a suitable formula, they are converted from raw ELISA figures into activity units on a continuous scale.

A number of methods for processing and reporting of ELISA data have been evaluated in terms of fulfilling certain criteria (9). No single method satisfies all criteria, including

the one reported here. However, the method allows for continuous monitoring of sensitivity and reproducibility at the highly significant and normal limits of specific antibody levels. It is not dependent on any single reference serum or antigen batch and facilitates comparative studies undertaken over a considerable period of time.

METHODS

Antigenic Fractions of A. fumigatus Mycelium

A. fumigatus strain NCPF 2109 was grown in stirred culture in neutral glucose peptone medium incubated at 30°C for 3 days (12). The supernatant obtained after mycelial breakage and centrifugation was reduced in volume by counter-dialysis against a 10% (w/v) aqueous solution of polyethylene glycol 6000 (13). This constituted the water-soluble preparation (WS); it was used to test all serum samples on counterimmunoelectrophoresis (CIE) and selected sera on ELISA.

A WS preparation was separated by affinity chromatography on ConA-Sepharose (14). Two peaks were obtained, one of which eluted with the void volume, shown to be unsuitable for ELISA, and a peak which was elutable only on application of methyl α-D-mannopyranoside and was active on ELISA (BF fraction).

In addition, WS material from A. fumigatus was resolved into 10-14 protein bands by preparative isoelectricfocusing in Sephadex G-75, using a pH gradient from 4-6.5. These bands

were eluted with water and pooled, on the basis of their antigenic activity in an ELISA system, into three fractions, viz., a, b and c (15). Fraction c consisted of slightly acidic components with isoelectric points in the range pH 5.5-6.5 (cf 16).

Human Sera

Sera were obtained from patients suffering from aspergillosis and other pulmonary diseases in which colonisation by aspergillus may occur. All gave positive precipitin tests on double-diffusion, and on that basis were used in this study. The reference positive sera were human pooled samples, shown to have high aspergillus titres on CIE. Of the negative control sera, 4 were from healthy laboratory personnel and the remainder were pooled samples, recorded as negative on CIE and ELISA. The sera were either submitted to this laboratory or donated by Dr. J. Edwards, Sully Hospital, Wales, U.K.

Double-diffusion

Antigens for double-diffusion were obtained from A. fumigatus strain no. I355, grown on visking tubing containing glycine and sodium chloride in nutrient broth (Oxoid, Basingstoke, Hampshire, England) at 37°C for 14 days using the double dialysis method of Edwards (17). Both mycelial and extracellular antigens were harvested and each used in the test at 10 mg dry wt/ml. Double-diffusion (DD) was done in 1.5% (w/v) purified agar

(Difco, West Molesey, Surrey, England) in normal saline containing 10 mM EDTA (disodium salt), as used routinely in the MRC Pneumoconiosis Unit, Penarth. The test employed a 1.2 mm layer of agar on a plate (10 x 15 cm) and a pattern of antigen wells of 4 mm diameter, serum wells of 1.1 cm diameter and centre-to-centre distances of 1 cm. Antigen (12.5 µl) and antiserum (105 µl) were applied to the appropriate wells. A maximum 2 day development period at room temperature was allowed and, after washing and fixing, the gels were stained with Naphthalene Black solution (0.06%, w/v).

Counterimmunoelectrophoresis

In a 2.5 mm diameter well 5 µl of antigen was applied with 15 µl of patients' serum in a 4.5 mm diameter well (18). Antigen migration was towards the cathode. After application of a constant current to the gel for approximately 1.5 hours, the slides were washed, fixed and stained with Coomassie Brilliant Blue R, C.I. no. 42660.

ELISA Method

Analyses were performed in polystyrene microtitre plates (Dynatech, Plochingen,West Germany), essentially as described in (19). The BF antigen was used at an optimum concentration determined by chequer-board titration against reference sera. The range was 10-30 µg dry wt/ml, depending on the batch. Each

serum sample was analysed in duplicate at a dilution of 1:100, chosen on the basis of previous experiments (14). Each test incorporated 5 reference positive and 5 reference negative sera. In this series of experiments, a total of 10 positive and 10 negative sera were used. In a given experiment, any 5 of each group were included as controls.

The conjugate used was goat antihuman IgG linked to peroxidase (Miles Laboratories, Slough, Berks., England); the substrate was o-phenylenediamine. The enzyme reaction was stopped after 30 minutes at room temperature by the addition of $2N\text{-}H_2SO_4$. The absorbance (A) values were read in a Titertek Multiskan Spectrophotometer (Organon Teknika, St. Neots, Cambs., England), at $A_{492\ nm}$ (14).

All wells in a plate were used and the application of samples to the wells varied according to the experimental design. Both conjugate and substrate controls should yield low extinction values, for the BF antigen a mean A = 0.12 $\pm$ 20% (coefficient of variation) was acceptable. Duplicate test samples which showed wide discrepancies were repeated. The uncorrected A readings and clinical details of the sera were used as data for a programme written in Basic on a Commodore PET 3042 (20), which calculated the results using control sera to determine rejection criteria. Readings at a single serum dilution were transformed into units on a linear scale and obtained as a print

out. A mean value for the positive control was obtained at the same time and used for comparative purposes.

RESULTS

The experimental data presented here are based on results obtained from 19 individual ELISA's performed over a period of several months. As detector antigen, four separate batch preparations of a carbohydrate-rich fraction from affinity chromatography (BF) were used. Each test included 5 reference positive and 5 reference negative sera whose mean A values and standard deviations were used to monitor the performance of the assay. In any given experiment, a maximum of 30 test sera were analysed on a single plate; a total of 230 sera were screened.

In a preliminary study it was found that the absolute difference between duplicate A readings tended to increase roughly proportionately to the size of the readings. Accordingly, all the A readings were transformed to logarithms (to the base 10) before further analysis. Using the transformed data, an estimated standard deviation (SD) was obtained for each experiment from the mean values for the negative sera. Two of the 19 experiments gave much higher variabilities than the rest. The remaining 17 experiments gave SDs which were consistent with a true value of $\pm$ 0.112 on the $\log_{10}$ scale.

The variability between negative sera provides the basic yardstick by which an unknown serum may be assessed. It can

only be very imprecisely estimated in a single experiment, and it was decided that the true value of 0.112 should be used for each subsequent experiment provided that the estimated SD for that experiment was not too large. A suitable upper limit of 0.180 for an SD obtained from 5 negative sera was calculated by multiplying 0.112 by the square root of the 95% value of the appropriate F distribution (= 1.61). This ruled out as aberrant 2 experiments with SD values of 0.235 and 0.259; 17 experiments had acceptable SDs with readings < 0.180.

With this methodology, a test serum may be assessed as positive if its mean log A value exceeds m + k x 0.112, where m is the mean of the negative sera in the relevant experiment and k is a suitable numerical factor. To obtain a false-positive rate of p%, k should be the corresponding quantile of the Normal distribution multiplied by $(1 + 1/n)^{\frac{1}{2}}$, where n is the number of negative sera. Noting that a one-sided quantile is appropriate, the value of k for p = 5%, n = 5 is $1.645 \times (6/5)^{\frac{1}{2}} = 1.802$. Thus, sera whose mean log A values exceed the mean of the negative sera by 1.802 x 0.112 = 0.202 may be assessed as positive.

For this method to be satisfactory, it is desirable that the mean log A values for known positive sera should exceed the mean for negative sera by an adequate margin. The ratios between the mean A values of 5 reference positive and 5 reference negative sera was measured for each of the 17 experiments (Fig. 1). This

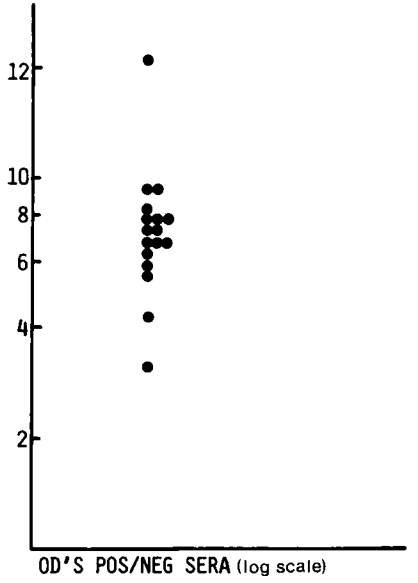

FIGURE 1 Ratios of Reference Positive to Reference Negative Sera in an ELISA System (each ratio was derived from the mean A value of 5 positive and 5 negative sera).

suggests that for a satisfactory experiment, the ratio should exceed 5, corresponding to a difference of 0.7 on the log scale.

Rather than a simple discrimination into positive and negative, a serum can be allotted a quantitative score giving its difference from the negative mean in SD units. Because of the small reference sample, allowance for the uncertainty in the negative mean is made by calculating

$$\frac{\text{test log A} - \text{mean negative log A}}{0.123}$$

where the denominator is $0.112 \times (6/5)^{\frac{1}{2}}$. A score greater than + 1.645 corresponds to a positive serum on the simpler classification. A representative experiment is shown (Table 1).

Using this method of quantitation, the scores from 230 serum specimens were compared with the coarser titres obtained from parallel experiments using the techniques of CIE and DD (Figs. 2 and 3). The majority of the specimens which gave a strong reaction on CIE (i.e. titre of 1:8 or greater) showed a correspondingly high value on ELISA. In this group of 34 sera, 94% gave SDU values > 1.65 (i.e., two patients gave negative readings). The weak-moderate (titres of 1:2 to 1:4) group of patients (total of 16) had a mean value of 4.3 SDUs, with 100% giving an SDU value of > 1.65. The trace-positive (undiluted serum) group of patients (total of 8) had a mean value of 2.7; 75% of this group gave positive SDU values. The number of sera which gave negative readings on CIE was very large (total of 82); of these, only 48% were correspondingly negative on ELISA. The mean value for the negative group was 1.65 SDUs.

On DD, where 24 sera showed themselves strongly-positive, 100% had SDU values > 1.65, of the very large weak-moderate group (total of 104), 74 samples or 71% were positive by ELISA. Of the 30 samples which were negative by ELISA, 28 of these were also negative by CIE with 2 specimens which gave a positive result only when tested undiluted. In the trace-positive group (total of 10), only 20% were positive on ELISA and also on CIE. In

TABLE 1

Reactivity in an ELISA System of IgG Class Antibodies to *Aspergillus fumigatus* in Patients' Sera Expressed as Standard Deviation Units (SDU) Relative to a Reference Positive Sample

Sample	Mean Value ($\log_{10}$ A)	SDUs**	Form in which results are+ reported to the clinician (a)	(b)
Patient 1	-0.185	4.4	4.4 SD units =	moderate
2	-0.347	3.1	3.1 " " =	moderate
3	-0.409	2.6	2.6 " " =	trace
4	+0.156	7.2	7.2 " " =	strong
5	-0.588	1.1	1.1 " " =	negative
ref. neg.	-0.726 ± 118*			
ref. pos.	+0.114 ± .085*	6.8	6.8 " " =	positive control
ratio pos./ neg.	6.9			

* Standard deviation

** Standard Deviation Units

\+ Readings < 1.65 = negative; 1.65-3.0 units = trace; 3.0-5.0 = weak/moderate; > 5.0 units = strong. Positive control = mean value obtained for 5 positive reference sera included in each test.

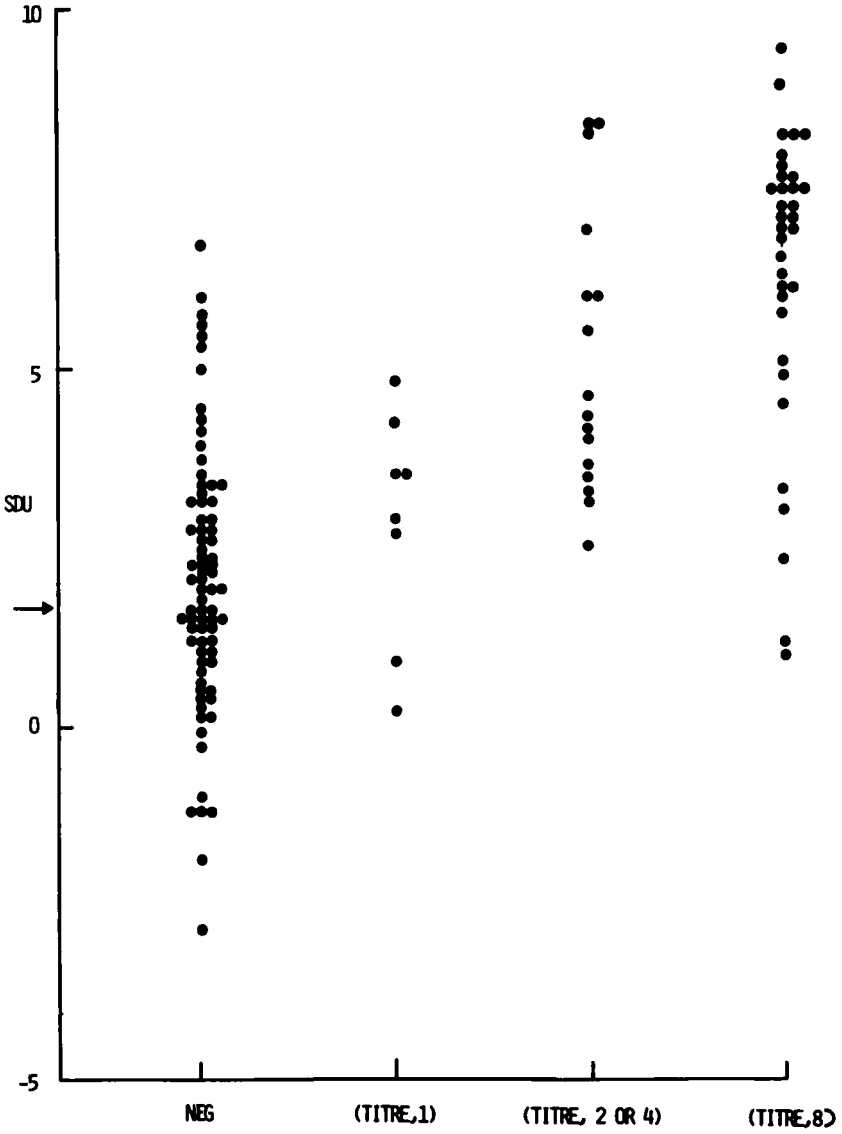

FIGURE 2 Comparison of ELISA and CIE Results obtained with Patients' Sera. The arrow indicates the cut-off point between positive and negative samples.

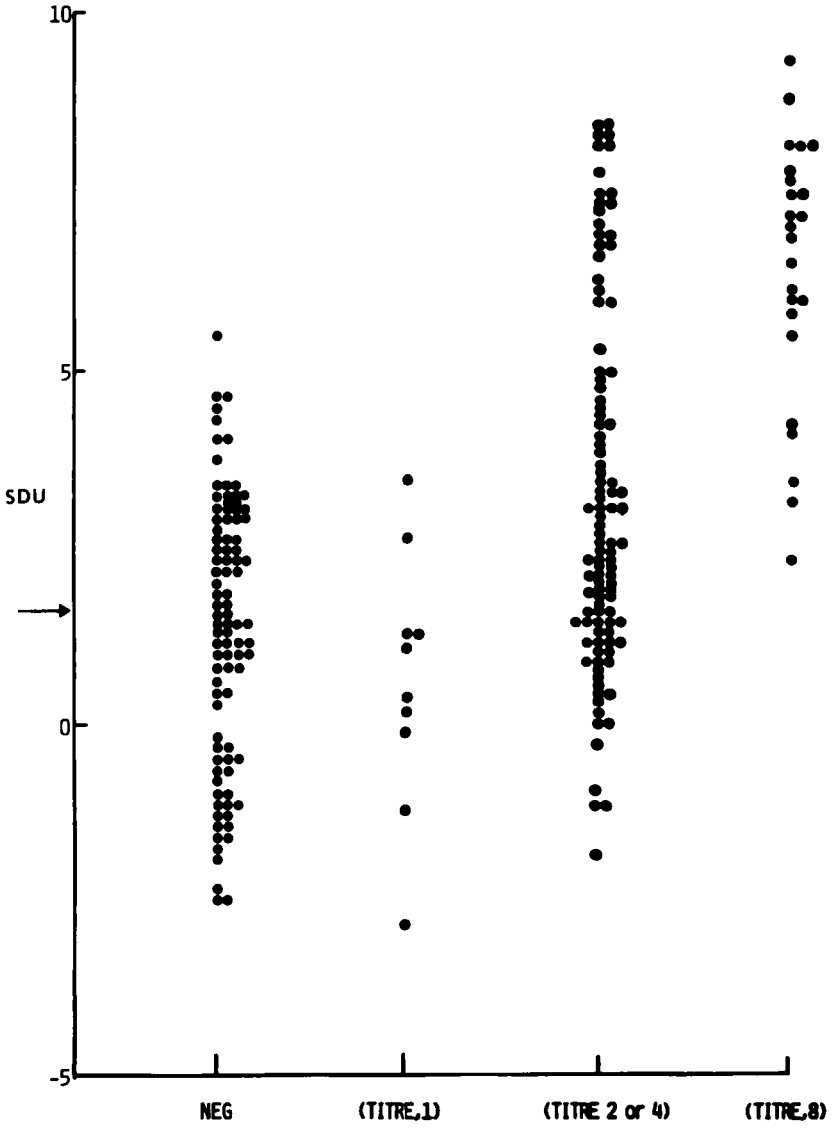

FIGURE 3 Comparison of ELISA and DD Results obtained with Patients' Sera. The arrow indicates the cut-off point between positive and negative samples.

a group of 92 sera, negative on DD, only 52% were correspondingly negative on ELISA.

Sera which gave SDU values $\geq$ 5.0 but with titres < 1:8 on CIE (total of 14) were not investigated further. Sera with titres of 1:8 on CIE which gave either low SDU values or negative readings (total of 3) on ELISA when the BF fraction was used as detector antigen, were re-tested with two additional antigenic preparations. These were the water-soluble components from total, fragmented mycelium (WS) and a fraction obtained from this material by isoelectricfocusing (fraction c). Serum from a patient, diagnosed as a case of old TB, which showed a low level (2.3 SDUs) of antibodies with the BF antigen, showed a high level (6.5 SDUs) when tested with fraction c. The status of the negative cases (one a dyspnoea patient, one an asthma patient) remained unchanged.

DISCUSSION

Studies which assess the reliability of the different methods used to detect significant serum levels of antibody to fungal pathogens are of considerable importance. There have been a number of reports which document a marked increase in the rate of detection of Aspergillus antibodies in patients with aspergillosis, when ELISA was compared with the more conventional methods of CIE and DD (4, 6, 7). In tests where the double dialysis antigen of Edwards (17) was used to detect A. fumigatus antibodies in both DD and ELISA systems, a group of 11 sera which gave OD values

at A_{492} > 1.00 (at a dilution of 1:100) were actually negative by DD (J. Evans, personal communication). On the other hand, very close correlation of results was obtained from ELISA and DD in their ability to detect Aspergillus antibodies in a small group of asthma patients (5). Eleven samples were negative by both tests, while one of the 15 samples which gave a positive result on DD gave a negative reading in the ELISA system. More recently, however, in another study of chronic asthma sufferers, these authors cite a group of 13 patients who gave a positive precipitin result on CIE, only 4 of whom gave a positive reading in the ELISA (21). In an extensive study of patients with suspected candidiasis, Kostiala & Kostiala (22) measured serum antibody levels by ELISA, CIE and DD. Of 26 serum samples which gave a positive reaction when tested undiluted on DD, 54% were negative for IgG on ELISA (42% were negative for all classes of Ig). They also found that, of 21 sera positive on CIE when tested against mycelial and somatic antigens, 24% were negative on ELISA. Thus, there is no absolute correlation between these techniques, and this must be due in part to the fact that they measure different physical phenomena.

Our ELISA results indicate that the test in our hands is considerably more sensitive than the CIE method, where of 82 negative sera, 42 (or 51%) were positive by both ELISA and DD. Of the 92 samples negative on DD, 91% were also negative by CIE, while only 52% were negative in the ELISA system. This supports

the general consensus that the sensitivity of ELISA is much greater than either DD or CIE, and is due in part to the enzyme "cascade" effect of the method. It may also indicate the predominance of non-precipitating antibodies in some aspergillosis patients (cf 23). The absence of precipitating antibodies is known to occur in certain clinical conditions, including systemic lupus erythematosus, where sera from a group of patients positive by the Farr test gave no precipitation reaction on CIE (24).

On the other hand, 29% of samples which gave a positive result at a 1:2 or 1:4 dilution on DD were negative on ELISA. Clinical diagnoses were of little use in these cases because the patients had suspected, but not proven aspergillus involvement. The discrepancy may be due, in part, to the fact that ELISA in our system measured only IgG. Some of these antigen-antibody reactions may represent the presence in these sera of precipitating antibodies of the IgM class. It is also possible that these sera may contain predominantly low affinity antibodies which have been reported to be less readily detected than high affinity antibodies in an ELISA system (25). In addition, the antigens used in ELISA are mixtures, and competition for the available binding sites on the support surface is known to occur under these circumstances (26). The possibilty exists that some patients may have antibodies to antigens not present on the plastic surface in large enough proportion to give a positive reaction. This theory has also been advanced by Kauffman et al., (23) in connection with their findings

that a small group of patients with suspected aspergillosis (n = 16) whose sera gave weak precipitates on DD were negative by ELISA. In this context, the results obtained by Kostiala & Kostiala (22) for suspected candidiasis patients are also of interest. They reported negative results by ELISA for 32% of cases positive at a 1:2 dilution on DD and for 17% of cases positive at a 1:4 dilution.

In most of these studies, the only distinction made was between positive and negative results. Kauffman et al., on the other hand, showed in general that increasing numbers of precipitation lines found by DD were significantly related with increasing IgG titres on ELISA. However, they also found that the results of these two serological assays on individual sera may deviate considerably, one from the other (23). Sepulveda and his co-workers also reported a good correlation between the number of precipitin lines seen on DD and absorbance values in ELISA, when used to detect aspergillus antibodies in cases of ABPA (6). However, in this test, results were selected on the basis of the maximum number of lines detected by any one of 5 different batches of antigen reacted with each test serum. In addition, the positive/negative cut-off used by these authors, while not explicitly stated, appears from their data to be set immediately above the highest negative reading and may possibly include false positive results.

The selection of control negative sera is of paramount importance because IgG antibodies to aspergillus can be detected in

a high proportion of normal individuals (27, 28). The method of discrimination between positive and negative is a crucial factor in a design for the analysis of ELISA data. In some of the studies referred to here, the minimum positive absorbance was selected as the mean negative value of a large group of apparently healthy donors plus 2 standard deviations of the mean (7, 22, 23). This method is rarely valid because it assumes that ELISA values in the normal population are normally distributed while they are actually distributed with a positive skew. Therefore, type I errors (false positives) can occur at plus 2 (and plus 3) the standard deviation (9). Another procedure is to set the minimum positive response value at plus 2 or plus 3 the mean absorbance of a negative reference serum (4). This method is used only when small numbers of reference normal sera (< 10) are available, and is not recommended because of inadequate data (9). We have made allowance for the fact that the mean negative is subject to error, by the introduction of a suitable correction factor. With this system, we have found that the distribution of ELISA results is essentially a continuous one without a discernible gap between high and low readings. A similar distribution was observed in a previous study (14), when results were presented as OD values and compared with CIE titres. Any classification of sera into positive and negative is thus bound to be an arbitrary one.

It would appear, both from our work and that of others, that no one test is 100% reliable. One of the problems with DD-type

tests has been the difficulty in interpretation of ill-defined and non-specific precipitin lines (6, 7), while the determination of cutt-off points and the problems of cross-reactivity present difficulties with ELISA. We have found that, while there is an obvious correlation between the different serological methods, the relations are not particularly close. This is hardly surprising, in our view, given that the tests depend on different physical phenomena and that measurements made on a continuous scale (ELISA) are being compared with doubling dilution methods (DD and CIE), which are separated into groups on this basis.

The method of evaluation suggested here satisfies several of the criteria listed in (9). Results are presented on a continuous numerical scale, at a single serum dilution and relative to both normal and pathological samples. It makes use of the fact that the determinations will usually be done routinely, so that a long-term estimate of experimental variability can be used, while incorporating adjustments for changes in level of absorbance and checks on the sensitivity and reproducibility of the method. The basic standard deviation of the log A values for negative sera, here taken to be 0.112, may vary from one laboratory to another; it can be re-estimated quite simply from experimental data, and updated if desirable from the data obtained while the assay is in routine use. The routine inclusion of a number of reference positive and negative sera which satisfy the parameters outlined above offers, in general, the most reliable estimate

of what constitutes a significant level of IgG antibodies in serum to Aspergillus antigens. That a number of false negative results may occur, possibly for one or more of the reasons outlined above, is a problem which merits further investigation.

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