

Letters to the Editor

CHROMOGENS FOR THE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) USING HORSE-RADISH PEROXIDASE

Sir,

Horse-radish peroxidase (EC1.11.1.7) is rapidly gaining popularity as a cheap and stable enzyme for use in the enzyme-linked immunosorbent assay (ELISA). There are at least three easy and efficient ways to conjugate it onto immunoglobulin, or antigens.¹⁻³ However, there is still some confusion as to the best way to quantitate the enzyme.

The presence of peroxidase is revealed by the development of a coloured product, when the enzyme interacts with hydrogen peroxide (H_2O_2) and a chromogen. The chemistry involved is not fully understood and is bewilderingly complex.⁴ The ideal chromogen should be cheap, stable, easily used and non-carcinogenic and the change in optical density should be large in relation to the amount of enzyme present (e.g. the extinction co-efficient should be high).

The last point deserves amplification. If the optical density achieved in the presence of a certain quantity of enzyme is low, the sensitivity of the assay is obviously also low. At first sight this might seem unimportant for most purposes, because in ELISA assays it is usually possible to increase the concentration of enzyme conjugate used until readable results are obtained. However, not only does this waste reagents, but it is not even a satisfactory solution to the problem because the use of an insensitive chromogen has another more serious disadvantage. This becomes apparent when the optical density achieved is plotted against the quantity of enzyme present. With a poor chromogen this dose-response curve is relatively *flat* and the test therefore achieves very poor discrimination between 'positive' and 'negative' samples.^{1-2, 5}

The available chromogens

A number of possible chromogens have become obsolete because of carcinogenicity and insolubility (o-dianisidine), or instability of the coloured product, (o-tolidine), and need not be considered further.

Those currently in use are ortho-phenylene diamine (OPD), 2,2'-azino-di (3 ethyl) benzothiazoline-6-sulphonic acid (ABTS) and 5-amino salicylate (5-AS).

Of these three 5-AS should probably now be regarded as obsolete. When compared with OPD¹⁻² or o-tolidine⁵ it proves so insensitive and gives such a flat dose-response curve that its use for the type of assay described by Huikeshoven and his colleagues⁶⁻⁷ cannot be recommended. The ratio of positive to negative results in a number of assays can be 5 to 20 times lower with 5-AS than with more efficient chromogens^{1, 5} and higher concentration of conjugate and longer incubation periods are needed.⁵ Moreover, the relative insolubility both of 5-AS and its coloured reaction product is a major problem with many batches of the compound.

There is now general agreement that the greatest sensitivity is achieved with OPD¹⁻² (own unpublished data). This compound is available cheaply from Sigma (Cat. No. P-3888). Optimal results are obtained when 34 mg of OPD are dissolved immediately before use, in 100 ml of citrate/phosphate buffer (0.15 M, pH 5.0) containing 30 μ l of 6% ('20 vol') H₂O₂. The reaction can be stopped with 12.5% H₂SO₄ and the results are read at 492 nm. This chromogen does, however, have two minor disadvantages. First, it has been said to be mutagenic,⁸ but the author is not aware of any reports of carcinogenicity, and there are no restrictions on its use. It is recommended by most suppliers of reagents and equipment for ELISA assays. The second disadvantage is its photosensitivity and the tendency for the colour to darken slowly even after the reaction has been stopped. This means that tests should be read immediately.²

An alternative to OPD is ABTS (Sigma, Cat. No. A-1888). This was developed by Boehringer as a non-toxic chromogen, and no reports of toxicity were revealed by a recent computer search of the Cancerline and Toxline data bases (although there is a report⁸ that like OPD, ABTS is mutagenic in the fluctuation test and the Ames test, when used at high concentrations). Fifty milligrams of ABTS should be dissolved in 100 ml of citrate/phosphate buffer (0.1 M, pH 4.0) with 30 μ l of 6% H₂O₂. The reaction can be stopped with sodium fluoride (final concentration of 0.64 mg/ml). Used in this way, both the substrate and the reaction product are remarkably stable and the tests do not have to be read at once. There are two absorption peaks, one at 414 nm and one at 650 nm. The latter results in a blue colour which is particularly easy to read visually. It also allows the design of a very simple battery-powered photometer for accurate through-the-well reading of the plates,⁹ because monochromatic light of approximately this wavelength can be obtained with a red light-emitting diode. Most workshops should be able to construct such a device.

In spite of all these advantages, ABTS is somewhat less sensitive than OPD, which in our hands gives absorbance values two to four times higher. This difference is not enough to matter and it is vastly superior to 5-AS. Its other advantages may lead to acceptance of ABTS as the chromogen of choice for many applications.

In summary, there are now two safe, soluble, inexpensive and sensitive chromogens for use in peroxidase-based ELISA systems. These are o-phenylene diamine (OPD), and 2,2'-azino-di (3 ethyl) benzothiazoline-6-sulphonic acid (ABTS).

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BACTERIAL AND MORPHOLOGICAL INDICES IN LEPROSY

Sir,

We wish to make some comments on the letter published in *Leprosy Review*, 1980, **51**, 361 by Schreuder and Colpa.

It appears that some time can be saved for an experienced laboratory assistant by recording his findings along the line of Ridley's SFG index (*Leprosy Review*, 1971, **42**, 96–7) because if, for example, the proportion of solids is (at one glance) between 1 and 19%, then he does not have to decide in each doubtful case whether he sees a solid AFB or a fragmented AFB. We feel, however, that by using Ridley's SFG index a great deal of potentially useful information is lost. For instance, one would no longer know whether the proportion of solids was 2% or 20% (both would give the value 1 for Ridley's index) or whether a patient has 25% fragmented AFB and 75% granules or 75% fragmented AFB and 25% granules (both situations would give the value 2 for the SFG index), which surely is of interest.

Secondly, it appears useful to us to observe a shift to the right (i.e. from solid towards granular) in the bacterial morphology during the course of treatment, as a sign that the compliance of the patient is adequate and that there is no primary resistance. This is only possible if the relative percentages of solid, fragmented and granular AFB are recorded in detail, as has been done for the past 15 years in Malawi in a standardized way. A typical smear result of a new, well-established lepromatous patient would be recorded by us as follows: 5+, 15, 47, 38 (BI SFG%). In the case of a low BI we record the actual numbers of S, of F and of G seen, rather than their percentages.

Ridley's SFG index would only allow a relatively rough monitoring of the patient's progress, although it would probably pick up the emergence of secondary resistance as quickly as a more detailed recording of the proportion of solids, fragments and granules.

Another question we have been asking ourselves recently is whether it is more meaningful to record the *average* of the BI and of the proportion of S and F (and G) AFB, or the *highest value*, if smears are taken from several sites, which is the usual case. In relapsed patients with new lesions, the results of slit-skin smears from these new lesions are of critical importance. We should not allow the results of concurrent smears from routine sites (such as the earlobes, which might still be low in BI and might have no solid AFB yet) to depress the alarming facts into a smoothing average. We would very much welcome comments on this question.

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'SFG INDEX'. REPLY TO 'BACTERIAL AND MORPHOLOGICAL INDICES IN LEPROSY'

Sir,

Drs Boerrigter and Ponnighaus are correct in saying that each of the code values (2, 1, 0) for solid, fragmented and granular bacilli represents a wide range of percentages of each of those forms. Any one who needs to know the actual percentage of particular forms of bacillus is recommended to do an arithmetic count, always provided that he has the time to do so, that he has accurately defined the 3 forms and has demonstrated reproducibility of counts among observers and reproducibility of morphology in spite of vagaries of stain technique. This would be more accurate and there may be research requirements which would demand it, but it is not easy.

The rationale of the SFG index is that the latitude of any code value is limited by the other two code values. This may operate through the obvious fact that the actual percentages have to add up to 100, and if one or two go up something else must come down. And it may operate through the probability that a redistribution between, say, fragmented and granular forms will not come about without some change also in the number of solids. In the example given by your correspondents, the number of solids would be unlikely to remain at 20% or 2% while a preponderance of fragmented forms was replaced by a preponderance of granular forms. This would be the situation when the index fell from 5 to 3. Thus the SFG index is most useful for showing a shift to the right, during treatment, or the left in relapse. This is just what Drs Boerrigter and Ponnighaus say requires an actual percentage count, which makes one wonder if they have ever tried the SFG index in practice. The index was originally proposed for routine clinical use, but during 20 years experience it has been found satisfactory by us for at least some research purposes.

As regards the last point raised in the letter, it is correct to record indices as the average of all sites, but in the special situation of a reappearance of solid forms at one site, indicative of relapse, most people would surely draw attention to the fact and record it separately.

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TRAINING IN LEPROSY OF MEDICAL STUDENTS IN ETHIOPIA

Sir,

As long as leprosy control depends mainly upon early detection and adequate treatment of patients the battle cannot be won until a satisfactory distribution of health services and a proper training of all health personnel in the recognition and management of leprosy patients has been established. The training of such personnel is particularly important where leprosy work is rapidly becoming integrated into the general health services. In the training programmes high priority should no doubt be given to the medical students.

Most students will, after their graduation, be posted to rural hospitals and health centres. For many of them this will sooner or later imply a teaching task in the district — or regional training schools for other cadres of health personnel such as nurses, medical assistants, health assistants, rural medical aids, sanitarians, etc. The young doctor will also become responsible for the proper management of leprosy patients referred to the hospital because of complications of the disease.

It follows, therefore, that the medical schools should provide a thorough training in leprosy not only to prepare future doctors for this clinical task but also to enable them to teach various cadres of health personnel, upon whose participation the success of leprosy control so much depends.

In 1978 the Medical Faculty of Addis Ababa University took the decision to have the medical undergraduates trained in leprosy and dermatology at the All Africa Leprosy and Rehabilitation Training Centre (ALERT) for a period of 4 weeks instead of the usual 2 weeks. In addition, training in leprosy field work was included in their 6 weeks assignment to a rural health centre, so that they could study community health activities at first hand.

During the 4 weeks at ALERT the students undergo an intensive course consisting of supervised clinical practice, lectures with case demonstrations, and slide presentations.

The objectives of the course primarily aim at enabling the students to satisfactorily deal with the great majority of leprosy complications and all forms of uncomplicated leprosy. That ability is an indispensable prerequisite for their teaching leprosy to other cadres of health personnel. As regards dermatology, the emphasis is more on the principles of examination, diagnosis and treatment than on the pathology and treatment of individual skin diseases, with the exception of the most common dermatoses.

In the outpatients department the students are therefore from the start made to take histories, examine the skin and record their findings. These and their proposals as to treatment are subsequently discussed with each of them by one of the staff dermatologist-leprologists.

In classroom sessions during the first 2 weeks a great variety of leprosy patients is demonstrated to the class and extensively discussed. Much attention is given in those sessions to the early recognition and the prevention of disabilities. During the second 2 weeks the students themselves demonstrate and discuss cases to the group and members of staff. To this end the students are given inpatients to examine without access to the clinical records. During the 2 weeks in the field the students participate in the running of rural leprosy clinics and undertake school, village and contact surveys. Up to 1981, 92 students were trained according to this new programme.

Going by their scores in the final test and by their evaluation reports, it seems to produce satisfactory results. The students particularly appreciate their active participation in the running of the various clinics. The 4 weeks of intensive involvement with a good many leprosy patients not only gives them the required confidence and abilities but also liberates them from the fear of the disease with which, many admit, they entered the course. The change of attitude is a major achievement of the course and also a major prerequisite for becoming sound teachers of leprosy.

An important criticism from the students on the courses is about the large amount of time spent on leprosy as compared to dermatology. This criticism is valid. The students are very much aware of the fact that although patients with skin diseases form a great proportion of the patients attending clinics and hospitals many if not most of the medical officers and other health professionals are ill- or not at all trained in the management of those patients. They therefore see the need for a better gearing of their medical education to the actual needs of the community. It is, however, not the intention to make alterations in the ALERT courses in favour of dermatology as this would not solve their problem and only detract from their leprosy training. To meet the criticism of the students, lectures and case demonstrations on dermatology are going to be held in the University hospital for the students while they are doing their medical internships. The medical faculty will release the students for those sessions for 2 hours each week.

Ethiopia seems to be setting a commendable example which might well be followed by others.

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