TITRATION OF CHOLERA ANTI-TOXIN BY RABBIT SKIN ASSAY AT THE CHOLERA RESEARCH LABORATORY

Kh. Abdullah Al-Mahmud
George Curlin

Summary

Assay of cholera toxin and anti-toxin in rabbit skin offers a convenient, accurate, and sensitive assay of cholera toxin activity. Since the discovery of cholera toxin in the stools of cholera patients at the CRL, the development of the rabbit skin assay has been carried out in Dacca, although majority of the work was done both in Dr. Craig's and other laboratories abroad. The cholera toxoid field trial of 1974 called for cholera anti-toxin titrations of large numbers of sera. Of necessity, the serological survey samples from the field trial population were finger-stick samples. The rabbit-skin assay offered a convenient and accurate assessment of anti-toxin titers in the small, dilute samples because of its great sensitivity, and therefore the rabbit-skin assay was used to titer a systematic sample of 20% of the sera from the vaccine field trial serological survey. The data indicate a higher median baseline titer for children aged 1-4 than children aged 5 to 14, or adults. The lower limits of a detectable end-point was found in the neighborhood of 3 AU/ml in samples of 100 lambda volume of blood diluted 1-10 in normal saline in the field. Although children aged 1-4 have a higher average base-line titer, their response to two injections of 100 mcg of Wyeth 21201 toxoid were less on the average than older children or adults. The geometric mean titer for each age group was considerably less than comparable titers observed in the Phase II study in village Meharan, summer 1973 using Wyeth 11201 toxoid. By comparing antitoxin titers from simultaneously drawn finger-stick and venepuncture sample, it was established the difference was due to an erroneous estimate of the initial dilution which, in fact, was always greater than 1 to 10 because of average hematocrits considerably less than 50% in the field trial population.

Introduction

Cholera is a disease caused by action on the small bowel by an extremely active protein toxin called cholera entero-toxin. The entero-toxic properties of the cholera toxin were established from cultures by Datta et al.(1) in India and was confirmed to be present in cholera steel of patients at the Dacca Cholera Research Laboratory by Dr. John. P. Craig (2,3) who also established a vascular permeability property of the toxin called Pf. The development of rabbit and guinea pig skin for the assay of cholera toxin and, subsequently, cholera anti-toxin was further developed at Dr. Craig's laboratory (4) and abroad, but the Cholera Research Laboratory maintained technical capabilities to perform these tasks in Dacca.

The first large scale field trial of cholera toxoid which occurred in the latter part of the calendar in 1974 required serological survey of anto-toxin titer in addition to vibriocidal titers which had been a common accompaniment of previous field trials. A serological survey of 3% of the field trial population demands that finger-stick samples be obtained in the field. This dictates that the initial dilution of serum for assay of antitoxin antibody be at least greater than 1 to 10. Should low titers of anti-toxin be significant in protecting against cholera, it is desirable to use a sensitive assay for anti-toxin. Two antitoxin assays were thought to be feasible in this setting. The hemagglutination assay was cheaper and could be scaled-up more easily, but it offered reduced sensitivity. The rabbit skin assay of Pf offered sensitivity, but it was not as efficient or cheap as hemagglutination. Therefore, it was decided that the hemagglutination assay to monitor the entire serological survey would be established concurrently with the Pf assay to monitor a sample of sera from the survey.

Methods

The toxoid field trial called for finger-stick bleeding of every member of every 33rd family prior to the first and second injections, and six weeks following the second injection. Samples were obtained as 100 lambda whole-blood collected from a finger stick into 100 lambda heparinized

capillary tubes which were evacuated immediately into .9 ml. of sterile normal saline in the field. In cases when it was not possible to get a good sample of 100 lambda, 50 lambda were obtained, and evacuated into .45 ml. of normal saline in the field. Patient's identification and the date were made on the diluted whole blood. Samples were returned to Matlab and were forwarded to Dacca the following day on ice. Immediately on arrival in Dacca the cells were spun down and diluted plasma decanted and frozen.

Dr. Ansaruddin Ahmed will detail the methodology and results of the hemagglutination assay which was performed on every sample. Permeability factor assay in rabbit skin was performed on every 5th sample in a serial order beginning with a random start.

The finger-stick blood samples received from the field were considered to have a initial dilution of 1-10 neglecting Hct.% of the patients. Depending on the volume, the sera were first screened at the dilution of 1-80 and 1-640.

Standard Toxin: Finkelstein Purified Cholera Toxin lot No. 0572 were used throughout the tests. The Lb. of the toxin was found to contain 27 Lb/mcg as determined both in Dacca CRL and in Brooklyn by Dr. Craig. A 5 mgm vial of toxin was rehydrated in 1 ml distilled water and then diluted to a volume of 200 ml of 1 mcg/ml in phosphate buffered saline diluent containing 0.1% bovine serum albumin and 0.01% merthiolate (PBSSM) as per manufacturer's direction. Aliquots of 1 ml vial was quick forzen and stored at -60°C. One aliquote of the toxin was thawed and diluted to 1 mcg/ml and kept at 4°C until it was used within a maximum of five days.

Standard Antitoxin: The serum used is the provision standard antitoxin "Purified Cholera Antitoxin Lot No. EC3(A-2/67)-B" prepared by the Swiss Serum Vaccine Institute, Switzerland. The material is hence referred to as SSVI-B. When rehydrated as per instruction of the manufacturer, the serum has been assigned to contain 4470 AU/ml in Dr. Craig's laboratory. Rehydrated 1 vial of SSVI-B with distilled water and then in normal saline containing 0.01% merthiolate in a 25 ml volumetric flask, the final dilution being 357.6 AU/ml (say 358 AU/ml) and kept at stock at 4°C. For use in our assay it was diluted in BG to 10 AU/ml and

then to 1 AU/ml in BG.

As the test dose of toxin depends upon the sensitivity of antitoxin detection desired, 1/10 Lb/ml was found to be convenient. This dilution tests at the Lb/200 level since 1/200 Lb. of toxin enters each injection site.

Serial 2-fold dilution begining from 1-80 or 1-640 of the test sera in BG were dispensed in 0.3 ml volume in Wasserman tube. The initial dilution was chosen based on the result of the screening tests. SSVI-B, which was used as the standard Antitoxin control, was prepared in serial 2-fold dilutions in BG buffer containing 1/4, 1/8, 1/16 and 1/32 AU/ml.

To each tube of both standard antitoxin and test sera we added an equal volume of toxin 0572 containing 1/10 Lb. i.e. 0.0037 mcg (4 mg)/ml. A positive control for toxin was included in each test which contained equal volumes of toxin (1/10 Lb/ml) and B.G. buffer. A negative control of BG buffer only was also included in all the tests. All the tubes were incubated at 37°C in water bath for one hour and injected intracutaneously in a volume of 0.1 ml in to one randomly chosen spot on each of the two rabbits.

Between 22-23 hours after the injection 1.2 ml of a 5% solution of Pontamine Sky Blue 6BX (DuPont) in 0.45% saline per Kg. body weight was administered intravenously. One hour after the intravenous dye injection the diameter (in mm) and the blueing intensity (on a graded arbitrary scale of ± to ++++* as defined by Dr. Craig) of the lesions were measured. The blueing scores were calculated by multiplying the mean lesion diameter in mm by the mean intensity value. The bluing scores were plotted against the log serum dilution and the interpolated concentration of antitoxin which yielded a mean bluing score of 20, which was considered the steepest part of the curve in our tests, was considered the end point of the assay. The log of the test serum dilution was then substracted from the log of the standard antitoxin in units, which represented the log of the AU/ml of the test serum.

In addition to finger-stick samples from the serological survey of the toxoid field trial, simultaneous collection of

*± = 1; + = 2; ±+ = 3; ++ = 4; ±++ = 5; +++ = 6; ±+++ = 7; ++++ = 8

venepuncture whole blood and finger-stick samples were conducted following the epidemic in 33 individuals who had received toxoid or who were convalescing from cholera. Serum was obtained by vein puncture, and finger-stick samples were obtained in 100 lambda quantities and were diluted in .9 ml saline exactly as in the field. The pairs of undiluted serum and diluted plasma were assayed in the rabbit skin by the same techniques as described above.

A further set of 50 paired sera of Phase II study of Wyeth 11201 toxoid in Meharan was assayed on the rabbit skin by the same technique as described above to show the correlation of the results obtained in Dr. Craig's lab and in Dacca CRL.

Results

Figure 1 illustrates a frequency distribution of titers obtained in serological surveys in Pf assay. The median and significant percent of observations are indicated for each of the 3 age groups on day 0, day 42 (just prior to the second injection); day 84 (six weeks following the second injection). It is seen that children aged 1-4 have a higher median titer and lower percentage in the undetectable titer range. Sensitivity was usually 3 antitoxin units per ml. depending on the particular run on the sensitivity of the rabbit skin. It is also noteworthy that the placebo-immunized group did not change significantly. A few individuals were seen to raise their titer considerably, and although these individuals have not been matched with hospitalized cases, we have already heard that there is more than sufficient cholera in the area to explain the low proportion of placebo immunized patients showing a significant rise in the antitoxin.

Table 1 presents median antitoxin units per ml. as determined by the permeability factor assay among the toxid field trial survey population. It seemed that children 1-4 started with a higher median titer, but responded less than older children or adults.

Figure 2 is a correlation diagram of antitoxin units per ml. determined by the permeability factor assay using pairs of whole serum and diluted finger-stick plasma.

Table III shows the results obtained in Dacca CRL and in Dr. Craig's lab, Brooklyn, U.S.A., and Figure 3 is a correlation diagram of antitoxin units per ml. determined by the permeability factor assay of the 50 paired serum of Phase II study of Wyeth 11201 toxoid in Meharan.

Discussion

The accuracy of the rabbit skin assay for permeability factor makes it a convenient reference to use between laboratories. In a situation at the CRL in 1974 when large numbers of finger-stick samples of diluted human plasma were to be assayed for antitoxin, the increased sensitivity of the rabbit skin assay made it possible to perform simultaneous assay by two techniques of antitoxin.

Because of the increased sensitivity in the rabbit skin assay, it was possible to estimate with greater accuracy the baseline titers of all the people included in the serological survey. The data contained no surprises in that children showed higher average base line titers than older children or adults. It was surprising, however, that children responded less well on the average to two injections of 100 micrograms Wyeth 21201 toxoid than did older children or adults. The placebo immunized patients did not change significantly with placebo immunization. Five percent did,however, increase their individual antitoxin titrations when the same person was followed serially. This is perfectly consistent with the attack rate of cholera which was seen in the area during the time of the serological survey.

Although there were significant numbers of patients who had undetectable end-points of antitoxin titrations because of the initial dilution of plasma for assay, a comparison of median responses to 100 micrograms of similar toxoids indicates that the response to Wyeth 21201 is less than the response to Wyeth 11201. This is opposite of the observation made in Texas volunteers in which the field trial lot was thought to be slightly more antigenic than the preliminary lot. In an effort to establish the magnitude of inaccuracy which occurs as a consequence of a variable initial dilution of finger-stick blood simultaneous corrections of finger-stick and venepunctures samples were obtained in toxoid

immunized or convalescent patients.

Conclusion

The rabbit skin assay per cholera antitoxin was shown to be a sensitive, feasible assay of cholera antitoxin. The data generated from this assay confirmed previous observation of higher average titers with younger people. The trend to an increased response to cholera antitoxin administered intramuscularly with increasing age was noted. We conclude that the rabbit-skin assay is a valuable part of the Cholera Research Laboratory capability in toxin and antitoxin research. Its usefulness lies in accuracy and increased sensitivity over hemagglutination. Its drawbacks are the expense of maintaining a rabbit colony and limited numbers of samples which can be handled on a day to day basis.

References

1. Datta, N.K., M.V. Pense and D.R. Kulkarni (1959). Role of choleratoxin in experimental cholera.

2. Craig, J.P. (1965). A permeability factor (toxin) found in cholera stools and culture filtrates and its neutralization by convalescent cholera sera. Nature (London), 207: 614-616.

3. Craig, J.P. (1965). The effect of cholera stool and culture filtrates on the skin of guinea pigs and rabbits. In proceedings of the Cholera Research Symposium, U.S.P.H.S. 1328. U.S. Govt. printing press Washington D.C. p-153-158.

4. Craig, J.P. Procedure for titration of cholera toxin and antitoxin by the rabbit intracutaneous method. Unpublished set of instructions through personal communication.

PROCEEDINGS OF THE 9TH MEETING OF THE SCIENTIFIC REVIEW AND TECHNICAL ADVISORY COMMITTEE OF THE CHOLERA RESEARCH LABORATORY

(a)

and

REPORTS OF THE COLLABORATIVE STUDIES BETWEEN CENTER FOR MEDICAL RESEARCH AND CHOLERA RESEARCH LABORATORY

For the

YEAR 1974

Dacca, Bangladesh