PASSIVE HEMAGGLUTINATION ASSAYS FOR QUANTITATION OF CHOLERA ANTITOXIN : GLUTERALDEHYDE AND CHROMIUM CHLORIDE USED AS COUPLING REAGENTS TO SENSITIZE HUMAN ERYTHROCYTES WITH PURIFIED CHOLERAGEN

74

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PREFACE

The International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) is an autonomous, international, philanthropic and non-profit centre for research, education and training as well as clinical service. The Centre is derived from the Cholera Research Laboratory (CRL). The activities of the institution are to undertake and promote study, research and dissemination of knowledge in diarrhoeal diseases and directly related subjects of nutrition and fertility with a view to develop improved methods of health care and for the prevention and control of diarrhoeal diseases and improvement of public health programmes with special relevance to developing countries. ICDDR,B issues two types of papers: scientific reports and working papers which demonstrate the type of research activity currently in progress at ICDDR,B. The views expressed in these papers are those of authors and do not necessarily represent views of International Centre for Diarrhoeal Disease Research, Bangladesh. They should not be quoted without the permission of the authors.

ABSTRACT

Previously described passive hemagglutination assays (PHA) for quantitation of cholera antitoxin in avoiding animal assay like rabbit intracutaneous test were found inadequate for use in large-scale serosurveys in connection with cholera toxoid field trials. This was because of non-specific agglutination of the unsensitized sheep cells and the nonfeasibility of decomplementation of numerous small volume (less than 0.5 ml.) finger-prick plasma samples prior to PHA to avoid hemolysis of sheep cells.

Two alternative PHA assays were developed using human 'O' group, Rh negative erythrocytes as carrier particles and gluteraldehyde and chromium chloride as bifunctional coupling reagents to sensitize the erythrocytes with purified choleragen. Both these in vitro assays were specific and showed excellent direct correlations with the corresponding in vivo rabbit intracutaneous test. The gluteraldehyde-PHA assay, finally selected for its additional ability of stabilizing the erythrocytes against hemolysis and simpler methodology, correlated with another PHA test, using tanned chicken erythrocytes with simple coating of toxin. By titrations of 53 plasma samples from Wyeth toxoid field trial in Bangladesh, 1974, this assay also showed an excellent correlation with the in vivo skin test ($r = 0.917$). It showed a good reproducibility and a sensitivity of measuring up to 0.35 antitoxin units/ml. By its inhibition modification the toxin antigen can also be measured up to 0.3125 microgram of a purified cholera toxin.

INTRODUCTION

Development of *in vitro* methods (11, 8) for titration of cholera antitoxin by passive hemagglutination (PHA) provides a convenient alternative to the more cumbersome *in vivo* assays by skin capillary permeability factor (3, 2, 6) and ligated rabbit ileal loop (12). The animal assays are not suitable for large numbers of sera and also are time consuming and expensive. In late 1969 one of the PHA tests, namely that of Hochstein et al. (11) using fresh sheep erythrocytes, was set up at the Cholera Research Laboratory (CRL) with the technical information obtained from the authors through a pre-publication communication. We planned to use this test for surveying the humoral antitoxin response in volunteers of the cholera toxoid vaccine field trial in Bangladesh by CRL during 1974, using toxoid lot No. 21201 prepared by Wyeth Laboratories.

Though the test fulfilled all the specifications obtained by Hochstein et al., we found it inadequate for supporting estimations of anti-cholera toxin antibody in this large-scale field trial due to the following disadvantages: (I) Non-specific agglutination was found in a considerable number of titrations against control sheep erythrocytes not sensitized with cholera toxin. This was probably due to the Forssman-type antigen-antibody reactions. It was almost impossible to adapt

the method of absorption of plasma with sheep erythrocytes prior to titration, when more than six thousand finger-prick samples, each of less than 0.5 ml. plasma, were to be handled.
(II) Decomplementation of plasma prior to the PHA test was necessary to prevent complement-mediated lysis, because of the use of fresh sheep erythrocytes.

In quest of alternative methods obviating these disadvantages, two PHA assays were developed using human 'O' Rh negative erythrocytes as carrier particles and gluteraldehyde and chromium chloride as bifunctional coupling reagents to sensitize the erythrocytes with purified choleragen. This paper describes these two PHA assays for quantitation of cholera antitoxin establishing their sensitivity, specificity, reproducibility and correlation with the corresponding rabbit intracutaneous assay.

MATERIALS & METHODS

Sensitizing Toxin

Dr. Finkelstein's purified lyophilized choleragen, Lot No. 0572 (7) having 27 limit-of-bluing (Lb) doses per microgram protein, was used as the antigen. The Lb dose content was determined according to the results of titrations done at CRL and at Brooklyn by Dr. J.P. Craig, prior to this

investigation. Five mg toxin lyphile in a vial, after reconstitution with 1 ml. of distilled water as specified by its producer, was carefully diluted to a concentration of 200 µg/ml. with a 0.15 m phosphate buffer saline, pH 7.2, containing 0.1% bovine serum albumin (BSA) and 0.01% thimerosal. Aliquotes of 0.2 ml. in 1 dr. screw-capped bottles of this 200 microgram/ml. choleragen were snap-frozen and kept at -20°C in deep freeze. One such vial was quickly thawed at a time, and mixed with equal volume of physiologic saline to be used as a working solution of antigen in a final concentration of 100 microgram/ml. for not more than two days.

This same batch of choleragen was used as the Standard Toxin in the rabbit skin assay, stored as stock at -20°C in 1 ml. aliquotes in a concentration of 25 microgram/ml. in the same BSA-fortified PBS. One vial was thawed at a time, diluted to a concentration of 1 microgram/ml. in borate-gelatin buffered saline, pH 7.5 (8), and kept refrigerated to be used for a maximum of five days.

Carrier Particle

Human 0 group Rh negative-type (14, 17) blood (isohemagglutinogen CDE-negative), 50 ml. at each bleeding, was collected asceptically in a sterile container by venipuncture from a single donor, mixing with approximately one tenth of

its volume of anticoagulant acid citrate dextrose solution and the erythrocytes were used between sixth through twentieth day of storage at 4°C.

Coupling Reagents

(a) Gluteraldehyde (GA): (25% in H_2O), OHC $(CH_2)_3$ CHO. F.W. 100.12, J.T. Baker Chemical Co., Phillipsburg, New Jersey 08865. For use of the GA solution, it was purified (15) by two serial treatments (20 g/100 ml.) with activated bone charcoal and in the process was further diluted approximately 1:5 having a 0.5 M final concentration.

(b) Chromium chloride: Cr Cl_3, 6 H_2O, F.W. 266.18, analytical reagent, Mallinckrodt Chemical Works, U.S.A. The working solution of 1.25 millimolar strength is made in physiologic saline from an initial 0.1M aquous solution prepared for one-day use only and kept out of contact with light before use.

Buffer

The working solution of a phosphate-buffered saline, specially prepared for passive hemagglutination work (10), is mixed with 0.15M saline in equal parts for erythrocyte sensitization, pH 7.2 to 7.4, which will be simply called as PBS.

Standard Antitoxin

This was used as a reference for calculating antitoxin unitage of unknown sera: "Purified Cholera Antitoxin, Lot No. EC3(A-2/67)-B," prepared by the Swiss Serum and Vaccine Institute (SSVI-B) Berne, Switzerland. When rehydrated according to the instructions of its producer, the serum was assigned to contain 4470 AU/ml. in Dr. J.P. Craig's laboratory.

The same SSVI-B antiserum was also used in the rabbit intracutaneous assay (Skin Permeability Factor assay) as the standard antitoxin at a concentration of 10 AU/ml. and 1 AU/ml. in borate-gelatin-buffered saline, pH 7.5 containing 0.01% thimerosal (4). In addition, this serum was titered as the reference positive antibody, at a starting dilution of 1:200, six times by each technician in each run of PHA assay in the 1974 toxoid field trial (53 titrations) and 1978 pilot trial for acceptibility of the Wellcome toxoid vaccine (11 titrations) for checking the reproducibility of the PHA assay, as well as for determining the lowest limit of detection of antitoxin in each day run with the help of the mean value of the six titrations.

Antisera Tested

(a) For showing correlation between the two PHA assays and the rabbit intracutaneous assay, a panel of 36 lyophilized

immune anti-toxoid human sera from American volunteers of the H-8 Texas study* (16) were tested by both methods at a starting dilution of 1:2 and 1:20 in the first well of microtiter plate. These sera were titred earlier by J.W. Peterson, independently in Texas, by another method of PHA using tanned chicken erythrocytes coated with choleragen by non-covalent simple adsorption in PBS, pH 6.4 (8). The results of these sera by gluteraldehyde-human erythrocyte PHA are compared and correlated with those of chicken-cell PHA, sent to us through personal communication by Dr. Peterson.

(b) Fifty-three finger-prick plasma samples from volunteers of the 1974 Wyeth toxoid field trial, selected on the basis of a wide range of antitoxin contents, were titred by gluteraldehyde-PHA and rabbit intracutaneous assay. The results between the assays were correlated in retrospect at a later date than the time when the tests were performed as routine procedures.

(c) Five hundred finger-prick samples from the vaccinees of this trial were titred by control unsensitized erythrocytes undergoing all the treatment of sensiti-

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* Briefly, the American volunteers were immunized with subcutaneous injection with two doses of gluteraldehyde treated (for toxoiding) Wyeth toxoid lot No.11201. The doses of toxoid varied from 625 to 100 micrograms, with or without protamine aluminum phosphate adjuvant. The interval between injections was 4 weeks.

zation except contact with the toxin antigen which was replaced by same volume of saline.

The results of antitoxin titrations of about 6,700 finger-prick plasma samples from the volunteers of this study by this PHA and rabbit intracutaneous assay, will be presented in a separate paper (Ahmed, A., Curlin, G., Merson, M. and Black, R. Efficacy of a PHA assay using HRBC and gluteraldehyde as coupling reagent for quantitation of cholera antitoxin in toxoid field trials).

All the above mentioned plasma samples were obtained by the standard CRL method (12) of taking 50 microliter blood by finger-prick, mixing it immediately with nine times its volume of sterile saline in the field, sending them in ice box to the laboratory, separating the supernatant and keeping frozen until titration of antitoxin. The dilution of the plasma was considered as 1:10 irrespective of the hematocrit value of the blood sample.

Sensitization of Erythrocytes Using Gluteraldehyde as coupling Reagent

Sensitization is done by a modification of the method of E. Onkelinx et al., 1969, for the use of gluteraldehyde as a coupling reagent in PHA (15). Briefly, human erythrocytes from the refrigerated stock are washed thrice in PBS. Conditions of the third centrifugation are set in such a way that the loosely

packed cells will have a hematocrit of approximately 80 percent. Once the centrifuging conditions standardized, there is no necessity of estimating the hematocrit for daily titration. 0.25 ml. of these loose-packed cells are added to 0.4 ml. of the working solution of antigen (100 microgram/ml.) in a 15 ml. conical centrifuge tube. After mixing, 60 microliter of the purified gluteraldehyde is added, mixed thoroughly for 60 seconds in a cyclo-mixer, and the mixture is allowed to react at room temperature (24°C to 27°C) by gently shaking on a Thomas clinical rotator. After one hour the mixture is centrifuged at low speed, the supernatant discarded, deposited cells washed once with 6 ml. of PBS and finally re-suspended in 40 ml. of PBS containing 1% BSA to make 0.5% final suspension of the sensitized erythrocytes, to be used as antigen in the microtitre PHA.

Sensitization of Erythrocytes Using Chromium Chloride as Coupling Reagent

The sensitization of erythrocytes is done by a modification of the method of Vyas and Shulman, 1970, for the assay of antigen or antibody associated with viral hepatitis by hemagglutination (17). Briefly, one volume of 40 percent triple-washed human erythrocytes in PBS is mixed with two volumes of the sensitizing choleragen antigen in PBS (100 microgram/ml.), followed by one volume of freshly prepared 1.25 millimolar chromium chloride solution. After mixing thoroughly for 30 seconds with a cyclo-

mixer, the mixture is incubated for 15 minutes at room temperature with constant gentle shaking on a rotator. After being washed four times in saline (each wash in 50 volumes) the erythrocytes are suspended in a PVP-Tween-BSA diluent (17) to make a final antigen suspension containing 0.5 percent sensitized erythrocytes. In all PHA titrations in connection with the present work, the media for suspending the sensitized erythrocytes was a PVP-Tween-BSA buffer. Later on, we found (data not presented) that a replacement of this one by a simple PBS-BSA (1 percent) buffer is equally effective.

Antitoxin Titration

The titration technique using microtitre equipment was described in detail elsewhere (Ahmed, A., Aziz, K.M.S. and Rahaman, M.M. 1979. An antibody assay in shiga dysentery by microtiter passive hemagglutination using human erythrocytes and chromium chloride as a coupling reagent. Manuscript submitted). Briefly, 25 microlitres of serially twofold diluted unknown antisera in PBS were mixed with an equal volume of the antigen suspension, incubated for half-an-hour at 37°C water bath and kept at room temperature for two hours. The end point is read as the highest dilution which shows characteristic hemagglutination pattern at the bottom of the well of microtitre U-plate. Starting dilutions of the reference antisera mentioned in this paper include the volume increase caused by the addition

of antigen suspension in the first well of titration. The antitoxin unitage of the unknown serum is calculated by multiplying the end point unitage (in AU/ml.) of the reference antitoxin SSVI-B with the end point dilution-factor of the unknown serum.

Rabbit Intracutaneous Assay

This assay technique for quantitation of cholera antitoxin on rabbit skin is exactly the same as that of Dr. J.P. Craig (4), (in addition: Craig, J.P. Procedures for titration of cholera toxin and antitoxin by the rabbit intracutaneous method. Unpublished set of instructions through personal communication).

RESULTS

Sensitization of human erythrocytes with the purified choleragen by the help of either of the two coupling reagents, gluteraldehyde and chromium chloride, was successful. Figure I shows that both methods bear excellent correlation with the corresponding rabbit intracutaneous assay, ($r=0.949$ for gluderaldehyde, and $r=0.928$ for chromium chloride) when the antitoxin unitage of the panel of 36 hyperimmune human sera from American volunteers of the Texas study were determined on reference to the SSVI-B standard antitoxin. (Number of sera shown in the figure is 26, because the antitoxin content of

10 sera were below the level of detection). The almost complete parallelism of the two regression lines stresses the fact that either of the two methods can be used with equal facility for quantitation of cholera antitoxin.

All of the above 36 sera, even with a starting dilution of 1:2 in the titration, did not show non-specific agglutination against control unsensitized erythrocytes. Finger-prick plasma samples from 500 different vaccinees from the 1974 Wyeth toxoid field trial also did not show any non-specific agglutination against unsensitized erythrocytes with 1:20 starting dilution in the titration. More than 100 serial samples from the 1978 Wellcome pilot toxoid trial also did not have agglutination with control erythrocytes on titrations with 1:10 starting dilutions.

The two anti-whole cell vaccine and two anti-purified-ogawa-antigen-hyperimmune rabbit sera did not show any agglutination with the toxin-sensitized erythrocytes in either of the methods.

Figure 2 shows an excellent correlation between the gluteraldehyde PHA at CRL and the chicken cell-PHA done by Dr. J. W. Peterson in Texas (8) [r=0.970], when the same panel of 36 sera from H-8 volunteers were tested. The antitoxin contents of nine samples were not detectable by both laboratories, and one sample was not detectable at CRL probably because of a higher starting dilution, such as 1:20 as opposed to 1:10 with the chicken cell PHA assay.

Figure 3 shows a very good correlation of antitoxin units/ml. of 53 selected toxoid field trial samples by in-vivo rabbit intracutaneous assay with corresponding results obtained by in-vitro PHA assay, using gluteraldehyde, the correlation coefficient (r) being 0.917. The figure does not include the data for eight sera having antitoxin contents below the detectable level of this PHA technique.

The reproducibility of the assay during the pilot trial of the Wellcome toxoid, as estimated by the variability of the endpoints in number of tubes in 43 titrations of the reference serum of 8 days by one particular technician was (mean) 5.805, SD± 0.335. The level and variability of the lowest limit of antitoxin that were measured on each day with the unknown sera of this trial with 1:10 starting dilution was 3.729 AU/ml. SD± 0.792 in a total of 11 runs. This value was computed from the mean endpoint of six-titrations of the reference serum per day per technician. During Wyeth toxoid trial, four years back, with a starting dilution of 1:20 of the field samples this value was (mean) 7.195 AU/ml., SD± 1.562.

The lowest concentration of the antitoxin measurable by titrating the reference antitoxin, of course, is calculated to be 0.35 AU/ml. and the absolute amount of antitoxin in the end-point-well to be 0.00875 A.U.

DISCUSSION

The results of this investigation demonstrate that either of the two PHA assays for quantitation of cholera antitoxin has such a close correlation with the rabbit intracutaneous assay that the in vivo assay may be replaced by the in vitro one, without loss of sensitivity or accuracy, when the lowest limit of estimation does not go beyond 0.4 AU/ml. The gluteraldehyde-PHA assay, as used in the pilot trial of Wellcome toxoid has been shown to be dependably effective for measuring cholera antitoxin in plasma samples from the vaccinees at a mean antitoxin concentration of 3.729 AU/ml., SD±0.729 AU/ml. This value depends on the inherent initial dilution factor in the finger prick plasma collection from the toxoid field trial for which the assays were designed. Using undiluted serum samples obtained by venipuncture this limit of estimation is expected to be lowered by at least another fourfold.

The endpoint in rabbit intracutaneous assay, depends on the biologic activity of the toxin, while in PHA assay or other binding assays such as ELISA, this is not the case. One cruicial variable factor in the rabbit assay is that the toxin used in the neutralization must be toxoid free, but toxin is easily self-degradable. From this point of view, at least on theoretical consideration, PHA may be a better candidate for assaying

antibodies raised against the injection of a toxoid vaccine. It should be noted that a PHA assay may be set up by coating the erythrocytes even with a choleragenoid instead of choleragen (8). The level of sensitivity of the PHA test as done with the toxoid trial plasma samples seems to be quite satisfactory, especially, when a measure of the rise of anti-toxin titer in samples of toxoid-and placebo-injected age-matched vaccinees is the main requirement. That the correlation of the PHA with the intracutaneous assay may, inturn, be equated with its correlation with the rabbit ileal loop assay (12) depends on earlier indirect (13) and direct (9) evidences for the unitarian concept of the same biologic process occurring in these two bio-assays.

Our data indicate that for serologic surveys in connection with any cholera toxoid field trial this gluteraldehyde PHA may be used as the only serologic tool for the quantitation of antitoxin.

The reason for selecting the gluteraldehyde-PHA assay over the equally effective method using chromium chloride is that, in addition to coupling of choleragen, the gluteraldehyde stabilizes the erythrocytes in such a way that the sensitized erythrocytes, even in distilled water, do not undergo hemolysis. Hemolysisis due to complement activity and detergent contamination are also obviated. So, decomplementation of sera prior to titration,

becomes unnecessary. In contrast to brown discolouration of erythrocytes using tannic acid and/or formaldehyde the use of gluteraldehyde keeps the erythrocytes looking fresh and red for better visualization. After contact with the coupling reagent there are two steps of centrifuging including one wash in gluteraldehyde-PHA in comparison to five steps of centrifuging for 4 washes in chromium chloride-PHA.

The assay used in large scale toxoid field trials, twice at both the ends of an interval of four years, was found to have nearly identical levels of sensitivity and reproducibility; and consequently, in both the times, the lowest level of estimations of antitoxin in the field samples was almost identical when tallied after the values were corrected for starting dilutions of the samples.

We have been also quantitating cholera toxin by the inhibition modification of the PHA assay (PHAI) using a rabbit anti-purified choleragen antibody R-122 in a dilution of 1:2000 as the competiting dose of specific antibody. The lowest limit of toxin that is measurable by this method is approximately 0.3125 microgram of Finkelstein's 0572 batch of purified toxin.

ADDENDUM

For all the sera examined in this paper the antigen suspension used was 0.5% toxin-coated human erythrocytes; but recently it has been found that the use of a concentration of approximately 0.17% sensitized erythrocytes in PBS containing 0.75% BSA, makes the PHA titration one tube or two fold more sensitive. In this case, the use of microtiter V-plate is preferred and they should be read after keeping them overnight on a table at room temperature instead of two hours, needed by the use of 0.5% cell suspension.

With two experimental assays during the Wellcome toxoid pilot trial, late 1978, the lowest limit of antitoxin estimation with 1:10 starting dilution in the titration of the field samples was 1.66 AU/ml. (mean values of two runs).

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E. Monograph Series:

No. 1. Kinship in Bangladesh by K.M. Ashraful Aziz. May 1979. 250 p.

LEGEND OF FIGURE I

Correlation of two PHA assays with the rabbit intracutaneous test using 36 human sera from American volunteers immunized with cholera toxoid. The end points of the PHA titrations were read in number of tubes; when it is gradual, it is read in one-third tube more or less than the nearest positive tube. The results of ten specimens are not shown, because their antitoxin content was below the level of detection by the PHA assays.

LEGEND OF FIGURE 2

Correlation of gluteraldehyde-PHA with tanned-chicken cell-PHA done by Dr. J.W. Peterson, Ph.D. in Texas using 36 human sera from American volunteers immunized with cholera toxoid. The results of ten specimens are not shown, 9 of which were undetectable by both the assays and 1 of which was not detectable only by gluteraldehyde-PHA because of a twofold higher starting dilution.

LEGEND OF FIGURE 3

Correlation of Gluteraldehyde-PHA assay with rabbit intracutaneous test using 53 finger-prick plasma samples from Bangladeshi cholera toxoid vaccines. The result of 8 specimens are not included because their antitoxin contents were not detectable by the PHA assay.

FIGURE-1

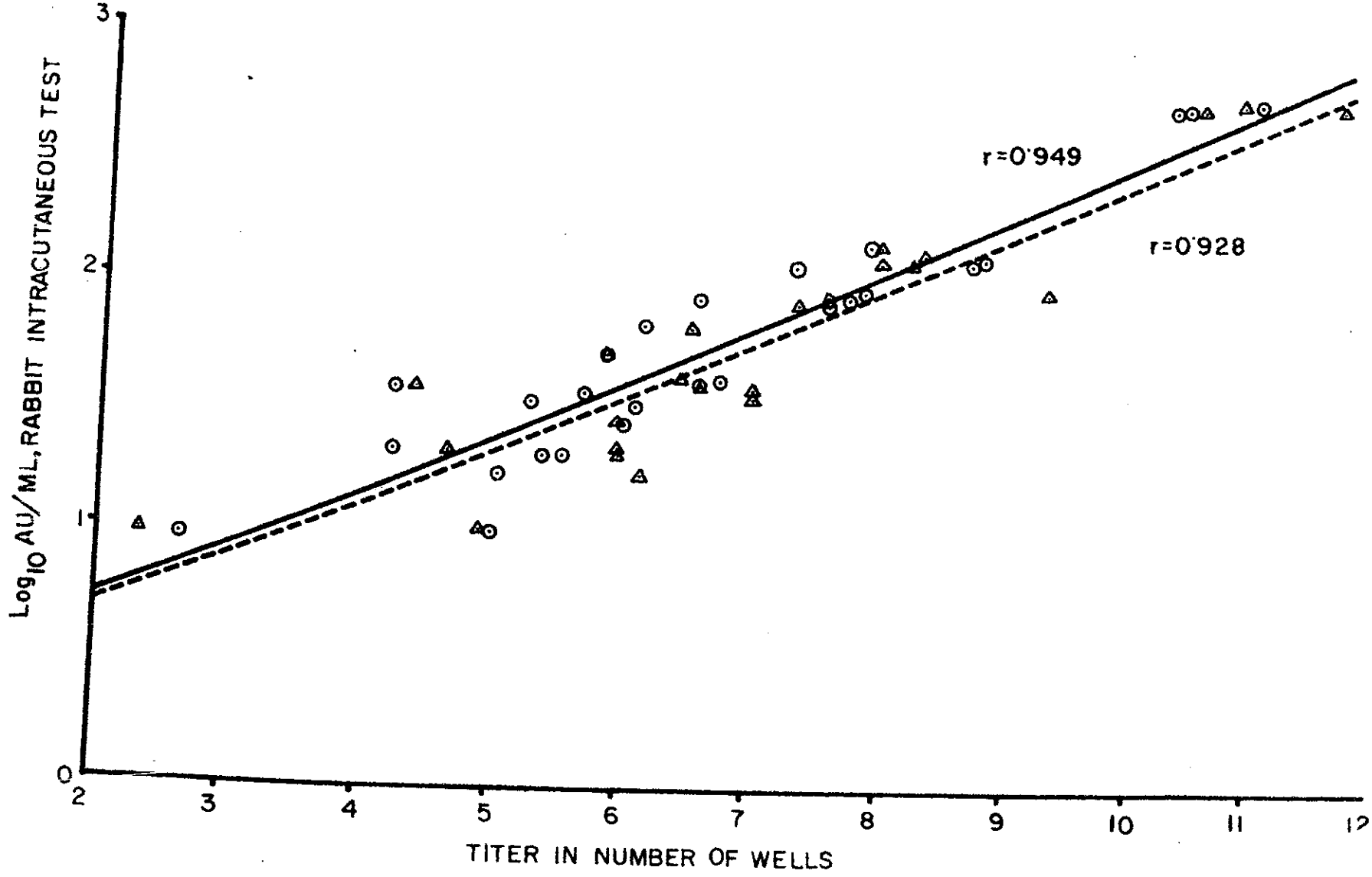

r=0·949
r=0·928
Log10 AU/ML, RABBIT INTRACUTANEOUS TEST
TITER IN NUMBER OF WELLS
0
1
2
3
2
3
4
5
6
7
8
9
10
11
12

FIGURE -2

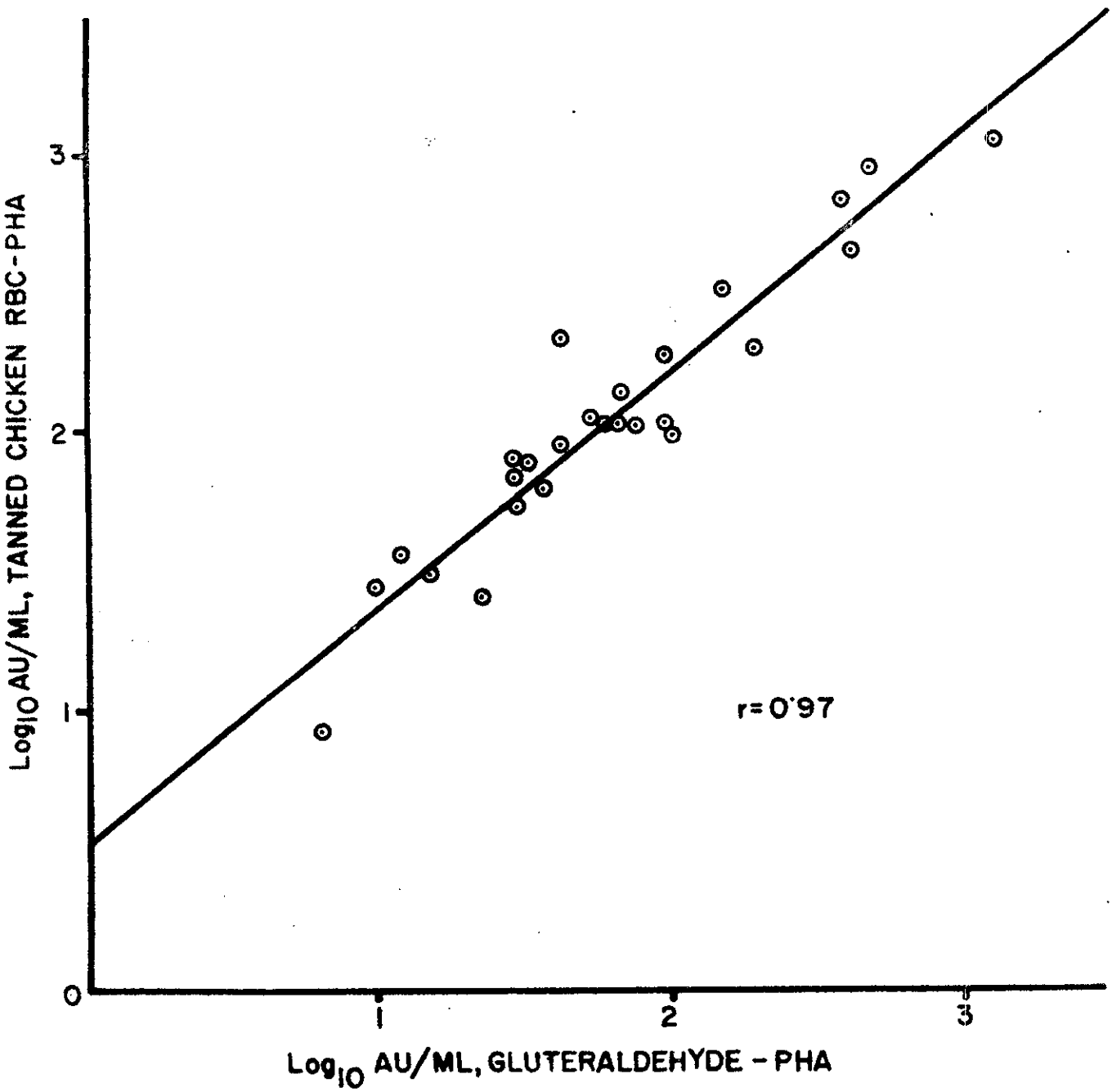
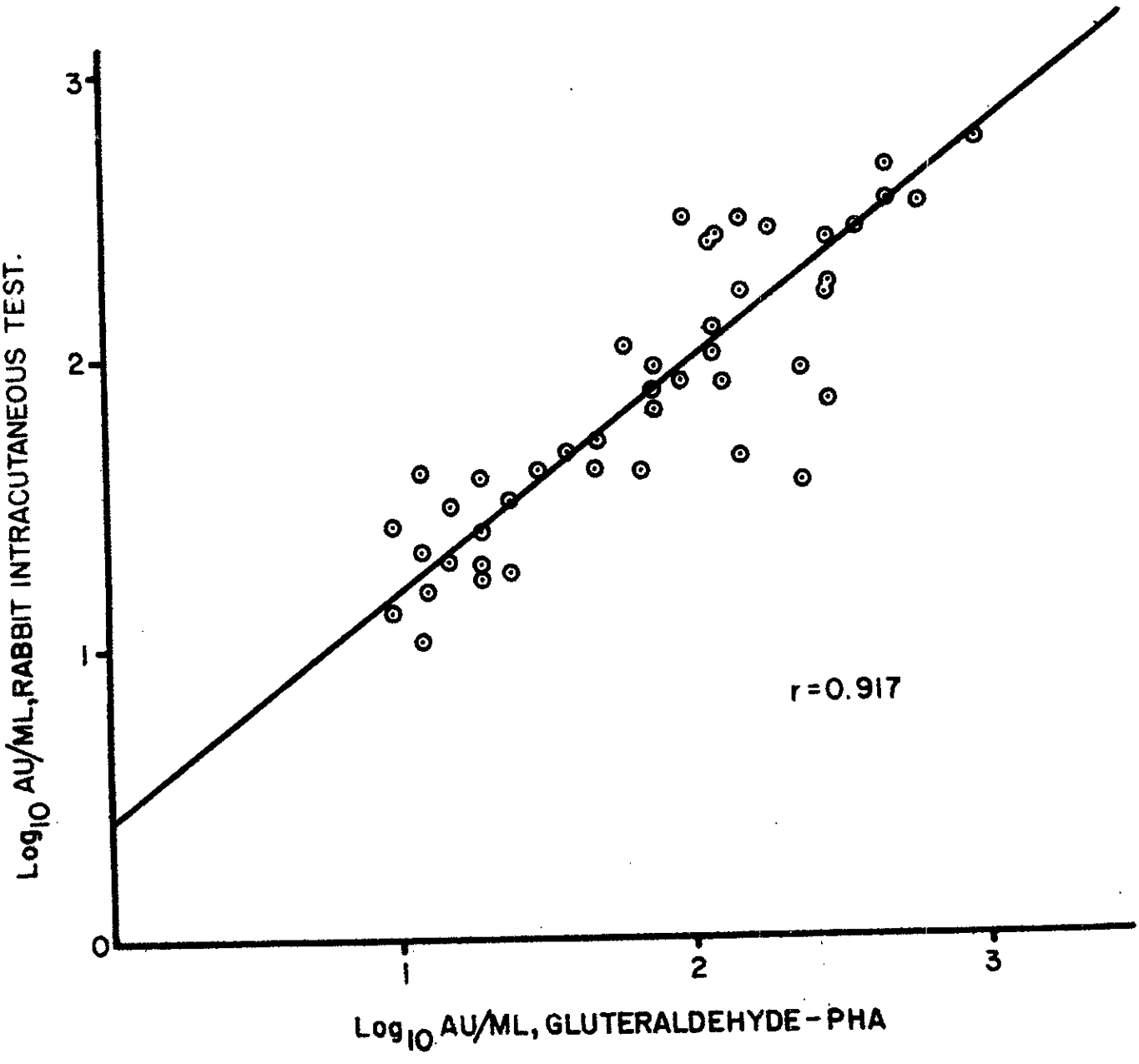

Log10 AU/ML, TANNED CHICKEN RBC-PHA
0
1
2
3
r=0·97
1
2
3
Log10 AU/ML, GLUTERALDEHYDE - PHA

FIGURE - 3


3
2
1
0
1
2
3
r = 0.917
Log10 AU/ML, RABBIT INTRACUTANEOUS TEST.
Log10 AU/ML, GLUTERALDEHYDE - PHA