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Radiobacteriolysis: a New Technique Using Chromium-51 for Assaying Anti-*Vibrio cholerae* Antibodies

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A new method for detecting and quantitating antibodies against *Vibrio cholerae* is described. The reaction involves the release of radiochromium from prelabeled vibrios in the presence of specific antibody and complement. The entire assay can be completed within 5 hr. The method is highly reproducible, immunologically specific, temperature- and complement-dependent, and significantly more sensitive than other methods currently used for titration of anti-*Vibrio cholerae* antibodies. The technique is also potentially applicable to titration of antibodies against other gram-negative bacteria.

A variety of bactericidal techniques have been described for assay of antibodies against *Vibrio cholerae* (2, 8, 15). All of these methods measure the ability of specific antibody and complement to inhibit growth of vibrios. Among those techniques frequently used are the macrovibriocidal plate technique of Finkelstein (8), the photometric assay of McIntyre and Feeley (15), and the microtechnique developed by Benenson et al. (2). Although *V. cholerae* has long been known to be highly susceptible to lysis by antibody and complement, and although a number of investigators have described bacteriolytic immune assays for gram-negative bacteria (1, 11, 14, 18), vibriolytic techniques have not been generally used for detecting and quantitating anti-*V. cholerae* antibodies.

Ever since the labeling of animal cells with ^{51}Cr was first described by Gray and Sterling (12), immunolytic release of radiochromium from a variety of labeled target cells has been successfully used as an indicator of immune damage in vitro (3-5, 20). Since the primary advantage of this technique in animal systems is its greatly increased sensitivity and technical simplicity, we elected to adapt it to our studies on the early immune response in experimental cholera. In this paper we describe the radiovibriolytic assay of anti-*V. cholerae* antibodies. Various parameters of the assay and its potential applications are discussed.

MATERIALS AND METHODS

Cultures. Smooth *V. cholerae* strain 569B (Inaba), originally obtained from N. K. Dutta (The Haffkine Institute, India), was stored in the lyophilized state. *Aeromonas hydrophila* was kindly supplied by E. K. Koneman of the Presbyterian Medical Center, Denver, Colo. *Escherichia coli* serotype O127:B8 was from our culture collection. For routine work the organisms were cultured and maintained on meat extract agar (MEA) plates or slants. Periodic serological and bacteriological tests were carried out to ascertain the biological characteristics of these organisms.

Sera and reagents. The same lot of lyophilized guinea pig complement (Baltimore Biological Laboratories, lot no. 1091276) was used throughout the study. Complement was reconstituted just prior to each experiment and then diluted to the desired concentration with cold peptone-saline (PS) consisting of 0.01% peptone (Difco) and 0.85% NaCl (9). The complement-PS mixture was designated C'-PS.

Sera were collected from a group of healthy rabbits, pooled, inactivated at 56 C for 30 min, absorbed with washed sheep red blood cells, sterilized by filtration, and frozen at −20 C in small samples. This serum pool was designated NRS. Its vibriocidal titer (8) against the 569B strain was less than 10.

A standard immune serum against *V. cholerae* was prepared in the same group of rabbits by biweekly immunization with increasing doses of heat-killed (56 C for 2 hr) and washed 569B strain. The rabbits were bled 1 week after the sixth intravenous injection, and sera with significant agglutinating antibody titers were pooled. This serum pool was processed in the same manner as the NRS and was designated anti-*Vibrio* serum. Its vibriocidal titer was 36,500.

Rabbit serum against *E. coli* 0127:B8 was purchased from Baltimore Biological Laboratories and was heat-inactivated before use.

Human cholera convalescent serum (HCS), catalog no. G-005-501-572, was kindly supplied by the Research Resources Branch of the National Institute of Allergy and Infectious Diseases (NIAID). This

serum is part of a pool of convalescent sera from Dacca, Bangladesh, and is being distributed as a reference standard for vibriocidal titrations. The serum has been assigned the following unitages by the NIAID, as determined by the microtechnique of Beneneson et al. (2): Ogawa, 160 vibriocidal units/ml; Inaba, 320 vibriocidal units/ml. In our hands, the anti-Inaba vibriocidal titer (8) was 7,680 units/ml.

A pool of normal human sera, vibriocidal titer less than 10, was always titrated together with HCS. Both sera were heat-inactivated before use.

Radioisotope. Chromium-51 was obtained as sodium chromate ($Na_2\ ^{51}CrO_4$) in sterile physiological saline, *p*H 7.0, from Amersham/Searle Corp., Arlington Heights, Ill. (catalog no. CJS. 1P). Its specific activity was 178 μCi/μg of ^{51}Cr (1 mCi/ml). Test samples were counted in an automatic gamma counting system (Nuclear-Chicago 1185 series). In all cases total counts were large enough to give no more than 3% error at the 95% confidence level.

Vibriocidal assay. The assay was performed as described by Finkelstein (8). Peptone-saline contained 0.01% peptone.

Technical details of the radiobacteriolytic assay. Because, to the best of our knowledge, the ^{51}Cr release assay has not been previously described for a bacteriolytic system, the technical aspects will be described here in some detail.

Pyrex test tubes, 13 by 100 mm, were used for the assays. Aseptic precautions were not required. Eppendorf pipettes were used for addition of serum, complement, and ^{51}Cr-labeled cells. Sera were diluted in C'-PS. Samples (0.5 ml) of the diluted sera were dispensed into test tubes and kept at 4 C. ^{51}Cr-labeled cells at the desired concentration were added to each tube to make a final volume of 1.0 ml. The tubes were then incubated with shaking at 250 rev/min and under predetermined conditions of time and temperature. At the end of the incubation period the tubes were centrifuged at 6,000 $\times$ *g* for 10 min at 4 C. In experiments in which time was the parameter tested, tubes were spun at 15,000 $\times$ *g* for 1 min. We observed no significant difference in the activity of the supernatant fluids when identical tubes were centrifuged under these two different conditions. After centrifugation, 0.5-ml samples of the supernatant fluids were carefully removed with a 500 μliter Eppendorf pipette and placed in separate disposable plastic tubes for measurement of radioactivity. The remaining supernatant fluids and sediments were discarded. The counts in the supernatant fluids were multiplied by two to correct for the volume left with the sediments. To determine the total number of counts incorporated into the bacteria, the radioactivities in replicate tubes each containing 0.5 ml of labeled, but otherwise untreated, bacteria were measured. Controls for each assay included (i) labeled bacteria in PS, (ii) labeled bacteria and complement, (iii) labeled bacteria and homologous antiserum without complement, and (iv) labeled bacteria and normal serum with complement. In all experiments the concentrations and dilutions given below were final. All assays were performed in duplicate and repeated on at least three different occasions.

Calculations. The raw data were first corrected for background radioactivity. The percentage of radiochromium released was then calculated according to the following formula, adapted from that of Brunner et al. (4): Percent specific ^{51}Cr release = [(counts per minute released in the presence of a particular dilution of immune serum and complement) – (counts per minute released in the presence of the same dilution of normal serum and complement)] $\div$ [(total counts per minute present in cells) – (counts per minute released in the presence of the same dilution of normal serum and complement)] $\times$ 100.

We define the titer as the reciprocal of the serum dilution at which 50% of maximum specific radiochromium release occurs. This serum dilution is referred to as $^{51}CrR_{50}$. This value is determined graphically by interpolation from the descending limb of the curve describing the release of label from cells as a function of serum concentration (see for example Fig. 4).

RESULTS

Labeling of vibrios with $Na_2\ ^{51}CrO_4$. In a preliminary series of experiments to determine optimal conditions for labeling vibrios with $Na_2\ ^{51}CrO_4$, it was found that vibrios taken from log-phase cultures incorporated more ^{51}Cr when labeled in a medium which did not support cell division (Fig. 1A) than when labeled in a nutritionally rich medium which did allow cell division (Fig. 1B). After 2 hr of labeling, the nondividing vibrios incorporated 10 times more ^{51}Cr than did growing cells. Furthermore, more label was taken up by nondividing vibrios obtained from log-phase cultures than by nondividing vibrios taken from stationary-phase cultures (harvested from overnight growth on MEA plates). The amount of isotope incorporated by 10^9 vibrios/ml in a 1-hr labeling period was found to be linear with concentration of chromate ion over the range 10 to 100 μCi/ml.

In light of the observations noted above, we adopted the following procedure for routine labeling of bacteria. A 20-ml amount of brain heart infusion (BHI) broth in a 250-ml flask was inoculated with about 1.0 mm^3 of bacterial paste obtained from an overnight culture on an MEA plate. The broth culture was incubated at 37 C for 1 hr with vigorous shaking (250 rev/min) and then washed twice with PS. An amount of these washed and sedimented bacteria to provide 10^9 to 10^{10} vibrios was resuspended in 1 ml of PS. Chromate was added to a final concentration of 100 or 200 μCi/ml, and the tube was incubated at 37 C with shaking (200 rev/min) for 2 or 1 hr, re-

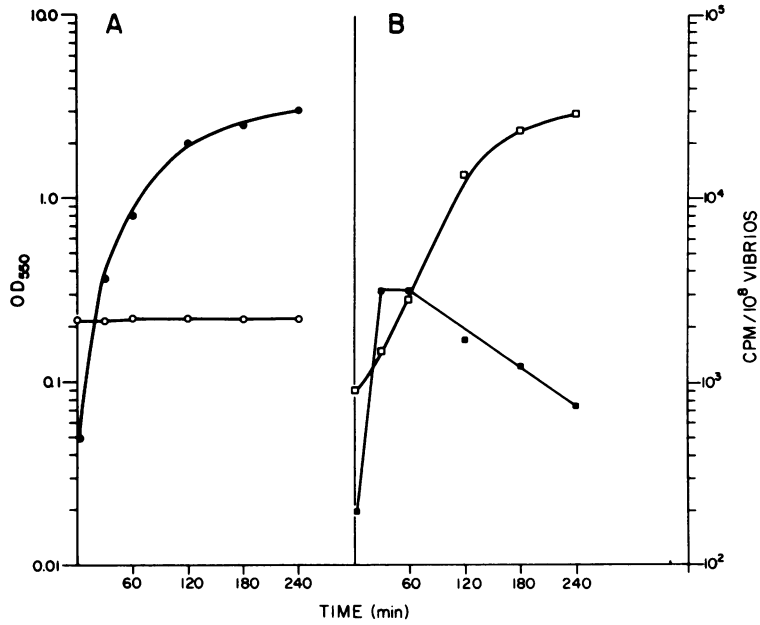

FIG. 1. *^{51}Cr uptake by nondividing (A) and dividing (B) Vibrio cholerae. Nondividing and dividing vibrios derived from log-phase cultures were labeled with $Na_2{}^{51}CrO_4$ (50 μCi/ml) as described in the Results section. ○, Optical density of PS suspension; ●, counts per min per 10^8 vibrios labeled in PS suspension; □, optical density of BHI culture; ■, counts per min per 10^8 vibrios labeled in BHI culture.*

spectively. The cells were then washed three times with PS and resuspended to the desired concentration with the aid of a standard curve relating cell concentration to optical density. The entire labeling procedure required about 2.5 hr. Under the preparative procedures just described there was no damage to the cells as judged by cultural characteristics, viability, morphology, and antigenic properties.

Release of ^{51}Cr from sonically treated vibrios. As an indirect measure of the maximun possible ^{51}Cr release from lysed vibrios, labeled cells were sonically treated for various periods of time. Sonic extracts were examined visually with the aid of a phase-contrast microscope. The sonic extracts were then centrifuged and washed, and both debris and supernatant fluids were assayed for radioactivity. Such assays disclosed that under conditions of sonic oscillation sufficient to disrupt > 99% of the vibrios, 10 to 30% of the initial radioactivity was retained in the cell debris.

Parameters affecting the radiovibriolytic assay. (i) Concentration of complement. In the complete absence of exogenous complement, lysis was negligible and agglutination was often observed at the lower serum dilutions. When complement was present at a final dilution of 1:80, lysis was extensive. At this dilution, complement was not in excess. Increasingly higher concentration of complement increased the extent of label released, as well as the apparent titer of the serum (Fig. 2A). However, an undesired effect in high concentration of complement was the high nonspecific release of chromium. For example, at NRS dilution of 10^{-4} and complement dilution of 1:80, 8% of the ^{51}Cr was released. When the concentration of complement was increased four- and eightfold, the percentage of ^{51}Cr released in NRS was 15 and 35%, respectively.

(ii) Temperature. The rate of lysis was markedly influenced by temperature (Fig. 2B). Whereas at 4 C only 26% of the label was released after 30 min, 51% was released at 37 C after the same period.

(iii) Cell concentration. The concentration of vibrios was found dramatically to influence the sensitivity of the assay, the apparent titer being inversely proportional to the concentration of bacteria (Fig. 2C and 7). For example, at a concentration of 10^6 vibrios/ml the apparent serum titer was 1.8×10^6. When the number of vibrios was increased 10-fold, the titer dropped to 2.9×10^5 (Table 1).

(iv) Reaction kinetics. The ^{51}Cr release in both immune and normal sera was measured over a 5-hr period. In immune serum, vibriolysis started immediately after addition

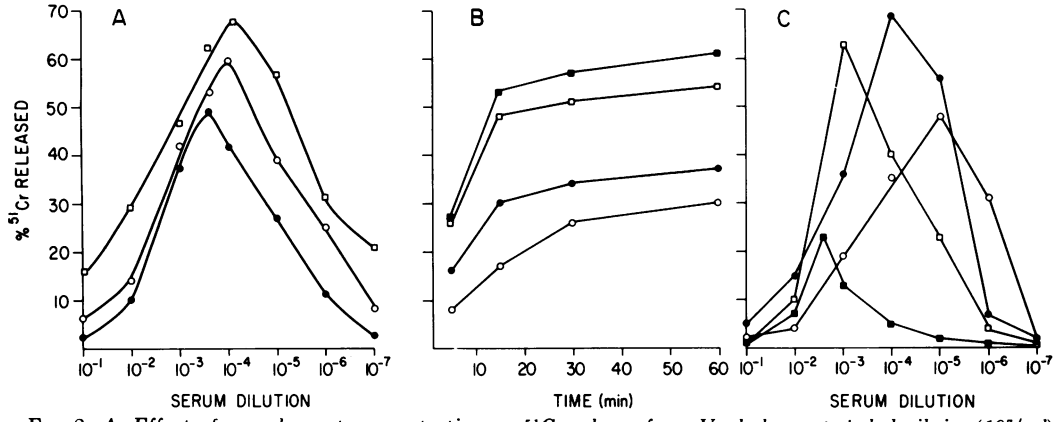

FIG. 2. *A, Effect of complement concentration on ^{51}Cr release from V. cholerae. Labeled vibrios (10^7/ml) were reacted for 30 min at 37 C with serially diluted rabbit anti-Vibrio serum in the presence of varying dilutions of guinea pig complement. ●, Complement diluted 1:80; ○, complement diluted 1:20; □, complement diluted 1:5. B, Effect of temperature on ^{51}Cr release from V. cholerae. Labeled vibrios (10^7/ml) were incubated in the presence of rabbit anti-Vibrio serum (10^{-4} dilution) and guinea pig complement (1:20). ○, 4 C; ●, 23 C; □, 37 C; ■, 47 C. C, Effect of concentration of vibrios on release of ^{51}Cr from V. cholerae. Varying concentrations of labeled vibrios were reacted for 30 min at 37 C with serially diluted rabbit anti-Vibrio serum and guinea pig complement (1:20). ○, 10^6 vibrios/ml; ●, 10^7 vibrios /ml; □, 10^8 vibrios/ml; ■, 10^9 vibrios/ml.*

TABLE 1. *Comparative sensitivites of vibriocidal and radiovibriolytic assays as functions of cell concentration*

Vibrios/ml	Titer	
	Vibriocidal[a]	Radiovibriolytic[b]
10^3	3.6×10^5	ND[c]
10^6	3.1×10^5	1.8×10^6
10^7	3.0×10^5	2.9×10^5

[a] Reciprocal of rabbit anti-*Vibrio* serum dilution required for 50% inhibition of colony-forming units.

[b] Reciprocal of rabbit anti-*Vibrio* serum dilution required for 50% of maximum ^{51}Cr release.

[c] Not done.

of cells to the reaction mixture. Lysis was rapid, and at 30 min most of the label was released into the medium. Beyond 30 min, ^{51}Cr continued to be released in the presence of immune serum at a much reduced rate (Fig. 3). Chromium was also lost from cells in the presence of NRS and complement; after the first 15 min the loss of label was approximately linear with time. At 5 hr the amount of chromium released in NRS was nearly that in immune serum. Maximum difference between specific and spontaneous release of label was observed at 30 min.

(v) Other variables. The vibriolytic reaction was carried out in several different buffers. Results were most satisfactory in peptone-saline. Wardlow's Tris-buffer (19) was apparently detrimental to the vibrios, spontaneous release of ^{51}Cr in this buffer being nearly equal

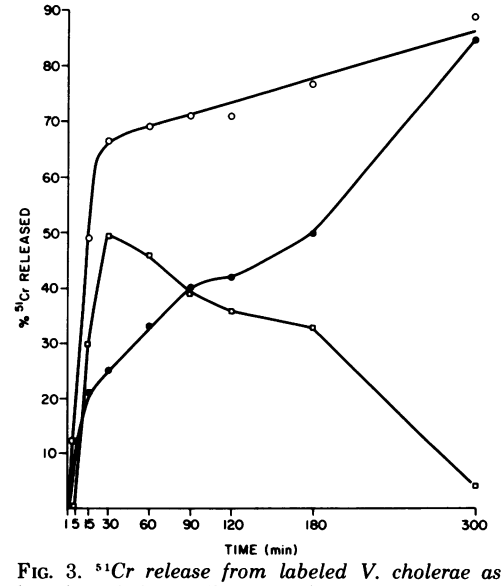

FIG. 3. *^{51}Cr release from labeled V. cholerae as a function of time. Vibrios (10^7/ml) were incubated at 37 C with a 10^{-4} dilution of either rabbit anti-Vibrio serum (○) or normal rabbit serum (●) in the presence of a 1:20 dilution of guinea pig complement. □, Specific ^{51}Cr release calculated as described under Materials and Methods.*

to that in immune serum. Addition of Mg ions to PS did not affect test results. This is in agreement with the findings of Sack et al. (17), who used the vibriocidal procedure for assay.

Titration of rabbit anti-Vibrio serum by the radiovibriolytic method. On the basis

of the foregoing observations we adopted the following conditions for routine titrations of sera: concentration of vibrios, 10^6 to 10^8/ml, depending on the level of sensitivity required; complement, 1:20; temperature, 37 C; incubation time, 30 min.

A representative titration curve for rabbit anti-*Vibrio* serum is shown in Fig. 4. The maximal release shown there, about 70%, is corrected for the control; actual release was somewhat higher, 70 to 90%. The amount of isotope released varied from one experiment to another, the range usually being 50 to 80% (corrected for control). Nevertheless, the $^{51}CrR_{50}$ end point was acceptably reproducible for any given serum. In 14 out of 15 trials the titer of our standard rabbit anti-*Vibrio* serum was 2.0×10^5 to 2.9×10^5 when 10^7 vibrios/ml were used for the assay. A pronounced prozone was always observed with both rabbit and human immune sera, when either the vibriolytic (Fig. 4) or the vibriocidal test (Fig. 8) was used. A similar bactericidal zonal effect was observed in 1901 by Neisser and Wechsberg, and later, by Muschel and Treffers (16) and others. The amount of chromium released in the various controls was as follows: vibrios in PS, 7 to 14%; vibrios in complement, 8 to 19%; vibrios in immune serum (at 10^{-4} dilution) without complement, 5 to 11%; vibrios in normal serum (at 10^{-4} dilution) with complement, 11 to 28%.

Immunological specificity of the radiovibriolytic assay. *E. coli* and *A. hydrophila*, two gram-negative bacilli that do not cross-react with *V. cholerae*, were chosen for this part of the study. When labeled *V. cholerae*, *A. hydrophila*, and *E. coli* were separately incubated with anti-*Vibrio* serum, only *V. cholerae* significantly released radiochromium (Fig. 5). The amount of isotope released from *E. coli* and *A. hydrophila* in anti-*Vibrio* serum was not greater than the amount of label released from *V. cholerae* in NRS.

Comparative kinetics of the vibriocidal and radiovibriolytic reactions. ^{51}Cr-labeled vibrios (10^7/ml) were incubated with optimal dilutions of rabbit anti-*Vibrio* serum and complement. Samples were removed periodically and assayed for release of ^{51}Cr (vibriolytic assay) and for inhibition of colony-forming units (vibriocidal assay). Both inhibition and lysis were evident at 1 min (Fig. 6). At 5 min 84% of the vibrios were unable to form colonies, and at 10 min there was virtually 100% reduction in the number of colony-forming units. As compared with this inhibition, release of ^{51}Cr was slightly delayed and became maximal at about 30 min.

Sensitivity of the radiovibriolytic assay. A series of dilutions of rabbit anti-*Vibrio* serum was titrated against varying concentrations of vibrios both by the macrovibriocidal procedure of Finkelstein (8) and by radiovibriolytic assay; experimental conditions for the latter were those specified in Fig. 2C. It was found

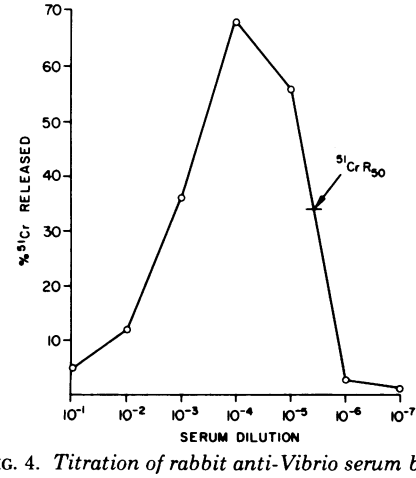

FIG. 4. *Titration of rabbit anti-Vibrio serum by the ^{51}Cr-release method. Under optimal conditions of assay derived from the experiments shown in Fig. 2, ^{51}Cr released from labeled vibrios was determined as a function of antiserum dilution. Vibrios, 10^7/ml; complement, 1:20; incubation time, 30 min; incubation temperature, 37 C.*

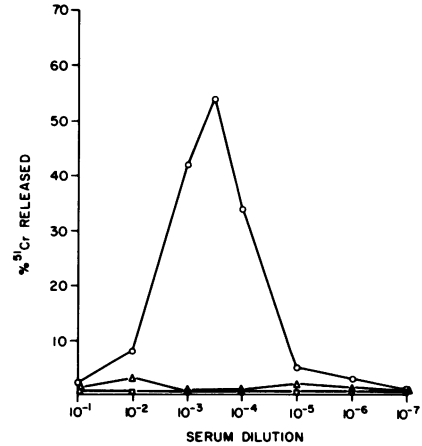

FIG. 5. *Immunological specificity of the ^{51}Cr-release assay. Labeled bacteria (10^8/ml) were incubated with serially diluted rabbit anti-Vibrio serum under the conditions described in Fig. 4. ○, Vibrio cholerae; □, Aeromonas hydrophila; △, Escherichia coli.*

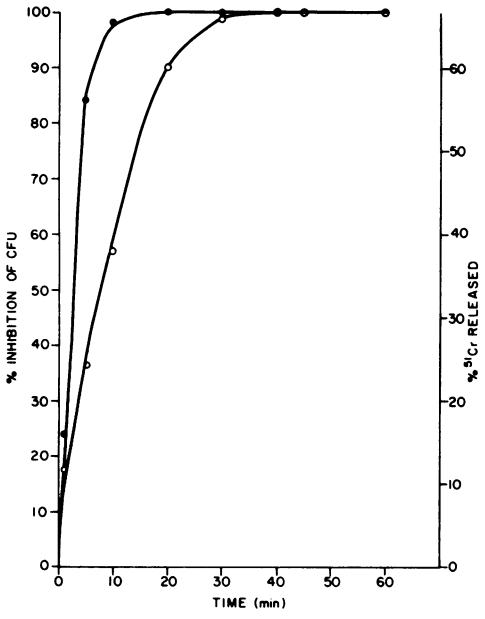

FIG. 6. *Comparative kinetics of the vibriocidal and radiovibriolytic reactions. ^{51}Cr-labeled vibrios (10^7/ml) were incubated at 37 C with rabbit anti-Vibrio serum (10^{-4} dilution) and guinea pig complement (1:20). At various times samples were removed and assayed for either ^{51}Cr released (O) or inhibition of colony-forming units (●).*

that, whereas the serum titer determined by the macrovibriocidal method was virtually independent of vibrio concentration across the range of 10^3 to 10^7 vibrios/ml, the radiovibriolytic titer was inversely proportional to vibrio concentration across the range 10^6 to 10^9 vibrios/ml (Table 1; Fig. 7). For example, at a vibrio concentration of 10^7/ml the serum titers by the two methods were nearly identical (3.0×10^5 and 2.9×10^5). However, when only 10^6 vibrios/ml were used for these assays, the same vibriocidal titer was obtained (Table 1), but the vibriolytic titer increased sixfold. A concentration of vibrios below 10^6/ml could not be used for the vibriolytic assay because of limiting radioactivity of the cells.

For further comparison we titrated a reference human cholera convalescent serum by these two techniques (Fig. 8). At a vibrio concentration of 10^7/ml, the vibriocidal titer against *V. cholerae* (Inaba) was 7,680; the radiovibriolytic titer was 26,500. The median macrovibriocidal titers for this serum as compiled by NIAID ranged between 2,560 and 5,120.

As noted above, more ^{51}Cr was taken up by log-phase than by stationary-phase vibrios. Log- and stationary-phase vibrios also differed in their release of ^{51}Cr. The titer of our reference serum was threefold higher with log-phase than with stationary-phase cells. Additionally, nonspecific release of ^{51}Cr was lower with the log-phase cells.

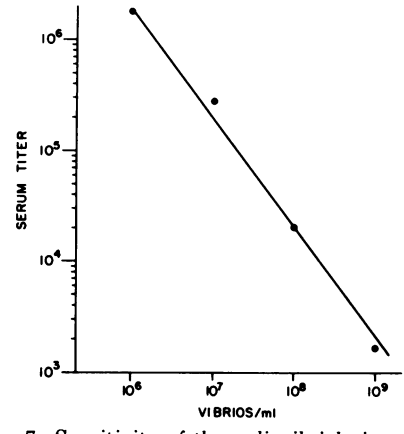

FIG. 7. *Sensitivity of the radiovibriolytic assay as a function of vibrio concentration. The titers given are the reciprocals of the rabbit anti-Vibrio serum dilutions at which 50% of maximum ^{51}Cr was released.*

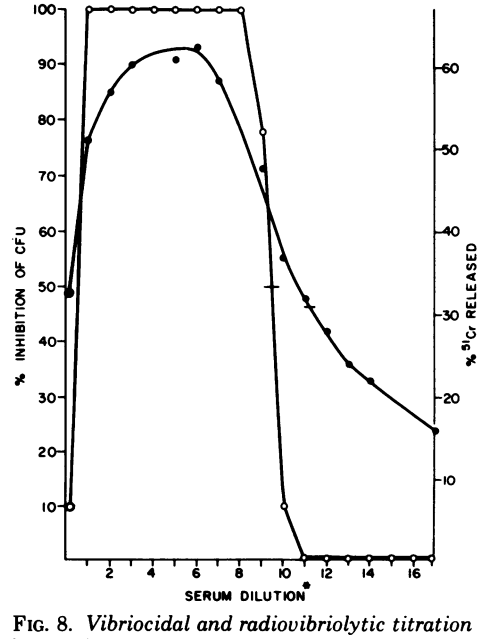

FIG. 8. *Vibriocidal and radiovibriolytic titration of reference human cholera convalescent serum (HCS). Labeled vibrios (10^7/ml) were incubated at 37 C for 30 min with guinea pig complement (1:20) and HCS. Each reaction tube was assayed for either ^{51}Cr released (●) or percent inhibition of colony-forming units (O). *Serum dilutions are $\log_2$ starting from 1:10 initial dilution, i.e., 1 = 1:20, 2 = 1:40, 3 = 1:80, etc.*

Application of the radiobacteriolytic assay to other gram-negative organisms. The radiobacteriolytic assay was successfully applied to titration of an anti-*E. coli* antiserum. *E. coli* O127:B8, labeled according to the procedure outlined for *V. cholerae*, released the label in the presence of specific antibody and complement. Only background release was observed when labeled *E. coli* were incubated in the presence of our standard anti-*Vibrio* serum or NRS (Fig. 9).

DISCUSSION

Labeling of living cells with ^{51}Cr without loss of their viability was first described by Gray and Sterling in 1950 (12). Recently, the release of ^{51}Cr from a variety of labeled target cells has been successfully used as an indicator of immune damage in animal cell systems (3–5, 20). The advantages of this procedure are its relative technical simplicity and its rapid, quantitative, and sensitive assessment of cell damage. Furthermore, chromium is not incorporated into any biological macromolecules.

The radiovibriolytic assay described here is very sensitive, highly specific, rapid, and simple to perform. The entire procedure, including labeling of cells, carrying out of the vibriolytic reaction, and preparation of samples for counting requires approximately 4 hr. Two of these hours are required for labeling of the cells, and this time may be utilized for preparing assay tubes and serum dilutions. Large numbers of samples can thus be processed and the results analyzed in one working day.

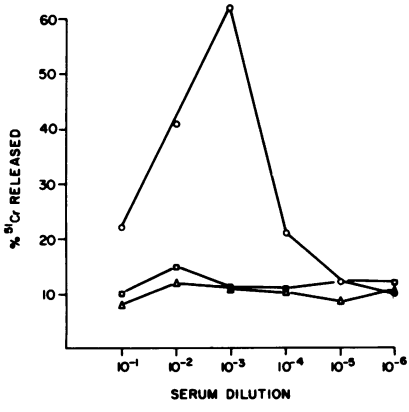

FIG. 9. *Application of the ^{51}Cr-release assay to Escherichia coli. E. coli O127:B8 (10^7/ml) were incubated at 37 C for 60 min with guinea pig complement (1:20) in the presence of rabbit anti-E. coli O127:B8 serum* (○), *rabbit anti-Vibrio serum* (□), *or normal rabbit serum* (△). *Values are not corrected for spontaneous release of ^{51}Cr.*

Three desirable features of a radiovibriolytic assay for antibody are (i) maximally labeled vibrios, (ii) maximal antibody-mediated lysis, and (iii) minimal nonspecific lysis. As judged from data presented here (Fig. 1), these features are best met by a 2-hr labeling of log-phase, nonreplicating vibrios. Prolonged labeling did increase the amount of label (Fig. 1), but this was at the expense of additional time required for completion of the assay, and of cells which were both decreasingly sensitive to specific lysis and also increasingly sensitive to nonspecific lysis. At the level of labeling achieved in this study, and with either a human or rabbit serum, this vibriolytic assay is somewhat more sensitive than the macrovibriocidal assay. However, it is reasonable to assume that by using more $Na_2{}^{51}CrO_4$ one could obtain still greater sensitivity, or comparable sensitivity in a "microassay."

Antibody-mediated release of ^{51}Cr from labeled vibrios is very rapid, being nearly complete within 30 min (Fig. 3). However, ^{51}Cr is also slowly released from vibrios in control tubes containing no specific serum. The loss of label from vibrios in the presence of NRS and complement could not be attributed to antibody-mediated cell death. NRS diluted 10^{-4} and complement do not inhibit growth of *V. cholerae*, at least not during an incubation period of 60 min. It seems more likely that ^{51}Cr is nonspecifically lost from vibrios incubated in NRS and C′ at 37 C for prolonged periods of time. We observed similar loss of label from vibrios incubated in peptone-saline at 37 C. The difference between the amount of ^{51}Cr released in the presence of immune serum and the amount of ^{51}Cr released in the presence of normal serum was maximal at 30 min. We found that results were most reproducible when corrections were made for nonspecific ^{51}Cr release. This was satisfactorily achieved by using the formula presented in Materials and Methods.

Specific release of ^{51}Cr is both complement- and temperature-dependent. There was virtually no release in the presence of heat-inactivated immune serum in the absence of exogenous complement. Over the range of dilutions which we examined, release of ^{51}Cr was directly proportional to concentration of exogenous complement. Other investigators (9, 16, 17) have also observed the enhancing effect of increasing concentrations of complement on bacteriolytic reactions. We found, however, that with increasing concentrations of complement there was concomitant increase in

nonspecific release of ^{51}Cr. Hence we adopted a 1:20 dilution of complement as an acceptable compromise which gave a satisfactory rate of specific release without entailing an unacceptable level of nonspecific release. With both immune and normal sera there was increasing release of ^{51}Cr with increasing temperature from 4 to 47 C (Fig. 2). Again, however, we adopted that experimental condition, 37 C, which gave the greatest specific isotope release. Furthermore, we feared that there might be some inactivation of complement at 47 C. Finally, we would note here that there can be significant release of ^{51}Cr at 4 C and therefore that lowering the temperature of the reactants during a vibriolytic assay may not be sufficient completely to halt cell lysis.

In the experiments reported here we never recorded complete release of ^{51}Cr from the labeled vibrios, the maximum being 60 to 90%. That some label is not readily released from vibrios is suggested from our examination of sonically treated cells. When more than 99% of the cells were visibly damaged by sonic disruption, only 70 to 90% of the ^{51}Cr label was released. Upon prolonged sonic treatment virtually 100% of the ^{51}Cr label was released; however, the extent of cell damage under these conditions far exceeded that observed in the antibody-mediated vibriolytic reaction. Berke et al. (3) similarly noted that in a completely lysed population of labeled fibroblast cells, 10 to 20% of the radioactivity was retained in the cell debris.

A comparison of the kinetics of vibriocidal and vibriolytic reactions disclosed (Fig. 6) that the former definitely preceded the latter. This may indicate that the first effect of the interaction of vibrios with immune serum and complement is the inhibition of the process leading to colony formation, and only after reaction with antibody and complement, and perhaps other serum compoents, does the actual process of vibriolysis begin with resultant release of ^{51}Cr from the damaged cells. Several investigators have suggested a similar sequence of events in immune damge to *E. coli* (6, 10, 11) and to *Neisseria meningitidis* (14). Our observation that under optimal conditions the bactericidal reaction is virtually complete within 10 min is in agreement with the results of Finkelstein, working with *V. cholerae* (8), and others who worked with *E. coli* (10, 11). Other workers, however, have reported that up to 45 min may be required for a complete vibriocidal reaction (9, 17).

Upon comparing the sensitivities of vibriocidal and vibriolytic assays, we found that the vibriocidal serum titer was virtually independent of cell concentration over the range 10^3 to 10^7 vibrios/ml (Table 1). Other investigators have also usually found in vibriocidal assays for antibody that the serum titer is relatively independent of cell concentration (15, 17). The vibriolytic titer, however, was inversely proportional to cell concentration over the range 10^6 to 10^9 vibrios/ml (Fig. 7). Thus, maximal sensitivity of the radiovibriolytic assay may be limited by the minimal number of labeled cells required for measuring radioactivity. This inverse relationship between serum titer and cell concentration has also been observed (16) in a bactericidal assay for antibody against *Salmonella typhi*. In our titrations of rabbit anti-*Vibrio* serum against vibrios at a concentration of 10^6/ml the radiovibriolytic assay was six times more sensitive than the conventional vibriocidal assay. Moreover, when we used human cholera convalescent serum and vibrios at a concentration of 10^7/ml, the radiovibriolytic assay was three to four times more sensitive than the vibriocidal test. Deb et al. (7) reported that Finkelstein's vibriocidal test (8) was significantly more sensitive than either the photometric (15) or the microtechnique (2) variations of this assay. Holmgren et al. (13) have recently suggested an agar plaque method for titration of vibriocidal antibodies. They reported that their method was more sensitive than the microtechnique of Benenson et al. (2), but unfortunately no direct comparison of sensitivity was made with the macrovibriocidal assay.

Finally, the vibriolytic assay appears to be immunologically highly specific (Fig. 5). Parenthetically, extension of this radiobacteriolytic technique to the assay of anti-*E. coli* serum showed (Fig. 9) a similar immunological specificity and suggests that this method may have general application to the assay of antibodies directed against other gram-negative bacteria.

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