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Simplified Radioimmunoassay for Diagnostic Serology

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A simplified, indirect radioimmunoassay is described for *Escherichia coli*, vaccinia virus, and herpesvirus. The antigens were affixed to glass cover slips; thus both the primary and secondary reactions take place on the cover slips, and the unbound antiserum is easily separated from the bound antiserum by rinsing. Rabbit or human immune sera were reacted with the antigens, and the primary immune complex was quantitated by a secondary reaction with ^{125}I-indicator globulin (anti-rabbit or anti-human). A direct relationship between the antiserum concentration and the ^{125}I absorption was established. Variations in titers were detectable, and the titers were comparable to complement fixation titers. Homologous and heterologous reactions were distinguishable. The method affords an objective, quantitative, and qualitative evaluation of antibody, and results are reproducible.

Radioassays have been used advantageously to measure a variety of biological materials. Methods were first developed for measuring polypeptide hormones such as insulin, growth hormones, and lactogenic hormones. The radioassays developed for hormones were immunoassays in which unlabeled hormones competed with labeled hormones for the antibody-binding sites (1).

The utility of radioassay methodology for measuring hormones led to application of the technique to measurement of antibodies to infectious agents. Several successful radioimmunoassay (RIA) methods have found little general acceptance for routine use because they require complicated and laborious methods for separating the radiolabeled antigen-antibody complex from the unbound, labeled materials.

Examples of these methods include the double antibody precipitation RIA for poliovirus described by Gerloff and co-workers (3). A similar microtiter precipitation RIA for measuring hepatitis-associated antigen was used by Lander et al. (7). Evans and Yohn (2) described a slightly less cumbersome method which used a paired radioiodine technique for detecting adenovirus 12 tumor antigen. They used adenovirus-infected tissue culture cells affixed to glass cover slips in Leighton tubes. Even this method, however, required manipulation of at least one tube for each sample.

These RIA procedures are conceptually feasible, but they all require time-consuming sample preparation and multistep procedures to remove unbound radiolabeled indicator globulins. Therefore, alternate procedures using less complicated methods were considered. Tanigaki, Yagi, and Pressman (11) have described a paired label radioantibody technique for tissue sections and cell smears. The tissue sections and cell smears were affixed to glass cover slips, and the antibody was allowed to react with the antigen affixed to the cover slips. This method was used to measure only cellular antigens rather than specific viral antigens. Henle and Henle (5) described an indirect fluorescent-antibody (FA) test for Epstein-Barr virus, in which a crude cell-associated antigen preparation was affixed to glass cover slips. These procedures were used as models for the development of an RIA procedure which is adaptable to routine serological tests.

In the RIA procedure to be described, the antigenic materials are affixed to replicate glass cover slips. Thus, the method affords a practical separation of unbound antibody from bound antibody. After the unbound antibody is removed, the antigen-antibody reaction is quantitated by using radioisotope-labeled indicator globulin, and the RIA results are re-

corded as the incorporated radioactivity for each antiserum dilution.

RIA is analogous to FA in concept and sensitivity, but measurement of incorporated radioactivity in RIA offers clearcut advantages over measurement of fluorochrome excitation in FA. Because of difficulties associated with the development of excitation scanners, reports of quantitation and automation of conventional FA techniques have been limited (9, 10, 12). In addition, the results in each of the methods are only semiquantitative. Since measurement of radioactivity is quantitative, RIA offers an alternative to attempts to quantitate and automate conventional FA methods.

MATERIALS AND METHODS

Viruses. Vaccinia virus, Connaught strain, was obtained from the Viral Exanthems Unit, Center for Disease Control, Atlanta, Ga. Stock virus was grown in human epithelial (HEp-2) tissue culture monolayers and harvested when approximately one-half of the cells exhibited cytopathic effects. *Herpesvirus hominis*, McIntyre strain, was obtained from the Respiratory Virology Unit, Center for Disease Control. Stock herpesvirus was grown in primary rabbit kidney (PRK) tissue culture monolayers, and it too was harvested before all cells exhibited cytopathic effects. The viruses were harvested by scraping the tissue culture cells from the glass and were washed twice with phosphate-buffered saline (PBS; *p*H 7.2; 0.0081 M Na_2HPO_4, 0.0015 M KH_2PO_4, 0.137 M NaCl, and 0.0027 M KCl). The cells from each 32-oz (ca. 0.97 liter) prescription bottle were resuspended in 10 ml of PBS. Samples (0.1 ml) of the virus suspensions were allowed to spread over the surface of cover slips (8 by 30 mm) attached to the edge of microscope slides. The cover slips were air dried, and the cells were acetone-fixed. The cover-slip cultures were stored at −20 C in desiccators containing a nitrogen atmosphere.

Bacteria. *Escherichia coli*, strains 01 and 0119, were obtained from the Biological Reagents Section, Center for Disease Control. The bacteria were grown in nutrient broth for 24 hr at 37 C. The cells were harvested by centrifugation at 1,000 × *g*, washed twice with PBS, and suspended in 10 ml of distilled water. Cover-slip cultures were prepared as indicated for the virus cultures.

Antisera. Human and rabbit globulins were prepared by precipitating human and rabbit sera successively with 18, 14, and 12.5% sodium sulfate (6). The precipitated globulins were suspended in borate saline buffer (0.15 M NaCl, 0.002 M H_3BO_3, *p*H 7.4). The globulins were dialyzed against 0.0175 M phosphate buffer to remove the sodium sulfate. The protein content was estimated spectrophotometrically at 280 nm.

The human and rabbit globulins were used to immunize 500-g male guinea pigs. The guinea pigs were initially inoculated intradermally with 2 mg of globulin diluted twofold with Freund complete adjuvant. The animals received intraperitoneal booster inoculations of 2 mg of globulin biweekly until they developed high antiglobulin titers. The titers were determined by the Ouchterlony procedure of double diffusion in agar. The specificity of the antiglobulins was tested by immunoelectrophoresis.

Iodination. Globulin was prepared from guinea pig anti-human and anti-rabbit sera by sodium sulfate precipitation (6) and dialysis against 0.0175 M phosphate buffer. Protein content was measured spectrophotometrically at 280 nm.

The globulin was iodinated by the McConahey and Dixon (8) modification of the Greenwood method (4), with chloramine-T as the oxidizing agent. ^{125}I in carrier free form was added at a concentration of 50 μCi/mg of protein. Unbound ^{125}I was removed by dialysis for 24 to 72 hr against several changes of 0.1 M phosphate buffer at 4 C.

RIA procedure. The RIA procedure was identical for both the viral and the bacterial assays. Specific antisera for vaccinia virus, herpesvirus, and *E. coli* were diluted serially in PBS containing 10% fetal calf serum. Samples (0.1 ml) of each dilution were added to duplicate cover-slip cultures of virus or bacteria. The cover slips were incubated at 37 C in a moist atmosphere. After incubation, the excess serum dilutions were drained from the slides, and the slides were washed twice with PBS and partially dried.

Guinea pig anti-rabbit or anti-human ^{125}I-labeled globulin was diluted in PBS. The globulin used was specific for the antiserum species used in the primary antigen-antibody reaction. Samples of 0.1 ml of ^{125}I-labeled globulin were added to each slide. The slides were again incubated at 37 C in a moist atmosphere. The slides were drained, washed twice with PBS, dehydrated with two changes of 95% ethanol, and air dried. The incorporated radioactivity was counted in a Packard autogamma scintillation spectrometer.

Individual variations of the above procedure were used to determine the optimal conditions for the assay. The variables included time of incubation, number of washes, and concentration of each reagent. The variations will be described below.

RESULTS AND DISCUSSION

Indirect immunological procedures involve two antigen-antibody reactions. In the first reaction, specific antibody combines with the antigen. In the secondary reaction, species-specific ^{125}I-labeled anti-immune globulin (^{125}I-indicator globulin) combines with the antibody in the initial antigen-antibody complex. Therefore, if the anti-immune globulin is radioactively labeled, the second antigen-antibody reaction can be used to quantitate the first reaction.

We have developed an indirect RIA in which the antigen is fixed on cover slips attached to microscope slides. Each of the immunological reactions occurs with the primary antigen af-

fixed to the glass surface. In both of the immunological reactions, the concentration of the reactants and the optimal conditions for the reaction were determined.

Antigen concentration. The effect of varying the concentration of antigen was considered for both bacteria and viruses. A stock suspension of *E. coli* was adjusted to an optical density of about 0.2 at 650 nm. This concentration of bacteria was designated as an undiluted suspension. Variations in the RIA due to antigen concentration were assessed by preparing 10-fold serial dilutions of this suspension, affixing the diluted antigen to separate cover slips, and reacting each preparation with a 1:10 dilution of specific antiserum. As a blank control for the antigen-antibody reaction, diluent was substituted for the 1:10 dilution of specific antiserum.

The specific antiserum dilution absorbed about four times as much ^{125}I-guinea pig anti-rabbit globulin as the corresponding control (20,200 counts/min: 5,200 counts/min) when an undiluted antigen preparation was used. However, the undiluted antigen preparation was the only concentration of antigen which differentiated between the specific antigen-antibody reaction and the control. As the antigen concentration was decreased, the nonspecific absorption of ^{125}I-indicator globulin increased. The reaction with undiluted antigen control absorbed 5,200 counts/min of ^{125}I-indicator globulin, whereas the same reaction with a 1:1,000 dilution of *E. coli* antigen absorbed 15,400 counts/min. Furthermore, as the antigen concentration was decreased, the specific antiserum absorption of ^{125}I-indicator globulin decreased. The undiluted antigen-antibody reaction absorbed 20,200 counts/min of ^{125}I-indicator globulin, and the same reaction with a 1:1,000 antigen dilution absorbed 10,600 counts/min.

These data suggest that it is imperative to affix excess antigen to the cover slips. This assures complete saturation of the primary antigen-antibody reaction. In addition, the use of excess antigen eliminated nonspecific binding of the indicator globulin. The results showed conclusively that, with sufficient antigen, specific antigen-antibody reactions are easily distinguishable from nonspecific ^{125}I absorption.

The optimal concentration of vaccinia antigen was also investigated. The vaccinia antigen consisted of three separate suspensions of virus-infected HEp-2 cells, each having a different viral titer. Each antigen preparation and the cell control were adjusted to contain equal cell concentrations. Samples of each suspension were spread on cover slips, air dried, and fixed with acetone.

Each cover slip with its affixed antigenic material was allowed to react with an equal volume of a single dilution of specific antiserum. Blank controls were included for each antigen concentration. Finally, all cover slips were reacted with ^{125}I-indicator globulin, and the radioactivity was determined.

An antigen preparation containing $10^{6.7}$ median tissue culture infective doses ($TCID_{50}$)/0.1 ml incorporated approximately 18,000 counts/min of ^{125}I-indicator (anti-rabbit) globulin after reaction with the rabbit anti-vaccinia serum. The corresponding serum controls for this inoculum incorporated approximately 4,800 counts/min of ^{125}I-indicator. Antigen preparations containing $10^{6.0}$ $TCID_{50}$/0.1 ml and $10^{4.5}$ $TCID_{50}$/0.1 ml absorbed 15,500 and 12,000 counts/min of ^{125}I-indicator globulin, respectively, when treated with specific antiserum. The corresponding controls for these inocula absorbed 5,300 and 7,200 counts/min of ^{125}I-indicator globulin, respectively.

These data indicated that the antigen preparations with the highest virus titer gave the maximal specific antigen-antibody reactivity with minimal nonspecific ^{125}I binding. Although the background ^{125}I absorption increased with decreasing antigen concentration, it was not as marked as that observed with the *E. coli* preparations. The protein concentrations were approximately equivalent in the vaccinia preparations; therefore, less change in the nonspecific binding would be anticipated.

Specificity of ^{125}I-antiglobulin. The specificity of the ^{125}I-indicator globulin was evaluated by studying the competition between radioactively labeled and unlabeled antiglobulin for the specific antibody bound to the antigen in the primary reaction. The effect of unlabeled anti-rabbit globulin on the binding of ^{125}I-indicator globulin was determined by maintaining a constant concentration of ^{125}I-indicator globulin and varying the concentration of unlabeled anti-rabbit globulin.

As the concentration of unlabeled anti-rabbit globulin was increased, the binding of ^{125}I-indicator globulin decreased (Fig. 1). The relationship was linear when the ratio of radioactively labeled to unlabeled globulin was between 1/2 and 1/8. However, at ratios greater than 1/2, the relationship deviated from linearity, and at a ratio of 2/1 the maximal absorption was attained. At ratios less than 1/8, the radioactivity of absorbed ^{125}I-indicator globulin approached the radioactivity of the control

slides. Despite these observed changes in the presence of antiserum, there were no concomitant changes in the blank control as unlabeled antiglobulin concentration was increased.

These results showed that ^{125}I labeling is competitively inhibited by unlabeled globulins and, therefore, that the ^{125}I absorption is a specific measure of the primary antigen-antibody reaction. They also provide evidence that iodination does not markedly denature the protein.

Concentration of ^{125}I-antiglobulin. In direct immunoassays, a secondary antigen-antibody complex is used to quantitate the primary immunological response. For accurate quantitation of the primary response, an excess of the secondary labeled antibody should be used. The effect of variations in the quantity of ^{125}I-labeled antiglobulin was, therefore, considered for both rabbit and human immune antisera.

The optimal concentration of ^{125}I guinea pig anti-rabbit globulin was determined by reacting constant concentrations of *E. coli* with a constant concentration of rabbit anti-*E. coli* serum (1:40 dilution) and a diluent control. The concentration of ^{125}I-indicator globulin (anti-rabbit) was varied from 4.2 to 83.3 μg. Each concentration was contained in 0.1-ml fractions.

As the concentration of the ^{125}I-indicator globulin was increased, the absorption of the labeled globulin increased in both the antiserum and control reactions. A marked rise in the ^{125}I absorption was observed in the antiserum reaction as the concentration of ^{125}I-indicator globulin was increased from 4.2 to 16.7 μg/slide. At a concentration of 16.7 μg/slide, the antiserum absorbed about three times as much ^{125}I-indicator globulin as the control. At concentrations greater than 16.7 μg/slide, the ^{125}I absorption continued to increase. However, as the concentration approached 83.3 μg/slide, the ratio of the specific to nonspecific reactions decreased to about two. Although maximal ^{125}I absorption was not attained even with 83.3 μg of the labeled protein, it was not feasible to use greater concentrations. Thus we have used concentrations between 16.7 and 50.0 μg/slide.

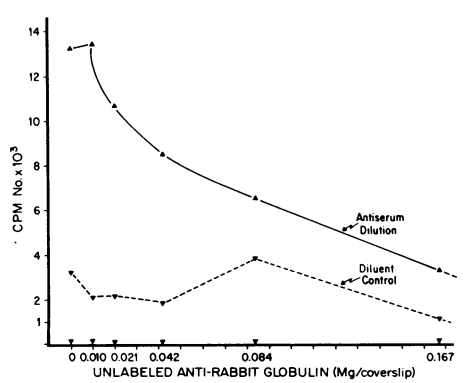

FIG. 1. *Competitive inhibition of ^{125}I guinea pig anti-rabbit globulin absorption by unlabeled anti-rabbit globulin. Each cover slip was reacted with a constant amount of ^{125}I-indicator globulin (0.021 mg/cover slip) plus the amount of unlabeled globulin indicated on the abscissa. Symbols: △, specific E. coli antiserum; ▽, diluent control.*

The optimal concentration of ^{125}I-indicator globulin (anti-human) was determined in a similar manner, except that the primary antigen-antibody reaction consisted of *Herpesvirus hominis* and a human hyperimmune herpes antiserum. Again, the concentrations of antigen and antigen-specific antibody were constant. The controls for this trial consisted of uninfected PRK cells absorbed with the herpes-immune serum. The concentration of ^{125}I guinea pig anti-human globulin was also varied from 4.2 to 83.3 μg/sample.

The amount of ^{125}I-indicator globulin bound by the specific antigen-antibody complex increased markedly as the concentration of the labeled globulin was increased from 4.2 to 16.7 μg/slide. At these concentrations, absorption by the specific reaction was approximately three times the absorption for the blank control cover slips. However, at concentrations between 16.7 and 83.3 μg/slide, the increased absorption of ^{125}I-indicator globulin by the specific reaction was equal to that of the control. We therefore chose a concentration range of 25 to 40 μg ^{125}I guinea pig anti-human globulin per cover slip for routine use in RIA.

Time of incubation. The optimal time of incubation was determined for the primary and secondary antigen-antibody reactions with both *E. coli* and herpesvirus. The time relationship for both the primary and secondary reactions was examined by incubating each reaction for 15, 30, 60, 90, and 120 min. The time of incubation giving maximal specific ^{125}I incorporation for the antiserum and minimal incorporation for controls was considered to be the optimal incubation time.

With *E. coli* as the primary antigen, absorption of ^{125}I-indicator globulin increased about 30% when the time of the primary reaction was increased from 15 min to 60 min. The concomitant increase in the control was approximately 20%. Incubation times of 90 and 120 min resulted in slight decreases in the specific

absorption with little change in absorption by the control antigens. Since 60 min of incubation showed the greatest ^{125}I incorporation, it was taken as the optimum and used in all subsequent experiments.

Herpesvirus hominis showed about a 50% increase in specific ^{125}I absorption when the primary incubation time was increased from 15 min to 90 min; beyond 90 min there was no further absorption of ^{125}I-indicator globulin. The controls at each incubation time gave nearly the same ^{125}I-indicator globulin absorption. An incubation time of 90 min was therefore used in all subsequent experiments with herpesvirus.

The secondary reaction for *E. coli* showed substantial increases (up to 3.3-fold) in the ^{125}I absorption when the incubation time was increased from 15 to 120 min. However, the antigen controls likewise showed large increases in the ^{125}I absorption. Thus the nonspecific, as well as the specific, binding increased with time. The ratios of specifically bound ^{125}I-indicator globulin to the amount of labeled globulin bound to antigen controls were greatest with incubation periods of 30 and 60 min (5.2 and 4.0, respectively, for the 1:10 antiserum dilution). Because of the very high blank controls, the ratios at 90 and 120 min were substantially reduced. Hence, the secondary *E. coli* antigen-antibody reaction was incubated for 30 to 60 min in all subsequent experiments.

The secondary reaction for herpesvirus, like that for *E. coli*, produced large increases (up to threefold) in the ^{125}I absorption as the time of incubation was increased from 15 min to 120 min. Unlike that of the *E. coli* system, the nonspecific binding did not increase substantially. Therefore the increased absorption with time can be regarded as specific antigen-antibody reactivity. Although the greatest binding of labeled globulin was attained after 120 min, the rate of binding decreased and approached completion during the period of 90 to 120 min. Longer incubation periods were not tested, and 90 to 120 min was used in all trials with herpesvirus.

Removal of unabsorbed immune globulins. Adequate rinsing to remove nonspecifically bound immune globulins is required after both the primary and secondary reactions. The optimal number of rinses was determined for both the primary and secondary *E. coli* antigen-antibody reactions. The two reactions were tested separately. A 1:10 antiserum dilution and a blank control were used for both. Reactants were added to antigen affixed on cover slips, absorbed, and finally rinsed zero, one, two, four, or six times in PBS. When the optimal number of rinses for either the primary or secondary reaction was determined, the reactant from the other reaction was rinsed twice.

The results of variation in the number of rinses after both the primary and secondary antigen-antibody reactions are shown in Tables 1 and 2, respectively. The results for the primary reaction show that the slides must be rinsed to remove nonspecific materials which prevent reaction of the indicator globulin with the antigen-antibody complex. A single rinse with PBS increased the specific absorption of ^{125}I-indicator globulin by about 50%. One or two rinses permitted maximal absorption of ^{125}I-indicator globulin by the antigen-antibody complex, whereas absorption of the radioactive indicator by the diluent controls was minimal with this treatment. Additional rinses caused decreased specific ^{125}I absorption, increased nonspecific ^{125}I absorption by the controls, and increased standard deviations for both the specific and nonspecific reactions. The errors associated with additional rinses were probably caused by loss of the antigen-antibody complex. Visual inspection confirmed loss of mate-

TABLE 1. *Radioactivity remaining after removal of unabsorbed E. coli immune globulin in the primary antigen-antibody reaction*

No. of rinses	Antiserum		Antigen control	
	Counts/min[a]	Coeff. var.[b]	Counts/min[a]	Coeff. var.[b]
0	6,737	2.3	2,612	6.2
1	13,560	2.4	2,630	10.6
2	13,209	4.8	3,002	8.7
4	12,076	9.0	3,864	9.0
6	8,516	9.8	5,004	14.7

[a] Mean of six replicates.
[b] Coefficient of variation.

TABLE 2. *Radioactivity remaining after removal of unabsorbed ^{125}I-indicator globulin in the secondary reaction from the E. coli antigen-antibody complex*

No. of rinses	Antiserum		Antigen control	
	Counts/min[a]	Coeff. var.[b]	Counts/min[a]	Coeff. var.[b]
0	28,501	26.6	16,035	29.3
1	13,944	16.1	5,301	36.5
2	13,223	4.3	3,505	6.6
4	11,216	13.6	2,155	16.1
6	11,223	20.9	3,303	32.0

[a] Mean of six replicates.
[b] Coefficient of variation.

rial from the cover slips. Hence, one or two rinses of the primary reaction were established as standard procedure.

Next, the rinse requirements for the secondary reaction were determined. These results (Table 2) show that a single rinse removed most of the excess ^{125}I-indicator globulin, but the standard deviation was abnormally high, indicating insufficient rinsing. One rinse removed about 50% of the radioactive iodine from the antiserum-treated cover slips and about 67% from the control cover slips. A second PBS rinse removed only an additional 5% of the radioactivity, but the standard deviation for six replicates was reduced considerably. The radioactivity of control cover slips treated identically was reduced 34%, and the standard deviation likewise was reduced substantially. Additional rinses caused increased standard deviations for both the antiserum dilutions and controls. Again, as in the primary reaction, the increased standard deviations with four to six rinses reflected a loss of antigen from the cover slips. We concluded from these results that two PBS rinses are optimal for removal of excess ^{125}I-indicator globulin in the secondary reaction.

Reproducibility. The reproducibility of this RIA was tested with *E. coli* 01 and its specific antiserum. The variables considered were those which could grossly affect the reproducibility of the method: different lots of antigen, different lots of ^{125}I-indicator globulin, and variations in the antiserum dilutions.

The same lot of antiserum was used in all of the tests, but fresh dilutions of antiserum were prepared for each experiment. Twofold antiserum dilutions (1:10 to 1:160) were used for replicate trials. The separate evaluations included trials with antisera diluted at different times but with the same antigen preparation and ^{125}I-indicator globulin preparations. Alternatively, separate trials were performed with different lots of antigen and ^{125}I-indicator globulin but with identical antiserum dilutions. When all of the variables were considered, the coefficient of variation ranged from 8.9 to 20.6% for the individual antiserum dilutions. The various conditions yielded an average titer of 1:54 (end points represented as the antiserum dilution twofold greater than control ^{125}I absorption). The range of titers for five trials was 1:48 to 1:73.

The greatest variation was observed when different lots of ^{125}I-indicator globulin were used with identical antiserum dilutions and antigen preparations. Since corrections were not made for differences in the specific radioactivity of the globulins, it is believed that differences in this parameter contributed to the differences in ^{125}I absorption observed in the experiments.

Application of RIA. To establish the feasibility of the RIA method for general use, we applied the procedure to three typical laboratory experiments. These experiments include the differentiation of two strains of *E. coli* and their respective antisera, evaluation of antiherpes titers in human antisera, and quantitation of the immunological response to vaccination.

(i) Bacterial strain differentiation. The reactivity of antibacterial antisera in RIA was determined with two strains of *E. coli* (01 and 0119) and their respective antisera. Twofold dilutions of rabbit antisera to the *E. coli* strain-specific somatic "O" antigen were allowed to react with homologous and heterologous cell cultures.

Figure 2 shows the results of RIA with homologous and heterologous reactions. The controls for which PBS diluent was substituted for the anti-*E. coli* antiserum dilutions absorbed less than 3,000 counts/min of ^{125}I-indicator globulin. The absorption by the control was approximately one-half the absorption by the lowest antiserum dilution (1:5) for the least reactive antiserum. Furthermore, the binding of ^{125}I-indicator globulin for each of the specific antigen-antibody reactions was linear over a 4- to 32-fold range of antiserum dilutions.

That the homologous reaction is distinguishable from the heterologous reaction is indicated by the differences in absorption of ^{125}I-indicator globulin. The reaction of *E. coli* 01

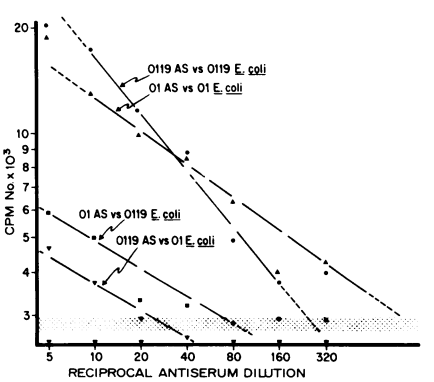

FIG. 2. *Reaction of homologous and heterologous antisera (AS) with E. coli strains 01 and 0119. Diluent was substituted for antiserum in the blank control. Blank control is represented by the cross-hatched area drawn parallel to the abscissa.*

antigen with its homologous antiserum was approximately 2.5-fold greater than the heterologous reaction with 0119 antiserum. The reaction of *E. coli* 0119 antigen with its homologous antiserum was approximately 4.4-fold greater than the heterologous reaction with *E. coli* 01 antiserum.

(ii) Survey of anti-herpesvirus activity in human sera. Four human sera (A, B, C, and D) were examined by the RIA method for anti-herpes activity. Twofold dilutions of each serum were prepared and allowed to react with duplicate herpes-coated cover slips.

The results show that at the highest concentration each of the antisera absorbed more ^{125}I-indicator (antihuman) globulin than either the antigen or antiserum controls (Fig. 3). The range of the blank controls is represented by broken lines drawn parallel to the abscissa. Antisera A and B absorbed between four and five times as much ^{125}I-indicator globulin at the highest serum concentrations as the antigen or antiserum controls. Furthermore, the relationship between the absorbed radioactivity and the antiserum dilutions was linear over a 32-fold range. Antiserum dilutions of lower reactivity (curves C and D, respectively) produced a similar linear response over approximately an eightfold range, and the maximal ^{125}I absorption was at least twice as great as the controls. The RIA method clearly distinguishes between sera of low and high reactivity.

When the counts/min of bound ^{125}I-indicator globulin is plotted against the reciprocal of the antiserum dilution, the slopes for different sera varied. In this study, the slope of curve A (Fig. 3) differed from that of the other three curves. This difference in slopes has not been investigated, but it may reflect a qualitative difference between sera of different antigenic specificity.

(iii) Immune response to vaccination in rabbits. Rabbits were immunized with vaccinia virus and bled sequentially twice weekly beginning 3 days after immunization. Booster doses of virus were given 28 and 63 days after the primary immunization. Five fold dilutions of preimmune sera and 7-, 69-, and 80-day postimmune sera were prepared. Duplicate samples of each serum dilution were absorbed to slides containing HEp-2 cells infected with vaccinia virus and uninfected HEp-2 cell controls. Additional blank controls were included in which diluent was substituted for antisera. RIA antiserum titers are plotted in Fig. 4.

The results show that vaccinia-infected cells reacted with rabbit anti-vaccinia sera absorbed significantly more ^{125}I-indicator globulin than the controls. The two types of background controls bound nearly equal amounts of ^{125}I-indicator globulin. Again, the range of the background radioactivity is represented in Fig. 4 by the broken lines drawn parallel to the abscissa.

An expression of the relationship between the absorption of the radioactivity and corresponding serum dilution was obtained by plotting the logarithm of the counts per min per cover slip on the ordinate and the logarithm of the reciprocal of the serum dilution factor on the abscissa (Fig. 4). A linear relationship was obtained over at least a 20-fold range. The ^{125}I-absorption curve of each antiserum dilution series reflects the anticipated antibody rises. The absorption of ^{125}I-indicator globulin by preimmune serum is about equivalent to that for the controls. However, 7 days after immunization, the antiserum shows an increased ^{125}I absorption, and antisera taken after two booster inoculations show even greater ^{125}I

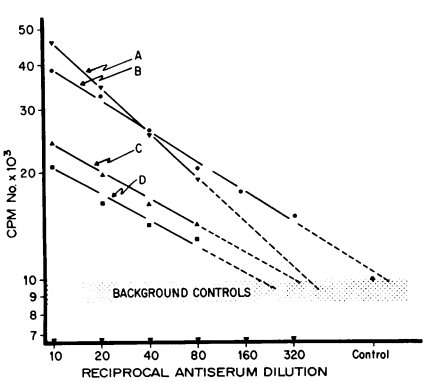

FIG. 3. *Reaction of randomly chosen human sera (A, B, C, and D) with Herpesvirus hominis. Antigen controls (uninfected cells) and blank controls (diluent substituted for antiserum) are represented by the cross-hatched area drawn parallel to the abscissa.*

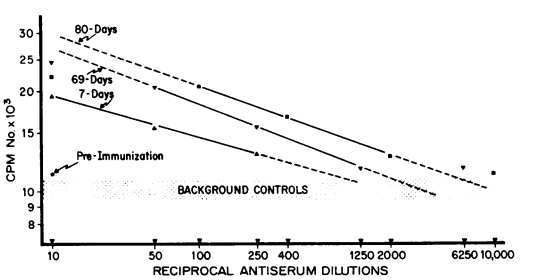

FIG. 4. *Determination of the sequential appearance of antibodies by vaccinated rabbits. Antigen controls (uninfected cells) and blank controls (diluent substituted for antiserum) are represented by the cross-hatched area drawn parallel to the abscissa.*

TABLE 3. *Comparison of antivaccinia activity of sera from vaccinated rabbits by complement fixation and RIA*

Days after immunization	Antisera dilution	
	Complement fixation[a]	RIA[b]
0	1:10	<1:10
7	1:40	1:10
69	≥1:320	1:60
80	≥1:320	1:120

[a] Highest serum dilution fixing complement.
[b] Serum dilution yielding twice the counts/min obtained with control material.

absorption. These results indicate that antibodies arising from specific immunization can be detected and quantitated by the differences in absorption of the radioactive antiglobulin.

Comparison of RIA and complement fixation methods. RIA and complement fixation titers for sera of vaccinated rabbits (Fig. 4) were compared (Table 3). The complement fixation titers increased progressively from 1:10 before immunization (0 day) to 1:320 or greater on the 80th day after immunization. In the RIA procedure, end-point dilutions were the antiserum dilutions causing twice as much ^{125}I-indicator globulin absorption as the blank controls. The RIA titers in this comparative study ranged from less than 1:10 on day 0 to 1:120 on the 80th day after immunization. The progression of complement fixation titers is in agreement with the increased binding of ^{125}I-labeled globulin in the RIA procedure with the same sera.

The limits of the antigen-antibody specificity detected by RIA have not been determined. RIA, which closely resembles fluorescent-antibody serological techniques, probably measures a variety of antigens including those measured by complement fixation, hemagglutination inhibition, precipitation, and neutralization. However, RIA is at least equally effective for qualitative and quantitative evaluation of antibody and is more objective than these conventional serological techniques.

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