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Detection by Radioimmunoassay of Antibodies in Human Smallpox Patients and Vaccinees

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A radioimmunoassay procedure was developed for determining smallpox and vaccinia antibodies in human sera. The test detected and measured both primary and secondary immune responses in persons infected with variola virus or vaccinia virus. The antibody titers obtained by complement fixation, hemagglutination inhibition, plaque reduction neutralization, and radioimmunoassay methods were compared. In sequential serum specimens, the radioimmunoassay test indicated fourfold or greater increases in all of the smallpox patients and in six of eight vaccinated persons. Both the complement fixation and the hemagglutination inhibition tests were less effective. In persons who had been vaccinated, radioimmunoassay and plaque reduction neutralization tests appeared to measure the same immune response. However, in smallpox patients the immune response was readily detected by radioimmunoassay, whereas an immune response was not detected by the plaque reduction neutralization test when vaccinia virus was the antigen in the test system. Radioimmunoassay is an operationally simple procedure which provides objective and quantitative end-point titers in serological determinations.

A variety of serological methods for determining smallpox antibody titers in human sera are used in clinical laboratories, but each of the methods has characteristic shortcomings. Hemagglutination inhibition (HI) tests frequently give false-positive results with certain human and wild-animal sera, complement fixation (CF) tests usually fail to detect low antibody levels during early immune response, and neutralization tests by plaque reduction involve tedious plaque or pock enumeration. Radioimmunoassay (RIA) methods may offer an improved alternate method; however, a practical RIA method for measuring smallpox antibodies in human sera has not been established.

The indirect solid phase RIA methods described by Hutchinson and Ziegler (10) and Rosenthal et al. (14) in which antigen preparations are affixed to planar glass or polymeric surfaces, offer a technically facile procedure. The methods satisfactorily measured antibody in potent antisera of hyperimmunized animals, and in this report provide the basis for development of a practical RIA method for determining smallpox antibodies in human sera. These RIA procedures were adapted for detecting antibodies in humans who were either naturally infected with variola virus or were immunized by vaccination. The RIA titers of the patients were compared with the titers obtained by CF, HI, and plaque-reduction neutralization (PRN) tests.

The RIA procedure used in this study is an operationally simple procedure. The antigen is affixed to planar surfaces; thus unbound antisera of both the primary and secondary antigen-antibody reactions are easily separated from the bound antisera by rinsing. The reaction of radioisotope-labeled antiglobulin with the primary antigen-antibody complex provides a measure of antibody in the serum specimens. Furthermore, the use of radionuclides in immunoassays affords a more definite expression of end-point titers than conventional serological assays, and RIA methods are potentially more readily adaptable to automation. Because of these considerations, the RIA determination is recommended as a routine diagnostic tool.

MATERIALS AND METHODS

Microtiter plates. Both the primary and secondary antigen-antibody reactions take place within the wells of disposable microtiter plates as reported by Rosenthal et al. (14) (Disposable Microtiter Plates, Flat Bottom, no. 220-29, Cooke Engineering Co., Alexandria, Va). To obtain more uniform spreading of small volumes of aqueous reaction mixtures, flat bottom plates were siliconized by immersing them in a 1.0% (vol/vol) aqueous mixture of silicone concen-

trate (Siliclad, Clay Adams Co., Division of Becton, Dickinson Co., Parsippany, N.J.). After treatment, the plates were air-dried.

Virus. A Wyeth strain of vaccinia virus was obtained from the Viral Vaccine Investigations Section, Virology Branch, Center for Disease Control, Atlanta, Ga. Stock virus was prepared in rabbit kidney (RK-13) tissue culture monolayers and stored as frozen tissue culture material.

Antigen preparation. Vaccinia antigens were prepared from virus grown in RK-13 tissue culture monolayers and harvested when approximately 50% of the cells showed cytopathic effect. An uninfected control bottle of RK-13 cells was included for each bottle of virus-infected cells. The cell monolayers were washed twice with phosphate-buffered saline (PBS; pH 7.2; 0.0081 M Na_2HPO_4, 0.0015 M KH_2PO_4, 0.137 M NaCl, and 0.0027 M KCl) and were harvested by scraping the cells from the glass. The cells from each 32-ounce (960 ml) prescription bottle were resuspended in 5 ml of PBS. The cells were frozen and thawed once and sonically treated for 2 min in a 10 Kc Raytheon sonic oscillator. The titers of the vaccinia antigen suspensions were approximately $10^{6.4}$ mean tissue culture infective dose per 0.05 ml. Aliquots (0.05 ml) of the infected and uninfected cell suspensions were added to the wells of siliconized microtiter plates. The plates were air-dried and stored at −20 C in a desiccator containing a nitrogen atmosphere. The antigens were stable at least 12 months. Immediately before use, the cells were fixed for 15 min with cold (4 C) 10% formaldehyde in pH 7.0 buffer. After fixation, the cells were rinsed once with PBS.

Human antisera. One or more serial serum specimens were obtained from 11 clinically diagnosed and virologically confirmed smallpox patients from Bangladesh, 1972. Seven of these patients stated that they had never been vaccinated. Four of the patients thought that they might have been vaccinated, however there was no evidence of scars or other indications of "takes." The day of onset of prodromal fever (≥102 F [38.7 C]) and the day of onset of rash was ascertained by interview. In addition to sera from smallpox patients, sequential serum specimens were obtained from eight persons who were immunized with vaccinia virus. Seven of these persons had been vaccinated previously, however, one person, vaccinee no. 8, did not have a record of previous vaccination. Aliquots of each serum were prepared and stored at −20 C.

Guinea pig anti-human globulin. Sodium sulfate-precipitated guinea pig anti-human globulin was iodinated by the chloramine-T procedure of Greenwood et al. (6) as described previously (9). The only alteration in the procedures was an increase in the concentration of ^{125}iodine ($Na^{125}I$) to 1,000 μCi per mg of protein.

RIA procedure. The RIA procedure used in this study was an adaptation of those described by Rosenthal et al. (14) and Hutchinson and Ziegler (10). The human sera were serially diluted in a diluent consisting of 10% fetal calf serum in PBS. Duplicate aliquots (0.025 ml) of each serum dilution were added to wells of microtiter antigen plates containing both virus-infected and uninfected RK-13 cells. As controls, diluent was added to duplicate wells of virus-infected cells. The microtiter antigen plates containing the antibody dilutions were incubated at 37 C for 1.5 h. After incubation, the serum dilutions were removed from the wells, and the plates were rinsed five times with PBS.

The use of appropriate concentrations of ^{125}I-indicator globulin was shown by Hutchinson and Ziegler (9) to be necessary to distinguish antisera of different potencies. Hence, the optimal concentration of each lot of antiglobulin must be determined. The optimal amount of the ^{125}I-labeled guinea pig anti-human globulin used in these studies was 0.65 μg of protein per well of the microtiter plate. Dilutions of the antiglobulin were adjusted to contain this amount in the 0.025-ml aliquots added to each well. The plates were again incubated at 37 C for 2 h. After incubation, the excess ^{125}I-labeled globulin was removed from the wells and the plates were rinsed five times with PBS, dehydrated with one rinse of 95% ethanol, and air-dried. The wells were clipped from the plates into scintillation vials and counted in a gamma scintillation spectrometer.

Serological procedures. The CF titers were determined for all sera by the Laboratory Branch Complement Fixation procedure (1). The HI titers were determined for all sera by using the procedure described by Hierholzer and Suggs (7) and Hierholzer et al. (8). The neutralization titers were determined by a plaque-reduction test described by Wulff et al. (16). In the PRN test both the virus and the sera were diluted in 0.02 M tris(hydroxymethyl)aminomethane buffer, pH 7.0. The antigen used in each of the serological procedures was the Wyeth strain of vaccinia. The validity of the RIA procedure and the other serological procedures was confirmed by testing positive and negative control sera.

RESULTS

Effect of fixation. Acetone was used for fixation of tissue culture cells in our original RIA procedure (10). Because polyvinyl microtiter plates are destroyed by acetone, an alternate fixation method was required. Air-dried, vaccinia-infected and -uninfected RK-13 cells contained in the wells of microtiter plates were treated by three methods. These were unfixed, cold 95% methanol-fixed, and cold-buffered formalin-fixed cells. The cells were maintained at 4 C for 15 min during treatment and were then washed once with PBS. The method of fixation was evaluated by reacting the cells with two sera, one known to contain vaccinia antibodies and one with no vaccinia antibodies.

One serum had no apparent antibody titer, regardless of the fixation method. With the vaccinia-positive antiserum the methanol-fixed antigen yielded the lowest titer of the three methods (Table 1). The antigenic materials which were either unfixed or formalin-fixed on

the plastic surface yielded approximately equal titers (77 and 80, respectively). Although the end-point titers were equal, the nonspecific adsorption was significantly lower in the cells fixed with formalin. Therefore, in all subsequent experiments the cells were formalin fixed.

Comparison of RIA, CF, HI, and PRN titers. Sera from 11 clinically and virologically diagnosed smallpox patients were tested by CF, HI, and PRN methods for comparison with RIA results. These sera were obtained at various intervals, beginning with the day the patients were admitted to the hospital. The elapsed time between onset of prodrome and day of hospitalization ranged from 5 to 13 days (mean 7.7 days). The elapsed time between appearance of eruption and day of hospitalization ranged from 2 to 6 days (mean 3.7 days). Two or more serum specimens were obtained from seven patients.

The comparative titers determined by CF, HI, PRN, and RIA revealed marked temporal and quantitative differences among the methods. The HI titers for sera collected on the day of hospitalization were positive (titer, ≥ 10) for 10 of 11 patients (91%). RIA results for the same specimens were positive (≥ 10) for 2 of 11 sera, and the CF titers for all of the initial sera were negative (titer, <10). Thus, the earliest apparent response to smallpox infection was detected by the HI test. Multiple serum specimens were collected 5 to 33 days after the onset of prodrome from 7 of the 11 patients. When tested by RIA or HI, all of the sera collected from each patient 4 or more days after admission to the hospital (S_2) were positive, but when tested by CF and PRN only four of seven (57%) and one of seven (17%), respectively, were positive. These results suggest that both RIA and HI would reliably detect smallpox antibodies in the second sera collected after hospitalization and in all subsequent sera. On the other hand, neither CF nor PRN tests appeared to be sufficiently sensitive to reliably detect the immune responses to smallpox infection during this period. The fact that the PRN test detected antibody titers in only 2 of 23 sera was unexpected. Hence, this test was of little value with smallpox antisera.

Of greater importance is the ability of a serological test to detect diagnostic fourfold increases in antibody titer. The sequential serum specimens from seven patients collected after hospitalization (Table 2) were examined by each of the serological tests for evidence of seroconversion. On day 8 after the mean onset of prodrome, most of the patients possessed no RIA-reacting titers. By day 9 to 17 (mean = 12 days) after mean onset (S_2 sera), each of the patients had significant titers (range 33 to 720, geometric mean titer 228). By day 18 after onset, the geometric mean titer had risen to 525, and in the only S_4 specimen obtained 33 days after onset, the elevated titer was maintained. The titers determined by RIA indicated fourfold or greater increases in each of the seven smallpox patients. The other serological tests were less effective. Both the HI and CF tests revealed diagnostically significant increases in four of seven patients, and the PRN test detected a significant increase in only one of seven patients.

TABLE 1. *Results obtained with different methods for fixation of antigen onto planar surface*

Fixation method	RIA titer[a]	
	Serum A	Serum B
Unfixed	77	<10
Methanol (95%)	38	<10
Formaldehyde (10%)	80	<10

[a] End-point dilution factor. Titers are averages of duplicate determinations.

These results indicate that in smallpox infections the apparent pattern of immune response was characteristic of the test procedure. Therefore, there was little agreement among the titers obtained by different procedures. It was noted, however, that agreement between RIA and CF tests was greatest with sera obtained during the early immune response (within 8 days of onset of prodrome), whereas agreement between RIA and HI tests was best with later sera.

A similar comparison of serological results with sera from recently vaccinated persons is shown in Table 3. The CF and HI tests detected antibody in four of eight persons and six of eight persons, respectively. However, RIA detected antibodies in each of the eight persons, and PRN detected antibodies in all but one (no. 8). It is important to note that unlike the results in the smallpox patients, the PRN test effectively detected antibodies in vaccinated persons. In contrast, the RIA test detected antibodies in both groups.

Diagnostic fourfold increases in titer were demonstrated in four of eight persons (50%) by CF; three of eight (38%) by HI; four of eight (50%) by PRN; and six of eight (75%) by RIA. The failure to demonstrate fourfold increases by PRN in vaccinees no. 1, 3, and 4, and by RIA in vaccinees no. 3 and 4, can be attributed to the existence of initial high titers in these vaccinees.

The RIA titers of sequentially obtained sera resembled the titers obtained by PRN but were generally higher. The RIA method also ap-

TABLE 2. *CF, HI, PRN, and RIA, assay titers of sera from smallpox patients*[a]

Patient code	Specimen code	Days after:			Titer[b]			
		Admission	Rash	Prodrome	CF	HI	PRN	RIA
B	S_1	0	5	7	<8	80	<4	<10
B	S_2	10	15	17	<8	40	<4	190
C	S_1	0	4	10	<8	10	<4	<10
C	S_2	4	8	14	<8	40	<4	480
C	S_3	10	14	20	8	320	<4	1000
C	S_4	23	27	33	16	80	<4	820
F	S_1	0	6	9	<8	20	1024	280
I	S_1	0	4	8	<8	<5	<4	<10
I	S_2	4	8	12	32	160	<4	540
I	S_3	10	14	18	64	320	<4	600
N	S_1	0	3	7	<8	40	<4	<10
N	S_2	4	7	11	<8	40	<4	33
N	S_3	10	13	17	32	320	<4	>1000
Q	S_1	0	4	13	<8	40	<4	<10
W	S_1	0	2	6	<8	20	<4	<10
Z	S_1	0	3	5	<8	40	<4	<10
Z	S_2	4	7	9	<8	20	<4	720
AA	S_1	0	4	7	<8	20	<4	<10
AA	S_2	4	8	11	<8	10	>1024	66
AC	S_1	0	3	7	<8	10	<4	<10
AC	S_2	4	7	11	<8	40	<4	420
AC	S_3	10	13	17	64	320	<4	>80
AE	S_1	0	3	9	<8	40	<4	15
Control positive					NT	20	300	580
Control negative					NT	<10	<10	<10

[a] The antigen used in each serological procedure was the Wyeth strain of vaccinia.
[b] End-point dilution factor.

peared to be somewhat more sensitive than the PRN method. This is shown by comparing the titers obtained with the sera of vaccinee no. 8 (Table 3). PRN failed to detect vaccinia antibodies, whereas RIA revealed the appearance of antibodies on day 4 after vaccination and an increased titer by day 14. In spite of these differences, the RIA and PRN titers were the same for 24 positive specimens and for 2 negative specimens. Thus, there was agreement between the two methods with 26 of the 30 specimens or 87%.

Reproducibility. The reproducibility of the titers obtained by the RIA procedure was determined with replicate serum titrations in a single experiment and in successive experiments. The same antiserum was used in all trials. The antiserum was diluted serially, and aliquots of each dilution were added to eight replicates of both infected and uninfected RK-13 cells. After the adsorbed radioactivity was counted, the eight replicate results for each dilution were randomized, and eight separate titration curves were plotted. Separate end-point titers were determined graphically for each of the curves. The results (Table 4) showed a range of titers from 145 to 220 and an arithmetic mean titer of 190.

Alternatively, the serum was titrated on successive days with either different cell preparations and/or different ^{125}I-guinea pig anti-human globulin. Seven successive trials yielded

TABLE 3. *CF, HI, PRN, and RIA titers of sera from vaccinated persons*[a]

Vaccinee code	Specimen code	Days after vaccination	Titer[b]			
			CF	HI	PRN	RIA
1	S_1	0	<8	5	256	44
1	S_2	4	<8	5	220	80
1	S_3	14	<8	10	180	510
1	S_4	36	<8	<5	220	220
2	S_1	0	<8	5	9	80
2	S_2	4	<8	10	6	80
2	S_3	14	16	20	180	850
2	S_4	36	8	5	100	1000
3	S_1	0	<8	<5	180	890
3	S_2	4	<8	<5	320	610
3	S_3	14	<8	<5	570	740
3	S_4	36	<8	<5	360	980
4	S_1	0	<8	40	50	700
4	S_2	4	<8	40	110	470
4	S_3	14	<8	80	110	650
4	S_4	36	<8	10	80	180
5	S_1	0	<8	<5	<4	<10
5	S_2	4	<8	<5	11	25
5	S_3	14	16	<5	120	620
5	S_4	36	<8	<5	22	580
6	S_1	0	<8	5	7	170
6	S_2	4	<8	5	10	200
6	S_3	14	64	160	2800	>10000
6	S_4	36	32	20	360	5200
7	S_1	0	<8	10	<4	40
7	S_2	4	<8	10	<4	38
7	S_3	14	32	20	25	1500
8	S_1	0	<8	<5	<4	<5
8	S_2	4	<8	<5	<4	13
8	S_3	14	<8	20	<4	110

[a] The antigen used in each serological procedure was the Wyeth strain of vaccinia.
[b] End-point dilution factor.

a range of titers from 180 to 320 and an arithmetic mean titer of 241.

DISCUSSION

Despite the continuing success of worldwide smallpox eradication efforts, there were over 120,000 cases of smallpox reported in 1973 in the endemic countries of Bangladesh, Ethiopia, and Pakistan (15). In addition to the classical cases of variola infection, there have been recent reports of cases of human monkeypox in West and Central Africa (5). Smallpox vaccination programs are a major component of preventive medicine in many parts of the world. Thus, accurate and convenient serological methods are required to identify susceptible populations and to measure immune responses to both vaccination and smallpox infections.

Most conventional serological assays yield results as end-point dilutions in terms of geometric incremental values arbitrarily selected for the dilution of the serum specimen. The proposed RIA procedure offers an alternate method involving interpolation which may overcome some of the deficiencies of conventional serological methods. In this report we have described an indirect RIA procedure which is operationally facile and yields definitive analytical results.

Conventional serological procedures (CF, HI, and PRN) appear to have different sensitivities which are characteristic of each procedure (4, 11, 13). Furthermore, the temporal and quantitative immune response of the patient may reflect the choice of the serological procedure. The HI antibodies appear soon after infection and diminish with time, whereas the CF antibodies appear later in the course of infection. The concentration of the CF antibodies increases and then may disappear entirely after vaccination. Secondary antigenic stimulation by vaccination may not produce a secondary rise in CF titer, and usually the response is minimal. Similarly, HI titers produced in response to secondary stimulation are somewhat variable. In contrast, neutralizing titers usually increase in response to secondary antigenic stimuli and persist for long periods after vaccination or infection (11). Thus, of the conventional serological procedures, the preferred method for detecting vaccinia or variola antibodies is a neutralization test.

In most neutralization tests either embryonated chicken eggs or tissue culture host systems are used; these tests are technically more difficult than CF or HI procedures. Therefore, radioimmunological procedures may pos-

TABLE 4. *RIA titers obtained in a single replicated experiment and in successive experiments*

Single experiment		Successive experiments	
Trial	Titer[a]	Trial	Titer[a]
1	200	23 May	190
2	220	4 June	300
3	220	18 June	190
4	180	20 June	190
5	190	26 June	320
6	195	6 July	320
7	145	9 July	180
8	170		
Mean	190	Mean	241

[a] End-point dilution factor.

sess advantageous characteristics which will make some form of RIA a satisfactory alternate serological procedure.

The simultaneous testing of serial serum specimens from patients with active smallpox and from vaccinees by four different serological procedures, CF, HI, PRN, and RIA, clearly showed the usefulness of RIA. The CF and HI antibodies appeared in the sera of most of the smallpox patients in a temporal sequence which suggested a primary immune response. In these sera PRN titers were absent in all but two of the specimens (Table 2). On the other hand the RIA titers in the same serially obtained sera were remarkably uniform and showed progressive increases in titer for most of the patients.

The failure to demonstrate in the smallpox patients a comparable immune response by the plaque neutralization test was unexpected and prompted us to investigate the response in persons who were vaccinated. All persons with low or negative vaccinia antibody titers on the day of immunization responded with increased titers. The titers as measured by RIA and PRN were similar except for one individual (no. 8) who failed to develop a PRN titer (Table 3). The failure of the PRN test to detect vaccinia antibodies by 14 days after immunization in this individual indicates that the PRN test is less sensitive than the RIA procedure. Most of the immune responses, determined by both RIA and PRN, were generally typical of secondary vaccinations (4, 11, 13). Thus it appears that in vaccinees antibody titers obtained by RIA are similar, temporally and qualitatively, to plaque neutralization titers.

The RIA and PRN titers obtained with vaccinated persons were compared with the titers obtained with the smallpox patients. With vaccinia used as the antigen, the specificities of the PRN and the RIA tests appear to be grossly different. The differences between RIA titers and PRN titers may reflect antigenic differences between variola and vaccinia. However, this is not likely because McCarthy and Downie (12), Downie and McCarthy (2), and Kitamura and Shinjo (11) have found no significant differences when variola virus was substituted for vaccinia virus in serological tests. A possible explanation for the apparent discrepancy may be the fact that the immune responses of the smallpox patients were primary antigenic responses, and, possibly, antibody titers did not increase sufficiently within the test period to be detected by the PRN test. This is consistent with the suggestion by Downie et al. (3) that in unvaccinated smallpox patients the antibody response is frequently delayed and the titers lower than those reached by previously vaccinated patients. It might also be argued that the sensitivity of the RIA test is much greater than that of the PRN test. Comparison of the titers obtained with vaccinated patients indicates that the RIA test is somewhat more sensitive than the PRN test, but the difference is probably not sufficient to account for the absence of measurable PRN titers in the sera of smallpox patients.

Diagnostic laboratories have established criteria for interpreting serological results obtained by CF, HI, and PRN tests. However, a similar basis for interpreting RIA results has not been established. RIA closely resembles fluorescent antibody serological techniques and probably measures a variety of immune globulins, including CF- , HI- , precipitation- , and neutralization-reacting antibodies. In these studies the RIA results did not agree with the results obtained by CF or HI tests. On the other hand, RIA and PRN appeared to detect the same immune response in sera of vaccinees. With sera from both vaccinees and smallpox-infected persons, RIA effectively detected primary and secondary immune responses. Despite these observations, interpretation of RIA results must await additional studies.

RIA will be especially advantageous for diagnostic laboratories which test large numbers of serological specimens. However, adoption of the procedure will be contingent upon the availability of a gamma scintillation spectrometer. In spite of this requirement the proposed RIA method is operationally simple, yet the radioactive labeling provides objective and quantitative determination of end-point titers. The RIA procedure can be used for the determination of antibodies to numerous infectious agents, and it can also be adapted for automated procedures. In its present form it is less time consuming than most neutralization tests and eliminates the need for trained observers to visually judge titration end points. The results of this study indicate that the RIA method may provide a convenient and sensitive serological procedure which accurately measures antibodies soon after both primary and secondary viral infections.

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