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Further Studies With Precipitation In Gel Test In Diagnosis Of Smallpox*—Part I—Studies On Detection Of Antibodies In Sera By Pig Test

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In detection of the precipitating antibodies in the sera of persons, results seem to vary considerably with the type of vaccinia-variola antigen used. Antigen prepared from variola infected chorioallantois as well as variola infected cell culture seem to behave almost similar to vaccinia antigen and hence give positives even in vaccines' sera, whereas unpassaged variola antigen prepared from vesicular fluid or scabs of smallpox cases does not give. Hence, for making diagnosis of smallpox retrospectively from a single specimen serum of completely recovered cases or for detecting subclinical (inapparent) infection in exposed contacts of smallpox cases, only unpassaged variola antigen has to be used, since use of cell passaged or egg passaged variola or vaccinia antigens are likely to give false positives in recently vaccinated persons.

Introduction

In our earlier studies (Rao **et al** 1970) we observed that the precipitation in gel (PIG) test can be usefully employed as a very simple tool in diagnosis of smallpox, not only in acute stage but even after complete recovery, by demonstrating precipitating antibodies using the smallpox vesicular fluid as antigen. We have also stated, that there was laboratory evidence of occurrence of subclinical (in apparent) infection in smallpox as can be detected by this test.

The PIG test can also be used for detection of antigen in the fluid of smallpox lesions, in the scabs and even in the blood of highly fatal cases belonging to the haemorrhagic types (Downie **et al** 1969a). Detection of antigen by this test is easier and more reliable, than detection of antibodies. Though the test is simple to do, yet the results of the test when used for detection of antibodies, vary greatly, depending upon the antigen used and perhaps even on its concentration. It appears, therefore, that there is a need for standardization of the technique in order to get reliable and accurate results so as to be helpful in diagnosis of smallpox. This paper presents results of study using different types of antigens of variola-vaccinia group.

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Material and Methods

Sera : Sera of recently vaccinated adults, cases of smallpox, and contacts of smallpox cases were tested in this study. In the case of vaccinees, blood was collected on 14th day of vaccination. In smallpox patients, blood was collected on different days of disease, and in contacts, it was collected on different days after exposure to primary case. Sera were separated and preserved in the deep freeze at —20°C till they were tested.

Antigen : Five types of antigens were used for comparative study and they are coded as A to E.

Antigen A : Vesicular fluid of smallpox case : Fluid from several vesicles of a case of Ordinary variety of smallpox was collected.

Antigen B : Scabs of smallpox case : Several scabs were collected from a case of Ordinary variety of smallpox, after they were completely dry and the antigen was prepared as described (WHO 1969).

Antigen C : First egg passaged vesicular fluid : Vesicular fluid (antigen A) was inoculated on to CAM as per standard techniques. The membranes were harvested, ground, and made into 50 per cent normal saline suspension.

Antigen D : First egg passaged scab material : Scab material (antigen B) was inoculated on to CAM as per standard techniques. The membranes were harvested, ground and made into 50 per cent normal saline suspension.

Antigen E : First egg passaged freeze dried smallpox vaccine : Freeze dried vaccine (obtained through the courtesy of Director of King Institute for Preventive Medicine, Guindy) was reconstituted with the diluent supplied along with the vaccine and inoculated on to CAM as per standard techniques. The membranes were harvested, ground, and made to 50 per cent normal saline suspension.

Eight hundred units of penicillin and 800 microgrammes of streptomycin per one ml of antigen were added to all these antigens and they were kept frozen in the deep freeze at —20°C till they were tested.

Pig test : The technique described by WHO (1969) was employed in general as far as the procedure is concerned. In the absence of pure agar capsule, difco bacto-agar was used. For the preparation of Agar, instead of phospate buffered saline, double distilled water was used, and instead of 1 per cent agar, 2·3 per cent was used.

In one study (Table I) all the sera available from the three categories of sources were tested with the 5 different antigens and the results were presented. In another study (Table II) a specific number of sera were taken from each of the categories and were tested with different sets of antigens and the comparative results so obtained were presented.

Results

Table I shows the results of the PIG test on the sera of vaccinees, smallpox patients, and contacts of smallpox cases, using the 5 different types of antigens A to B.

Table I. Results of PIG test for detection of antibodies in sera of vaccinees smallpox patients and contacts of smallpox cases using different types of antigens of variola and vaccinia viruses

Antigen used	Nature of sera tested								
	Vaccinees'			Patients'			Contacts'		
	Number tested	Number positive	Percentage positive	Number tested	Number positive	Percentage positive	Number tested	Number positive	Percentage positive
A	82	0	0·0	101	59	58·4	126	28	22·2
B	75	0	0·0	75	33	44·0	82	25	30·4
C	97	28	35·4	73	55	75·3	80	33	41·3
D	78	27	34·6	70	44	62·8	79	30	38·0
E	74	20	27·0	92	60	65·2	124	41	33·1

None of the vaccinees' sera gave positive precipitation either with vesicular fluid (A) or scab (B) antigens, whereas 20 to 35 per cent were positive with first egg passaged variola and vaccinia antigens (C, D and E). There were greater number of positives with the former. For control, we have found that healthy uninoculated CAM did not give positive precipitation with any antibodies.

As regards the patients' sera, about 58 per cent of them were positive with vesicular fluid (A), and about 44 per cent with scab antigen (B). However, there were greater number of positives with egg passaged variola (C and D) as well as vaccinia (E) ranging from 63 per cent to 71 per cent.

Almost same was the case with sera of contacts, though there were slightly greater number of positives with scab antigen (B) than vesicular fluid (A), yet the general pattern was the same that there were greater number of positives with egg passaged virus than the plain variola antigens.

Table II shows the results of PIG test on specific sera using different antigens. It may be mentioned for clarification that the same sera were tested against 2 antigens in each experiment for comparison, as can be seen from Table II. None of the vaccinees' sera were positive with plain variola antigens A and B. On the other hand about 31 to 35 per cent were positive with egg passaged variola antigens (C and D). Relatively there were greater number of positives with egg passaged variola (C and D) than egg passaged vaccinia (E).

In the case of patients' sera, about 55 per cent of them were positive with A as against about 42 per cent with B. With egg passaged variola as well as vaccinia, the positive rates were varying between 62 and 73 per cent.

Same was the case with the sera of contacts too ; egg passaged antigen gave greater number of positives.

Discussion

Downie **et al** (1969b) studying three groups of sera of vaccinees for vaccinia antibodies have found that all were negative for PIG. On such an assumption Downie **et al** (1969c) from the results of another study on the antibody response in non-haemorrhagic smallpox cases suggested, that a positive precipitation test in agar gel in a single specimen of serum, would be suggestive of a recent smallpox infection. We, (Rao **et al** 1970) on the other hand, have shown that from our study as high as 16 per cent of the vaccinees' sera gave positive precipitation test in agar gel using vaccinia antigen and therefore the assumption of Downie and others may not be wholly correct. From the results of the same study, we have also shown that none of the vaccinees' sera gave positive precipitation test in gel with variola antigen (vesicular fluid) and therefore we suggested that this test could be utilized to distinguish variola infection from vaccinia infection. A positive precipitation in gel using variola virus as antigen in a single specimen of serum, according to us, was highly suggestive of recent smallpox infection and, this test, we said, could be utilized not only for diagnosis of acute cases of small-

Table II. Comparative results of PIG test of sera of vaccinees, smallpox patients, and contacts of smallpox cases using different sets of antigens

Serial Number of the experiment	Antigen used	Nature of sera tested								
		Vaccinees'			Patients'			Contacts'		
		Number tested	Number positive	Percentage positive	Number tested	Number positive	Percentage positive	Number tested	Number positive	Percentage positive
1	A	65	0	0·0	67	37	55·2	73	21	28·8
	B	65	0	0·0	67	28	41·8	73	15	20·5
2	A	68	0	0·0	65	36	55·4	70	19	·27·0
	C	68	24	35·3	65	48	73·4	70	27	38·6
3	A	71	0	0 0	85	49	57·6	115	23	20·0
	E	71	18	25·3	85	53	62·3	115	34	27·3
4	B	72	0	0·0	70	31	44·3	70	25	31·6
	D	72	23	31·9	70	44	62·9	70	30	37·9
5	B	57	0	0·0	70	33	47·1	79	22	27·8
	E	57	6	10·5	70	45	64·3	79	32	40·5
6	C	61	21	34·4	68	50	73·5	77	30	38·9
	E	61	8	13·1	68	44	64·7	77	32	41·5
7	D	61	21	34·4	65	41	63·1	76	29	42·0
	E	61	8	13·1	65	43	66·1	76	32	46·4

pox but also for retrospective diagnosis in completely recovered cases, as well as for detecting subclinical (in apparent) smallpox infections.

Meanwhile, Heiner **et al** (1971) have found that vaccinees' sera gave positive precipitation in gel even with variola antigen. In their studies, they have used a purified concentrated antigen prepared from variola infected cell culture. With this antigen, they were able to detect in 19 out of 25 vaccinees, precipitins in their sera and they stated that 'it is, therefore, clear that precipitins may be formed after vaccination and may persist for an, as yet, undefined period, but their detection appears to depend upon the concentration of the antigen and consequently, sensitivity of the test'.

From the results of the present study of ours, we have also found that nearly 35 per cent of the vaccinees' sera gave positive precipitation with variola antigen prepared from variola infected chorioallantois whereas none of the sera gave positive precipitation with unpassaged vesicular fluid or scab antigen. Of course, we admit by passing through CAM, the concentration of antigen increases. Heiner further stated 'in Rao's study (Rao **et al** 1970) the use of pooled vesicular fluid apparently provided an antigen of relatively low potency and they allowed a quantitative distinction to be made between the reaction of smallpox cases including subclinical cases and those of vaccinia. In the present study in which a more concentrated antigen was used, no distinction could be made in individual case between the residual post-vaccinial precipitins and those to recent variola stimulus'.

While agreeing with Heiner and his associates that the concentration of antigen may play some role and influence the results of PIG test, it is difficult to understand why the vesicular fluid of smallpox cases, which we used, and which according to Heiner 'apparently provided an antigen of relatively low potency', gave a high proportion of positive results with sera of smallpox patients which are known to contain far higher concentration of antibodies than vaccinees. Further the results of PIG test, using vesicular fluid and vaccinia antigen with sera of smallpox patients, were more are less identical. There appears to be no reason, therefore to attribute the difference in the results, we had with different antigens, to the concentration of the antigen in the vesicular fluid alone. Instead, as we have suggested already in our earlier paper, antibodies formed as a result of vaccinia infection, may not precipitate variola antigen, whereas antibodies formed as a result of variola infection precipitate both variola as well as vaccinia antigen.

From the results of this present study of ours as well as that of Heiner and his associates, it is apparent that an antigen, prepared from either variola-infected chorioallantois, or variola infected cell culture, behaves almost in the same manner as vaccinia antigen as far as this antigen-antibody reaction is concerned. Apart from this observation of difference in the behaviour of this antigen between the unpassaged and cell-passaged, which requires a further detailed study, what we are at present most interested in, is what antigen has to be used, so as to see that we do not get false positives, in serological diagnosis of smallpox by PIG test retrospectively in those who have recently recovered from an eruptive fever and for detecting subclinical (inapparent) infections among the exposed contacts of smallpox cases.

If a person has never been vaccinated, or has not been recently successfully vaccinated, it is immaterial which antigen is used. Vaccinia or variola, either cell passaged or unpassaged. It is highly suggestive of a recent infection with variola, if serum of any such person gives a positive precipitation in gel with any of these antigens. But the same does not appear to be true in the case of persons who have evidence of a recent (within 3 months) successful vaccination. In such persons, only an unpassaged variola antigen (smallpox vesicular fluid or smallpox scabs) can detect infection with variola. It is diagnostic of recent infection with variola, if serum of any such persons gives a positive precipitation in gel either with vesicular fluid or scabs of smallpox case. The cell passaged variola antigen or vaccinia antigen, if used is likely to give false positives in such persons in view of the fact that they have been recently revaccinated. In all tests of course, a negative finding is absolutely of no significance.

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