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IMMUNOLOGICAL STUDIES IN SMALLPOX.

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IMMUNIZATION against smallpox by calf-lymph vaccine has been the best available prophylaxis in medical science since Jenner established its value in 1798, and there is no doubt that by this single measure it has been possible to eradicate the disease from many parts of the world. Nevertheless, from the experience of many recent outbreaks reported from time to time, e.g., the 1942 Glasgow outbreak (MacGregor and Peters, 1942), the outbreak on a New Zealand Hospital ship (D'Arcy, Moore and Whetter, 1943 ; Smith, 1943), the outbreak in the British Army Unit in India (Coleman, 1944), a small outbreak in U.K. (Jeans *et al.*, 1944) and the Kweilin outbreak in Kwangsi, China (Su, 1944), and from the field experience of several outbreaks investigated in India by the senior author and his staff, it appears that the knowledge about the level and character of immunity following the disease, or vaccination or revaccination is not yet complete. These workers quoted many cases that occurred in spite of supposedly successful vaccination and revaccination at different intervals of time prior to the attack, and stressed the need for further studies.

There are several field and experimental works in support of the above observations. Kii (1926), Kasai (1926), Horgan and Haseeb (1944) reported that the lymphs of borderline potency (League of Nations, 1928) completely failed to give reactions and even potent lymphs produced varying results in both animals and human volunteers.

There is still some confusion regarding the types of reaction after revaccination. Ellis and Boynton (1939) in connection with their study of the comparative immunizing potency of three types of vaccines found immune reactions in the non-immune group. Previously, Leake (1927) had remarked "the fact that the reactions otherwise indistinguishable from reactions of immunity may be given by heated vaccine indicate that at least a part of the visible phenomenon which we call reaction of immunity is due to the inert material and to that extent may be called a reaction of sensitivity." Even in some primary takes an early reaction may be seen, particularly if there was a previous unsuccessful attempt at vaccination. Moreover, a negative response to vaccine virus does not always mean immunity, for there are cases which are refractory to repeated vaccination but otherwise susceptible. Sir William Osler who never had a successful take contracted the disease (a mild attack), while working in smallpox wards in 1875. He, therefore, always cited his own case to illustrate the fallacy of 'no take' as an evidence of immunity.

There is also another belief that complete life-long protection against the disease is obtained after recovery from an attack of smallpox. But there are many instances

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in which smallpox and even deaths occurred in persons who were pock-marked, by previous attacks and many of them following primary or revaccination suddenly came down with primary type of reaction. Again, it has been observed that if the revaccination is performed at or near the same spot every year the reaction may be falsely negative due to local immunity, although in reality the individual may be susceptible to smallpox (Horgan and Haseeb, *loc. cit.*).

Some people are, however, of the opinion that revaccination irrespective of the type of reaction raises the immunity level (Blaxall, 1930 ; Horgan and Haseeb, *loc. cit.*). This assumption is neither supported by critical field observation nor by experimental results mentioned above. Moreover, it is a common experience that many persons who get revaccinated every year or fairly frequently and show a negative or the so-called immune reaction on the first, second or third occasion give positive reaction (primary take) on the second, third or fourth occasion (Smith, *loc. cit.*). The senior author made an extensive review on the subject in 1945 (Seal, 1945). From these observations he was strongly inclined to the view that immunity in smallpox is relative, and that, not infrequently, it is in some manner dependent upon race, age, sex, state of nutrition and genetic background of the host and upon the climatic conditions, etc., and that in certain cases the period of absolute protection may be even short.

It, therefore, raises the question whether revaccination ending in negative or the so-called immune reaction actually reinforces the immunity. Since in immunization against bacterial diseases by means of killed vaccines revaccination always pushes up the immunity level (booster action), it may be a general belief that smallpox revaccination must behave in the same manner. But after reviewing the situation in 1945 the senior author advanced the following hypothesis :

"...there is little or no rise of immunity in the immune reactors after revaccination ; an immunity or negative reaction simply indicates that the residual immunity in such persons is above certain critical level, not yet defined, as in the case of the Schick test in diphtheria or Dick test in scarlet fever. So long as this level of immunity does not fall below the critical level revaccination or for that matter vaccination does not take i.e., it gives negative or so-called immune reaction. Thus, it is possible that these people who are practically in the borderline may soon lose their immunity and become susceptible to the infection. The vaccinoid reactors may accordingly either be borderline cases or just above the borderline and are, therefore, nearly or as much susceptible to smallpox as the group belonging to the primary takes prior to vaccination."

An objective proof is, therefore, required for the above hypothesis to adequately explain the various observations referred to above. The presence of humoral antibodies, e.g., precipitins, agglutinins, complement-fixing and neutralizing antibodies, etc., according to Parker and Rivers (1936) failed to explain the nature and duration of vaccinial immunity which is sometimes enduring and sometimes variable or even short-lived. There are, therefore, sufficient reasons for carrying out some extensive and critical studies of the available serological methods along with fresh attempts at exploration of the newer fields of attack, such as, haemaggutination-inhibition test, neutralization technique

in developing chick embryo and in animal, etc. The present study was, therefore, undertaken to elicit three important issues in the first instance, namely :

(1) the duration of passive immunity in infants born of mothers vaccinated at different intervals of time before delivery ;

(2) the average level and duration of immunity after primary vaccination ; and

(3) the threshold level of immunity at which the revaccination yields reaction of immunity, negative or vaccinoid reaction respectively.

MATERIALS AND METHODS.

The study included (1) field observations, (2) smallpox patients in hospitals, (3) collection of cord and infant blood from hospital, (4) laboratory tests, and (5) field-cum-laboratory investigations.

(1) *Field observations* :

(*a*) *Intra-epidemic investigation.*—An intra-epidemic investigation was carried out by house to house visits to collect the information regarding history, primary and secondary cases in the family, age, sex, vaccination records and interval between vaccination or revaccination and attack. It involved 391 individuals including 132 smallpox cases.

(*b*) *Follow-up study.*—A follow-up study of 206 persons including school children was undertaken by collection of blood samples before and 4 weeks after primary or revaccination for laboratory tests, and by recording the results of revaccination. To get a group of people under observation for 4 weeks convicts in a jail were taken as a control group and also a group of patients admitted in the Police Hospital, Calcutta.

(2) *Study of smallpox cases in hospital.*—One hundred and nineteen cases of smallpox admitted to the Infectious Diseases Ward of Nilratan Sircar Hospital, Calcutta, were investigated. In these cases information was collected about their vaccination or revaccination history with results and the interval between the last vaccination and the attack. Vesicular fluids and scabs were collected from these patients for examination and for growing the virus in chorio-allantoic membrane of the living chick embryo.

(3) *Collection of samples of cord, infant and children's blood from Maternity Hospital.*—Altogether 23 samples of cord blood with mother's vaccination history and 7 samples from the corresponding babies were collected from a maternity hospital, followed by collection of samples from the same infants after primary vaccination. In addition to this, samples of blood were collected before and 4 weeks after primary vaccination from a group of 51 children belonging to these localities namely, Entally area of Calcutta, Serampore town and a jute mill in Howrah. All the samples were subjected to haemagglutination-inhibition tests to estimate the titres of the sera collected.

(4) *Laboratory tests* :

(*i*) *Neutralization test on rabbit's back.*—The neutralization test was carried out on rabbit's back according to the standard method. It was abandoned for two main reasons, namely, (1) irregular results, and (2) high cost of animals.

(*ii*) *Neutralization test in embryonic chicken eggs* :

(*a*) *Method of culture and of collection of specimens from embryonic chicken eggs.*—Fresh and fertilized eggs marked with date of laying were purchased from the Government Poultry Farm and incubated in an Egg Incubator at 102°F. for 11 days. After confirming that the embryo was living the chorio-allantoic membrane was inoculated with 0·1 mg. of inoculum or 0·2 ml. of serum-virus mixture according to the standard method by means of 1 ml. pipette. The triangular opening and the slit over the air sac were then closed with cellophane tape and the egg was sharply rotated to spread the inoculum and then placed in the incubator at 101°F. On the 4th day, the chorio-allantoic membrane adhering to the shell membrane underneath the triangular opening was removed with forceps and scissors and kept in a sterile Petri dish and washed clean with sterile normal saline and transferred to another Petri dish containing clean sterile normal saline and later preserved in 50 per cent glycerine-saline in the refrigerator (Plate LXIII).

(*b*) *Infecting or lethal dose of virus.*—Blood sera were collected from persons before and 4 weeks after vaccination. The vaccine lymph obtained from the Vaccine Institute of West Bengal and preserved in the cold was diluted to the strengths of 1/10, 1/100, 1/1000 etc. at the time of the test or the virus dilutions were made from the infected chorio-allantoic membrane preserved in the freezer. In the latter case, the membrane was thawed and weighed in tared container and grounded in a mortar into a smooth paste. To 1 part of this were added 9 parts of diluents and mixed thoroughly and centrifuged at 2,500 r.p.m. for 20 minutes. The supernatant was removed to another container and the sediment preserved in the refrigerator. Adequate quantities of ten-fold serial dilution from 10^{-2} to 10^{-6} were made as in the case of rabbit skin test. The ID_{50} for both these virus dilutions was estimated as follows :

For each dilution 5 eggs were inoculated and incubated up to 5 days. The eggs were candled every 24 hours up to the 5th day. Embryo mortality within the first 24 hours was taken as due to non-specific cause. The total number of mortality out of 5 eggs for each dilution was recorded, and the egg membrane removed and pock marks counted under a dark background and the average count for each dilution noted. The dilution that caused 50 per cent mortality was calculated by the method of Reed and Muench (1938) and the number of infecting doses in ID_{50} or LD_{50} calculated by taking the reciprocal of the dilution and by consulting the log and antilog tables. The sample protocol is given below :

Showing estimation of LD_{50} (lethal dose) of ID_{50} (infecting dose)

Dilution of virus.	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Preventive reaction (mortality rate)	5/5	5/5	5/5	5/5	3/5	0/5	0/5	0/5

50 per cent end-point calculated according to Reed and Muench = $10^{-5\cdot2}$.
Antilog of reciprocal of 50 per cent end-point = 1,58,500.
Titre of LD_{50} per 0·1 ml. = $1\cdot585 \times 10^5$.
Titre of LD_{50} per 1·0 ml. = $1\cdot585 \times 10^6$.

Alternate method.—The average number of pocks or foci given by the highest dilution was counted. This number multiplied by the reciprocal of the dilution represented the number of ID's in the original sample. For example, if 0·1 ml. of 10^{-5} dilution of the virus produced an average of 1·5 foci, the ID per 0·1 ml. would be $1{\cdot}5 \times 10^{-5}$ or per ml. $1{\cdot}5 \times 10^{-6}$. This method being easier and less complicated was preferred to the first method and was found good enough for comparative purposes.

Neutralization test procedures.—

(*a*) *Decreasing virus—constant serum method.*—Vaccine lymph of which ID_{50} or LD_{50} had been determined either by the mortality rate of chick embryo or by pock count method as above, was used for neutralization test in dilutions of 10^{-1} to 10^{-5} against a constant serum quantity. To each virus dilution of 0·4 ml. an equal amount of undiluted serum was mixed thoroughly and incubated at room temperature for ½ hour and 0·2 ml. of each mixture was inoculated into 3 eggs and the average pock count at the lowest dilution of the virus (*i.e.*, the highest quantity) giving complete neutralization (complete absence of pock marks) was noted. The pock counts in one step lower down would have given a more accurate neutralization point but the complete neutralization point being more important from the point of view of preventing an infection than the partial one, the latter was utilized for the purpose of the present investigation. The protocol of the test is given below :

Showing neutralization test in chorio-allantoic membrane of chicken eggs by decreasing virus—constant serum method.

	Virus dilution—0·1 ml.					ID per 0·1 ml. of original.	NI dose per 0·1 ml.	Neutralizing dose, per ml.
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}			
Lymph virus	+ + +	+ + +	+ +	14	15	15000	—	$1{\cdot}5 \times 10^{-5}$
Pre-infection serum	+ + +	+ + +	+ +	13	14	—	2	20
Convalescent serum	—	0	0	0	0	—	14980	$1{\cdot}498 \times 10^{-5}$

N.B.—The average of pock counts of 3 eggs was taken.

Neutralization titre of the convalescent serum

per 0·1 ml. = 15000 —20 = 14980

per 1·0 ml. = $1{\cdot}498 \times 10^{-5}$.

(*b*) *Constant virus—decreasing serum method.*—The quantum of infecting dose based on pock count method was first estimated in a sample of vaccine lymph ; 0·4 ml. of 1/100 dilution of this lymph was used as the constant virus dilution against the decreasing dilutions of the test serum e.g., 1/5, 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, and 1/640, separate pipette being used for each dilution. The mixture was incubated for 30 minutes at room temperature and the chorio-allantoic membranes of three eggs were each inoculated with 0·2 ml. of the mixture (0·1 ml. of virus dilution + 0·1 ml. of serum dilution). The highest dilution of serum at which no growth had been noted was taken as the

neutralizing dose, calculated as follows :

If the neutralizing point was at the level of 1/160 dilution of the serum the neutralizing dose per 0·1 ml. of the serum = 1500 × 160 and per 1·0 ml. = 1500 × 1600 = $2{\cdot}4 \times 10^6$.

(0·1 ml. of 1/100 virus dilution contained 1500 infecting dose)

(*iii*) *Haemagglutination-inhibition test* :

1. *Reagents* :

(*a*) *Virus-antigen suspension.*—The antigen was prepared by inoculating the dropped chorio-allantoic membrane of 11 to 13 days old embryonated hen's egg with 0·2 ml. of a 1 : 100 to 1 : 1,000 dilution of calf-lymph. Eggs were incubated for 3 days at 37°C. and the chorio-allantoic membrane with the virus growth were harvested, pooled and grounded in a mortar and diluted to 20 per cent suspension by weight in normal saline. The suspension was then centrifuged at 1,500 r.p.m. for 10 minutes and the supernatant fluid was removed as the stock virus suspension and kept in the cold room at 4°C.

(*b*) *Chicken cell suspension.*—Only red cells of certain adult chickens being agglutinable by the variola-vaccinia virus group appropriate donors were selected by testing with 0·5 ml. of known virus suspension or with 0·5 ml. of cardiolipin microflocculation antigen in dilution of 1 : 10,000. Properly washed chicken cells agglutinated by at least 1/32 dilution of 20 per cent chorio-allantoic membrane (CAM) suspension (0·25 ml. of 0·5 per cent cell suspension *plus* 0·5 ml. virus suspension) were considered appropriate.

(*c*) *Sera.*—Sera were diluted 1 : 5 in 0·85 per cent salt solution and incubated at 36°C. for 30 minutes. Hyper-immune and normal rabbit sera were used for positive and negative controls respectively.

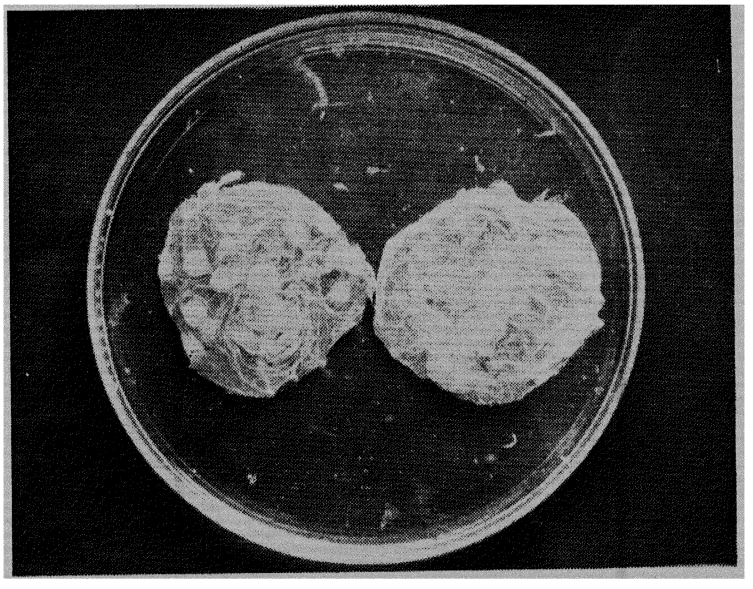
2. *Haemagglutination titre* (*HA*).—Haemagglutination titre of the CAM test virus suspension was initially determined according to the standard procedure. A titre of 1 : 640 or better 1 : 1280 was preferable for H-I test. One HA unit was defined as the least amount of antigen in 0·25 ml. that produced a definite agglutination indicated by a layer of uniformly agglutinated cells on the bottom of the tube, while the control showed a sharply compact disc of sedimented cells in the centre of the bottom of the tube. Haemagglutination intermediate between positive and negative patterns might occur in the range of minimal viral activity (Text-fig. 1). The highest dilution of the virus (before addition of serum and cells) was taken as the end-point of positive HA activity. The reciprocal of this end-point was taken as the virus titre of HA units contained in the undiluted sample of the virus suspension.

3. *Haemagglutination-inhibition test procedures* :

(*a*) Decreasing virus—Constant serum method (Alpha procedure)—not done.

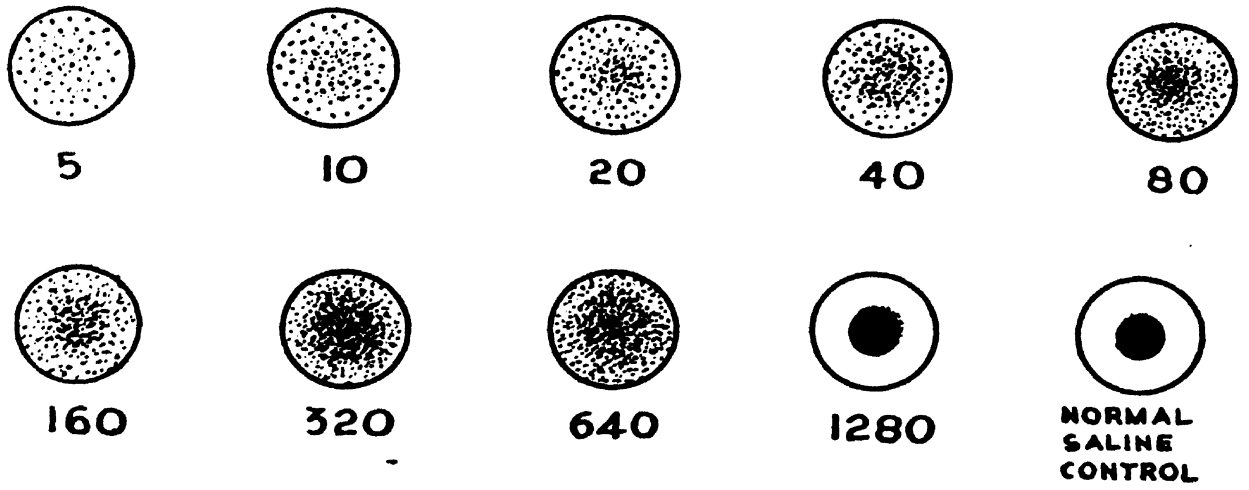
(*b*) Constant virus—Decreasing serum method (Beta procedure).

Two agglutinating units of previously titrated virus antigen suspension contained in 0·25 ml. was added to a row of ten tubes. In a parallel row of tubes two-fold serial dilutions of the test serum in saline were added starting from 1 : 5 dilution; 0·25 ml. of each dilution of serum was added to its parallel virus tube and then 0·25 ml. of 0·5



Chorio-allantoic cultivation of vaccinia virus showing pock vesicles in the membranes on petri dish.

TEXT-FIGURE 1.

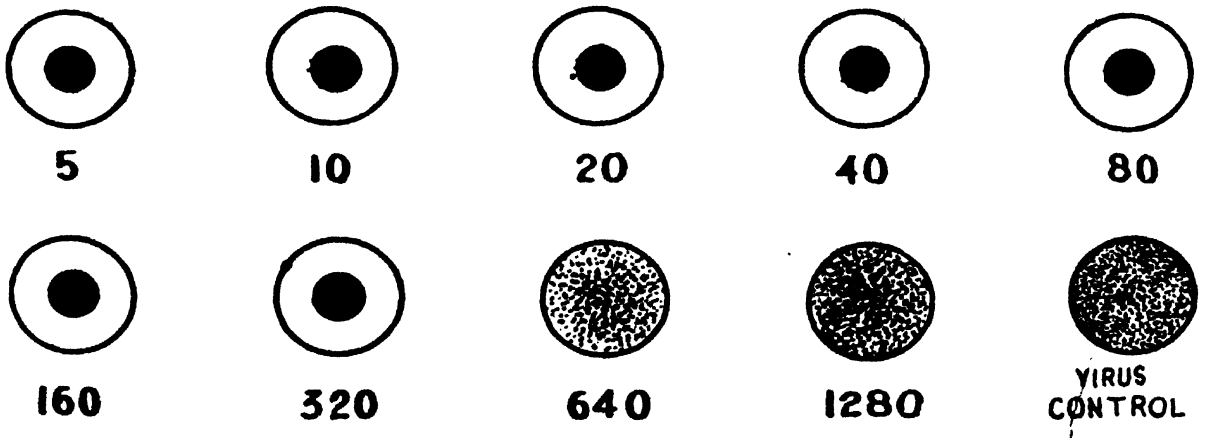


Hemagglutination test (HA) virus titration dilution of virus.

per cent chicken red cells suspension was added to each tube and the ingredients mixed by shaking the rack and then incubated for 30 minutes at 37°C. or till the cells were well settled.

Concurrently, haemagglutination test with the last three dilutions of virus giving positive haemagglutination and another tube for cell control with normal saline were set up. The end-point of the inhibitory activity of the serum was the highest dilution of serum at which haemagglutination was completely inhibited (Text-fig. 2). The HI titre of the serum was computed for the beta procedure by multiplying the reciprocal of the end-point of the serum by the number of HA units used in the test. The protocol

TEXT-FIGURE 2.



Hemagglutination-inhibition test (HI) serum titration-beta procedure dilution of serum.

of the test and the formula used for calculation of the end titre are given below :

Protocol of haemagglutination-inhibition test by constant virus-decreasing serum method.

	Serum dilution :									Virus control.	Cell control.
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560		
0·25 ml. serum	1	1	1	1	1	1	1	1	1	—	—
0·25 ml. of virus (2 units)	1	1	1	1	1	1	1	1	1	1	—
0·25 ml. of 0.5 per cent red cells	1	1	1	1	1	1	1	1	1	1	1
0·25 ml. of 0·85 per cent saline	—	—	—	—	—	—	—	—	—	—	1
Result	—	—	—	—	—	—	+	+	+	+	—

1 = 0·25 ml. containing 1 unit ; — = no agglutination ; + = haemagglutination.
The titre of the serum is 320 × 2 (HA units) = 640 Hl. units.

(5) *Field-cum-laboratory tests.*—After the laboratory tests were standardized samples of blood were collected from different sources and situations in the field along with the history of vaccination and revaccination. In view of the high cost involved in the purchase of animals and fertilized eggs, the actual laboratory tests were carried out in a part of the samples so collected. The results are noted and discussed in the following sections.

Results.

1. *Intra-epidemic investigations* :

(*a*) *Differences of incidence and fatality rates among the primary, revaccinated and unvaccinated groups.*—In the investigation of a smallpox outbreak in a rural area the differences of incidence and fatality rates noted between the primary and the revaccinated and between the vaccinated and the unvaccinated groups are given in Table I.

Table I.

Incidence and fatality rates of smallpox cases in the primary, revaccinated and unvaccinated groups.

Groups.	Number of persons.	Per cent of total.	Number of cases.	Incidence rates (per cent).	Number died.	Fatality rate.
Primary vaccinated	194	49·6	73	37·6	13	17·8
Revaccinated	156	39·9	30	19·2	2	6·7
Total vaccinated	350	89·5	103	29·4	15	14·6
Unvaccinated	41*	10·5	29	70·7	11	38·0
Total	391	..	132	33·8	25	19·0

* As many as 26 cases were below 7 years of age constituting 31·7 per cent of the population of 0-7 years.

In the epidemic concerned, there were 132 cases of smallpox among 391 individuals of the affected families giving an incidence rate of 33·8 per cent. Of them 49·6 per cent were only primary vaccinated and 39·9 per cent revaccinated (i.e., 89·5 per cent total vaccinated) and 10·5 per cent unvaccinated, as confirmed by inspection of pock marks in the subject concerned. The relative incidence rates among the vaccinated and the unvaccinated were 29·4 and 70·7 per cent and those among the revaccinated and the primary vaccinated groups were 19·2 and 37·6 per cent, respectively. The total fatality rate was 19·0 per cent but it was 14·0 per cent among the vaccinated (6·7 per cent in the revaccinated and 17·8 per cent in the primary vaccinated group) and 38·0 per cent in the unvaccinated group, giving an advantage of 2·6 times to the total vaccinated, 2·1 times to the primary vaccinated and 5·7 times to the revaccinated group.

Types of reactions noted after primary and revaccination.—The record shows that out of 391 persons 345 or 88·0 per cent were successfully vaccinated at least once, 5 or 1·3 per cent were unsuccessfully vaccinated and 10·5 per cent remained unvaccinated. Yet a large proportion (33·8 per cent) got the attack. The fact of this susceptibility was supported by the results of revaccination* at different intervals of time, 75 out of 156 revaccinated or 48 per cent giving primary type of reaction (Table II). Again, among the 30 cases of smallpox in the revaccinated group as many as 20 including two deaths gave negative or so-called immune reaction at different intervals of time prior to the attack, and 10 had positive takes, 7 of which, however, fell during the incubation period, and the remaining three gave vaccinoid reaction within less than 2 years before the attack, also indicating partial susceptibility (Table II). Thus, those who gave negative or immune reaction did not apparently give protection against the disease during an epidemic. But the redeeming feature was that none of the revaccinated persons who had positive takes including those revaccinated during the incubation period, died.

(*b*) *Interval between attack and successful or unsuccessful vaccination and revaccination.*—The intervals between attack and successful or unsuccessful vaccination and revaccination are given in Table II.

From column 2 of Table II it will be apparent that there was a progressive loss of immunity against smallpox with the increase of length of time after primary vaccination, particularly if the period exceeded 15 years, but there were certain persons who lost their immunity within even one year, and a few others within 2 years and 2 to 4 years. In this community all the 5 persons (1·4 per cent) who out of 350 vaccinated at least once, did not respond to primary vaccination, suffered from the disease. But the more important point which has emerged from this investigation was the uncertainty about the results of revaccination (columns 4 and 5). Apart from those who were revaccinated during the incubation period 22 cases who had given immune reaction, including three giving vaccinoid reaction, some within one month and others within 1 to 2 years, came down

*The batch of cowpox lymph vaccine obtained from the West Bengal Vaccine Institute that gave at least 10 pock marks in rabbit with 0·2 ml. of 1:10000 dilution was used in this investigation, but the potency of the vaccine used in the initial primary or for revaccination is not known, except that it was a lymph vaccine supplied by the above Institute.

TABLE II.

Intervals between attack and successful and unsuccessful vaccination and revaccination (103 cases with 15 deaths)

Interval.	PRIMARY VACCINATED GROUP :		REVACCINATED GROUP :		Total.
	Successful.	Unsuccessful.	Successful.	Unsuccessful.	
Incubation period	3	1	7	3	14
Less than 1 month	..	2 (1)	1*	2	5 (1)
Less than 1 year	1	..	..	7	8
1—2 years	3 (1)	1	2*	3 (1)	9 (2)
2—4 years	5	..	..	1	6
4—8 years	4	..	..	1 (1)	5 (1)
8—15 years	6	..	..	1	7
15—25 years	16 (4)	..	..	..	16 (4)
25 years and above	30 (7)	1	..	..	31 (7)
Not known	..	..	..	2	2
Total	68 (12)	5 (1)	10	20 (2)	103 (15)

* Vaccinoid reaction.

N.B. : The figures in the prenthesis are the number of deaths.

with smallpox. There was thus sufficient indication for a quantitative study of the actual levels of immunity which would ensure durable protection and the level at which negative or immune reaction is obtained.

(*c*) *Duration of immunity.*—From the preceding records, it became apparent that some persons lost their immunity within a short period of 1 to 2 years, others between 2 and 4 years after primary vaccination. To find out whether this is corroborated by the revaccination results among those who escaped the attack the data were further analysed and the results are given in Table III.

The records of 16 revaccinations among 76 non-cases upto 9 years of age in the present study show that the minimum duration was 2 years in 2 instances, 3 years in 5 instances, 5 years in 2 instances and 6 years in 4 others. These revaccinations, however, depended upon the available opportunity. It is not known what would have been the response if all of them were revaccinated within 3 years after primary vaccination. Similar results were also obtained by Bhattacharji (1956-57), the then Associate of the senior author, among 1,610 persons examined in an urban community field. Out of 94 smallpox cases 51 (54·3 per cent) occurred among the vaccinated persons, and out of the latter 33 or 64·7 per cent among persons who had been vaccinated or revaccinated within 3 years prior to the attack. Such a high percentage of attack among the vaccinated and revaccinated persons demanded an intensive study of the situation.

(*d*) *Second attack of smallpox.*—In the present epidemic, three instances of second attack of smallpox were recorded—one was aged 15 years who had the first

TABLE III.

Minimum duration of immunity as shown by the positive takes after revaccination in 0-3 years age group.

Age in years.	Age at primary vaccination.	Revaccinated at intervals of (years).	Observed reaction.
3	1	2	vaccinoid
8	6	2	vaccinoid
4	1	3	primary
5	2	3	primary
5	2	3	primary
5	2	3	primary
7	4	3	primary
5	1	4	primary
5	1	4	primary
6	2	4	primary
7	2	5	primary
7	2	5	primary
8	2	6	primary
8	2	6	primary
8	2	6	primary
9	3	6	primary

attack at the age of 5 years, the second was aged 22 years having had the first attack at the age of 1 year and the third case was aged 35 years with history of first attack at the age of 6 years.

(*e*) *Vaccination history of smallpox cases admitted to hospital in 1957.*—The intervals between the attack of smallpox and the last vaccination in 94 smallpox cases, with history of previous vaccination or revaccination, at the N.R.S. Hospital, Calcutta, as elicited from their histories, were as follows : Sixty-two cases had reaction of immunity —6 cases within 4 weeks, 1 within 1 year, 5 within 3 years, and 50 cases over 3 years ; 12 cases had history of vaccinoid reaction—one within 4 weeks, 4 within 3 years and 7 over 3 years, while 20 cases gave history of typical primary take (corroborated by checking pock marks in most cases) more than 3 years prior to the attack. There were, however, 25 other cases who had no primary vaccination.

(*f*) *Experimental revaccination of 206 persons.*—The results obtained from the field investigation described above included information generally given by the persons concerned or by the elderly relatives. It was, therefore, thought necessary to personally carry out the revaccination work with the West Bengal Vaccine Institute lymph, as mentioned earlier, to verify the results noted above. With this objective in view a contingent of 206 persons, largely school children, were revaccinated at two points and the results are noted in Table IV.

It will be seen from Table IV, that out of 206 individuals 18·0 per cent had primary type of reaction, 30·1 per cent vaccinoid type and 51·9 per cent reaction of immunity,

TABLE IV.

Reactions observed after revaccination of school children and some adults.

Interval between previous and revaccination.	Number of persons revaccinated.	TYPICAL PRIMARY :		VACCINOID TYPE :		REACTION OF IMMUNITY :	
		Number.	Per cent.	Number.	Per cent.	Number.	Per cent.
Less than 2 years	75	16	21·3	14	18·6	45	60·0
Less than 3 years	52	7	13·4	18	34·6	27	50·9
Less than 4 years	19	3	15·8	6	31·5	10	52·7
Less than 5 years	23	4	17·4	9	39·1	10	43·5
Over 5 years	37	7	19·6	5	40·5	15	40·5
Total	206	37	18·0	62	30·1	107	51·9

and that within 2 years 21·3 per cent had lost full immunity and 18·6 per cent partial immunity, i.e., as high as 40 per cent became fully or partially susceptible within 2 years of previous vaccination. Similarly, immunity was fully or partially lost by 49·1 per cent within 3 years, 47·3 per cent within 4 years, 56·5 per cent within 5 years and 59·5 per cent over 5 years. Besides, one person in the group who had smallpox at the age of 3 years and had given reaction of immunity 2 years earlier came down with a positive take at the age of 45 years. Another person who had smallpox at the age of 7 years and gave negative reaction when vaccinated at the age of 15 and 26 years had positive take at the age of 30 years. A third individual who had shown reaction of immunity only 2 years earlier had typical primary take on revaccination this time. These observations show that there is no certainty about the duration of immunity nor it can be predicted when it is likely to go down making him susceptible to the infection of smallpox.

2. *Field-cum-laboratory investigations* :

(*i*) *Neutralizing test on rabbit's back.*—In a preliminary test 12 rabbits were set up according to the standard procedure for testing three human sera collected 4 weeks after primary vaccination, against one rabbit convalescent serum, one normal rabbit serum and one virus control, using 2 rabbits for each test. As the method did not give consistent result for calculating the neutralizing dose with any reliability the experiment was discontinued. Besides the test requires a large number of animals involving heavy expenditure.

(*ii*) *Neutralizing test in embryonic chicken eggs* :

(*a*) *Decreasing virus—constant serum method.*—A series of 16 sera collected from persons before and 4 weeks after revaccination were tested according to the decreasing virus—constant serum method, described earlier, the quantity of serum used being 0·1 ml. per virus dilution. The maximum concentration of virus used in the present test was 1 in 10, i.e., containing $1{\cdot}5 \times 10^5$. The results are given in Table V which show that the primary type of reaction is given by those individuals whose neutralizing capacity of their

TABLE V.

Results of neutralization tests on embryonic chicken eggs of sera obtained before and after revaccination by decreasing virus—constant serum method.

Serial number.	Age in years.	Previous history of vaccination.	Reaction of revaccination.	NEUTRALIZATION TITRE OF SERA PER ml. :		Titre increased by
				Before revaccination.	4 weeks after revaccination.	
1*	25	Every year—no reaction	Immunity	$1{\cdot}5\times10^{4}$	$1{\cdot}5\times10^{4}$	No increase
2*	29	1 year—no reaction	Immunity	$1{\cdot}5\times10^{4}$	$1{\cdot}5\times10^{4}$	No increase
3*	30	1 year—no reaction	Immunity	$1{\cdot}5\times10^{5}$	$1{\cdot}5\times10^{5}$	No increase
4*	34	1 year—no reaction	Immunity	$1{\cdot}5\times10^{5}$	$1{\cdot}5\times10^{5}$	No increase
5	23	Every year—no reaction	Immunity	$1{\cdot}5\times10^{3}$	$1{\cdot}5\times10^{4}$	10 times
6	20	2 years—doubtful reaction	Vaccinoid	$1{\cdot}5\times10^{3}$	$8{\cdot}25\times10^{4}$	55 times
7	28	4 years—no reaction	Vaccinoid	$1{\cdot}5\times10^{3}$	$8{\cdot}25\times10^{4}$	55 times
8	29	1 year—no reaction	Vaccinoid	$1{\cdot}5\times10^{3}$	$8{\cdot}25\times10^{4}$	55 times
9	30	1 year—no reaction	Vaccinoid	$1{\cdot}5\times10^{3}$	$8{\cdot}25\times10^{4}$	55 times
10	37	2 years—no reaction	Vaccinoid	$1{\cdot}5\times10^{2}$	$1{\cdot}5\times10^{4}$	100 times
11	38	3 years—doubtful reaction	Vaccinoid	$1{\cdot}5\times10^{3}$	$1{\cdot}5\times10^{5}$	100 times
12	28	1 year—doubtful reaction	Primary take	$1{\cdot}5\times10^{2}$	$1{\cdot}5\times10^{5}$	1000 times
13	22	5 years—no reaction	Primary take	$1{\cdot}5\times10^{1}$	$1{\cdot}5\times10^{5}$	10000 times
14*	29	7 years—no reaction	Primary take	$1{\cdot}5\times10^{1}$	$1{\cdot}5\times10^{5}$	10000 times
15	25	3 years—no reaction	Primary take	$1{\cdot}5\times10^{1}$	$1{\cdot}5\times10^{5}$	10000 times
16*	20	4 years—no reaction	Primary take	$1{\cdot}5\times10^{1}$	$1{\cdot}5\times10^{5}$	10000 times

* Sera marked with asteriks are common with the sera Nos. 1, 2, 3, 4, 19 and 22 of Table VI.

sera fell below $1 \cdot 5 \times 10^2$ or less than 150 pock count units. In four of the 5 sera which were collected from persons giving primary type of reaction the neutralization titre per ml. was $1 \cdot 5 \times 10^1$ or 15 pock count units or less, and one serum which gave doubtful reaction 1 year earlier showed a neutralization titre of $1 \cdot 5 \times 10^2$ or 150 pock count units. This is a borderline case. In the other four the record of the last unsuccessful vaccination ranged between 3 and 7 years and their age varied between 20 and 29 years. The titres of the sera after primary vaccination reached the level of $1 \cdot 5 \times 10^5$ or 150,000 pock count units, an increase of 10,000 times. The titre of borderline case, however, increased by 1000 times.

The neutralizing titres of 6 individuals who gave vaccinoid reaction ranged between $1 \cdot 5 \times 10^2$ and $1 \cdot 5 \times 10^3$ or 150 to 1500 pock count units per ml. before revaccination, the intervals between their last unsuccessful vaccination and the present one varying between 1 to 4 years. The titres of the sera after revaccination reached the level between $1 \cdot 5 \times 10^4$ and $1 \cdot 5 \times 10^5$, 4 of them giving a titre of $8 \cdot 25 \times 10^4$ the increase varying between 55 and 100 times depending on the original titre. The neutralization titres of sera of 5 persons who gave reaction of immunity or immediate reaction before revaccination ranged between $1 \cdot 5 \times 10^3$ and $1 \cdot 5 \times 10^5$ and there was no rise of titre due to revaccination except 10 times in a particular case (No. 5) that had a basic titre of $1 \cdot 5 \times 10^3$. This was the titre which generally gave vaccinoid reaction (cases 6 to 11). In other words, case No. 5 may be an example of a borderline case which though giving a reaction of immunity or no reaction is likely to lose the residual immunity within a comparatively short time and if exposed to heavy infection, as in an epidemic, would run the risk of an attack of smallpox.

Another important finding was that those having neutralization titre of $1 \cdot 5 \times 10^4$ or $1 \cdot 5 \times 10^5$ did not show any increase in their immunity levels after revaccination. Thus the rise of titre was 1000 to 10,000 times in case of primary type of reaction and 55 to 100 times in case of vaccinoid type. In the latter case the level of immunity reached is also less than in fully susceptible persons. In sum, the rise of titre of immunity was inversely proportional to the existing level of immunity or directly proportional to the degree of susceptibility.

(*b*) *Constant virus—decreasing serum method.*—A series of 24 sera collected from persons before and 4 weeks after revaccination were tested according to the constant virus—decreasing serum method, described earlier, the virus dilution used being 1/100 containing $1 \cdot 5 \times 10^3$ pock count units (Table VI). These included six sera numbering 1, 2, 3, 4, 19 and 22 in Table VI corresponding to numbers 4, 3, 1, 2, 16 and 14 of Table V. There were seven sera from persons who had given no reaction one year earlier, maintained the same reaction and showed no increase in the titre of immunity after fresh revaccination, except in one case (No. 7) which recorded a 4-fold rise only. This was perhaps a borderline case like case No. 5 in Table V. The results, on the whole, are similar to those obtained by the constant serum—decreasing virus method.

There were 9 cases who showed *nil* or doubtful reaction following revaccination 1 to 4 years earlier and gave vaccinoid reaction after the present revaccination, the titre increasing 4-fold in 5 cases and 8-fold in 4 cases. It, thus, indicates that the limiting

TABLE VI.

Results of neutralization tests on embryonic chicken eggs of sera before and after revaccination by constant virus—decreasing serum method.

Serial number.	Age and sex. (year).	Previous history of vaccination.	Reaction of revaccination.	Titre of sera neutralized:		Titre increased by
				Before vaccination.	4 weeks after vaccination.	
*1	34 M	1 year—no reaction	Immunity	160	160	*Nil*
*2	30 M	1 year—no reaction	Immunity	160	160	*Nil*
*3	25 M	Every year—no reaction	Immunity	40	40	*Nil*
*4	29 M	1 year—no reaction	Immunity	80	80	*Nil*
5	30 M	1 year—no reaction	Immunity	160	160	*Nil*
6	34 M	1 year—no reaction	Immunity	80	80	*Nil*
7	27 M	1 year—no reaction	Immunity	20	80	4-fold
8	29 M	2 years—no reaction	Vaccinoid	20	80	4-fold
9	20 M	2 years—no reaction	Vaccinoid	20	80	4-fold
10	39 M	4 years—no reaction	Vaccinoid	20	80	4-fold
11	29 M	4 years—no reaction	Vaccinoid	20	80	4-fold
12	29 M	1 year—doubtful reaction	Vaccinoid	20	80	4-fold
13	58 M	1 year—doubtful reaction	Vaccinoid	10	80	8-fold
14	36 M	2 years—doubtful reaction	Vaccinoid	10	80	8-fold
15	28 M	1 year—doubtful reaction	Vaccinoid	10	80	8-fold
16	27 M	1 year—doubtful reaction	Vaccinoid	10	80	8-fold
17	26 M	1 year—no reaction	Primary take	5	320	64-fold
18	32 F	Suffered from Smallpox at the age of 8 years	Primary take	<5	320	>64-fold
*19	20 M	4 years—no reaction	Primary take	1	320	320-fold
20	24 M	3 years—doubtful reaction	Primary take	1	160	160-fold
21	23 M	5 years—no reaction	Primary take	1	160	160-fold
*22	29 M	7 years—no reaction	Primary take	1	320	320-fold
23	Infant (cord blood)	Mother vaccinated 1 year back	..	5	..	..
24	Infant blood on 4th day	..	..	5	..	..

A titre of $1=1{\cdot}5\times10^3$; $10=1{\cdot}5\times10^4$; $20=3{\cdot}0\times10^4$; $40=6{\cdot}0\times10^4$; $80=1{\cdot}5\times10^5$; $160=$m $3{\cdot}0\times10^5$; $320=4{\cdot}8\times10^5$; pock units.

*Sera marked with asteriks are common with sera Nos. 4, 3, 1, 2, 16 and 14 of Table V.

serum dilution against 1/100 virus dilution (containing 1500 pock count units per ml.) was 10 to 20 and the rise of titre was 4 to 8 times. On the other hand, the basic immunity titre in those giving primary type of reaction did not exceed 5 (usually 1) but the rise in titre after vaccination ranged between 64 and 320 times.

The neutralizing titre of a sample of cord blood serum of a baby and of his serum collected on the 4th day of his birth were found to be 5 against the virus dilution of 1/100. This in terms of neutralizing pock count units per ml. of serum is $150 \times 5 \times 10 = 7500$ or $7{\cdot}5 \times 10^3$. It is also seen from both Tables V and VI that these persons who had given *nil* or immunity reaction 1 to 7 years before the test revaccination showed three types of reaction depending largely upon the length of time elapsed after the reaction of immunity, namely :

(*i*) No change in reaction within 1 year ;

(*ii*) partial loss of immunity (vaccinoid reaction) within 1 to 4 years ;

(*iii*) full loss of immunity (primary take) within 1 to 7 years.

The above results show that revaccination giving negative or the so-called *reaction of immunity* is not usually followed by a rise of titre of immunity unless it is a borderline case and that any one of them may revert to complete susceptibility sooner than expected with the full risk of contracting the disease, particularly in an epidemic area.

This series also included a female case who had suffered from smallpox at the age of 8 years, became fully susceptible again at the age of 32 years and showed a good response to vaccination.

(*iii*) *Haemagglutination-inhibition test* (*by constant virus—decreasing serum method*).—After determining the haemagglutinating antigen (HA) titre of the virus suspension made out of the growth in chicken allantoic membrane following inoculation with calflymph virus, two haemagglutinating units of the virus suspension were used for each dilution of the test serum according to the technique of beta-haemagglutination-hibition (HI) described earlier in this paper. The results are given in Table VII.

The haemagglutination-inhibition test gave a fairly consistent result in respect of the three types of reaction after revaccination, namely, (1) primary take, (2) vaccinoid or so-called accelerated reaction, and (3) negative or so-called reaction of immunity.

Altogether 39 samples were examined by this method. Twelve persons gave primary type of reaction. The titre before revaccination varied between 10 and 40, the usual titre being 20 or less. After revaccination the highest titre reached was 2560 or more than 256 times that of the original titre and the lowest was 640 i.e., 16 times the original titre and this happened when the limiting titre prior to revaccination was only 40.

Nine persons gave vaccinoid reaction, the range of titre prior to revaccination being 40 to 160 and the lowest titre overlapping with the highest limiting titre giving primary type of reaction ; the lowest titre giving reaction of immunity was, however, 320. The individual among the vaccinoid reactors giving a titre of 80 prior to revaccination did not show any increase of titre, while in 7 others the increase of titre was only 4 to 8 times.

TABLE VII.

Results of beta-haemagglutination-inhibition tests of sera obtained before and 4 weeks after vaccination and revaccination.

Serial number.	Serial number of experimental subjects.	Reaction of revaccination.	HAEMAGGLUTINATION-INHIBITION TITRE :		Titre increased by
			Before test revaccination.	After test revaccination.	
1	1	Primary type	<10	2560	>256-fold
2	2	Primary type	<10	1280	>128-fold
3	10	Primary type	<10	1280	>128-fold
4	11	Primary type	<10	1280	>128-fold
5	17	Primary type	<10	1280	>128-fold
6	20	Primary type	<10	1280	>128-fold
7	47	Primary type	20	1280	64-fold
8	43	Primary type	20	640	32-fold
9	52	Primary type	20	640	32-fold
10	53	Primary type	20	640	32-fold
11	16	Primary type	40	640	16-fold
12	22	Primary type	40	640	16-fold
13	3	Vaccinoid type	80	80	No increase
14	4	Vaccinoid type	80	80	No increase
15	7	Vaccinoid type	40	160	4-fold
16	23	Vaccinoid type	160	640	4-fold
17	24	Vaccinoid type	160	640	4-fold
18	29	Vaccinoid type	160	640	4-fold
19	32	Vaccinoid type	160	640	4-fold
20	37	Vaccinoid type	160	640	4-fold
21	21	Vaccinoid type	40	320	8-fold
22	6	Reaction of immunity	2560	2560	No increase
23	8	Reaction of immunity	640	640	No increase
24	15	Reaction of immunity	640	640	No increase
25	27	Reaction of immunity	2560	2560	No increase
26	30	No reaction	2560	2560	No increase
27	31	Reaction of immunity	2560	2560	No increase
28	34	Reaction of immunity	2560	2560	No increase
29	41	Reaction of immunity	320	320	No increase
30	45	Reaction of immunity	320	320	No increase
31	51	Reaction of immunity	640	640	No increase
32	S_1	Reaction of immunity	1280	1280	No increase
33	S_2	Reaction of immunity	640	640	No increase
34	S_3	Reaction of immunity	640	640	No increase
35	28	Reaction of immunity	640	1280	2-fold
36	46	Reaction of immunity	320	640	2-fold
37	5	Reaction of immunity	<10	20	Refractory
38	9	No reaction	<10	10	Refractory
39	13	No reaction	<10	10	Refractory

N.B. S_1, S_2 and S_3 are samples from the laboratory staff.

Nineteen individuals had reaction of immunity or negative reaction prior to revaccination, the haemagglutination-inhibition titre varying between 320 to 2560. In case of 13 of them there was no increase of titre after revaccination irrespective of whether the original titre was high or low. A two-fold rise was, however, noted in case of two individuals while three others proved refractory, among the latter, one had shown reaction of immunity although the basic titre was less than 10 in both cases. After revaccination the rise in titre was very negligible being 20, 10, and 10 respectively.

The above results, therefore, fully corroborated the findings of the neutralization tests in embryonic chicken eggs with the difference that the haemagglutination-inhibition test proved to be much simpler, easier to perform and perhaps more consistent and dependable.

(*iv*) *Estimation of haemagglution-inhibitination titre in samples of cord and infant blood.*—Twenty-three samples of cord blood were collected but six of these being haemolysed, 17 samples were available for HI test. The corresponding babies blood could be collected from 7 infants only. History of revaccination of mothers was obtained for all babies and primary vaccination was given to all except two who were left out to watch the behaviour of the serum titre, passively obtained from the mother, with the passage of time. Blood samples for retesting of the HI titre 4 weeks after vaccination or revaccination were collected from 16 babies, only 5 of whom could be followed to study the progress of immunity in them. The results are summarized in Tables VIIIA and VIIIB.

The haemagglutination-inhibition titres of the first 5 samples of blood in Table VIIIA indicate that the titre of the cord blood serum depends upon mother's immunity status and the length of time elapsed after the positive take in them. Secondly, the titre falls fairly rapidly, even within the first week, and by the third month or earlier it comes to a level which permits successful take after primary vaccination. In the majority of cases, however, the immunity titre of the cord blood was very low to allow a positive take after primary vaccination within 1 week of birth (Table VIIIB). In all such cases it may be stated that the mothers did not possess sufficient immunity in th mselves. Also, the titre did not reach the expected high level in all cases of positive take after primary vaccination. It varied between 320 and 2560.

It is interesting to note that in these babies which had some passive immunity at the time of primary vaccination the titre did not rise high ; in a few others the cause of low rise was not known but it explains why in certain cases immunity is lost within a short time as noted in the present field investigation as well as previously reported by many workers. Furthermore, a negative reaction may be obtained in infants with as low a HI titre as 80 when the primary vaccination is undertaken within 1 week of birth. Also, the tissue response may not be well-developed at this stage, which partly explains the low rise of titre in serial Nos. 1, 4 and 5 (Table VIIIA). It is therefore a question whether it would be advantageous to undertake primary vaccination earlier than 3 months except in situations where it would by risky to wait as in case of a community in which a large proportion of adults, particularly mothers, are not fully protected by vaccination and revaccination and thereby maintains a local endemic conditions.

TABLE VIII (*a*).

Haemagglutination-inhibition test of cord blood and corresponding infant sera before and after primary or revaccination against 1/100 virus dilution (1500 pock units per ml.) with follow-up records.

Serial number.	Cord blood number.	Mother's last vaccination history.	HI TITRE OF :		FIRST PRIMARY VACCINATION :			FOLLOW-UP RECORD :		FINAL VACCINATION OR REVACCINATION :		
			Cord blood.	Baby's blood*.	Given at.	Reaction.	HI titre.	Interval.	HI titre residual.	Given at.	Nature.	HI titre final.
1	2	Revaccination 1 year positive take	320	..	Not given	..	..	(i) 1 m (ii) 6 m	160 40	.. 6 m	.. primary	.. 640
2	6	Revaccination 2 years Reaction of Immunity	80	80 (4th day)	Not given	..	..	3 m	10	3 m	primary	1280
3	9	Revaccination 1 year Vaccinoid type	80	..	1 week after birth	Negative	..	..	..	2 m	revaccina-tion	1280
4	14	Revaccination 2 years Vaccinoid type	40	20 (7th day)	1 week after birth	Vaccinoid	320	6 m	320	..	..	320
5	17	Revaccination 1 year positive take	160	80 (5th day)	1 week after birth	Negative	..	3 m	20	3 m	revaccina-tion	640

*Within 1 week of birth.

TABLE VIII (*b*).

Haemagglutination-inhibition test of cord blood and corresponding infant sera before and after primary vaccination against 1/100 dilution of virus (1500 pock count units).

Serial number.	Cord blood number.	Mother's last vaccination history.	HI TITRE OF :		FIRST PRIMARY VACCINATION :		
			Cord blood.	Baby's blood*.	When given.	Reaction.	HI titre.
6	3	Positive primary take at early age	<10	..	1 week after birth	Primary type	2560
7	13	Positive primary take at early age	<10	<10	1-2 weeks after birth	Primary type	2560
8	16	Positive primary take at early age	<10	..	1-2 weeks after birth	Primary type	1280
9	19	Positive primary take at early age	<10	..	1-2 weeks after birth	Primary type	1280
10	20	Positive primary take at early age	<10	<10	1-2 weeks after birth	Primary type	1280
11	21	Positive primary take at early age	<10	..	1-2 weeks after birth	Primary type	1280
12	4	Revaccination 3 years	<10	<10	1-2 weeks after birth	Primary type	1280
13	7	Revaccination 4 years	<10	..	1-2 weeks after birth	Primary type	1280
14	18	Revaccination 4 years	10	10	1-2 weeks after birth	Primary type	320
15	10	Revaccination 3 years	10	..	1-2 weeks after birth	Primary type	640
16	23	Revaccination 3 years	10	..	1-2 weeks after birth	Primary type	640
17	22	Revaccination 2 years	10	..	1-2 weeks after birth	Primary type	320

* Within 1 week after birth

N.B. Cord blood Nos. 1, 5, 8, 11, 12 and 15 were haemolysed.

Reaction after revaccination in mothers of serial Nos. 12 to 17 could not be elicited.

(*b*) *Testing of infant and child blood after primary vaccination.*—Fifty-one babies of different ages and residing in Calcutta, Howrah and Serampore were given primary vaccination with the tested lymph of the West Bengal Vaccine Institute and blood samples were collected before and 4 weeks after vaccination from 19 of them for estimation of HI titre of their sera. Mother's history of vaccination was available for most of them. The results are given in Table IX.

TABLE IX.

Summary results of primary vaccination of 51 children of different age groups at Calcutta, Howrah and Serampore (of greater Calcutta)

(Number of children : Calcutta — 20 ; Howrah — 3 and Serampore — 28)

Number of children tested.	MOTHER'S LAST VACCINATION RESULTS :		Age of children at the time of primary vaccination.	Results of primary vaccination.
	Type.	Reaction.		
	A. Primary			
19	(*a*) In childhood	Positive take	4m-2; 5m-3; 6m-9; 7m-1; 8m-1 9m-1; 1 yr-1; 1½yr-1	Successful
1	(*b*) 1 year ago	Positive take	5m	Vaccinoid type
	B. Revaccination			
1	(*a*) Every year*	Positive take	3m	Unsuccessful
2	(*b*) 1 year ago	Positive take	5m-1; 6m-1	Unsuccessful
1	(*c*) 1 year ago	Positive take	10m	Successful
2	(*d*) 1 year ago	Vaccinoid type	1m-1; 2m-1	Unsuccessful
3	(*e*) 1 year ago	Reaction of immunity	7 day-1; 1m-1; 3m-1	Unsuccessful
5	(*f*) 1-2 years ago	Reaction of immunity	6m-1; 1⅓ yr-1; 1½ yr-1 2yrs-2	Successful
1	(*g*) 2 years ago	Positive take	2m	Unsuccessful
1	(*h*) 3 years ago	Positive take	1½ yr	Successful
3	(*i*) 3 years ago	Reaction of immunity	9m-1; 1 yr-1; 2 yrs-1	Successful
8	(*j*) Over 3 years	Not known	3m-1; 4m-1; 5m-5; 6m-1	Successful
4	Not known	..	4m-1; 6m-1; 3 yrs-1; 6 yrs-1	Successful

* She had positive reaction in her last revaccination.

Successful—41 (80·4 per cent) ; Vaccinoid—1 (2 per cent) ; Unsuccessful—9 (17·6 per cent).

Among 51 children primary vaccinated, 9 children gave negative reaction—2 at 1 month, 2 at 2 months, 2 at 3 months and one each at 7 days, 5 months and 6 months respectively, the immunity status was high in practically all the 9 mothers, 3 of them having history of revaccination with reaction of immunity, 4 mothers had positive reaction after revaccination with 1 to 2 years and 2 others had vaccinoid reaction within 1 year. In one infant which gave vaccinoid reaction at 5 months the mother had a positive take only one year before delivery. In the rest of the children either the mother's

immunity was at low level due to the absence of reinforcing the immunity after primary vaccination or to the loss of the passive immunity in the infants due to delay in taking the primary vaccination. The results of estimation of HI titres in the sera of these children are given in Table X.

The results given in Table X have corroborated the results obtained with the samples of cord blood, and have also supported the laboratory findings described earlier in this paper. The level of HI titre giving positive take is 20 or less and that giving vaccinoid reaction is 40 to 80 and the one giving negative or the so-called immune reaction is 160 or above. The maximum titre in a positive take recorded in the present investigation is 2,560, more commonly 1280, but in those subjects which maintained some residual immunity to give vaccinoid reaction the level of HI titre reached after primary or revaccination is on the lower side, e.g., 640 and even 320 in some cases. The immunity level giving negative or immune reaction either showed no rise or only one step in HI titre. These findings thus supported the hypothesis enunciated by the senior author (Seal, *loc. cit.*). There were, however, a few cases like the serial Nos. 11 and 14 in which the response to successful primary vaccination was of low order. Such cases may be due to their constitution or to the state of health at the time of vaccination or any other cause not known.

Discussion.

Although smallpox vaccination has been the best prophylaxis available in Medical Science, certain lacunae in knowledge still exist as to the duration and qualitative and quantitative response it stimulates after primary vaccination and more particularly after revaccination. During the Second World War, several incidences of smallpox were brought to light by different authors, occurring among persons who were supposed to have been protected by vaccination or revaccination short time earlier. In the Indian experience such cases were rather fairly common, for it is well known that the percentage of the primary vaccinated being much too high compared to the unvaccinated the number of smallpox cases among the former group far outnumbers that among the latter during an epidemic. This is quite understandable because the immunity acquired by primary vaccination during early infancy, if not reinforced by revaccination, deteriorate with increasing age and such persons may revert to partial or complete susceptibility at variable intervals of time. But the problem arises when the vaccinated individuals come down with smallpox within 3 years or even lesser time after a successful take or when revaccinated persons giving negative or so-called immunity reaction, only a year or even shorter period earlier, meet with the same fate and also when persons getting themselves revaccinated every year suddenly give positive reaction or even get an attack of smallpox. Such situations naturally give rise to serious epidemiological problems that needed a thorough investigation. The senior author while reviewing the situation in 1945 highlighted the problem and advanced some hypotheses based on the published reports, personal field observation and his laboratory experience under Dr. Craigie of the Connaught Laboratories at Toronto, Canada. The main difficulty in carrying out an objective study at that time was the absence of any reliable serological method or well-established cultural technique.

TABLE X.

Haemagglutination-inhibition titre of sera collected from 19 children of different ages before and primary vaccination.

Serial number.	Area.	Age.	Mother's last vaccination history* :		Children's vaccination results :			
			Type.	Result.	Reaction after primary vaccination.	HI titre. before vaccination.	HI titre. after vaccination.	Titre increased by
1	Entally, Calcutta	9 months	Revaccination 12 years	Positive take	Successful	< 10	640	> 64 times
2	Entally, Calcutta	1½ years	Primary at early age	Positive take	Successful	< 10	1280	> 128 times
3	Entally, Calcutta	10 months	Revaccination 1 year	Positive take	Successful	40	640	16 times
4	Entally, Calcutta	2 years	Revaccination 5 years	Reaction of Immunity	Successful	10	1280	128 times
5	Serampore Hooghly District	3 months	Revaccination 4 years	Not known	Successful	< 10	2560	> 256 times
6	Serampore Hooghly District	5 months	Revaccination over 3 years	Not known	Successful	20	640	32 times
7	Serampore Hooghly District	4 months	Primary at early age	Positive take	Successful	< 10	1280	> 128 times
8	Serampore Hooghly District	6 months	Primary at early age	Positive take	Successful	< 10	1280	> 128 times
9	Serampore Hooghly District	6 months	Revaccination over 3 years	Not known	Successful	20	640	32 times
10	Howrah	7 days	Revaccination 1 year	Reaction of Immunity	1 of 4 marks positive	80	320	4 times
11	Howrah	2 months	Revaccination 2 years	Positive take	1 of 4 marks positive	80	160	2 times
12	Entally, Calcutta	5 months	Revaccination 1 year	Positive take	Vaccinoid reaction	40	320	8 times
13	Entally, Calcutta	6 months	Revaccination 1 year	Positive take	Vaccinoid reaction	80	320	4 times
14	Howrah	2 months	Revaccination 1 year	Vaccinoid reaction	Doubtful reaction	160	320	2 times
15	Entally, Calcutta	3 months	Revaccination 1 year	Reaction of Immunity	Negative reaction	160	160	No increase
16	Entally, Calcutta	3 months	Revaccination 1 year	Reaction of Immunity	Negative reaction	160	160	No increase
17	Entally, Calcutta	5 months	Primary at early age	Positive take	Negative reaction	640	640	No increase
18	Serampore Hooghly District	1 month	Revaccination 1 year	Reaction of Immunity	Negative reaction	160	160	No increase
19	Serampore Hooghly District	1 month	Revaccination 1 year	Vaccinoid reaction	Negative reaction	160	320	2 times

*Confirmed by other family members and checked by inspection of pock marks.

The present investigation is the outcome of a joint field-cum-laboratory investigation to find out the average durations of passive and active immunisations in infants and threshold levels of immunity at which revaccination yields negative, vaccinoid or the primary type of reaction. These needed a field epidemiological study, re-examination of smallpox cases in hospitals, testing of samples of cord, infant, child and adult blood before and after vaccination and revaccination following the establishment of suitable laboratory methods for quantitative estimation of antibody levels at different situations. This pilot study extended over several years and the results so far obtained have yielded some useful information and thrown light on some of the unsolved problems related to vaccination and revaccination.

(1) *Relative merits of the laboratory tests.*—The various tests carried out for the estimation of the serum antibody levels before and after vaccination and revaccination were : (*i*) neutralization test on rabbit's skin, (*ii*) neutralization test in embryonic chicken eggs by (*a*) decreasing virus—constant serum method and (*b*) constant virus—decreasing serum method, and (*iii*) beta haemagglutination-inhibition test by constant virus —decreasing serum method. Of these the haemagglutination-inhibition test is the easiest, less complicated and fairly dependable, the neutralization test on rabbit's flanks is expensive and not so consistent, while the same in the embryonic chicken eggs is fairly reliable but requires very careful handling by skilled hands and is more expensive than the HI test. In this test constant virus-decreasing serum method gives more consistent and slightly higher reading than the decreasing virus—constant serum method due perhaps to the better dispersion of antibodies in the diluted than in the undiluted serum.

(2) *Study of a smallpox epidemic, hospital cases and test revaccinations.*—In a fresh intra-epidemic study the incidence rate among the primary vaccinated group was as high as 37·6 per cent and among the revaccinated group 19·6 per cent (29·4 among the total vaccinated). On the whole, 33·8 per cent suffered from smallpox although as high as 88 per cent of the population was either primary or revaccinated (Table I). A more important point that emerged was that 30 out of 156 revaccinated persons got the attack —3 with history of positive take (excluding 7 during the incubation period), and 20 with 2 deaths with history of immunity reaction, within 1 year in 9 cases and within 2 years in 12 cases, before the attack (Table II). In the same epidemic the result of revaccination among the 76 non-cases in 0 to 9 years group 16 had been revaccinated after the epidemic was on, and the results indicated loss of immunity in 7 cases within 3 years including 2 cases within 2 years and the remaining 9 within 4 to 6 years. There were also 3 cases of second attack of smallpox. Similar history was also obtained in the 94 smallpox cases admitted to the hospital. These observations were also fully corroborated by test revaccination in 206 persons including school children. As high as 40 per cent among them gave either primary type (18·0 per cent) and vaccinoid reaction (partial immunity) within 2 years and 49·1 per cent within 3 years of preceding vaccination or revaccination, indicating fairly rapid deterioration of immunity status of the population.

(3) *Field-cum-laboratory tests.*—Altogether 115 paired samples of blood (i.e. 230 samples)—one before and another 4 weeks after primary or revaccination were examined for antibody quantum by the different methods mentioned above. This included 16 pairs of blood from adult persons tested by neutralization method in chicken embryonic eggs using decreasing virus—constant serum method and another 24 pairs including a pair of cord-cum-infant blood using constant virus—decreasing serum method, 39 pairs of blood from the mixed age groups, 17 pairs of cord and corresponding infant blood and 19 pairs of infant and children blood tested by beta haemagglutination-inhibition technique. All the methods gave similar results, as summarised below, thus conferring greater validity to the conclusions drawn.

(4) *Summary of the laboratory tests.*—The strength of antibody titre in cord blood broadly depended on the mother's antibody titre during her antenatal period (Table VIIIA). It started deteriorating even within the first week (cases 4 and 5) and practically disappeared by the third month (in exceptional cases 5 to 6 months). Primary vaccination done in the presence of some residual titre led to low rise of antibody titre whereas if done on a fully susceptible child it was followed by a high rise of titre to a maximum of 2,560 HI units or more commonly 1,280 units (Table VIIIB). Accordingly, the best time to immunise the child would be from 3 months onward, particularly if the mothers have sufficient immunity during the antenatal period i.e. when the vaccination status of the general population is fairly well maintained. The results of primary vaccination of the infants and children as given in Table IX and X show the same result. The increase of HI titre in case of primary positives ranged between 32 and 256 times, in the case of vaccinoid reaction 4 to 8 times, and in cases of immune reaction little or no rise ; the low rise after primary vaccination occurred in cases where the residual titre was between 20 and 40, the final titre rising to 640 or 16 to 32 times. The results were more clear cut in cases of adult revaccination as given in Table VII. The lowest HI titre at which revaccination gave immunity reaction was 320 and that of vaccinoid reaction 40, below which there was always primary type of reaction. According to this, the HI titre of 160 was a borderline case between vaccinoid and immunity reactions and a titre of 40 between primary and vaccinoid reactions.

In case of neutralization test on embryonic chicken eggs (Table VI) titres were registered 1 or 2 steps lower down. For immunity reaction it ranged between 40 and 320, for vaccinoid reaction between 10 and 20, and for primary positives 5 and below, but the rise of titre as with the other tests, was *nil* in case of immunity reaction, 4 to 8 times in cases of vaccinoid reaction and 64 to 320 times in case of primary reaction. The same test when carried out by decreasing virus—constant serum method the results were also similar though the range of titres different, namely, there was no rise of titre in immunity reaction, after revaccination, it was 10 to 100 fold in case of vaccinoid reaction and 1,000 to 10,000 times in case of primary positives. But the constant virus—decreasing serum method is preferrable to this latter method. Briefly, the rise of titre was inversely proportional to the existing level of immunity or directly proportional to the degree of susceptibility. These results indicate that in the presence of partial immunity the live virus vaccine cannot exercise its full intensity to stimulate the

highest level of immunity. Besides, the basic or residual immunity being already at a certain upper level the ratio of increase would naturally be less than that given by the fully susceptible person with no or low level of immunity.

SUMMARY.

1. The above results lead to the conclusion that revaccination giving negative or immune reaction is not followed by a rise of titre, which continues to fall with the lapse of time and may come down to vaccinoid and fully susceptible stage, the time required for the purpose varying with the level of the residual titre. The loss of titre from borderline to partial or full susceptibility may even be within one year or less time and this provides the reason why persons showing immunity reaction after revaccination more than once or even in several consecutive years suddenly give primary type or vaccinoid reaction within one year of the last vaccination and if exposed to an epidemic of smallpox sometimes get an attack though it may turn out to be a mild one with better chance of recovery.

2. The rise of titre following vaccinoid reaction is much less than that following the primary type and hence both the immune and the vaccinoid reactors are not in as advantageous or secure position as those who have recently had typical positive takes. It was also observed that all the babies given primary vaccination may not uniformly respond with the same degree of high titre and may lose their immunity within as short a period as 3 years against an average period of 5 years. The first revaccination should not therefore be delayed more than 3 years on any account. For the adults the better procedure of revaccination would be to repeat it every year till a positive response is obtained and then a gap of several years (5 years) may be allowed. The limiting serum titres by HI test for primary reaction is 40 or less, for vaccinoid reaction 40 to 160 and for immunity reaction 160 and above (upto 2560). The same by the neutralization test in embryonic chicken eggs using constant virus—decreasing serum method are 5 or less, 10 to 20 and 20 to 160 respectively.

REFERENCES.

BHATTACHARJI, L.M., and SEAL, S.C. (1956-57) — Possible natural immunity against vaccinia. Report of the Section of Epidemiology, All-India Institute of Hygiene & Public Health, Calcutta, for the year 1956-57, **34**.

BLAXALL, F.R. (1930) .. .. — 'A System of Bacteriology', **7**, 115. Her Majesty's Stationary Office, London.

COLEMAN, P.N. (1944) .. .. — Revaccination of an Army Unit. *Brit. Med., Jour.* **i**, 191.

D'ARCY, P.N.S., MOORE, P.A.H., and WHETTER, C.W. (1943) — Smallpox on a New Zealand Hospital Ship. *New Zealand Med. Jour.*, **42**, 195-199.

ELLIS, R.V., and BOYNTON, R.E. (1939) .. — Smallpox Vaccination. A comparison of vaccines and techniques. *Pub. Hlth. Rep.*, **54**, (Part I), 1012-1025.

HORGAN, E.S., and HASEEB, M.A. (1944) .. — Revaccination as a measure of immunity in smallpox. *Jour. Hyg.*, **43**, 337-340.

JEANS, W.D., JEFFREY, J.S., and GUNDERS, K. (1944) — Penicillin and smallpox. *Lancet*, **ii**, 44-45.

KASAI, H. (1926) .. .. .. Titration of lymph potency in semi-immune animals. Scientific Ref. *Gov. Inst. Inf. Dis. Tokyo*, **5**, 113.

KII, N. (1926) .. .. .. Titration of lymph potency in semi-immune animals. Scientific Ref. *Ibid.*, **5**, 63.

LEAGUE OF NATIONS (1928) .. .. Report of Commission on Smallpox Vaccination, Geneva.

LEAKE, J.L. (1927) .. .. .. Questions and answers on smallpox vaccination. *Pub. Hlth. Reports.*, **42**, 221-238.

MACGREGOR, A., and PETERS, R.J. (1942) .. The outbreak of smallpox in Glasgow, 1942. *Brit. Med. Jour.*, **ii**, 627-629.

PARKER, R.E., and RIVERS, T.M. (1936) .. Immunological and chemical investigations of vaccinia virus. III. Response of rabbits to inactive elementary bodies of vaccinia and to virus-free extracts of vaccine virus. *Jour. Exp. Med.*, **63**, 69-94.

REED, L.J., and MUENCH, H. (1938) .. A simple method of estimating 50 per cent end-points. *Amer. Jour. Hyg.*, **27**, 493-497.

SEAL, S.C. (1945) .. .. .. Prevention of smallpox by vaccination and revaccination : A critical study. *Ind. Med. Gaz.*, **80**, 313-318.

SMITH, H. (1943) .. .. .. Smallpox and vaccination. *New Zealand Med. Jour.*, **42**, 200-205.

SU, T.L. 1944) .. .. .. Smallpox in Kweilin, Kwangsi, 1940-1941. *Ind. Med. Gaz.*, **79**, 332.