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Immunity to vaccinia induced by small doses of active virus

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INTRODUCTION

Active immunity to an infective agent is best attained as the result of sub-clinical infection. In the case of immunization against smallpox this objective is approached but not completely realized. Jennerian vaccination of infants is usually carried out at the cost of no more than a mild indisposition but in adults the resulting local and general condition may cause considerable distress. Moreover, the complications of vaccination, though rare, are a constant anxiety both to the producing laboratory and to the medical officer. It would be a great advantage if the local skin reaction could be avoided altogether. This would to a large extent remove the objection of the layman to vaccination and it would prevent those complications that are the result of direct spread of the virus to adjacent skin areas. These are good reasons for attempting to improve the presently available smallpox vaccines and the traditional method of using them. This communication is concerned with attempts to develop such a vaccine, using partly purified suspensions of living vaccinia elementary bodies derived from skin pulp. The experiments have led to the discovery that an extremely small dose of the purified virus, far below the amount required to produce a clinical infection, is sufficient to evoke an immune response. The possibility of applying this knowledge to the production of an improved smallpox vaccine is discussed.

The present studies are a continuation of experiments recently reported from this laboratory (Amies, 1961) on the kinetics of inactivation of vaccinia virus by formaldehyde. It was found that the rate of inactivation did not remain constant but decreased continuously in the course of the process. Similar findings with respect to poliovirus have been described and discussed at considerable length by Gard and his associates (Gard, 1957; Gard & Maaløe, 1959). My own experiments also showed that samples of HCHO-treated virus which still contained minimal amounts of active agent were immunogenic whereas later samples were neither infective nor capable of producing active immunity in rabbits. On this evidence it was stated unequivocally that HCHO-inactivated vaccinia virus is of no practical value as an immunizing agent. Similar findings have been reported by many other investigators (Hallauer, 1938).

In opposition to the view expressed above, some authorities in Europe believe that HCHO-inactivated vaccinia virus inoculated subcutaneously in man is of value in establishing a basal immunity which can be augmented later by vaccinating

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in the normal manner. Clinical and experimental data in support of this thesis have been published by Verlinde (1942), Herrlich (1959), Mahnel (1960), Beunders, Driessen & van Hoek (1960) and Ehrengut (1961). Ramon and his colleagues (Ramon, Boquet & Richou, 1942; Ramon, Richou, Thiéry, Salomon & Doucet, 1954) have also published a number of articles on HCHO-treated vaccinia ('anavirus'). In none of these articles, with the possible exception of that by Mahnel, is there any acceptable evidence that the vaccine was completely devoid of infectivity. Indeed, Beunders and his co-workers admit that their preparations were reactivated by neutralizing the residual free HCHO with bisulphite and adding histidine. They also found that their supposedly inactivated material lost its antigenic properties when stored for a few weeks. These facts strongly suggest that the results obtained by all the investigators quoted above were due to small amounts of residual active virus. This in no way detracts from the value of their work: it only offers an alternative definition of the biological material which they used. If this view is correct, further research could be carried out on the preparation of a stabilized living vaccinia suspension which could be issued at a potency suitable for subcutaneous inoculation. This would represent not an entirely new procedure but an improvement on a method of vaccination which has apparently proved successful in the hands of those who developed it.

A historical background for the present studies is provided by the writings of Calmette & Guérin (1901) more than 60 years ago. These men, better known as the originators of BCG, described the method of inoculating vaccinia virus percutaneously in rabbits and they were among the first to carry out experiments on immunity resulting from experimental infection. Their most important findings are summarized here because they have a direct bearing on the work to be described. In the rabbit, immunity to vaccinia could be demonstrated on the 6th day following percutaneous or subcutaneous inoculation, and on the 5th day after intravenous inoculation. After intracerebral inoculation, fragments of brain removed 4 days later were infective according to the skin scarification test but fragments removed on the 7th day or later were not infective. The same strain of virus when introduced directly into the trachea, lung or pleural cavity produced immunity without any apparent lesions; and when these tissues were removed 4 days after inoculation they were found to be non-infective. After intravenous injection of virus, shaving the skin of the inoculated animal 24 hr. later produced a crop of vaccinial lesions at the site of the slight trauma produced by the razor. This localizing effect could not be demonstrated when the interval between infection and trauma by shaving was increased to 48 hr. In other experiments, the rabbits were killed 2–5 days after receiving a fairly large dose of virus by the intravenous route. Specimens of defibrinated blood, liver, kidney, spleen, lung and bone marrow were removed, pulped and applied to the newly shaved skin of normal rabbits. No skin lesions developed. These experiments of Calmette & Guérin very clearly demonstrate that vaccinia virus is rapidly removed from the blood stream and tissues with subsequent immunity to re-infection. Thus, immunity and not overt infection is the result of parenteral inoculation of vaccinia virus.

Later investigators have confirmed the fact that both in man and animals

successful infection by vaccinia virus is followed by viraemia (Rivers & Tillett, 1923; Herzberg-Kremmer & Herzberg, 1930). Usually, this is not accompanied by any signs and symptoms of disease and it is of short duration, though Alivisatos & Violaki-Paraskeva (1959) claim to have isolated the virus from the blood of vaccinated people for periods up to 73 days after inoculation. Thus there can be no valid objection to subcutaneous injection of the virus on the grounds that this may lead to generalized vaccinia. Viraemia is a normal event after jennerian vaccination.

In the course of his valuable contribution to this subject Herrlich (1959) mentions that he carried out primary vaccination of more than 2000 elderly people without encountering any complications. He abandoned this method, however, after the report by Berger (1954) of a single case of encephalitis occurring after subcutaneous vaccination. The exact nature of the vaccine used by these investigators was not disclosed: presumably it was a bacteria-free lymph.

METHODS

Vaccinia virus

All experiments were carried out with a strain of vaccinia virus propagated exclusively on the rabbit skin. This originally came from the Lister Institute, London, and it has been maintained for the last 3 years by serial passage of purified material. The crude skin pulp was triturated with 4 mM McIlvaine buffer of pH 7·0, and partly purified suspensions of elementary bodies (E.Bs) were prepared by fractional centrifugation as described by Craigie (1932). A centrifugal force of 4340g (angle centrifuge) for 30 min. was sufficient to deposit the virus. The intermediate clarification stages were carried out at 1000g, using a swinging bucket type of centrifuge. The dilute McIlvaine buffer must be used as the dispersing medium: physiological saline and buffers of equivalent ionic strength cause the virus particles to flocculate. In order to maintain their potency over long periods at refrigerator temperature the stock suspensions were stabilized by addition of an equal volume of glycerol. New suspending media have recently been developed: These have better preservative qualities than those containing glycerol (Amies, 1962).

The infective potency of the virus suspensions was determined by plaque count assays on monkey kidney cell monolayers (Cutchins, Warren & Jones, 1958). In the hands of an experienced worker with the resources of a large virological laboratory available to him this method of assay has an experimental error of about $\pm 17\%$. Infectivity was also measured by intradermal titration in the rabbit skin. Provided that several rabbits are used for each assay in order to allow for the occasional poor reactor this method gives reliable information and is at least as sensitive as the monkey kidney tissue culture method.

Response to infection

The immune response to minimal amounts of active virus was studied in young adult rabbits inoculated by the subcutaneous route. The infecting dose was measured at the same time by the two methods indicated above. Usually this

proved to be not far from the intended dose of 10 ED_{50} units (effective dose units). The suspending medium was either the 4 mM buffer or one of the depot suspensions to be described later. The volume of the inoculum was always 1·0 ml. Samples of blood were obtained from each animal prior to infection and at intervals thereafter. All sera were inactivated by heating at 56° C. for 30 min: they were then stored in the frozen state. At varying intervals after infection the animals were inoculated intradermally or by scarification with a series of tenfold dilutions of the same virus suspension that had been used for the initial injection. Daily examination and measurement of the resulting lesions made it possible to interpret the response as *normal susceptible*, *immune reaction* or *hypersensitive*. The accepted criterion of immunity was a reduction of the infective titre by at least 2 log dilutions.

Serological tests

The samples of rabbit serum were examined for vaccinial antibody by two different methods. Complement-fixation tests were carried out according to the procedure recommended by Kempe (1956). A complete titration was done in every case. The antigen was the supernate from a suspension of infected skin pulp which had been clarified by centrifugation for 30 min. at 10,000*g*. No difficulties were encountered with these tests. In some experiments serum-virus neutralization tests were performed with monkey kidney cell cultures, using a modification of the technique described by Cutchins *et al.* (1958). By employing a procedure which is as standardized as possible it is possible to assign to each serum a figure which expresses its neutralizing potency. For comparative purposes these methods may have some value but it is doubtful whether such quantitative information has much meaning outside the laboratory where the work is done. For the present studies, therefore, it will be sufficient to record the presence or absence of neutralizing antibody.

Nine separate experiments were carried out, involving the study of 180 rabbits by the methods outlined above.

RESULTS

The first experiment to be presented is concerned with the response to inoculation of a very small dose of purified living vaccinia virus suspended in dilute buffer solution. Twenty young adult rabbits each received, by subcutaneous injection, 1·0 ml. of a 10^{-5} dilution of the stock E.BS in 4 mM McIlvaine buffer. This virus suspension, titrated at the same time, had an infective potency of $4{\cdot}6 \pm 10^5$ ED_{50}/ml. on monkey kidney cells and $1{\cdot}0 \pm 10^6$ ED50/ml. when tested in the rabbit skin. The inoculum or *sensitizing dose* thus contained approximately 10 ED50 doses of virus. It produced no recognizable local or general effect upon the animals. After an interval of 34 days each animal was inoculated intradermally with 0·1 ml. volumes of a series of tenfold dilutions of the same stock E.BS. The resulting skin lesions were examined daily and samples of blood serum were obtained and tested as already described. The results of this experiment are summarized in Table 1.

A hypersensitive skin reaction was characterized by the development, within 36–48 hr., of a circumscribed raised indurated lesion measuring 30 to 60 mm. in diameter at the site of inoculation. At this time, the overlying skin appeared

Table 1. *Results of infecting* 20 *rabbits by injection of* 10 ED_{50} *doses of purified vaccinia virus in buffer*

(1) Response to challenge 34 days later (skin test)

Normal response	9 animals
Immune response	6 animals
Hypersensitive response	2 animals
Excluded (rough skin)	3 animals

(2) Complement-fixation tests

	Negative	Positive	Mean titre
Before sensitization	20	0	—
33 days after sensitization	7	13	1/28
6 days after challenge	0	20	1/55
13 days after challenge	0	20	1/100

(3) Neutralization tests

	Negative	Positive
Before sensitization (not tested)	—	—
33 days after sensitization	18	2
6 days after challenge	6	14
13 days after challenge	1	19

oedematous and had a pale blue-purple colour. By the 4th day this induration had decreased but an area of haemorrhagic necrosis had formed in the centre. This lesion slowly resolved during the following 10–15 days. Reactions of this type were confined to the 3 lowest dilutions of virus suspension. The higher dilutions produced lesions nearly resembling normal reactions to virus inoculation but the titration end point was always below that shown by control animals which had not received a sensitizing dose. When the results of the skin tests and of the serological reactions were compared it was found that there was a negative correlation between skin sensitivity and the presence of serum antibody. The experiment, taken as a whole, demonstrates that a very small amount of living virus is capable of producing an immune response in more than one half of the animals inoculated. Absence of immune response may reasonably be explained as a failure of this minimal quantity of virus to establish itself and undergo at least some degree of proliferation in the tissues.

The next experiment was similar to the first one except that the challenge dose of virus was introduced into the skin by scarification instead of intradermal inoculation. Twenty rabbits each received 10 ED_{50} doses of vaccinia E.Bs by subcutaneous inoculation. The challenge virus was given to 10 of these animals on the 18th day and to the remaining 10 animals on the 24th day after sensitization. The skin of the flanks was carefully shaved and then inoculated percutaneously with a series of tenfold dilutions of the stock vaccinia E.Bs. In this manner, 0·1 ml. volumes of each dilution were applied to an area of shaved skin measuring approximately 25×15 mm. The same procedure was followed with 3 normal animals and with 3 others which had developed immunity to vaccinia as the result of a heavy infection by skin scarification. In the group of 20 sensitized animals,

5 showed a hypersensitive response to challenge, 10 had some degree of immunity, and 5 were fully susceptible. Hypersensitivity was demonstrated by the development, within 24 hr., of a red raised and indurated lesion extending over the whole of the inoculated area. By the second day following inoculation this area showed many small haemorrhagic points surmounting the general erythema and induration. Regression occurred rapidly and was almost complete by the 7th day. In the other animals immunity was recognized by the early development of pink, slightly indurated lesions which quickly regressed. The titration end point in these animals, as in the immune controls, was 2 or 3 log dilutions below the titre shown by the normal controls.

Table 2. *Summary of four experiments in which a small dose of active vaccinia virus was incorporated in an adjuvant* (10 ED_{50} *doses of virus in* 0·1 *ml. buffer plus* 0·9 *ml. DPT-polio vaccine*)

Experiment no.	No. of animals	Interval before challenge (days)	Response to challenge		
			Normal	Immune	Hypersensitive
1	9	18	2	3	4
2	11	24	3	7	1
4	10	17	0	4	6
5	22+1	44	4	12	6
	52		9	26	17

* One rabbit in Experiment 5 was excluded on account of a rough skin.

Serological results (Experiment 5 only)

Serum sample	Neutralization test		Complement-fixation test		Mean titre
	Negative	Positive	Negative	Positive	
Before sensitization	23	0	23	0	—
43 days after sensitization	13	10	4	19	1/92
6 days after challenge	7	16	1	22	1/76
13 days after challenge	7	16	0	23	1/81

The effect of adjuvants

These early experiments suggested that an important feature of the immune response to very small amounts of vaccinia virus was the development of a state of hypersensitivity. In the next series of experiments, therefore, an attempt was made to increase this hypersensitivity by incorporating the virus in an adjuvant. It seemed undesirable to use a substance which produced a marked cellular reaction, such as Freund's complete adjuvant or alum: the choice, therefore, fell on a multivalent prophylactic containing Salk poliovirus vaccine, diphtheria toxoid, tetanus toxoid and *Bordetella pertussis* vaccine. This quadruple vaccine (DPT-polio vaccine: Connaught Laboratories) has been used with success in Canada and is known to cause little reaction in children. Four experiments of this type were undertaken. The essential details and the results obtained are summarized in Table 2. As the findings were similar in each experiment in spite of some

variation in the experimental conditions it is permissible to accept this as one composite experiment. It then appears that 43 of the 52 animals included in the experiment (82 %) developed an appreciable degree of immunity as the result of receiving approximately 10 ED_{50} doses of vaccinia virus suspended in multivalent vaccine. A hypersensitive type of reaction was produced in 32 % of these animals.

These results emphasize the importance of using a large number of animals for experiments of this kind. Although the response to inoculation of a small quantity of active virus was not uniform there is clear evidence that most of the animals developed a level of immunity high enough to be measured by conventional methods. Further analysis of the results showed that an immune reaction in the challenge test was associated with the presence of circulating antibody. In contrast to this, a hypersensitive response was shown by those animals which had failed to produce complement fixing and neutralizing antibody. It will be recalled that a similar phenomenon occurs in other infective diseases in which hypersensitivity is a prominent feature of the immune response.

The adjuvant effect of the quadruple vaccine cannot be attributed wholly to the *B. pertussis* component. This was shown by the results of another experiment in which the same small amount of vaccinia virus (10 ED_{50} doses) was incorporated in quadruple vaccine from which the *B. pertussis* cells had been removed by centrifugation. Seven of the ten rabbits inoculated with this material showed some degree of immunity when they were subsequently challenged, one was hypersensitive and 2 were normally susceptible. Complement-fixing antibody was present in the sera of 4 of these 7 immune animals. The control for this experiment consisted of 23 rabbits: these received the same amount of virus suspended in buffer. Of this group, 16 were fully susceptible to the subsequent challenge, 4 showed some degree of immunity and 3 were hypersensitive.

In the course of some other investigations it was found that polyvinylpyrrolidone (PVP) is a valuable immunological adjuvant. When particulate antigens such as bacterial cells or vaccinia elementary bodies are mixed with a solution of this substance each particle becomes coated with a layer of the polymer. As a consequence the forces responsible for maintaining suspension stability are reduced and the particles begin to form microscopic aggregates. This slowly progresses to the stage of flocculation; but even when a deposit has formed a satisfactory degree of dispersion is readily obtained by gentle shaking. Solutions of PVP have been widely used as a plasma substitute: the extensive literature on this subject testifies to the harmless nature of such solutions when introduced parenterally. For use as an adjuvant, equal volumes of a 25 % aqueous solution of PVP* previously sterilized by autoclaving, and an appropriate dilution of the antigen are mixed and inoculated subcutaneously. A needle of medium bore can be used for giving the injection because the solution has a low viscosity. The adjuvant effect of the PVP is attributed to delayed absorption whereby a slow and steady antigenic

* A purified polyvinylpyrrolidone suitable for parental injection is manufactured by the General Aniline and Film Corporation, New York, N.Y. This is sold under the name of Plasdone C.

stimulus is obtained. The subcutaneous inoculation of a 12·5% solution of PVP into man causes practically no pain.

Two experiments were undertaken to determine whether PVP used in the manner indicated above would influence the immune response to a small dose of active vaccinia virus. The infecting dose was 10 ED_{50} units of vaccinia EBS incorporated in 1·0 ml. of 12·5% PVP inoculated subcutaneously. There were no local or general reactions. The challenge test was made 18 days later and blood samples were taken at the usual intervals. A control group of rabbits received the same infecting dose of virus suspended in buffer solution. The 2 experiments differed only in minor details: it is permissible, therefore, to present them as one composite experiment. The essential data are given in Table 3.

Table 3. *Adjuvant effect of PVP on the immune response of rabbits to* 10 ED_{50} *doses of purified vaccinia virus*

(*a*) 41 rabbits received 10 ED_{50} doses of vaccinia E.BS incorporated in 1·0 ml. of 12·5% PVP solution. Results of challenge test given 18 days later:

Normal response	1 rabbit
Immune response	33 rabbits
Hypersensitive response	7 rabbits

(*b*) 32 rabbits received 10 ED_{50} doses of vaccinia E.BS in buffer. Results of challenge test given 18 days later:

Normal response	16 rabbits
Immune response	9 rabbits
Hypersensitive response	7 rabbits

The results of the serological tests need not be given in full. Only 2 of the 32 rabbits inoculated with the virus in buffer solution produced complement-fixing antibodies in response to the infecting dose whereas 21 of the 41 rabbits included in the PVP group showed a positive reaction.

From the examples given it is evident that the immune response to a very small amount of living vaccinia virus is considerably increased by the use of adjuvants. Solutions of PVP have the additional advantage of being powerful protective colloids. Vaccinia elementary bodies suspended in PVP survive for several weeks at 37°C. whereas the same virus suspended in 50% glycerol-buffer solution is inactivated within 2 weeks at this temperature (Amies, 1962).

There can be no doubt that multiplication of virus took place in the tissues of the inoculated rabbits. A simple calculation* based on the known size and density of the infective particle shows that the mass of virus protein injected was of the order of 10^{-5} μg: this is below the minimal amount of antigen required to produce a primary immune response. It is equally certain, however, that viral proliferation

* For the purpose of this calculation let it be assumed that one living vaccinia elementary body measures $250\mu \times 200\mu \times 200\mu$. It is further assumed that 1 ED_{50} dose of virus is equivalent to 100 elementary bodies. Then 10 ED_{50} doses, the amount usually employed in these experiments = 1000 elementary bodies. The mean density of the virus particle is taken as 1·3.

1000 E.BS have a volume of $10^3 \times 0{\cdot}25 \times 0{\cdot}2 \times 0{\cdot}2\ \mu^3 = 10\ \mu^3$; $1\ \mu^3 = 10^{-12}$ cm^3.

So the weight of 1000 E.BS is $1{\cdot}3 \times 10^{-12}\ g. = 1{\cdot}3 \times 10^{-5} \mu$g.

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was greatly restricted. The experiments of Calmette & Guérin, already quoted, bear witness to the truth of this statement. Moreover, all attempts made in this laboratory to demonstrate the presence of virus in the tissues of minimally infected rabbits were unsuccessful.

DISCUSSION

These experiments show that the subcutaneous injection of purified vaccinia virus in minimal amounts will evoke an immune response which can be defined in terms of hypersensitivity and antibody formation. This immune response can be materially improved by incorporating the virus in an adjuvant such as PVP or one of the prophylactics now administered as a routine in infancy. More information is needed, particularly with regard to the duration of immunity achieved by this method. Some of this information can be obtained by further animal experimentation but clinical trials will ultimately have to be undertaken in order to determine whether the new vaccine is likely to be of practical value. The present studies, supported as they are by the work of the other investigators already quoted, are ample justification for such clinical trials. The evidence provided by the animal experiments suggests that the primary sensitization produced by a very small dose of living purified virus may need to be reinforced by smallpox vaccination performed in the normal manner. It is probable that the result of the latter procedure will be an accelerated reaction with no general symptoms and a minimum of scar formation.

The advantages of this method of vaccination will be readily appreciated: an entirely subclinical primary infection with no danger of spread of virus either to adjacent skin areas or to other persons. Subcutaneous inoculation also avoids variable results due to differences in vaccination technique and differences in the texture of the skin. No guarantee can be given about the prevention of post-vaccinial encephalitis because the aetiology of this condition is not yet established. Nevertheless, there is evidence that this condition usually follows a severe local reaction with considerable proliferation of virus. Conversely, a history of a poor 'take' is seldom obtained in post-vaccinial encephalitis. This grave disease is, therefore, very unlikely to follow the minimal proliferation that occurs when the new vaccine is employed.

Objections to this method of vaccination against smallpox are likely to be formidable in spite of good evidence to the contrary. One can readily understand the reluctance of a medical officer to alter a procedure which has proved on the whole extremely successful for more than a century. The argument that subcutaneously inoculated virus will be followed by viraemia leading to generalized vaccinia has already been considered. A viraemia of short duration, usually unrecognized or marked only by a febrile reaction, occurs as a normal event after jennerian vaccination. Generalization is a rare event even when, as in normal vaccination, a large dose of virus is applied directly to the highly susceptible epidermal cells. Unsatisfactory results, if they occur, are more likely to be due to a failure of the virus to proliferate sufficiently. Herrlich (1959) whose work has already been quoted, abandoned subcutaneous inoculation partly because he found that his virus preparations had a low immunizing capacity. A probable explana-

tion is provided by experiments performed in this laboratory. In the course of purification of crude vaccinia suspensions by fractional centrifugation it was found that the final E.BS, after making allowance for alterations in volume, had a higher infective potency than that of the original skin pulp suspension in spite of mechanical losses at each step of purification. Mixing the purified virus suspension with crude pulp extracts freed of virus by ultracentrifugation produced an appreciable decrease of infectivity for the rabbit skin. This is perhaps an example of auto-interference or it may be attributed to non-specific effects; but whatever the explanation may be these findings provide another point in favour of using purified virus for immunization.

The proposal to introduce a new biological preparation for general use raises many problems of technical, administrative and commercial importance. It may be objected that in spite of the advantages claimed for it a two-injection method of immunization against smallpox is not acceptable for administrative reasons. This is not a valid argument. Incorporation of purified vaccinia in one of the universally employed combined prophylactics will ensure that the infant population receives the primary sensitizing dose of virus without additional work on the part of the medical officer. The second or reinforcing dose can be carried out a few weeks later by a normal vaccination technique. Further clinical experience may show that this second dose can also be given as a minimal quantity of purified virus incorporated in a combined prophylactic. If this proves to be the case, vaccination against smallpox could be secured automatically during the course of immunization with accepted prophylactics. The immunization of adults constitutes a different problem. In this group the incidence of severe reactions is higher and there is a greater risk of post-vaccinial encephalitis. If these hazards can be avoided by a two-injection immunization this should become the method of choice even though it entails two attendances at a clinic.

With respect to cost of production, stability and acceptability the purified vaccine has many points in its favour. Further discussion of these matters, however, can be deferred until clinical evaluation studies have been completed.

SUMMARY

Minimal quantities of active vaccinia virus in the form of a purified elementary body suspension were injected subcutaneously into rabbits. The amount administered was about 10 minimal infective doses as measured by intracutaneous titration and tissue culture assay: this is equivalent to about $1{\cdot}3 \pm 10^{-5}\mu$g. of virus protein. The immune response to this minimal quantity of virus was determined by complement-fixation and virus neutralization tests and also by skin sensitivity tests. The results indicated that viral proliferation occurred to an extent sufficient to sensitize the tissues to the virus protein. Measurable amounts of antibody could also be detected in about one half of the experimental animals. A second inoculation of virus produced a considerable augmentation of the immune response. Viral proliferation must have taken place in order to evoke this immunity, but infection always remained at the subclinical level. The results were much improved

when the infecting dose of virus was incorporated in an adjuvant. DPT-polio vaccine functioned well as adjuvant but equally good results were obtained with a solution of polyvinylpyrrolidone. The latter substance also has valuable properties as a protective colloid and is thus able to maintain the viability of the virus over long periods of time.

On the evidence provided by these experiments it is suggested that an adequate immunity to smallpox might be achieved in man by similar methods. A primary sensitization could be achieved by incorporating a very small amount of active virus in one of the prophylactics given as a routine in the early months of life. This could be followed by normal jennerian vaccination a few months later; or the second inoculation of virus could also be incorporated in amultivalent prophylactic. For adults, a somewhat similar two-stage immunization would be required.

It is hoped that the publication of these preliminary experiments will encourage other investigators to continue what promises to be an important line of research.

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