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SMALLPOX

Observations on the reliability of rapid electron microscopic diagnosis

by

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As a result of the increased contact of our population with countries outside Europe and the reduction in travel time brought about by air transport, smallpox has repeatedly been imported into Europe in recent years. In Germany during the post-war years there have been an isolated case in Hamburg in 1957 (Andres et al.); an outbreak affecting 20 persons in Heidelberg in 1958/1959 (Bingel & Kruse); another isolated case in Berlin in 1959 (Dumjahn et al.); and four cases in Ansbach in 1961 (Herrlich et al.). The disease was introduced on each occasion by persons showing only a clinical picture of varioloid, a form of smallpox seen in partly immune subjects.

The clinical diagnosis of varioloid which in some circumstances can follow a very mild course, is difficult, as is also diagnosis of the initial stage of true smallpox (variola vera). The clinicians will therefore always call on the laboratory for assistance and, because of the serious issues involved, the rapidity and reliability of diagnosis are of the greatest importance.

This paper describes laboratory experience gained during two other outbreaks induced in Düsseldorf and Simmerath (Monschau district) at the beginning of 1962, the disease being introduced from Liberia and India respectively. The work discussed includes direct electron microscopic detection of the elementary bodies and the culture of the causative agent on the chorio-allantoic membrane of the fertilized hen egg.

Egg culture is generally regarded as the most sensitive and therefore most reliable method of detection. By this means the presence of the virus can be shown from the initial phase until the late (crust) stage; culture in the fertilized hen egg permits of differentiation between the variola, vaccinia and

herpes simplex viruses and gives negative results with varicella and measles. Furthermore, under certain conditions it makes possible a distinction between variola major and variola minor (see below). It has proved to be appreciably better than the Paul test and the sole, although serious, drawback of the method is that it requires a relatively long time. After 48 hours the readings give only provisional results, reliable findings being possible only after 72 hours.

The most rapid test is the morphological one, although it does not allow differentiation between variola and vaccinia viruses. The results of examination with the normal light microscope of stained elementary bodies (which in favourable cases can be done within 30-60 minutes) are uncertain when, as for example in the early stage, there are only a few particles, or when cell debris hampers recognition. In the electron microscope, on the other hand, the typical quadrangular shape of the elementary bodies is visible; even a few particles suffice for a positive finding and cell debris is recognized as such. However, so far experience with this method has been limited.

After Nagler & Rake, as well as Van Rooyen & Scott had indicated in 1948 the possibility of using the electron microscope to diagnose smallpox, Lépine & Croissant described the electron microscopic appearance of the pathogen on the occasion of an outbreak in France in 1952. When the Hamburg case occurred in 1957 (Andres et al.) we were able for the first time directly to confirm a presumptive clinical diagnosis within two hours with the aid of the electron microscope. In connexion with the Heidelberg epidemic in 1958-1959, Bingel & Kruse using specimens from 20 smallpox patients (throat washings and content of pustules) made comparative examinations by using the electron microscope and fertilized egg or tissue culture methods. They found a good agreement in 16 cases. With some specimens morphological confirmation was achieved within a few hours, and with others later on. In the Berlin case too (1959), the electron microscope was called in to confirm the diagnosis (Dumjahn et al.).

The aim of the investigations described below was to determine how far the immediate electron microscopic results agreed with those obtained in the fertilized egg test later on, particularly when, as in these cases, the specimens could not be taken by laboratory personnel directly and when, as was perforce often the case with varioloid patients, only a small amount of material was available.

Specimens and methods

(a) Specimens

The specimens examined came without exception from the skin and had been taken by the doctors giving treatment themselves. They were either "excitation sera" (as far as possible blood-free, taken during the rash stage, which appears for a short time during the initial phase, or during the maculo-papular stage) or the contents of vesicles or pustules (occasionally together with the tops of the vesicles and with scabs). These were transferred in a thick layer, without any special spreading out, to a grease-free slide and dried in the air. The use of heat to speed up drying prevents both morphological and cultural virus detection. When specimens from different vesicles were dried closely together on the same slide, this proved to be particularly favourable for subsequent egg inoculation; furthermore, the marking of the specimen side with a greased pencil or in some similar way was very useful.

Usually wooden slide holders were employed for sending in specimens. However, because of possible contamination with shavings it is preferable to cover the specimen slide with a second slide, separating the two with blotting-paper or match-sticks and surrounding them with sticking plaster. As a rule, specimens were brought in directly by messengers, by the police or by special delivery. Experience showed that it was extremely important to inform the laboratory at the same time by telephone (night service!). In most cases the packing was in accordance with the highly-infectious contents, the package being designated on the outside as "highly infectious matter".

Thus the requirements for obtaining the specimens are extremely small. Cooling is not necessary, since the air-dried smallpox virus remains active for a long time at ordinary temperatures.

Naturally, the laboratory must endeavour to obtain as much material as possible from several eruptions. This not only increases the chances of detecting the virus but also prevents false diagnosis based on matter from non-specific lesions. In varioloid cases, it is advisable to take specimens from eruptions in different stages. Even if only little matter can be obtained in a very early stage of the disease, this need not be any hindrance to obtaining a positive result (see below).

(b) Electron microscopic method

The "indirect dabbing method" (Peters & Nasemann) which has already repeatedly proved successful, was used for the preparation. The air-dried specimen on the slide was moistened with distilled water, ground up and, after preparing at least two different concentrations, transferred by spotting to about eight platinum carriers with seven holes (Siemens type). The collodion films were first stabilized by depositing a layer of vaporized carbon on them. After fixation with one per cent. OsO_4 (10 minutes) and washing, the preparation was finally heated for half an hour at 90°C in the drying oven so as to prevent any introduction of active virus from the isolation laboratory.

All these steps, which are capable of yielding good preparations, took less than an hour to carry out. Further treatment with concentrated uranyl acetate for 10 minutes was found to be advantageous but not essential. It produced specific contrasting of the internal virus structure, particularly of the DNA-containing inner body, thus making easily recognizable even those elementary bodies which were embedded in diffuse impurities. We finally decided not to use shadow casting, since this offered no special advantage.

The preparations were examined in the Elmiskop I under a magnification of 15 000. We often employed two electron microscopes simultaneously, thus saving valuable time.

(c) Virus culture

We used as culture medium 11-12 day fertilized hen eggs (in exceptional cases 10 and 13 day eggs). (Since the Hamburg case in 1957, we have made sure that eggs in this stage are always available for diagnostic purposes.) After preparing the

specimen for the electron microscope, we took up the matter remaining on the glass slides in 0.3-1.0 ml of Earle's solution (according to the amount involved) to which five per cent. horse serum and antibiotics had been added, and used this suspension in at least two dilutions (1:10 and 1:100) to inoculate the lowered chorio-allantoic membranes (0.1 ml in each case). At least two and usually more eggs were employed for each dilution stage. The first half were read after two days and the second after three days. In positive cases we regularly found typical lesions at least in one dilution stage, already after two days. Only in two of the 22 cases found positive on the third day had the result still been read as negative on the second day.

Incubation was always carried out at 36°C. Using a method based on one recently described by Nizamuddin & Dumbell, we also tested both epidemic strains after the first isolation for multiplication at 38.3°C, so as to distinguish between variola major and variola minor.

Results

On the occasion of the two outbreaks in Düsseldorf and Simmerath we examined 85 specimens in the manner described between January and April 1962. Only the specimens from 23 cases finally diagnosed by the physicians as variola or varioloid call for special attention. Table 1 shows the corresponding results in so far as they are of diagnostic significance. Check tests were not included.

It may be stated straight away that both epidemic strains were variola major ones. Both multiplied at 38.3°C whereas according to Nizamuddin & Dumbell this is not the case with variola minor strains. (We were able to confirm this using a variola minor strain known to us.)

The data in Table 1 have been arranged, neglecting the actual order of the investigations, so as clearly to show the relationship between the day of the disease, the day of the exanthema and the results obtained. Since in our investigations only matter from the skin was used, the beginning of the eruptive phase (exanthema day) has been placed in the forefront. For comparative purposes, serological results obtained by other workers have been included, provided they refer to the same period of the disease.

The fact that there was excellent agreement between the electron microscope and egg-culture results can be regarded as the most important finding. In all 21 cases in which the culture gave definitely positive results, quadrangular viruses had already been detected morphologically at least two days previously. In two cases the culture results were negative or doubtful after a few typical elementary bodies had been recognized with the electron microscope (Nos. 5 and 11). However, fresh specimens from the same patients, taken two and four days later, respectively, proved to be positive with both methods, as had been expected (Nos. 19 and 23).

In the case of three patients (Nos. 20, 24 and 26) both tests were negative, in part even when repeated with new specimens (No. 27). In view of the agreement of the negative findings, however, it is probable that the specimens were unsuitable or non-specific rather than that the methods of detection failed. Thus, specimens Nos. 26 and 27 came from a chronic eczema patient and, in addition, were obtained at a very late stage of the disease.

It should be stressed that precisely in the important early stages of skin lesions, positive results were regularly obtained, in a stage of the disease where, as shown by Table 1, antibody cannot yet be definitely detected by the haemagglutination test. A significant increase in antibody titre is generally held to occur as from the seventh day of the disease, at the earliest. An exception to this was seen in only one case (see Nos. 8 and 21) where the disease was primary haemorrhagic smallpox with a fatal conclusion. The relatively high titre, at first 1:128 and then 1:320 is, however, in contradiction to the findings of other authors in similar cases of primary haemorrhagic smallpox (see Herrlich et al.).

As already mentioned under Specimens and Methods, less than one hour is necessary to prepare the specimens for the electron microscopic preparation, including inactivation. Quite often, virus was then detected already in the first minute of observation. Naturally, a longer period of examination was required with negative specimens. Nevertheless, we repeatedly found that a result definitely negative after examination for two hours still remained negative on further observation.

Difficulties in judging the electron microscopic preparations occurred only when specimens consisting of the purulent contents of pustules were received, as well as in the case of matter from acne and pyodermia cases. Cellular fragments of leukocytes which occasionally were of about the same order of size and showed about the same contrast as the elementary bodies, might be taken for virus particles on superficial examination; usually, however, their polymorphism served to distinguish them and, as a rule, they could be recognized as non-specific material when the photographic plate was examined and the internal structure considered, if not sooner. In difficult cases where doubt still persists, breakdown treatment with pepsin or trypsin can assist in differentiation, since this reveals characteristic internal structures within the surrounding membrane (Peters). These difficulties did not give rise to any false identifications in our case. Thorough experience in the recognition of quadrangular viruses is, of course, necessary in dealing with such specimens.

Other suspect matter which we found to be negative, came - according to information supplied later on by the doctors concerned - from generalized vaccinia (one case), molluscum contagiosum (one case), varicella (15), acne (five), pyodermia (five), exanthema caused by drugs (two), as well as one case each of generalized herpes simplex, septicaemia, dermatostomatitis, erythema exudatum, Seidlmeyer's purpura, Besnier's prurigo and Stevens-Johnson syndrome. In eight cases the diagnosis remained unknown to us. Repeatedly specimens from the same patient which had been found negative were examined, at intervals, twice or even three times.

In the generalized vaccinia case examined, which was observed in close relation to the Düsseldorf outbreak, skin specimens from the third exanthema day (fourth to fifth day of the disease) showed no quadrangular viruses under the electron microscope for reasons which have not yet been explained. Nevertheless, foci were seen on reading the egg culture test. On morphological reasons they could at first not be attributed without reservations to vaccinia virus alone. The lesions further exhibited considerable signs of haemorrhages. The following passages showed the same picture. The situation only became clarified when we carried out comparative tests with the Bern vaccinal strain used in this case, for the said strain behaved

in practically the same way. This experience shows that it would be advisable in future outbreaks to supply the laboratory making the investigations with a sample of the vaccine used at the beginning of the outbreak for any necessary comparisons.

Because of its different clinical course, suspicion of smallpox will only rarely arise in relation to molluscum contagiosum. Under the electron microscope we found in the case mentioned above, as on many earlier occasions (Peters & Stoeckenius), a large number of quadrangular elementary bodies. Outstanding purity of the preparations is regularly observed accompanied by the presence of virus particles on a large scale. It is true that this is no adequate criterion for distinction from variola and vaccinia viruses but nevertheless, it is a valuable indication. In any event, the fact that it cannot be cultured makes it possible to distinguish the molluscum contagiosum virus from the smallpox virus quite easily and reliably.

The large number of varicella specimens sent in is indicative of the difficulties repeatedly facing the physician during epidemics, when he must distinguish this clinical picture from that of varioloid. The diameter of the elementary bodies of varicella is about 180 mμ and they are thus smaller than the smallpox virus, in addition to differing from the latter in their shape, which is spherical. Like earlier workers (Nagler & Rake, Lépine & Croissant, Bingel & Kruse) we did not find the elementary bodies in all specimens, but only in eight out of 15 and even then in a relatively low concentration. Variola virus could be excluded in such suspect cases on the basis of electron microscope examination alone, while the fact that varicella virus cannot be cultured in the hen egg supplied a final confirmation.

DISCUSSION

The investigations described above, covering 23 smallpox cases show that the electron microscope test for the causative agent is just as reliable as the culture in the fertilized hen egg, regarded as the most sensitive test for variola virus. Against the disadvantage that the variola virus cannot be distinguished from the vaccinia virus under the electron microscope, must be set the compensating

advantage that the result is very rapidly obtained. When the laboratory has been informed in good time, a positive result can be available within one hour; on the other hand, the egg culture test requires two to three days. In considering what quarantine measures to take, however, every hour counts.

There can be no doubt that the electron microscope is superior to the light microscope for the recognition of the typical form of the elementary bodies. The higher cost is justified by the possibility of recognizing the virus when only a few elementary bodies are present or when recognition is hampered by the presence of cellular debris. So as not to waste the usually scanty specimens, we normally did not make use of ordinary light microscopy.

The fact that the results with specimens taken during the vesicular stage were positive is in good agreement with what has long been known in light microscopy. The important point is, however, that detection was invariably successful in the cases examined in the early stages also, namely already in the pre-eruptive stage (one day after the rash), on the first day of exanthema or the fourth day of the disease. If specimens taken even earlier had been sent in, then it might have been possible to reach a positive result even sooner.

Bingel & Kruse based their investigations to a large extent on throat washings and reported that in four favourable cases they had been able to detect the virus by culture already in the pre-eruptive phase. Downie et al. later observed in a large number of smallpox cases in Madras, that the pathogen could be shown by egg culture to be present in throat washings only from the third day of the disease onwards. The percentage of positive results increased from 50 per cent. on the third day, however, to not more than 65 per cent. on the sixth to ninth days. Thus, in the light of present knowledge, throat washings do not present any definite diagnostic advantage over material from the skin. In addition, the method is little suited to the dispatch of specimens, since in a liquid medium the virus is relatively instable as compared with the dry state.

On these grounds, and bearing in mind the need to make the method simple, we gave preference to examination of air-dried serum as well as the contents of vesicles and pustules, where the virus is very stable. In the cases described, the specimens were taken by the doctors in attendance, using methods they had chosen themselves. Clearly, no highly refined technique is necessary for this. As a rule in positive cases it was only necessary to send specimens once. However, in all test methods the success and possibility of obtaining readable results is dependent on the quantity and quality of the specimen examined. Therefore it is essential to comply with the regulations for dispatch (see Specimens and Methods) and, in particular, to send adequate material (as far as possible the contents of six pustules). Whenever circumstances permit, the laboratory should be questioned in advance by telephone, regarding the most suitable way of taking and sending in the specimens. Inadequate quality or quantity of the specimens sent in was probably responsible for the fact that three patients (Nos. 20, 24 and 26/27 in Table 1) whose illness had been diagnosed as smallpox on the basis of clinical and epidemiological data (in one case also virological findings from Munich), gave negative results both in the electron microscopic examination and in the egg culture test. In one of these cases (No. 26/27) it is true, the circumstances were particularly complicated, since the patient was a sufferer from eczema and the specimens were taken in a very late stage of the disease.

The electron microscope results were immediately communicated by telephone in all cases. In conjunction with a critical evaluation of the clinical findings they enabled the medical authorities and the clinic to take the appropriate measures and institute proper treatment without any appreciable loss of time. The egg culture results read two or three days later represented a further control and a confirmation of the morphological findings; in addition, they indicated the type of virus.

The serological methods for detection of virus antigen, namely the complement-fixation test and the agar-gel precipitation test recently developed by Dumbell & Nizamuddin have the same disadvantage as the electron microscopic method, i.e.

they cannot distinguish between variola and vaccinia virus. Under favourable conditions they require four to six hours; experience has shown that a relatively large specimen is necessary. They should only be used as an adjunct to electron microscopy and egg culture when adequate material is available.

The serological detection of complement-fixing antibody succeeds as a rule only in relatively late stages of the disease, since such antibodies normally appear only from the seventh day of the disease onwards. It is true that haemagglutination inhibiting antibody appears somewhat earlier but there are considerable differences of opinions regarding the diagnostic value of this titre, unless it is extremely high, especially in regard to its significance in epidemics, when a distinction must be made between genuine consequences of the disease and vaccinal reactions. On the other hand, detection of antibody is of great importance in the retrospective diagnosis of varioloid patients, who at first go undetected because of the abortive clinical course of the illness. Both outbreaks resulted from the introduction of the disease by such cases; on both occasions the antibody detection tests carried out with these persons after the occurrence of the first contact cases were undoubtedly of great diagnostic importance. At this late stage (19th-25th day of the disease) electron microscopic examination would probably have been unsuccessful.

During the last European smallpox epidemic it was found that the course of the disease in vaccinated subjects frequently deviated from that generally expected (different incubation periods, difference in the quality and quantity of the exanthema, etc.). As a result, clinical diagnosis can seldom be sufficiently definite and rapid. Bearing in mind the importance of tracking down suspect cases immediately and also the equal importance of rapid diagnosis of late cases, further comparative tests in the very early and in the late stages of the disease - both in variola vera and varioloid - would be very desirable. The investigations were carried out with the support of the Deutsche Forschungsgemeinschaft. The exemplary technical assistance of Miss U. Lehmann, Miss C. Sievers and Mr H. Giese was of great help. For important information supplied by them we should like to thank in particular Drs Schwonzen, Knab and Linzenich (Simmerath); Professor Stüttgen and Dr Richter (Düsseldorf); Professor Herrlich (Munich); Professor Mohr, Dr Ehrengut and Dr Bonitz (Hamburg).

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SUMMARY

In 23 smallpox cases occurring during the outbreaks in Düsseldorf and Simmerath, specimens from the skin were examined and excellent agreement obtained between the results of electron microscope examination for the pathogen and culture of the virus in the fertilized hen egg. Because of its rapidity (results one to two hours after receiving specimens) electron microscopic diagnosis has proved valuable in dealing with suspected cases. Both methods regularly gave good results, even in the early stages of the disease, e.g. already in the pre-eruptive stage, on the first day of exanthema or the fourth day of the disease. The advantages and disadvantages as compared with serological methods are briefly discussed.

TABLE 1. RESULTS OF DIAGNOSTIC TESTS OF SPECIMENS FROM SMALLPOX PATIENTS

Specimen number	Patient	Day of exanthema[1] (beginning of eruptive phase)	Day of disease[1]	Matter from the skin		Blood	Comments
				electron microscope	egg culture (1 passage)	haemagglutination inhibition test,[2] either simultaneously, previously, or afterwards (d = days)	
1	M. Hü	-	5	+	+	1:8	rash one day previously
2	G. G.	1	5	+	+	-	
3	M. He	1	5	+	+	negative 10 d previously 1:80 6 d afterwards	rash one day previously
4	J. O.	1	6	+	+	negative 2 d previously 1:320 10 d afterwards	rash two days previously
5	C. A.	2	4	(+)[3]	ø	1:16	cf No. 19
6	A. G.	2	4	+	+	1:10 11 d previously 1:1280 5 d afterwards	
7	T. G.	2	5	+	+	negative	
8	A. S.	2	5	+	+	1:128	cf No. 21
9	H. F.	2	6	+	+	1:4	
10	K. P.	2	7	+	+	1:80 3 d afterwards	
11	K. S.	2	10	(+)[4]	?+	1:4 1 d previously	cf No. 23
12	I. J.	3	4	+	+	1:8 1 d previously	
13	H. J. S.	3	5	+	+	1:20	
14	M. L.	3	6	+	+	1:32	
15	L. P.	3	6	+	+	1:8 1 d previously	
16	U. S.	3	6	+	+	1:40	
17	R. J.	3	7	+	+	1:80	
18	G. N.	3	7	+	+	1:320 7 d afterwards	

TABLE 1. RESULTS OF DIAGNOSTIC TESTS OF SPECIMENS FROM SMALLPOX PATIENTS (continued)

Specimen number	Patient	Day of exanthema[1] (beginning of eruptive phase)	Day of disease[1]	Matter from the skin		Blood	Comments
				electron microscope	egg culture (1 passage)	haemagglutination inhibition test,[2] either simultaneously, previously, or afterwards (d = days)	
19	C. A.	4	6	+	+	1:16 2 d previously	repetition; see No. 5
20	K. M.	4	8	ø	ø	1:16 3 d previously 1:128 6 d afterwards	
21	A. S.	5	8	+	+	1:320	repetition; see No. 8
22	E. S.	6	9	+	+	negative	
23	K. S.	6	14	+	+	1:640	repetition; see No. 11
24	J. G.	9	13	ø	ø	1:64 3 d previously 1:64 6 d afterwards	
25	W. B.	10	13	+	+	no blood could be obtained	
26	J. S.	15	18	ø	ø	1:160	cf No. 27
27	J. S.	17	20	ø	ø	1:160 2 d previously	repetition; see No. 26

[1] According to information from the physicians in attendance.

[2] Results of the Hamburg Vaccine Institute and the Bavarian Provincial Vaccine Institute, Munich. (Because of differences in the mode of calculation, the scale of titres is not absolutely identical.)

[3] Only two elementary bodies found.

[4] Only seven elementary bodies found.