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A virological study of post-vaccinal encephalitis

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SUMMARY

In the province of Voyvodina 18 cases of post-vaccinal encephalitis have been recorded, three of which were fatal.

The estimated morbidity rate was 0·6 per 10^5 after revaccination, and 3·9 per 10^5 after primary vaccination. The virological studies described, as well as other findings referred to in this report, are consistent with the possibility of an auto-immune aetiology of the post-vaccinal encephalitis cases observed.

INTRODUCTION

During the last smallpox outbreak in Yugoslavia, in the province of Voyvodina, 91 % of the total population of 1,950,268 was successfully vaccinated during April and May 1972. According to the official data (Vuković, Miškov & Mudrić, 1973) 60,896 persons missed vaccination; in 50,392 persons vaccination was contra-indicated; in 68,212 vaccination failed; 1,770,750 persons were successfully vaccinated, and the remaining 18 persons developed post-vaccinal encephalitis.

Of the 18 encephalitis cases 11 (0·6 per 10^5 vaccinees) occurred after revaccination and seven cases (3·9 per 10^5 vaccinees) after primary vaccination. Details of these 18 cases are shown in Table 1. From the nine surviving patients a total of 16 serum samples were collected (at the 3rd to 17th day of illness, i.e. at the 14th to 29th day after vaccination), and from five of these patients six CSF samples were taken.

The 18 encephalitis cases were all admitted to hospital. The clinical findings, treatment and results have been fully reported by Orovačanec, Mudrić, Vučković & Vuković (1973).

The origin of the samples tested, and some data relating to the history of survivors donating samples of CSF and serum, are shown in Table 2. In the three patients who died the onset of illness occurred on the 11th to the 19th day after vaccination, and the patients died on the 1st or 2nd day after the onset of manifest illness.

CF titres in the serum samples ranged from 1/4 to 1/32 and the samples of CSF were negative even in dilution 1/1. The results of serological studies will be fully discussed elsewhere; here we present only the results of our attempts to recover virus from the samples of CSF.

MATERIALS AND METHODS

Before the addition of antibiotics, samples of undiluted CSF and serum were seeded on blood agar plates and into thioglycollate and plain broth media. After 48 hr. incubation all media remained sterile. Samples of CSF intended for inoculation were preserved with penicillin 1000 units/ml. and streptomycin 2 mg./ml. and stored at $-70°$ C. for 1–14 days.

Seed virus was prepared from 96 hr. old, once frozen and thawed cultures of vaccinia virus in primary rabbit kidney cells.

Media and diluents contained penicillin 100 units/ml. and streptomycin 100 μg./ml. Maintenance medium was medium 199 (pH 7·4–7·6) with 5 % calf serum and antibiotics. Growth medium was medium 199 (pH 7·2–7·3) with 20 % calf serum and antibiotics.

If not stated otherwise, inocula were both prepared and diluted with the maintenance medium.

Inoculation of eggs

Before inoculation, eggs were incubated at 38–39° C. Chorioallantoic membranes (CAM) of 12-day-old chick embryos (CE) were seeded with 0·2 ml. of inoculum per CAM, incubated at 36° C and candled twice daily.

Sluggish and dead CE within 24 hr. after inoculation were discarded. Two days after inoculation dead CE were harvested, and 3 days after inoculation both dead and live CE were harvested and the number of typical pocks was counted on each CAM. The sensitivity of the procedure used for isolation of vaccinia virus was tested by inoculation of CAM with tenfold dilutions of seed virus. The LD50 of the seed virus was a dilution of 10^{-5}, and a 10^{-7} dilution of the same suspension produced 5 to 15 pocks per CAM, with no death of embryos until the 72nd hour of incubation.

Primary cultures of trypsinized kidney cells

These were prepared by distribution of 1 ml. volumes of freshly made suspensions into tubes which were incubated without rolling. Cell cultures were kept in growth medium for 4–6 days at 37° C, with one fluid change after 48 hr. Confluent monolayers of cells were inoculated by adding 0·2 ml. inoculum to each tube.

The sensitivity of RK cell cultures for propagation of vaccinia virus was tested by inoculation of the cultures with dilutions of seed virus. Depending upon the dilution of the inoculum, CPE appeared within 24 hr. to 4 days after infection with vaccinia virus. Rounded-up cells of darker appearance were scattered throughout the field, first single and later aggregated. As the CPE spread, degenerated cells fell off the glass, leaving only small isolated aggregations of rounded cells. At the 4th day of incubation a $10^{-5·3}$ dilution of the seed virus induced CPE in 50 % of the tubes.

Herpes simplex virus gave an ID50 mostly in dilutions of $10^{-6·3}$. It is known that primary cultures of RK cells are sensitive also for other members of the herpes group, as well as for simian foamy virus and for some arboviruses. It

Table 1. *Post-vaccinal encephalitis in Voyvodina*

Age group (years)	Number of cases after: Primary vaccination	Number of cases after: Revaccination	Total cases	Fatal cases
5–14	2 (1 S+F)*	3 (1 S+F)	5	1
20–68	5 (2 S+F)	8 (1 S+F) (4S)	13	2
All ages	7	11	18	3

* The figures in parentheses indicate numbers of patients from whom specimens were taken for examination. S+F = serum and cerebrospinal fluid; S = serum only.

should be noted that in cases of herpes simplex encephalitis the chances are especially poor for the isolation of the virus from lumbar CSF (MacCallum, 1969).

Inoculation of baby mice

Inoculation of 1-day-old sucklings with the seed virus (0·01 ml. cerebrally or 0·05 ml. peritoneally), produced pronounced runting, decreased motility, tremor, cyanosis and death of all animals within 2–6 days.

For detection of vaccinia virus, the inoculation of suckling mice proved to be inferior to the inoculation of cell cultures or CAM of embryos. Moreover, 10-day-old sucklings, or older animals, proved to be significantly less sensitive to cerebral or peritoneal infection with vaccinia than 1- or 2-day-old sucklings.

In our laboratory herpes simplex virus has been easily recovered by cerebral inoculation of suckling or adult mice with vesicular fluid of herpetic eruptions or with 10^{-5} dilutions of herpes simplex virus-infected tissue culture fluids. It is known that baby mice are a sensitive host also for the isolation of Coxsackie A and B, LCM, rabies and of many strains of arboviruses.

RESULTS AND CONCLUSIONS

Six samples of undiluted CSF were inoculated onto CAM. Each sample was inoculated onto six membranes, and after 72 hr. incubation the membranes were searched for pocks. Suspensions of membranes previously inoculated with single CSF were pooled, and 20% suspensions of the six membrane pools were sub-inoculated, each onto six membranes.

No suspect lesions could be detected on any of the 72 membranes inoculated.

Three CSF were inoculated in dilutions 1/4, each into four tubes. Samples of the other three CSF were inoculated undiluted, into four tubes each.

After allowing 2 hr. for viral adsorption, the growth medium was replaced by maintenance medium and the cultures were incubated at 35° C.

After 4 days no CPE could be observed in any of the 24 tubes. Two of each group of four tubes seeded with single fluids were frozen to minus 70° C., and the other two tubes were observed for a further 3 days. None showed signs of CPE.

Pairs of frozen cultures, seeded with the same CSF, were thawed, pooled, and 0·2 ml. of the undiluted pools were subinoculated, each into a new group of four cell cultures, in an attempt to recover virus through blind passage. After 8 days

Table 2. *Origin of the material tested*

Designation of samples (patients)	Illness: Onset at p.v. day	Illness: Duration in days	CS fluid drawn at day: Of illness	CS fluid drawn at day: P.v.	CF titres: In CS fluid	CF titres: In serum**
1 (1)*	11	33	3	14	< 1/1	1/16
2 (2)	12	32	5	17	< 1/1	1/16
3 (3)	8	15	6	14	< 1/1	1/32
4 (4)	12	15	7	19	< 1/1	1/32
5 (5)	7	26	15	22	< 1/1	1/4
6 (1)*	11	33	17	28	< 1/1	1/8
Average	10	20	11	19	< 1/1	1/14

P.v. = post-vaccination.
* Two samples obtained from the same patient.
** Drawn on the same day as the CS fluid.

no signs of CPE could be detected in any of the second series of 24 cell cultures. Thus, from none of the six CSF could either vaccinia or other viruses be recovered.

Each sample of the six CSF was inoculated into four suckling mice. Three groups of 1- to 5-day-old animals were inoculated with samples of three fluids diluted 1/4, both cerebrally (0·01 ml. per mouse) and peritoneally (0·05 ml. per mouse). Each of these sucklings received thus 0·06 ml. of CSF. Undiluted samples of the other three fluids were inoculated cerebrally into groups of 9-day-old mice (0·03 ml. per mouse). None of the mice showed signs of infection. Four to six baby mice from each of the six inoculated litters were left uninoculated to serve as controls.

Thus, from none of the CSF samples could either vaccinia or other viruses be recovered.

DISCUSSION

No virus could be recovered from any of the acute phase CSF specimens and this is consistent with the possibility of allergic encephalomyelitis.

Our patients had not been skin-tested with brain material, but the histopathological findings of Dožić (personal communication) on fatal cases in Belgrade, were suggestive of allergic encephalomyelitis and this is the currently accepted view in post-vaccinal encephalitis (Dixon, 1962).

REFERENCES

DIXON, C. W. (1962). *Smallpox*. London: J. and A. Churchill Ltd.

MACCALLUM, F. O. (1969). Herpes simplex virus encephalitis. In Whitty, C. W. M., Hughes, J. T. & MacCallum, F. O., *Virus Diseases and the Nervous System*. Oxford and Edinburgh: Blackwell Science Publications.

OROVAČANEC, K., MUDRIĆ, V., VUČKOVIĆ, S. & VUKOVIĆ, B. (1973). Neuropsihijatrijske komplikacije u vezi sa antivariolicnom vakcinacijom. *Medicinski Pregled* (in the Press).

VUKOVIĆ, B., MIŠKOV, D. & MUDRIĆ, V. (1973). Postvakcinalne komplikacije u Vojvodini posle masovne vakcinacije protiv velikih boginja 1972 godine. Jugoslovenski simpozijum o varioli Primošten 1972. *Zbornik radova* (in the Press).