THE CULTIVATION OF THE GRANULES OF VACCINIA VIRUS.

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An attempt was made in the following experiments to understand more thoroughly the nature of vaccinia virus. The literature has been increased yearly with contradictory ideas and theories as to the cause of the potency of the virus, yet no one has been able to produce with certainty this potent factor in unadulterated form. Some have succeeded in passing a potent virus through Berkefeld filters under special conditions, while most authors have failed. Others have concerned themselves especially with efforts to recognize in certain granules a visible virus.

Upon the suggestion of Dr. W. G. MacCallum, we endeavored to maintain the rôle of the so called "granules," seen in tissues and smears from vaccinia and smallpox by Calmette and Guérin (1), Prowazek (2), Paschen (3), Hallenberger (4), and many later observers, and which appear in such myriads in vaccinia virus. These "granules" are easily recognized by dark-field illumination. It was definitely ascertained that whenever sufficient numbers of "granules" were seen in material separated from the whole vaccine by various methods, it would give a typical vaccinia lesion on the scarified cornea of a rabbit. When no "granules" were seen, or when present in very small numbers, no lesion occurred after similar inoculation of the material.

As the "granules" seemed to form such an important part of the virus, an attempt was made to grow them (using as a control the fluid part of the vaccine) in tissue cultures, because of the successful growth of the whole vaccinia virus in connection with living tissue both *in vivo* and *in vitro*. *In vivo*, it has been cultivated in other tissues besides the skin.

Noguchi (5) succeeded in passing the virus from one rabbit to another by intratesticular inoculations. The brain, too, presents a good medium, according to Marie (6), Levaditi, Harvier, and Nicolau (7). Furthermore, Levaditi and Nicolau (8) showed that the virus will grow in epithelial tumors although not in connective tissue tumors. *In vitro*, it has been grown successfully by several groups of workers. Steinhardt, Israeli, and Lambert (9) used cornea immersed for a short time in dialyzed virus, while Gins (10), Parker (11), and Hach (12) used respectively cornea, testis, testis and spleen of already infected animals. Parker and Nye (13) grew normal testis in the plasma of an infected animal whose blood is virulent for a period of time as shown by Levaditi (14) and Ohtawara (15).

Procedure.

The "granules" were obtained in as pure a state as possible by separating them from glycerolated vaccinia calf lymph (kindly sent us by Dr. F. M. Huntoon of the H. K. Mulford Laboratories) according to the differential centrifugalization method of MacCallum and Oppenheimer (16) with sterile technique. The virus was always centrifugalized at rapid speed for 40 to 60 minutes. About 0.1 cc. of the top layer was carefully removed without disturbing the contents of the lower part of the small tube. This was transferred to another tube and sterile Locke solution added. After mixing thoroughly, the tube was centrifugalized for 20 minutes and the "granules" thrown to the bottom in a solid layer. The supernatant fluid was all removed, and this was the material used as the control. It consisted of the serum part of the virus, glycerol, and Locke solution. The "granules" were subsequently washed twice more in a similar fashion with Locke solution, the resulting supernatant fluid being entirely discarded after each centrifugalization. The final "granules" obtained were diluted to their original concentration in 0.1 cc. Locke solution. This was the material called by us "washed granules," which we endeavored to grow. When examined under the dark-field microscope, it showed a mass of tiny granules, moving with what appeared to be Brownian movement. No tissue fragments or bacteria were seen as in the whole virus by this method.

The aim of our technique was to use only sterile materials, necessitating our using only embryonic tissues. These also seemed superior because of the facility with which they grew. At first we used embryonic rabbit cornea in homologous iced plasma. The latter we changed for the heparin plasma of Craciun (17). Even though we obtained pure epithelial growth, we could not avail ourselves of rabbit tissue because it was difficult and expensive to get rabbits far enough advanced in pregnancy to furnish embryos with large corneæ, and because without the time-consuming operation of making a skin incision to examine the mammary glands, it was impossible to differentiate with certainty pregnant from non-pregnant animals. We, therefore, changed to the guinea pig which proved to be an ideal animal. The size of its embryos could be ascertained easily by palpation

and we could, therefore, always get sufficient embryonic cornea. With this tissue we used homologous heparin plasma, prepared in advance in ampoules. We always obtained enough cultures with pure epithelial growth to continue transferring them throughout the series. All cultures contaminated with bacteria were immediately discarded.

The embryonic cornea was cut into tiny pieces in Locke solution. The main cultures were made with cornea, Locke solution, plasma, and a tiny drop of "washed granules" from the tip of the smallest possible capillary pipette. Other cultures consisted of cornea, plasma, and "control material." Here no Locke solution was added as it was already contained in the material. To control the materials used, cultures were made with pieces of cornea, Locke solution, and plasma. Most of the cultures were made on cover-slips which were inverted and sealed over rather deep hollow, ground glass slides and incubated at 38°C. In a few series the Carrel D flasks and the Gabritschewsky dishes were used. Every 3 to 7 days the cultures were washed in Locke solution and changed to a new medium of fresh cornea and plasma. In the intervals, that is every 2 or 3 days, the fluid part of the cultures was pipetted off and the cultures received a fresh drop of plasma. About every 8 days the presence of the virus was tested by inoculation on a scarified rabbit's cornea. The "washed granules" and "control material" used for the series were, of course, similarly tested beforehand, the "granules" always and the "control material" never giving the vaccinia lesion.

Two different types of control series were made. From each set of cultures several were set aside in the incubator in their original state and never transferred to a new medium. These were inoculated at the same time as the last transferred cultures of the series. This served to see if "granules" or "control material," if suitably incubated, could live or multiply in connection with cultures that were no longer living. Some cultures were allowed to remain untransferred for 2 weeks, others for 3 weeks, and then transferred every 7 days, to discover at what period the "granules" died. It was found unnecessary to do this with the "control material."

Another control series was made to discover, if possible, the location of the "granules"—whether they are transferred from the old to the new tissue, or whether they merely remain alive in the plasma and will, therefore, disappear if the original piece of tissue is removed after being in contact with the new medium for a few days. The cultures were made as above and transferred to the new medium in 7 days. At this time a camera lucida sketch of the culture was made; the original inoculated piece of tissue was labeled "A" and the fresh piece of cornea (to which no "granules" were ever added) labeled "B." At the end of 7 days, both "A" and "B" were removed, washed separately in Locke solution, and each added to a new medium of fresh cornea and plasma—thus giving rise to an "A" and "B" subdivision of the same series. Both of these branches were treated as always about every 7 days, and each inoculated separately to test for the presence of the vaccine.

RESULTS.

Table I gives the results typical of each of the examples described above. 722 cultures were made in all. There were twenty-six series, some of them including several of the above types of experiments.

TABLE I.

Series No.	Age of culture when tested.	No. of transfers.	No. of cultures inoculated.	Material in culture.	Result.
	days				
IV	7	0	4	Granules.	Negative.
IV	13	1	5	"	"
IV	21	2	4	"	Positive.
XX	26	4	3	"	"
XX	25	0	7	"	Negative.
XXIII	21	2	3	"	"
XXIII	71	9	2	"	Positive.
XXIV	28	3	3	"A"	"
XXIV	28	3	3	"B"	"
XXV	21	2	4	"A"	"
XXV	21	2	4	"B"	"
XXV	49	6	3	"A"	"
XXV	49	6	3	"B"	"
XXV	56	7	2	"A"	"
XXV	56	7	2	"B"	"
XXVI	17	2	5	Control material.	Negative.
XXVI	17	0	4	" "	"
XXVI	17	2	3	Granules.	"
XXVI	53	7	4	Control material.	"
XXVI	53	0	4	" "	"
XXVI	53	7	4	Granules.	Positive.
XXVII	34	4	4	"	"
XXVII	34	3*	4	"	Negative.
XXVII	34	2†	4	"	"

* Kept 14 days before making first transfer.
† Kept 21 days before making first transfer.

Table I shows that whereas the fluid content of vaccinia virus will not produce a lesion typical of vaccinia even after being in contact

with growing epithelial cultures (Series XXVI), the "granules" found in the virus not only remain alive for so long a time as 71 days (Series XXIII) in sufficient numbers to produce a lesion when inoculated, but must actually increase in potency in connection with the cultures. This is shown by the fact that cultures in the first transfer did not contain enough "granules" to give rise to the lesion, but after growing for a longer time, they would produce the typical corneal reaction (Series IV). Likewise, two cultures in the late transfers would give a lesion whereas in the second transfer of the same series four cultures were needed to produce a similar lesion (Series XXV). This can be readily explained by the gradual removal of the fluid part of the cultures which contained some inoculated "granules," leaving in the cultures only those "granules" already attached to the tissue. These were never present in sufficient numbers to produce a lesion until they grew for at least 21 days under the conditions of our experiments.

It was already known that only living cells will keep the virus alive. Indeed, Steinhardt and her coworkers found that if corneal tissue first be killed by freezing or treating with hypotonic salt solution, the virus will soon die out. Furthermore, one can conclude from the work of Williams and Flournoy (18), Krumbach (19), and Hirano (20) that only tissue which is actually growing will permit the maintenance of the virulence of the virus. From our experiments it is seen that the living growing cell is also absolutely necessary to the growth of the virus "granules." They always died when left with untransferred tissue cultures that had also died (Series XX).

The fact that the "B" series as well as the "A" series produced vaccinia lesions (Series XXIV and XXV) indicates that the "granules" must, in part, leave the older "A" tissue for the more favorable soil to be found in the fresher "B" tissue. This is plain from the fact that the plasma containing the inoculated "granules" was always washed away before the "A" was added to the "B" cornea. It is interesting to note that after inoculation on a rabbit's cornea, "B" cultures always had a shorter incubation period and gave a larger vaccinia lesion than did the "A" cultures. This indicated that the cells probably have to be in a fairly good condition because in our experiments no more virus was present in the cultures in which neither fresh plasma nor new tissue was added until after 2 weeks and which therefore did

not show any further growth when transferred after this lapse of time (Series XXVII). But it was still present, even though weakened, in the "A" pieces which received new medium four or five times in the meanwhile and were situated near the fresh "B" tissue.

We have from these studies no morphological proof of an increase in the number of the "granules" since they cannot readily be distinguished from other granules normally seen in the cells of tissue cultures. But the experiments differ from others which have been concerned with the cultivation of the virus of vaccinia in that the "granules" have been washed free from all adhering fluid and then cultivated in increasing potency while the fluid in which they were formerly suspended shows no power to infect the rabbit's cornea even after cultivation. They show at least that the power of the vaccine to infect an animal is inherent in the granules even though further analysis of their nature must be deferred.

SUMMARY.

The technique is described whereby vaccinia "granules" are separated from all other material of glycerolated vaccinia calf lymph and cultivated *in vitro* with embryonic tissues.

These "granules" remain alive, as tested by rabbit corneal inoculation, for as long as 71 days, when grown in connection with growing tissue; they fail to remain potent if cultivated with dying cells, showing in this way the same characteristics as whole vaccine virus. The potency of the "granules" increases with the age of the culture under the first mentioned conditions.

The fluid part of the vaccinia lymph which remains after the removal of all "granules," is impotent under all conditions.

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