THE FATE OF VACCINIA VIRUS ON CULTIVATION IN VITRO WITH KUPFFER CELLS (RETICULO-ENDOTHELIAL CELLS)

By JOSEPH W. BEARD, M.D., and PEYTON ROUS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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Many facts indicate that the cells constituting the "reticulo-endothelial system" play a rôle in combatting the invasion of the mammalian organism by bacteria and viruses. Much there is also to suggest that such cells are the source of some antibodies at least. The work done on these problems has been inconclusive, however, because it has been impossible to exclude the participation of other elements of the animal body in the responses obtained. The development of techniques whereby the reticulo-endothelial cells lining the liver sinusoids (Kupffer cells) can be isolated in quantity (1) and cultured at will *in vitro* (2) has seemed to provide an opportunity for decisive tests of their abilities. We have attempted to learn whether they have any effect upon vaccinia virus.

General Methods

To obtain the Kupffer cells advantage was taken of their activity in the phagocytosis of foreign particles circulating in the blood stream. A suspension of very finely divided, strongly magnetic iron oxide was injected into rabbits intravenously, and some days later Kupffer cells were dislodged from the liver by perfusion with Tyrode, combined with massage, and they were sorted out of the washings by means of a powerful electro-magnet. For *in vitro* cultivation the cells were transferred to dishes containing lens paper immersed in rabbit serum. They distributed themselves along the fibres of the paper and were kept in good condition by changing the serum often.

The necessary cells and serum were procured from large brown-gray (agouti) rabbits, and animals of this breed were utilized for the ultimate tests of the effects of the cultivated cells upon the virus.

Homologous serum is the medium in which the Kupffer cells fare best. That procured from presumably normal, "agouti" rabbits which have been kept for some

time under the conditions prevailing in the animal house of The Rockefeller Institute not infrequently exerts some neutralizing effect on vaccinia virus exposed to it *in vitro*. For this reason animals freshly procured from breeders were used for serum purposes in the cultivation experiments. By preliminary neutralization tests with serum specimens and vaccinia in high dilution, individuals were selected which yielded serum devoid of neutralizing effect on the virus; and they were bled to death into chilled tubes containing just enough sterile heparin solution to delay clotting until after the cells had been brought down with the centrifuge and the plasma taken off. A bit of sterile, voluntary muscle was now introduced into the latter to precipitate clotting, and by twisting the clot with the aid of a pipette serum free from hemoglobin, or but faintly tinged with it, was obtained. The yield from several animals was pooled, distributed in tubes, and kept in the refrigerator. Each of the experiments was carried out with a single batch of pooled serum. This was employed within a few days, that is to say while alexin was still present. The strain of vaccinia used was that of the New York Board of Health, in the form of glycerolated vaccine lymph.[1] Rabbits were inoculated with it intratesticularly, and after 3 to 4 days the infected tissue was excised, hashed, a portion used for titration tests, and the remainder tubed in 1.0 cc. quantities and frozen for storage. When virus was needed for an experiment a tube was thawed at room temperature, and the tissue was ground with sand, diluted with Tyrode and "decelled," or in other words freed of particulate matter by a method already described (3). The material titrated high, yielding characteristic lesions on intradermal inoculation of 0.2 cc. at dilutions of 1:1,000,000 or more in Tyrode. Throughout the titrations in the experiments calibrated record tuberculin syringes were employed, and 0.2 cc. was injected as the standard amount. Only this quantity was taken up in the syringe for each inoculation, and these were made into the shaved sides of 2 to 4 rabbits, with variation in the arrangement of the several inocula to cancel out the influence of local differences in their position. The lesions they induced were outlined each day with a wax pencil on a superimposed sheet of transparent celluloid and then directly traced on the record cards. Only the findings of the early days are given in the charts, before necrosis had complicated the picture.

Tyrode solution was employed for washing and dilution, and specially calibrated micropipettes for the handling of minute quantities of material.

Immediate Tests with Kupffer Cells

In some initial experiments washed Kupffer cells, freshly obtained by means of the magnet, were mixed with a suspension of virus and injected into the skin of rabbits.

Experiment 1.—A normal rabbit weighing about 2,300 gm. was given intravenously 20 cc. of iron oxide-acacia suspension daily in two injections of 10 cc. on

[1] Kindly provided by Dr. Rivers.

each of 3 successive days, and 72 hours later the cells were flushed from the liver with about 300 cc. of Tyrode, collected on a collodion surface with the magnet, and washed by running about 100 cc. of Tyrode slowly past the brown pellicle of cells while this was still held fast. The magnetization was then discontinued and the material shaken off into 0.9 cc. of Tyrode. Of the resulting turbid, ruddy brown suspension, 0.35 cc. was put into each of two tubes, and one was heated in a water bath at 53°C. for 15 minutes to kill the cells. When it had been cooled to room temperature, 0.01 cc. of 1 per cent virus was added to it, as also to the tube containing the living cells, and to a control with 0.35 cc. of Tyrode, and after 5 minutes of gentle agitation, 0.01 cc. more of virus was introduced into them all. The usual difficulty was experienced in suspending the living Kupffer cells, which are extraordinarily sticky, soon forming clumps that cannot be broken up. In heated suspensions this does not happen. All of the tubes were agitated for 10

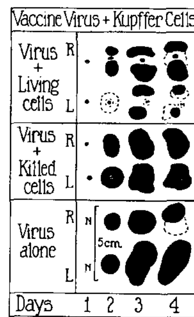

CHART 1. In this and the succeeding charts the lesions indicated by dotted lines were pink swellings slightly raised above the skin surface; those represented in black were elevated, purplish, indurated plateaus or mounds. Cross-hatching signifies necrosis.

minutes more at room temperature; then 0.6 cc. of Tyrode was added to all; and intradermal inoculations were made forthwith. 2 test rabbits were employed, with intradermal injections of all three materials on each of their sides.

One of the animals proved extremely susceptible to the action of the virus, enormous, confluent lesions resulting from the inocula. Chart 1 shows the results obtained in the other animal. The control mixture of Tyrode and virus gave rise to large areas of swelling, acute inflammation, and eventual necrosis such as vaccinia ordinarily causes, and so too did the material containing killed cells. Where the inoculum of living cells had been put the situation of the injected cells could be readily discerned by reason of the iron in them, which produced a brown spot several millimeters across. For some distance around this, the skin remained normal (Chart 1), but further away lesions developed like those caused by the other inocula but much smaller, and with less tendency to necrose.

In this experiment the virus injected together with the living Kupffer cells gave rise to relatively small lesions, and while the difference in the results with the inocula seemed significant the possibility that serum antibodies had been carried over into the mixture with the cells was not wholly excluded by the washing to which they were subjected. The following experiment was carried out in the same way except for the introduction of fresh normal serum into the tubes prior to the addition of virus. The activity of Kupffer cells for bacterial phagocytosis in normal animals is known to depend largely upon the presence in the blood of a thermolabile principle, or principles, an opsonin, so called. A very little fresh serum enables them to take up bacteria perfused through the liver (4).

Vaccine Virus Alone			+ Kupffer Cells		
+ serum	Rab. A	R / L	Living + serum	Rab. A	
	B	R / L		B	
+ Tyrode	A	5cm.	Killed + serum	A	
	B			B	
Days		2 3 4 5			2 3 4 5

CHART 2

Experiment 2.—An especially large yield of Kupffer cells was procured from a normal rabbit, and they were thoroughly washed with more than 100 cc. Tyrode while held with the magnet, and distributed in portions of 0.35 cc. to two tubes, one of which was heated as in Experiment 1. To each tube there was then added by means of a micropipette 0.04 cc. of fresh normal rabbit serum, previously cleared of any cells with the centrifuge, and after the mixtures had been shaken briefly 0.02 cc. of 0.4 per cent virus was added, after which the tubes were gently shaken at intervals during 30 minutes at room temperature. Two control tubes had been set up containing 0.35 cc. Tyrode plus 0.04 cc. serum and Tyrode respectively, and 0.02 cc. of virus. After all had stood for 30 minutes at room temperature intradermal injections were made into each side of 2 test rabbits.

The results of this test are depicted in Chart 2. It will be seen that the serum added to the mixtures had some neutralizing influence on the virus, the lesions produced by the control material containing it being smaller than those from the

control with Tyrode only. The heated Kupffer cells had no effect, whereas the presence of living ones, together with serum, resulted in an almost complete suppression of the virus activity in one rabbit (B), which developed mere dubious, transitory thickenings of the skin, while in the other rabbit lesions appeared only at a distance from the brown spot where the Kupffer cells lay, just as happened in Experiment 1.

In several further experiments of the sort, similar results were obtained. For one of them Kupffer cells were employed from an animal recently recovered from vaccinia. They had been washed as usual and they neutralized the virus no more effectually than did the cells from normal rabbits.

Tests with the Cells of Peritoneal Exudates

The experiments were now extended to determine the effect of other cells of the reticulo-endothelial system. The chief representatives of this system, outside of the liver, spleen, lymph nodes, and bone marrow, are the clasmatocytes. It is common knowledge that these cells largely replace the polymorphonuclear leucocytes which at first assemble after the injection of a sterile irritant into the peritoneal cavity. This happens, as we have found, when the injected material is an iron-acacia suspension of the sort used to procure Kupffer cells from the liver. After 72 hours clasmatocytes largely predominate in the exudate. They can be obtained from the exudate in considerable quantity and they proved much more hardy than Kupffer cells, living many hours in Tyrode solution, as evidenced by the trypan blue test of viability (5). For the experiments which follow, 72 hour and 24 hour exudates were utilized, the one clasmatocytic, that is to say consisting mostly of large mononuclear cells, with clasmatocytes predominating, the other polymorphonuclear, consisting almost entirely of such elements. Differential counts were done as routine. The clasmatocytes had taken up much more iron.

Experiment 3.—Rabbit A, which had recovered from an experimental intradermal infection with vaccinia 21 days previously, was injected intraperitoneally with 10 cc. of iron-acacia suspension on 2 successive days. Another recovered animal, B, of the same lot, received in a single injection 20 cc. of the suspension. On the morning of the experiment, that is to say 72 and 24 hours, respectively, after the last injection of the two animals, they were bled to death from the heart into containers in which 2 cc. of heparin (1:1000 in Tyrode) was already present. From rabbit A 60 cc. of blood was obtained, from B, 45 cc., and after centrifugation of it the leucocytic pellicles were taken off practically intact, washed 3 times in

19 cc. of Tyrode, and finally suspended in 0.8 cc. Meanwhile the abdomens of the animals had been opened aseptically and the peritoneal exudates washed out with 50 cc. of Tyrode in each case, containing 2 cc. of 1 per cent heparin. Examination with neutral red of the material thus obtained from rabbit A, which had been injected 72 hours before, revealed great numbers of clasmatocytes and a few monocytes, most of them containing iron particles in greater or less quantity. Not many polymorphonuclear cells were present, but some of these held one or several fine iron particles. To reduce the number of polymorphonuclear cells still

TABLE I

	Material		Serum	Virus	Iron	Heated iron	Tyrode
		cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Rabbit A	Living blood leukocytes	0.35	0.04	0.02			0.7
	Heated " "	"	"	"			"
	Living " "	"	"	"	0.33		0.38
	Heated " "	"	"	"		0.33	"
	Living exudate cells (mostly clasmatocytes)	"	"	"			0.7
	Heated " "	"	"	"			"
Rabbit B	Living blood leukocytes	"	"	"			"
	Heated " "	"	"	"			"
	Living " "	"	"	"	"		0.38
	Heated " "	"	"	"		"	"
	Living exudate cells (mostly polymorphonuclears)	"	"	"			0.7
	Heated " "	"	"	"			"
	Iron suspension	"	"	"			"
	Heated iron suspension	"	"	"			"
	Tyrode solution	"	"	"			"
	" "	0.39	—	"			"

further the suspension was passed by the magnet, and the iron-containing elements thus sorted out were washed *in situ* with more than 100 cc. of Tyrode.

The exudate from rabbit B, recently injected, consisted predominantly of polymorphonuclear cells carrying little or no iron. It was centrifuged and the deposit was washed by centrifugation in 3 changes of 40 cc. each Tyrode. While this was being done, the particles from 2 cc. of the standard iron-acacia suspension were washed 3 times in Tyrode, and a 0.4 per cent suspension of vaccinia virus was prepared. On the preceding day serum from a normal rabbit had been procured, and some of it heated at 53° for 10 minutes. Mixtures were now made as listed in Table I. The cell materials were first distributed to the tubes in the order

given, then serum was added, with agitation, and then virus, the whole taking about 10 minutes, after which the tubes were gently agitated by hand for 20 minutes at room temperature. Iron suspension, heated or unheated, plus Tyrode, or Tyrode alone, was then introduced as indicated, and the injection was begun at once of 0.2 cc. portions of the various mixtures into 4 rabbits. All the inoculations with each mixture were carried out at one time, in the order of their prepara-

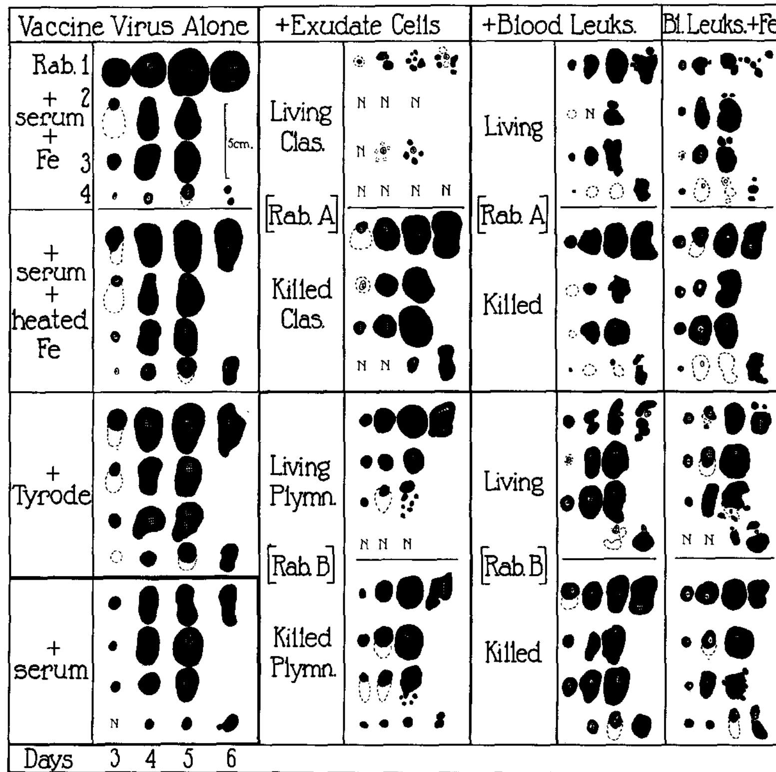

CHART 3

tion, and the intervals were such that the mixtures had been in existence for approximately the same period prior to their injection. In 2 of the animals some of the lesions coalesced after the 5th day. Consequently these are not charted thereafter.

It will be seen from Chart 3 that virus plus Tyrode caused pronounced lesions in all of the animals. When normal serum was also present somewhat smaller lesions resulted. Virus plus normal serum

plus iron caused lesions larger, if anything, than those due to virus alone, and the same held true when the iron had been heated. Virus plus serum plus blood leukocytes, heated or unheated, with or without iron (some of which was promptly phagocyted by the unheated cells), gave rise in general to lesions nearly as large as those produced by the control of virus plus serum. Evidently the blood leukocytes, though derived from immune animals, had but a slight effect on vaccinia under the circumstances of the experiment. This was true as well of the cells of the more recent of the two exudates, that in which polymorphonuclear elements predominated. The heated cell material of the older, clasmatocytic exudate seemed actually to favor the virus as compared with the other materials. This was not true of the living, unheated clasmatocytic material, however. It acted to neutralize the virus almost or quite completely in contrast to all the other inocula. Furthermore, in those instances in which lesions did arise they were situated at some distance from the brown spots marking the location of the cells, just as had happened in Experiments 1 and 2.

In the next experiment, similar exudate materials were utilized, from rabbits which had not been subjected to vaccinial infection, and the conditions were varied in other ways. Aleuronat instead of iron acacia was used to induce exudate formation, inactivated as well as fresh serum was employed, and no iron was added to any of the mixtures. The blood leukocytes were not tested.

Experiment 4.—2 normal rabbits, A and B, were injected intraperitoneally with 5 cc. each of a sterile suspension of aleuronat in starch solution, 24 and 72 hours respectively before the test was to be carried out. The exudates were washed from the peritoneal cavity in the same way as in Experiment 3 and the cells were thrown down with the centrifuge, washed twice with Tyrode, and made up to 1.6 cc. Counts showed them to be mainly polymorphonuclear leukocytes in one instance and clasmatocytes in the other. 0.5 cc. of the original aleuronat material was washed twice in the same way.

The 2 rabbits providing the exudates had been bled 5 cc. each on the previous day. The sera thus procured were united in equal amount, after they had been twice centrifuged to exclude cells, and a portion was heated at 56°C. for 30 minutes. Mixtures were then made with a 0.2 per cent suspension of virus, as shown in Table II. All of the mixtures were gently agitated at intervals by hand for 30 minutes at room temperature, prior to injection of 0.2 cc. portions into 4 rabbits. The order of preparation and injection was as given in the table.

It will be seen from Chart 4 and Figs. 1 and 2 that the control material of virus plus Tyrode gave rise to large lesions in all the animals. The control with heated serum caused equally large ones, but there was a slight reduction in their size when unheated serum was used instead. The cells of the exudate that consisted mostly of polymorphonuclears had some adverse effect upon the virus, and this was more considerable when the cells had not been killed by heat; but whether the serum was fresh or heated made no difference in the result.

TABLE II

	Material		Serum	Heated serum	Virus	Tyrode
		cc.	*cc.*	*cc*	*cc.*	*cc.*
	Aleuronat suspension	0.35		0.04	0.02	0.7
	Heated aleuronat suspension	"			"	"
	Aleuronat suspension	"			"	"
Rabbit A	Living exudate cells (mostly clasmatocytes)	"		"	"	"
	Heated " "	"		"	"	"
	Living " "	"	0.04		"	"
	Heated " "	"	"		"	"
Rabbit B	Living exudate cells (mostly polymorphonuclears)	"		"	"	"
	Heated " "	"		"	"	"
	Living " "	"	"		"	"
	Heated " "	"	"		"	"
	Tyrode solution	"	"		"	"
	" "	"		"	"	"
	" "		—	—	"	"

As in Experiment 3, large lesions resulted from the mixtures containing the killed material of the clasmatocytic exudate; and again it seemed to be immaterial whether the serum was heated or fresh. The living clasmatocytes, however, had a pronounced effect to suppress the action of the virus, and in the presence of unheated serum this was nearly or quite complete. The results with the aleuronat mixtures are not charted since the size of the lesions showed the substance to be wholly devoid of effect on the virus.

In another experiment (Chart 5), carried out in a precisely similar way but with aleuronat exudates from animals that had recovered from experimental vaccinial infection 20 days previously, the living material that was predominantly polymorphonuclear in content suppressed the virus to the same extent as did that which was mostly clasmatocytic, and this whether the normal serum added was fresh or had been heated. Control mixtures of virus with the last wash

CHART 4

fluid from the cells proved the latter to be devoid of effect. The routine examination of the polymorphonuclear exudate with neutral red had shown its cells to be, as usual, nearly all of this type; and the reason for its effectiveness,—which constituted a difference from the previous findings,—is not clear. The total bulk of polymorphonuclears in the mixtures with virus was somewhat larger than in the case of the clasmatocytes, but this had been true in the other experiments also.

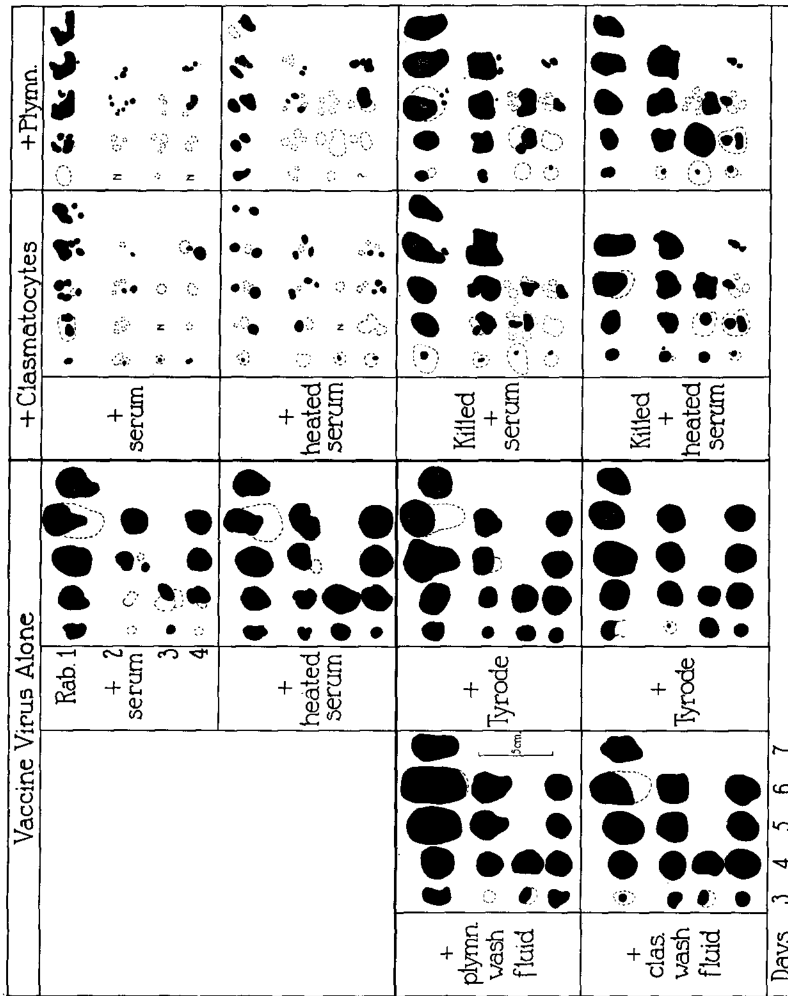

CHART 5

The Fate of Vaccine Virus in Cultures of Kupffer Cells

In the work just described the conditions had been simplified by removal of the reticulo-endothelial cells from the body for the purpose of *in vitro* mixture with the virus and serum; yet while the tests were still in progress fresh complications were brought into play by the introduction of the materials into the skin of new hosts. To avoid this difficulty resort was now had to determinations of the fate of vaccine virus in cultures of Kupffer cells *in vitro*. Two experiments of the sort will be reported which are representative of the results obtained in several others of less comprehensive character.

Following methods already described (1, 2), the Kupffer cells were sorted out with the magnet and washed with Tyrode, suspended in homologous serum, and transferred to flat dishes containing two layers of lens paper. They clambered up on the fibres of this paper, distributed themselves along its fibres, and were readily maintained in immense number by replacing the serum every 24 to 48 hours. It has been found that under such circumstances the cells do not become fatty or show other evident degeneration during 10 days at least, though no significant increase takes place in their number. When the serum is diluted with Tyrode the cells fare less well.

In the cultures employed for the present experiments Kupffer cells were so abundant that the layers of paper on which they were maintained appeared rusty brown, owing to the iron particles contained within them. A few monocytes and polymorphonuclear leukocytes that had taken up such particles, and hence been attracted by the magnet, were always present at first in the cultures, but within 2 or 3 days the polymorphonuclears died. The fate of the monocytes is uncertain, since cultivated Kupffer cells come to approximate them in morphology after a week or more has elapsed. Many of the cells contained very little iron, and the excellent condition of those holding more was shown by the alacrity with which they took up and segregated neutral red when exposed to it in slide and cover slip preparations after a week of cultivation or longer. The phagocyted iron particles were not visibly broken down or dissolved during the period of cultivation. Indeed they have been found in their first abundance, and with the ferromagnetic character retained, in the Kupffer cells of animals killed many months after their injection into the blood stream.

Experiment 5.—The iron suspension employed to procure the Kupffer cells for this test, and for Experiment 6, consisted of 4 gm. of gamma ferric oxide and 14 gm. of gum acacia, twice the ordinary amounts, in 1,000 cc. of distilled water. It was found to be of considerable advantage to keep the suspension in the ice box, autoclaving the necessary quantity just prior to use, since autoclaving reduces the capacity of the gum to hold up finely divided material for long periods of time.

The Kupffer cells from a normal rabbit injected intravenously with the suspension in the usual way were collected, washed with Tyrode while still held by the magnet, and suspended in 2 cc. of a freshly prepared mixture of 4.8 cc. pooled normal serum with 1.2 cc. of 1 per cent virus. Carrel dishes 3 cm. in diameter were employed for the cultures, each dish containing two discs of lens paper that covered its bottom. Since some iron particles always escaped from the cells during the manipulations, control cultures were set up containing the same or a slightly greater amount of such particles from the original suspension, washed free from acacia. Six dishes were prepared as follows, all with lens paper, and incubated at 37°C.

Dish A. 0.6 cc. cell suspension in serum-virus mixture + 1.4 cc. pooled normal serum.
B. 0.6 cc. cell suspension in serum-virus mixture + 1.4 cc. Tyrode.
C. Like B.
D. 0.6 cc. serum-virus mixture as such + 1.4 cc. serum + trace washed iron.
E. 0.6 cc. serum-virus mixture as such + 1.4 cc. Tyrode + trace washed iron.
F. Like E.

The following mixtures, standards for eventual titration of the virus in the cultures, were preserved in sealed pyrex tubes at −5° to −8°C.:—
G. 0.6 cc. serum-virus mixture as such + 1.4 cc. serum.
H. 0.6 cc. serum-virus mixture as such + 1.4 cc. Tyrode.
I. Several cc. of the 1 per cent virus suspension.

Daily 1.7 cc. of fluid, practically all that could be procured, was withdrawn with a pipette from each of the cultures. It came away water clear but was centrifuged to remove the few cells present, transferred to a tube, and stored in the freezing chest with the virus standards already there. To replace it a like amount of the original batch of pooled normal serum was added to cultures A and D, and serum plus Tyrode in the proportion of 6:14 to cultures B, C, E, and F. Each daily change involved dilution of the culture virus with new material in the ratio of 3:17.

The cells as seen under the microscope in the warm box appeared very active. Soon after the plating into whole serum they distributed themselves upon the fibre of the lens paper in the customary way (2). In the serum-Tyrode, on the other hand, most of them remained grouped, and soon many had rounded up and some appeared dead. None of the fluids was changed on the 5th day. On the 6th the cells cultured in serum appeared as numerous as before and showed no fat globules. Many in the Tyrode culture, B, had dropped from the fibres, obviously dead, and this culture was discarded; but those in C were in better state though with some fatty granulation. On the 6th day the culture fluids were pipetted off as usual; the lens papers were withdrawn from A and C; the cells were shaken free from the fibres into Tyrode, so far as this was possible, and washed twice, each time with 45 cc. of it, and made up to 0.75 cc. for injection. Many could not be dislodged from the lens fibres and hence were lost.

Culture fluid Days	One		Four		Six		Cells Six	
Calc. virus dil.	1 – 1,666		1 – 493,600		1 – 3,290,666			
Culture	Rab.	Days 3 4 5 7	Rab.	Days 3 4 5 7	Rab.	Days 3 4 5 7	Rab.	3 4 5 7
Cells + serum	1		4		4		4	
	2		5		5		5	
	3		6		6		6	
Cells + serum – Tyrode	1		4		4		4	
	2		5		5		5	
	3		6		6		6	
Free iron + serum	1		4		4			
	2		5		5			
	3		6		6			
Free iron + serum - Tyrode	1		4		4			
	2		5		5			
	3		6		6			

5 cm.

Approximately 0.3 cc. of culture fluid had been present with the cells when they were shaken into the Tyrode. The theoretical dilution of virus free in the culture fluid and carried with it through the progressive replacements to the final cell inoculum was of the order of 10^{-12}.

The fluids from A, C, D, and F, that had been stored after 1 and 4 days incubation were now thawed and injected intradermally in 0.2 cc. quantities into 3 rabbits, and so too were the final, or 6 day, fluids and the controls G and H. Material I, diluted repeatedly just as the virus had been in the cultures, but with Tyrode, was also injected. Similar inoculations were made into 3 other rabbits after dilution of the materials to 1:10 with Tyrode.

Vaccine virus of the strain employed retains its activity unimpaired for many weeks when in the frozen state. Just prior to inoculation the frozen control specimen of virus (I) was thawed, and diluted repeatedly in accordance with the successive culture dilutions. In our experience no virus-serum mixture has ever given larger lesions on immediate injection than has a similar mixture with Tyrode alone. In the present instance Tyrode had to be employed as the diluting medium instead of the stock serum, since the latter had been used up. The dilution sufficed to attenuate the virus so greatly that it caused no lesions, a finding which is not charted.

In this experiment (Chart 6) a considerable quantity of virus was added to the fluids in which the cells were maintained during the first 24 hours, and thereafter most of these fluids were almost entirely replaced on several occasions. Appropriate control preparations were made to determine the fate of the virus when no cells were present. The reason the incubation was kept up for 6 days and the fluid replacements repeated, was to get rid by dilution or inactivation of the virus originally free in the fluid, and to afford time for the cells to elaborate an antiviral principle in case this was one of their functions. The fluid introduced into the cultures after 4 days was left on them for 48 hours with a view to the accumulation in it of antiviral principles.

The cells nourished by whole serum flourished, showing no signs of the degeneration to be noted in connective tissue cultures into which vaccinia has been introduced (3). Only the usual few cells came away at the pipetting off of each day, and when the test was terminated the generality were in excellent state, free from fat, segregating neutral red promptly, and clinging so fast to the lens paper that many could not be dislodged by vigorous shaking in Tyrode. The cells in the dish receiving serum diluted with Tyrode, on the other hand, did as badly as usual in such a medium, but no worse than when no

virus was present. The successive replacements of the latter had been sufficient on calculation to have rendered it ineffective by dilution, and whether for this reason or because of inactivation during incubation the fluid removed from the control cultures on the 6th day failed to cause lesions.

All of the fluids removed from the cultures after one day of incubation gave rise to pronounced and characteristic lesions, those from the cultures containing the cells being slightly smaller than from those that had none, as might have been expected had some of the virus been taken out of the fluid by fixation on the cells. That the serum, as such, had no neutralizing effect upon the virus of significance in the present relation was indicated by the fact that the culture with whole serum yielded as large lesions as the one in which it had been diluted with Tyrode in the proportion of 14 to 6.

After 4 days of incubation, with daily replacement of the culture fluid to so great an extent that the initial virus quantity underwent a calculated dilution to about 1 in 500,000, two of the fluids failed to give rise to lesions, and two others, one from a culture with cells, yielded very small and late ones. These results can be explained on the assumption that the virus had been diluted or incubated into inactivity: there is no need to invoke any participation of the cells. After 6 days both of the cultures devoid of cells failed to yield virus, whereas it was present in the fluid from those containing them, notably in the culture with whole serum which had failed to yield it on the 4th day. This contained many Kupffer cells in excellent condition, and the fluid from it now gave rise to large necrotizing lesions, whereas that from the culture in serum-Tyrode, in which the cells had undergone fatty change, was nearly devoid of activity. Some influence of these degenerating cells to maintain the virus was manifest nevertheless, both the culture fluid and the washed cells themselves causing small, late lesions, whereas the cell-free controls yielded none. The healthy-looking Kupffer cells from the culture in whole serum containing virus gave rise to large, necrotizing lesions on injection after repeated washings. It should be remarked that iron-containing Kupffer cells, as such, living or killed with heat, give rise to no lesions on intradermal injection.

It was plain from the results as a whole that the Kupffer cells, instead of neutralizing vaccinia, had served to maintain it under conditions which led to its disappearance when they were absent. In the

culture in which the cells had done well the virus had actually increased. This cannot have been merely because they provided conditions favorable to such virus as was free in the medium and out of their reach, so to speak. For active virus existed in association with the cells themselves, as proven by the necrotizing lesions that resulted from injection of them after thorough washing with Tyrode.

A more comprehensive experiment was next undertaken in which the Kupffer cells were cultivated for 7 days, with replacement of most of the fluid each day, and with immediate titration of the specimens removed at this time, and comparison with some of the initial serum-virus mixture that had been kept in the frozen state.

Experiment 6.—A very abundant yield of rabbit Kupffer cells, procured as usual, was suspended in 1.5 cc. of a mixture of 3 cc. of 1 per cent virus in Tyrode and 12 cc. of pooled, normal serum; and the following cultures were made:

Dish A. 0.6 cc. of cell suspension + 1.4 cc. of serum-virus mixture.
B. The same.
C. 2 cc. of the serum-virus mixture alone.
D. The same.

The remainder of the serum-virus mixture, distributed in small tubes in 0.5 cc. portions was preserved frozen, and so too was some of the 1 per cent virus.

As in the previous experiment 1.7 cc. of water clear fluid was drawn off from each of the cultures daily, but this time in every instance it was replaced with undiluted normal serum of the original batch. The specimens from the duplicate cell cultures were pooled, centrifuged to remove any cells, and the supernatant fluids taken off; and the controls of serum incubated with lens paper were similarly treated. While this was being done a small tube of the original serum-virus mixture was allowed to thaw at room temperature, and now it and the culture fluids were serially diluted with Tyrode; and when the dilution had on calculation reached 1 in 100,000, 1 in 1,000,000 and 1 in 10,000,000 in terms of the original virus, inoculations were made of 0.2 cc. quantities into 4 normal rabbits, a new group of animals being used each day.

It has just been stated that at each successive replacement of the culture medium the residual 0.3 cc. was diluted by the addition of 1.7 cc. of the original batch of serum. Similar successive dilutions, but with Tyrode, were carried out with the specimens of thawed virus-serum mixture when these were utilized for the control inoculations. In doing this no allowance was made for the Tyrode accompanying the cells when they were first introduced into the cultures as 0.6 cc. of suspension,—whence it follows that the original serum-virus mixture, kept frozen and now utilized for the titrations, underwent less dilution than did the portions of it that had been introduced into the cultures.

After 4 days of cultivation, when the virus in the culture fluid would have had

a titre of 1 in 148,000 if it had persisted throughout the successive dilutions, some of this fluid was injected as such intradermally, for comparison with control material diluted in the same way; and the rest was made to 1 in 1,000,000 and compared with control fluid similarly diluted. For the purpose of the titrations made after 5 days and 6 days, the control material was diluted to 1 in 989,000 and 1 in 6,000,000 respectively and compared with the culture fluids as such; but after 7 days the control was diluted only to 1 in 6,600,000, through inadvertence, as compared with the 1 in 44,000,000 obtaining in the culture fluids.

The results of the comparison at 1 in 100,000 of the culture fluids of the early days are given in Chart 7, while those at 1 in 1,000,000 will be found in Chart 8. Because of the successive replacements of the culture medium, a comparison at the lower dilution could be carried out with the material of only the first 3 culture days. The results of the single set of comparative titrations, made later with the materials of the 5th, 6th, and 7th days, have been inserted into both charts. They are based on the calculated dilutions attained at these times by the virus in the culture, as has been stated in the preceding paragraph.

Some slight differences were encountered in the individual susceptibilities of the 4 normal rabbits injected intradermally each day for the purposes of the titration; but since every animal received all of the materials of the culture day for which it was employed, these did not seriously complicate the findings. The latter are consistent. They show a progressive diminution in the amount of active virus in the fluid procured from the control cultures, which after 6 and 7 days gave rise only to negligible lesions. The fluids that were procured from the cultures with Kupffer cells after 24 and 48 hours exhibited a diminished pathogenicity, as compared with those from the control cultures, as would have happened if some virus had been removed by fixation on the cells; but thereafter the specimens from the cell cultures steadily became more pathogenic, and those procured after 6 and 7 days gave rise to very large, necrotizing, vaccinial lesions. There can be no doubt that the virus had not merely persisted in active form in the cultures with the Kupffer cells, but had undergone so great an increase as to have much more than made up for successive dilutions by replacement that sufficed to render the incubated cell-free control practically innocuous. Unfortunately the frozen portion of the original cell-serum mixture that was utilized for comparison with the material procured from the cultures after 7 days was diluted only to 1 in 6,600,000, not to 1 in 44,000,000 as the latter had been through successive replacements. But though about seven times the stronger,

Days of culture	Rab.	Fluid from cell culture: Calculated virus dilution	Fluid from control culture: Calculated virus dilution	Frozen control (serum+virus): Calculated virus dilution
One	1, 2, 3, 4	1–100,000	Same	Same
Two	5, 6, 7, 8	1–100,000	Same	Same
Three	9, 10, 11, 12	1–100,000	Same	Same
Four	13, 14, 15, 16	1–148,000	Same	Same
Five	17, 18, 19, 20	1–990,000	Same	Same
Six	21, 22, 23	1–6,000,000	Same	Same
Seven	24, 25, 26, 27	1–44,000,000	Same	1–6,600,000

5 cm.

CHART 7

Days of culture	Rab.	Fluid from cell culture: Calculated virus dilution	Days 3 4 5 6	Fluid from control culture: Calculated virus dilution	Days 3 4 5 6	Frozen control (serum + virus): Calculated virus dilution	Days 3 4 5 6
One (5 cm.)	1, 2, 3, 4	1-1,000,000		Same		Same	
Two	5, 6, 7, 8	1-1,000,000		Same		Same	
Three	9, 10, 11, 12	1-1,000,000		Same		Same	
Four	13, 14, 15, 16	1-1,000,000		Same		Same	
Five	17, 18, 19, 20	1-990,000		Same		Same	
Six	21, 22, 23	1-6,000,000		Same		Same	
Seven	24, 25, 26, 27	1-44,000,000		Same		1-6,600,000	

Days of culture	Rab.	Cells A: Days 3 4 5 6	Cells B: Days 3 4 5 6
Seven	24, 25, 26, 27		

Chart 8

relatively speaking, it gave rise to negligible changes in 2 animals and to none in 2 others, whereas the culture fluid caused extensive and characteristic lesions in all of them.

During the 7 days of cultivation the cells appeared to be in excellent condition, though at its end a few fatty granules were present in some of them. Now as many as possible were shaken off the lens paper and inoculated intradermally. Inspection of a slide preparation with neutral red showed the generality to be alive, and they segregated the stain actively. Those from each culture were washed twice with about 15 cc. of Tyrode and suspended in 1 cc., with the injection of 0.2 cc. into each of the test rabbits of the final day. Large necrotizing lesions resulted, characteristically those of vaccinia (Chart 8). As a control to the possible presence and influence of minute fragments of lens paper carried along with the cells, the discs of this paper from the cell-free cultures were treated like those from which the cells had been dislodged. Tyrode was forcibly pipetted upon them and, although nothing was seen to come away, the hypothetical residues were centrifuged, made up with Tyrode, and injected like the cells. No lesions resulted.

In a preliminary experiment which calls for only brief mention, the conditions were varied by exposing freshly obtained Kupffer cells to the virus, and then washing and culturing them in serum. On the basis of previous findings which have demonstrated a fixation of vaccinia on connective tissue cells (3), it was assumed that some of it could be fixed upon Kupffer cells, which, after washing, would have only this amount to cope with. The serum employed as culture medium had been inactivated by heat, a procedure known to have no evident adverse effect upon the maintenance of Kupffer cells *in vitro*.

Experiment 7.—30.0 cc. of 1 per cent vaccine virus was mixed with 260 cc. of freshly obtained liver perfusate from an animal previously injected with iron acacia. The perfusate contained many sorts of cells in Tyrode. Selection of the Kupffer cells with the magnet was at once begun. It required 3 hours, and the large yield thus got was washed by slowly flowing 100 cc. of Tyrode past the brown cell layer. The magnetization was then stopped, and the cells were shaken into 3.3 cc. of normal rabbit serum,—which had been inactivated 2 days previously by heating at 56°C. for 30 minutes. Duplicate cultures were prepared, each containing 0.5 cc. cell suspension + 1.5 cc. of inactivated normal rabbit serum.

Each day 1.7 cc. from each culture was replaced with the same amount of inactivated serum. The fluid removed was stored frozen after centrifugation. That taken off after 4 days,—when the cultivation was discontinued,—was pooled, centrifuged, and injected as such into 2 rabbits and also after dilution to 1 in 10 and

1 in 100. At this time the specimens procured after the first 24 hours were thawed and similarly treated. The cells were dislodged from the lens paper by forcible pipetting with a little Tyrode, and those from the two cultures were united, made to a 1 cc. suspension without washing, and injected as such and in the further dilutions just mentioned.

The outcome of this test was essentially the same as in Experiments 5 and 6. The fluid removed from the cell cultures after the first 24 hours incubation, even when undiluted yielded only very small nodular lesions, whereas the specimens obtained on the 4th day gave rise to large and characteristic ones. The biggest lesions caused by any of the materials tested at the end of the cultivation were produced by the cell inocula.

In this experiment the amount of free virus introduced into the cultures must have been very slight; and the considerable increase that took place between the first and 4th days in the quantity present in the fluid media can scarcely be attributed to the release of virus originally fixed upon the Kupffer cells, since the fluid of the first 24 hours incubation yielded small indication of any such release, while thereafter the culture fluids had been six-sevenths replaced on two occasions. The inference seems warranted that events took the same course as in the two experiments already detailed, the virus increasing in association with the cells.

DISCUSSION

The findings leave no doubt that the activity of vaccinia virus is lessened or suppressed when it is mixed in the test tube with living reticulo-endothelial cells (Kupffer cells or clasmatocytes) and injected intradermally after some minutes at room temperature. The effect of these elements is far greater than that of polymorphonuclear cells. In those instances in which some suppression of the virus was observed, in mixtures with the cells of exudates in which polymorphonuclears predominated, clasmatocytes were also present, and they may have been a responsible factor.

Ledingham found that when vaccinia was inoculated into skin that had been injected with India ink, either no lesions developed or small ones situated beyond the edge of the inky patch (6). He concluded that the reticulo-endothelial cells, marshalled or multiplying in the cutaneous tissue as a result of the presence of the ink, were the cause of the virus suppression. Our finding that vaccinial lesions develop only at a considerable distance from reticulo-endothelial cells when

mixed with such cells and injected intradermally adds support to this view.

There are several ways in which the association of the virus with reticulo-endothelial cells might conceivably result in its suppression. The first possibility requiring consideration is that these cells might have failed to survive transfer, and the virus have been destroyed incidentally to their autolysis. As already mentioned, vaccinia becomes fixed rapidly and firmly upon connective tissue cells exposed to it *in vitro;* and Experiment 8 of the present work shows that this happens with living Kupffer cells also, as was to have been expected from their enormous, sticky, surface membranes (2) and activity in phagocytosis.

To learn whether rabbit Kupffer cells will survive transfer to the cutaneous tissue of another individual, as was done in the tests, a suspension of them obtained with the magnet in the usual way was injected intradermally into a new host in the 0.2 cc. amount employed in the experiments, and 18 and 48 hours later the brown spots showing where they lay were excised together with the surrounding skin, fixed in Zenker's fluid and sectioned in series. Under the microscope the precise point of introduction of the material could be readily discerned, for some debris lay there, including a little free iron. The Kupffer cells appeared in excellent condition after 18 hours though still mostly rounded, as when procured with the magnet, and they were at or near the immediate injection site; but after the lapse of 48 hours many had become stellate or lay spread out against connective tissue fibrils, and some had migrated to a considerable distance (Figs. 3 and 4). The course of events was, in other words, remarkably like that when Kupffer cells are introduced into cultures containing lens paper, and they ruled out the possibility that these elements had died *en masse* shortly after introduction into the skin. de Haan and Hoekstra (7) have found that clasmatocytes marked by a content of trypan blue will survive transfer to other hosts.

The Kupffer cells of this experiment had not been exposed to vaccine virus; but Experiments 5, 6, and 7 have sufficiently demonstrated that they flourish in association with it on cultivation *in vitro,* even when the test is so arranged as to insure much preliminary fixation of the virus upon them (Experiment 9). When mixed with the virus and introduced with it into the skin they did not suppress its activity

unless they were alive, as shown by Experiments 1 and 2. When they or the clasmatocytes of Experiments 3 and 4 had been killed by heating at 53°C.,—a treatment which does not prevent virus fixation (3),—large lesions resulted. For all these reasons it seems unlikely that the observed suppression was incidental to cell necrosis.

The antiviral principle circulating in animals recovered from vaccinia may be carried through washings by blood leukocytes, and retains under such circumstances the neutralizing capacity (8). It has seemed possible that the marked influence of exudate clasmatocytes to suppress the virus, noted in the present work, might have been due to some such happening; but the outcome of Experiment 4 is against this conception, the sera of the animal furnishing the exudate which neutralized the virus being devoid of any significant power of the sort. Furthermore, in Experiment 3 only the clasmatocytes suppressed the virus and this to no unusual extent, although both the clasmatocytic and the polymorphonuclear exudates, and the blood leukocytes as well, were derived from rabbits recently recovered from vaccinia, and hence undoubtedly possessed of potent circulating antibodies. But it should be remarked that the clasmatocytes had been washed by a less searching method than was employed in the case of the other cells.

The antiviral activity of clasmatocytes falls in with Gay's observations on the effect of these cells to combat bacteria (9). Ledingham's histological studies convinced him that "comminution of the reticulo-endothelial cells is the cardinal symptom of virus attack," the surviving elements of this sort rendering the virus inert. Granting, as our facts warrant, that the deterring influence of the reticulo-endothelial cells upon vaccinia resulted from some vital activity on their part, one still cannot conclude that it was the manifestation of a defense mechanism. Local changes incidental to the life of the cells implanted in the intradermal situation may merely have rendered the milieu unfavorable to the virus. But the extent of their influence, as indicated by the space about them in which no virus lesions developed, suggests that more was involved than this.

Our findings with polymorphonuclear cells, both those of exudates and of the blood, corroborate those of Sabin (8), who could obtain no evidence that blood leukocytes exert a destructive effect on vaccinia over and above that due to immune principles derived from the plasma

but carried by them. The observed slight reduction in size of the dermal lesions when blood leukocytes were mixed with the virus, may have been due to some fixation of the latter upon the cells.

The cultivation tests yielded results diametrically opposed to those just considered. The virus underwent a great increase during incubation for 6 and 7 days in cultures containing a large number of Kupffer cells (Experiments 5 and 6), even though most of the fluid medium was replaced daily. This cannot have been because the cells were in poor state,—for they flourished, and rapidly segregated neutral red when tested therewith at the end of the cultivation period; nor was it due to a "blocking" of them with iron,—of which many contained very little. Furthermore, it cannot be attributed to a situation of the virus out of reach of the cells. True, some free virus was present in the cultures when these were first set up, in addition to such as became fixed upon the cells; but this free portion lessened to the point of disappearance during the first few days, as was to have been expected from the combined effects of incubating it at 37°C. and diluting it by replacement of the fluid medium. That the later presence of free virus, in greater and greater amount, was due to an increase in that portion of it which had originally become associated with the Kupffer cells is evident from the large lesions resulting from the inoculation of these cells when the cultivations were discontinued. The findings obtained in this way were wholly against any destructive phagocytosis of the virus by the cells.

The cultivations were kept up long enough for antiviral principles to have appeared in the culture fluid, had forming them been one of the cell activities under the circumstances of the experiments; yet no evidence of any such activity was obtained. It might be urged that so much virus had become fixed upon the cells in the beginning that they had been overpowered. But their great number in the crowded cultures and their excellent first and last state are against this assumption.

Our negative findings as concerns the production of antibodies *in vitro* by Kupffer cells were paralleled by the outcome of numerous experiments made to determine whether these elements will form hemolysins and hemagglutinins. Washed Kupffer cells procured by the magnet method from rabbits and dogs were employed, in some cases from normal animals and in others from animals highly immun-

ized against the test objects (dog, rabbit, rat, and goat corpuscles). Most of the tests were made with fluids drawn off after 24 to 48 hours cultivation of the Kupffer cells, but in some instances the red corpuscles were introduced directly into the cultures. It will suffice to say that the results with these were wholly negative, the failure to demonstrate a formation of hemolysins or hemagglutinins being complete, though the cultured cells fared well to all appearance. In their natural situation within the liver Kupffer cells often manifest a prodigious activity in the phagocytosis of strange red corpuscles, and we had no difficulty in demonstrating that they retained this activity even when containing many iron particles. It was only necessary to run a suspension of rabbit corpuscles into the washed liver of a dog that had some days previously been injected with ferro-magnetic iron, clamp the hepatic vessels, let the organ remain for half an hour at body temperature, and then forcibly flush it out by the usual combination of Tyrode and massage. Numerous Kupffer cells were obtained in this way which held iron particles and in addition had stuffed themselves with the foreign corpuscles. Yet when rabbit corpuscles were introduced into cultures containing great hosts of dog Kupffer cells living on lens paper in fresh normal dog serum, to all practical intents and purposes no phagocytosis occurred. The corpuscles fell past the host of Kupffer cells to the bottom of the dish and even here, where many of them were slowly crawling about, there was no demonstrable phagocytosis. Nor were hemagglutinins formed during the next few days of cultivation in the presence of the strange corpuscles (which gradually broke down).

Extensive tests were made to determine whether Kupffer cells would reactivate normal, homologous serum heated to 56°C. for 30 minutes. The cells do well when cultivated with this as the medium, and it was left on for 24 to 48 hours. The fluid removed from one set of cultures after 24 hours, and centrifuged free from all cells, was found to activate a heated hemolytic serum procured from a rabbit immunized with guinea pig erythrocytes, while furthermore it lost this property on renewed heating to 56°C. for 30 minutes. In control tests with normal, inactivated serum such as had been put on the cultures, as well as with portions of it that had been incubated 24 hours in cell-free culture dishes containing lens paper, no activation took place.

In a succeeding experiment some of the heat-inactivated immune serum employed for the test just described was utilized as the medium of Kupffer cell cultivation, and when it was recovered after 24 hours and centrifuged it was found to hemolyze guinea pig cells without any addition of alexin, and to be inactivated when heated again at 56°C. for 30 minutes. Similar results were obtained in one other such experiment, though the reactivation was less pronounced. In a large, subsequent series of later cultivations, however, with other cells and other specimens of immune rabbit serum these results could not be duplicated; and wholly negative findings were obtained when heated anti-goat rabbit serum of very high titre was utilized in similar experiments. In some final tests cultures were made of Kupffer cells in the inactivated anti-goat serum, and after 24 hours of incubation goat corpuscles were directly introduced into the dishes. They underwent gross agglutination, as was to have been expected, but were not phagocyted or hemolyzed during several hours incubation.

Does the negative outcome of the cultivation experiments with vaccinia, and of nearly all of those with erythrocytes, mean that the Kupffer cells play no rôle in the production of antiviral substances, alexin, hemolysins, hemopsonins, and hemagglutinins? It seems far more likely, in view of the accumulated facts indicating an important rôle for these elements in some of the relations mentioned, that when they are maintained *in vitro* with serum as their culture medium and sole resource, they are unable to carry out tasks of which they are capable under the ordinary circumstances of their life. Parker (10) has recently found that the splenic tissue of an animal injected intravenously some days beforehand with foreign red cells will yield agglutinins for these latter on cultivation *in vitro*, whereas this will not happen if the spleen has been injected with the foreign red cells by way of its artery just prior to explantation. The physiological isolation inevitable to cell cultivation *in vitro*, as at present practiced, is not always an advantage from the experimental point of view. It can very well be a source of misinformation.

McMaster and Kidd (11) have demonstrated that the lymph glands are active in the formation of a neutralizing principle for vaccinia, presumably identical with that which appears in the blood of the animals infected with the virus. Whether the lymphocytes are the cells concerned in its formation has not been determined.

SUMMARY

The pathogenic activity of vaccinia virus is in large part suppressed when it is mixed with living Kupffer cells or clasmatocytes in the test-tube and injected intradermally.

Vaccinia increases in quantity when introduced into cultures of Kupffer cells *in vitro*, and survives in immediate association with these elements. No antiviral principle is elaborated by them under such conditions.

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EXPLANATION OF PLATE 39

FIGS. 1 and 2. Vaccinia lesions photographed 5 days after the injection of the materials of Experiment 4.

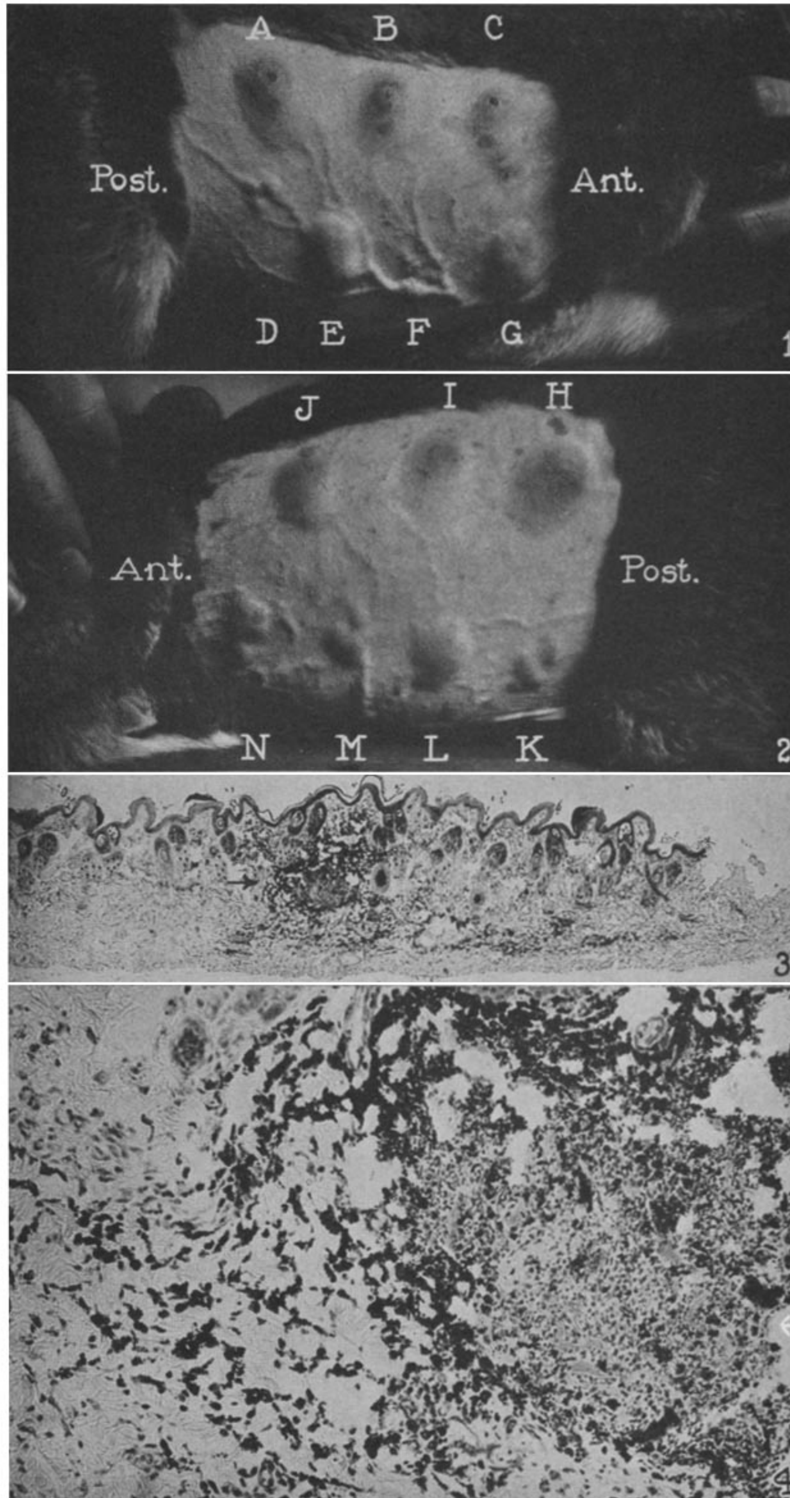
Virus + (A) Tyrode, (B) fresh serum, (C) heated serum.

" + (D) clasmatocytes, (E) heated clasmatocytes, (F) clasmatocytes and heated serum, (G) heated clasmatocytes and heated serum.

" + (H) aleuronat, (I) heated aleuronat, (J) aleuronat and heated serum.

" + (K) polymorphonuclears, (L) heated polymorphonuclears, (M) polymorphonuclears and heated serum, (N) heated polymorphonuclears and heated serum. × 1/2.

FIGS. 3 and 4. To illustrate the survival of iron-containing Kupffer cells after intradermal injection into a different rabbit: specimens procured 48 hours after the injection. Most of the Kupffer cells contain so much iron as to appear black. It will be seen that they lie scattered to a considerable distance from the immediate site of injection, which is marked by detritus (arrow), and that many have flattened out against the connective tissue fibrils. × 22 and × 153.



Photographed by Joseph B. Haulenbeek and Louis Schmidt

(Beard and Rous: Vaccinia virus cultivation with Kupffer cells)