Effect of Hyaluronidase on Reaction of Rabbits to Intradermal Inoculations of Vaccinia Virus.* (19608)

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When rabbits were injected intradermally with spreading factor from testicular extracts (hyaluronidase) together with concentrated suspensions of bacteria, the resulting lesions were more widespread than those produced by the inoculation of equally concentrated suspensions of bacteria in saline. Later observations, however, indicated that inoculations of testicular extract with dilute bacterial suspensions either induced lesions which were less widespread than those induced by saline suspensions or failed to induce a lesion (1,2). These data led to the conclusion that in the case of bacteria there could be determined a *critical concentration,* the minimal effective concentration per unit area of infection, below which no lesions would occur, and above which the intensity of the lesions would be roughly proportional to the concentration. However, the lesions induced by highly virulent bacteria such as some pneumococcus strains failed to be suppressed by spreading, even if injected in amounts nearing the minimal infective dose (presumably a single bacterial cell), but if antiserum was injected intravenously then pronounced suppression of the lesions by spreading occurred. When several viruses were examined, it was observed that no suppression took place as a result of the dispersion of the virus either in normal rabbits, or in rabbits injected with immune serum. It was then concluded that there is not a *critical concentration* for the viruses studied as there is for bacteria, or that the *critical concentration* of virus per unit area of tissue is an infective dose of virus.

The data recorded in the present paper indicate that when the resistance of rabbits is increased by virtue of a previous infection with vaccinia virus, subsequent intradermal inoculation of the virus together with hyaluronidase or testicular extract results in partial suppression of the lesions. This finding is supported by a decrease in the endpoint of intradermal infectivity for the rabbit of the lesions of the hyaluronidase series as contrasted with the saline series and by an increase in the ratio of the diameter of the lesions of the saline series of virus dilutions to the diameter of the lesions of the hyaluronidase series in rabbits with a partial, active immunity as contrasted with the ratio in non-immunized and passively immunized rabbits.

Materials and methods. Ten-fold serial dilutions of the commercial "calf lymph" strain of vaccinia virus were made in saline, and each dilution divided into 2 portions. To one portion was added an equal volume of saline buffered at pH 7.2 with M/15 phosphate buffer, and to the other an equal volume of 2% purified hyaluronidase (Wyeth) or a 10% extract of rat testes also buffered at pH 7.2. These 2 sets of virus dilutions were then inoculated intradermally in a volume of 0.1 ml in non-immunized rabbits, in rabbits passively immunized with hyperimmune anti-vaccinia serum, and in rabbits which had been infected intradermally with vaccinia virus 2 to 16 months previously. The saline series of virus dilutions were injected on the right side, and the hyaluronidase or testicular extract series on the left side of the rabbit. The hyaluronidase usually contained between 1000 and 1500 turbidity reducing units per mg. The extract of rat testes was prepared and used on the same day. The passively immunized group of rabbits received, depending on the lot of serum and the weight of the rabbit, from 4 to 20 ml of immune serum intraperitoneally from 1 to 6 days prior to the virus. Each lot of serum represented a pool obtained from 3 or more rabbits which were convalescing from a second infection. The animals were bled from 2 weeks to one month following the second infection. The titer of

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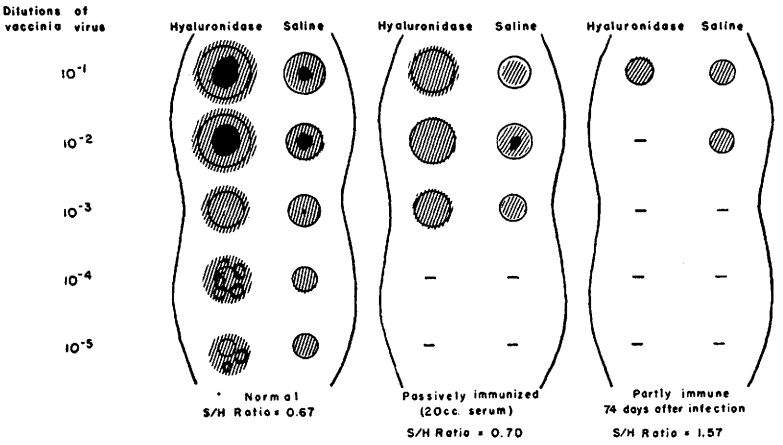

FIG. 1. Vaccinial lesions in rabbits recorded after five days.

hemagglutination-inhibiting antibodies varied from 320 to 2560. The dose of serum was adjusted so that each rabbit received approximately the same concentration of anti-vaccinia antibodies. The resulting lesions in all groups of rabbits were calibrated at frequent intervals following inoculation. The diameter of the lesion was ascertained by adding the smallest and largest diameters and dividing by two. Only those lesions which were at least 5 mm in diameter were considered to be produced specifically by vaccinia virus. Due to the marked variability in resistance of rabbits to intradermal inoculations of vaccinia virus, the experimental results are expressed largely in terms of the ratio of the diameter of the lesions of the saline series to the diameter of the lesions of the hyaluronidase series (S/H ratio). With this method it was found that the ratios within a particular group of inoculated rabbits was remarkably constant despite the fact that there might be a 2 or 3 log difference in the endpoint of intradermal infectivity for the rabbits included in the group.

Results. Despite the use of different lots of hyaluronidase, a number of testicular extracts, several samples of hyperimmune serum administered from 1 to 6 days prior to the virus, and rabbits which had been infected with vaccinia virus 2 to 16 months previously, the data obtained in each of the 8 experiments proved to be remarkably consistent. The results of a typical experiment are illustrated in Fig. 1. In the normal and the passively immunized rabbits hyaluronidase enhanced the vaccinial lesions induced by every dilution of the virus. In the previously infected rabbit hyaluronidase actually suppressed the development of the lesion when the 10^{-2} dilution of vaccinia virus was employed, and did not enhance or frankly suppress the lesion induced by the 10^{-1} dilution of virus. It should be noted that the diameter of the lesions in the saline series of virus dilutions was essentially 70% of that found in the hyaluronidase series both in the normal and in the passively immunized rabbits despite the fact that there was a 2 log decrease in the virus titer in the passively immunized rabbit. Thus, although the resistance of the rabbit was appreciably increased by the administration of the hyperimmune serum, the effect of hyaluronidase on the reaction of the rabbit to intradermal inoculation of vaccinia virus was the same as that observed in the non-immunized rabbit. On the other hand, when the resistance of the rabbit was increased by a previous infection with the virus, the diameter of the lesions in the saline series of virus dilutions was 157% of that encountered in the hyaluronidase series indicating a suppressive effect of hyaluronidase on the development of the lesions.

The data obtained in the 8 experiments are summarized in Fig. 2. When the reactions of the rabbits are expressed in terms of the ratio of the diameter of the lesions of the saline series to the diameter of the lesions of the

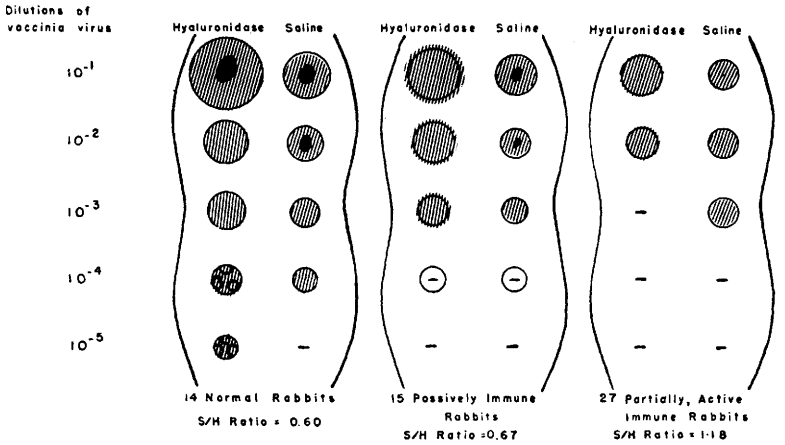

FIG. 2. Summary of vaccinial lesions in non-immune and partially immune rabbits.

hyaluronidase or testicular extract series (S/H ratio), the ratios are 0.60, 0.67, and 1.18 for the non-immunized, the passively immunized, and the partially, active immune groups of rabbits, respectively. In addition to the enhancing effect of hyaluronidase on the development of vaccinial lesions in the non-immunized and passively immunized rabbits and its suppressive effect in the previously infected rabbits, it was observed that hyaluronidase decreased the extent of necrosis which is depicted in Fig. 2 as the solid black area in the center of the lesion. The ability of hyaluronidase to give rise to isolated lesions in the non-immunized rabbits (10^{-4} and 10^{-5} dilutions of virus) is indicated by the smaller circles within the larger area of the lesion. As indicated by the open circle the lesions in the passively immunized rabbits induced by the 10^{-4} dilution of virus had a tendency after the 5th day to fade rapidly.

When the reactions of the rabbits to intradermal inoculations of vaccinia virus are tabulated in terms of days after infection, the S/H ratios remain relatively constant (Table I and Table II). The possibility cannot be excluded that the 2 passively immunized rabbits listed in Table I which had an S/H ratio of 0.89 also had a contact infection with vaccinia virus within the week prior to inoculation. Otherwise, the S/H ratios of the non-immunized and the passively immunized groups of rabbits are similar. In the previously infected rabbits in Table II the S/H ratios are consistently higher than the controls. The markedly higher S/H ratios found on the 8th and 9th days are a reflection of the fact that the lesions in the hyaluronidase series tend to fade much more rapidly than the lesions in the saline series of virus dilutions. Although the lesions in the passively immunized rabbits induced by the higher dilution of virus also fade, there is no marked difference between the rate of disappearance of the lesions in the hyaluronidase series as compared with the saline series of virus dilutions.

That the lesions in the partially, active immune rabbits are the result of an actual infection rather than an allergic manifestation due to the previous infection is indicated by the following facts. The evolution of the lesion, especially in the saline series of virus dilutions, follows the same pattern as that observed in the non-immunized and the passively immunized rabbits. Virus has been isolated from the lesions in the partially, active immune rabbits in approximaely the same concentration as in comparable lesions in the passively immunized rabbits. Finally, heat inactivated or partially heat inactivated vaccinia virus has failed to induce lesions in the partially, active immune rabbits.

The regularity with which vaccinial lesions were induced in rabbits which had been infected intradermally with vaccinia virus 2 to 3 months previously was an interesting finding. This was somewhat surprising in view of the

TABLE I. Reaction of Passively Immunized Rabbits to Intradermal Inoculations of Vaccinia Virus.

Time, days	No. rabbits		S/H ratio*	
	Immunized	Controls	Immunized	Controls
2	3	2	.56	.70
3	11	6	.68	.64
4	2†	2	.89	.57
5	14	8	.64	.57
6	3	2	.53	.48
7	9	4	.62	.54
8	3	2	.66	.43
9	3	2	.57	.43

* The ratio of diameter of lesions of the saline series of virus dilutions over the diameter of the lesions of the hyaluronidase or testicular extract series.

† These 2 rabbits may have had a contact infection within the week prior to inoculation since they were mistakenly placed in a room with rabbits infected with vaccinia virus.

TABLE II. Reaction of Previously Infected Rabbits to Intradermal Inoculations of Vaccinia Virus.

Time, days	No. rabbits		S/H ratio*	
	Immunized	Controls	Immunized	Controls
2	8	3	1.20	.63
3	19	7	1.07	.63
4	5	2	1.20	.57
5	15	6	1.18	.53
6	8	3	1.54	.50
7	11	4	1.06	.64
8	5	2	3.69	.43
9	10	4	2.27	.51

* The ratio of diameter of lesions of the saline series of virus dilutions over the diameter of the lesions of the hyaluronidase or testicular extract series.

high titers of neutralizing antibodies present in the serum of the rabbits, and especially in view of the continued presence of the virus in the testes of these rabbits. In 2 experiments testes were removed from rabbits which had been infected intradermally with vaccinia virus 53 to 86 days previously. In 9 of 10 cases vaccinia virus was isolated from the testes by inoculation of the chorioallantois of the chick embryo. Three isolations of virus were made on the first egg passage and 6 on the second egg passage. Virus was not isolated from 4 control testes after 3 egg passages. These rabbits developed typical lesions when inoculated with vaccinia virus on the day following the removal of the testes. Pearce(3) has isolated vaccinia virus from rabbits as long as 17 weeks after infection.

Comment. The suppressive effect of hyaluronidase on the intradermal inoculation of vaccinia virus in partially, active immune rabbits may be due to a spreading of the virus over a larger area and thus effectively increasing the concentration of neutralizing antibodies per unit of virus. If this explanation is correct, then it appears necessary to assume that the distribution of antibody is different in the passively immunized rabbit than in the partially, active immune rabbit for it was observed that hyaluronidase enhances vaccinial lesions in passively immunized rabbits. The possibility that other defensive agencies are acting in concert with the antibody has to be considered. The recent demonstration that hyaluronic acid inactivates vaccinia virus(4) suggests the intriguing possibility that a direct toxic effect on the virus by altered hyaluronic acid may play a role in the observed phenomenon. Whatever the mechanism involved in the inactivation of the virus, the important point shown by the present investigation is that there is a *critical concentration* for at least one virus, as there is for bacteria. However, in contrast to what is observed with the latter, the reality of this *critical concentration* can only be shown in hosts endowed with an active, partial immunity.

Summary. 1. Hyaluronidase or testicular extract enhances the lesions produced in non-immunized and passively immunized rabbits following intradermal inoculation of vaccinia virus. 2. On the contrary, in rabbits with a partial active immunity the lesions induced by the virus are partially suppressed by the spreading effect of hyaluronidase. 3. When the reactions of the rabbits are expressed in terms of the ratio of the diameter of the lesions of the saline series of virus dilutions to the diameter of the hyaluronidase or testicular extract series, the ratios are 0.60, 0.67, and 1.18 for the non-immunized, the passively immunized, and the partially, active immune groups of rabbits, respectively.

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